Imaging hypoxic stress and the treatment of amyotrophic lateral sclerosis with dimethyloxalylglycine in a mice model

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Abbreviations

ALS, amyotrophic lateral sclerosis; BOLD, blood oxygen level-dependent; BRET, bioluminescence resonance energy transfer; BW, body weight; ChAT, choline acetyltransferase; DMOG, dimethyloxalylglycine; EPO, erythropoietin; EPR, electron paramagnetic resonance; FJC, Fluoro-Jade C; GFAP, glial fibrillary acidic protein; G93A, G93A-human SOD1 transgenic; HE, hematoxylin and eosin; HIF-1α, hypoxia inducible factor-1α; Iba-1, ionized calcium-binding adapter molecule-1; NADH, nicotinamide adenine dinucleotide; NeuN, neuronal nuclear antigen; PBS, phosphate-buffered saline; PHD, prolyl hydroxylase; POL-AF, PTD-ODD-Luciferase labeled with a NIRF dye AF 680; ROI, regions of interest; SOD1, Cu/Zn superoxide dismutase; TDP-43, TAR DNA-binding protein 43; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; VEGF, vascular endothelial growth factor; WB, Western blot; WT, wild type.

Abstract

Hypoxia inducible factor-1α (HIF-1α) is a key transcription factor that maintains oxygen homeostasis. Hypoxic stress is related to the pathogenesis of amyotrophic lateral sclerosis (ALS), and impaired HIF-1α induces motor neuron degeneration in ALS. Dimethyloxalylglycine (DMOG) upregulates the stability of HIF-1α expression and shows neuroprotective effects, but has not been used in ALS as an anti-hypoxic stress treatment. In the present study, we investigated hypoxic stress in ALS model mice bearing G93A-human Cu/Zn superoxide dismutase by in vivo HIF-1α imaging, and treated the ALS mice with DMOG. In vivo HIF-1α imaging analysis showed enhanced hypoxic stress in both the spinal cord and muscles of lower limbs of ALS mice, even at the pre-symptomatic stage. HIF-1α expression decreased as the disease progressed until 126 days of age. DMOG treatment significantly ameliorated the decrease in HIF-1α expression, the degeneration of both spinal motor neurons and myofibers in lower limbs, gliosis and apoptosis in the spinal cord. This was accompanied by prolonged survival. The present study suggests that in vivo bioluminescence resonance energy transfer (BRET) HIF-1α imaging is useful for evaluating hypoxic stress in ALS, and that the enhancement of HIF-1α is a therapeutic target for ALS patients.

Key words: ALS, hypoxic stress, in vivo imaging, HIF-1α, DMOG

Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive and fatal disease that is caused by the selective death of motor neurons. About 10 % of patients have a genetically inherited form associated with mutations in Cu/Zn superoxide dismutase (SOD1) (Aoki et al., 1993; Rosen et al., 1993; Gurney et al., 1994), TAR DNA binding protein 43 (TDP-43) (Arai et al., 2006; Kabashi et al., 2008), and a hexanucleotide repeat expansion of the *C9orf72* gene (DeJesus-Hernandez et al., 2011; Renton et al., 2011). There are many reports about the pathological mechanisms of ALS, but the true pathogenesis of ALS is unclear. Hypoxic stress and an impaired response to hypoxia are related to the pathogenesis of ALS (Ilieva et al., 2003; Tankersley et al., 2007; Vanacore et al., 2010; Zhang et al., 2011; Kim et al., 2013). Moreover, the lack of oxygen plus flow-metabolism uncoupling induce motor neuron death in ALS (Tankersley et al., 2007; Miyazaki et al., 2012; Kim et al., 2013).

Hypoxia inducible factor-1α (HIF-1α) is a key transcription factor that maintains oxygen homeostasis (Huang et al., 1996; Semenza, 1998). In normoxia, HIF-1α was degraded by prolyl hydroxylase (PHD) when O_2 was used as a co-substrate (Maxwell et al., 1999; Berra et al., 2006). In hypoxia, HIF-1α is stabilized because of the dysfunction of PHD, inducing transcriptional activation in nuclear DNA (Semenza, 1998; Berra et al., 2006). Dysregulation of both HIF-1α expression and the downstream pathway in response to hypoxia induces motor neuron degeneration in ALS (Oosthuyse et al., 2001; Moreau et al., 2011; Sato et al.,

2012; Nagara et al., 2013). Even though ALS skeletal muscles are involved in oxidative stress (Ohta et al., 2019) and the HIF-1α pathway plays an important role in muscle activity (Mason et al., 2004; Mason and Johnson, 2007), hypoxic stress has not been investigated in ALS skeletal muscles.

Dimethyloxalylglycine (DMOG) is an inhibitor of PHD that upregulates the stability of HIF-1α expression (Milkiewicz et al., 2004; Sinha et al., 2017). DMOG treatment shows neuroprotective and anti-inflammatory effects on cerebral ischemia by increasing HIF-1α expression (Cummins et al., 2008; Nagel et al., 2011; Ogle et al., 2012; Selvan et al., 2017; Yang et al., 2018), but has not been used in ALS as an anti-hypoxic stress treatment. Therefore, in the present study, we investigated the involvement of hypoxia stress in ALS pathology by in vivo bioluminescence resonance energy transfer (BRET) HIF-1α imaging, and assessed the therapeutic effects of DMOG treatment in ALS model mice.

Experimental procedures

Animals

All experimental procedures were carried out according to the guidelines of the Animal Care and Use Committee of the Graduate School of Medicine, Dentistry, and Pharmaceutical Science of Okayama University (approval #OKU-2018084). G93A-human SOD1 transgenic (G93A) mice (Gurney et al., 1994) were obtained from Jackson Laboratories (Bar Harbor, ME, USA). This line was maintained as hemizygotes

by mating G93A males with C57BL/6J females. Our previous reports showed that G93A mice show disease onset around 98 days of age and die 21-28 days later (Sato et al., 2012; Ohta et al., 2019). G93A mice were divided into two experimental groups: G93A mice treated with vehicle (n=15; 9 males and 6 females), or DMOG (MedChem Express, Monmouth Junction, NJ, USA, n=15; 8 males and 7 females). We carried out a pilot study of DMOG treatment with a dose of 0.1 mg, 1 mg, and 10 mg per G93A mouse starting at 112 days (early symptomatic stage) for 10 days (every second day, total 5 times intraperitoneal administration). The trial showed that the best amount was 0.1 mg based on results of clinical (body weight (BW) and rotarod), in vivo bioluminescence of HIF-1α, and pathological analyses (hematoxylin and eosin (HE)-stained quadriceps muscle myofibers, Nissl-stained motor neurons in the lumbar spinal cord, and immunofluorescent analysis of HIF-1α in quadriceps muscle and the lumbar spinal cord) (data not shown). Every second day intraperitoneal administration of vehicle (phosphate-buffered saline (PBS), 0.5 ml per mouse) or DMOG (0.1 mg dissolved in 0.5 ml PBS per mouse) was initiated at 98 days (disease onset), over a period of 28 days (total of 14 injections). The two groups were assessed by clinical analysis, in vivo imaging of HIF-1α, and histological analysis. Age-matched non-transgenic control wild type (WT) C57BL/6J littermates were used as the control.

Clinical analysis

For the clinical analysis, survival was checked every day from 70 days of age, and BW and the rotarod score were measured once a week from 70 to 126 days of age in G93A mice treated with vehicle (n=10) or DMOG (n=10). In the rotarod test, the best results from three trials were recorded. The time at which a mouse could not right itself within 30 sec when placed on its side was recorded as the 'dead' point (Ohta et al., 2019). The investigators were blinded to the treatment conditions.

In vivo bioluminescence resonance energy transfer (BRET) imaging

In vivo BRET imaging was performed in WT and G93A mice treated with vehicle or DMOG (for each, n=3-5) using the IVIS spectrum imaging system (PerkinElmer Inc., Billerica, MA, USA) at 84 (pre-symptomatic stage), 105 (early symptomatic stage), and 126 (end stage) days of age. One nmol of PTD-ODD-Luciferase labeled with a NIRF dye AF 680 (POL-AF) probe in 100 μL of PBS was intravenously injected through the tail vein to detect HIF-1α expression by BRET imaging (Kuchimaru et al., 2016), in which HIF-1α specificity is based on the oxygen-dependent degradation regulation of HIF-1α (Harada et al., 2002). Under anesthesia with an oxygen: isoflurane mixture (98.5 %: 1.5 %), the skin of back and lower limbs was opened to reveal the vertebrae and muscles of lower limbs (Ohta et al., 2019). After 30 min POL-AF probe injection, 10 μg coelenterazine (Wako, Japan, dissolved in 10 μL of 100 % ethanol and 90 μL of PBS) was injected via the tail vein, and 5 min later HIF-1α signal imaging was detected. BRET signals of

areas of the spinal cord and bilateral lower limbs muscles were measured using regions of interest (ROI) exposed every 15 sec. Emission intensity was expressed as the total flux of photons (photons/s) by LivingImage software (PerkinElmer) (Kuchimaru et al., 2016; Nakano et al., 2017; Ohta et al., 2019). The investigators were blinded to the treatment conditions.

Immunofluorescent analysis of the quadriceps muscles and lumbar spinal cord

At 84, 105, and 126 days, WT and G93A mice with vehicle or DMOG (n=5 each) were deeply anesthetized by intraperitoneal injection of pentobarbital (40 mg/kg), and then transcardially perfused with chilled PBS. The quadriceps muscles and lumbar spinal cord (L4-5) were removed from WT and G93A mice, fixed in 4 % paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h and placed in PBS containing 30 % sucrose. The samples were cut on a cryostat (Thermo Scientific, Waltham, MA, USA) into 10 μm sections.

For immunofluorescent analysis, frozen sections were incubated in 10 mM citric acid and heated in a microwave oven for 5 min to activate the antigen. After washing in PBS, sections were blocked in 5 % bovine serum albumin for 1 h. Then, the sections were incubated with primary antibodies at 4 ℃ overnight. The following primary antibodies were used: goat anti-HIF-1α antibody (1:100; R&D Systems, Minneapolis, MN, USA, AF1935), mouse anti-neuronal nuclear antigen (NeuN) antibody (1:250; Millipore, Billerica, MA, USA,

MAB377), goat anti-choline acetyltransferase (ChAT) antibody (1:250; Millipore, AB144P), mouse anti-glial fibrillary acidic protein (GFAP) antibody (1:200; Millipore, MAB3402), and rabbit anti-ionized calcium-binding adapter molecule-1 (Iba-1) antibody (1:400; Wako, Japan, 019-19741). After overnight incubation, the sections were incubated again with corresponding secondary antibodies (1:500, Alexa Fluor™, Invitrogen, Carlsbad, CA, USA). Samples were observed by confocal laser microscopy (LSM780, Zeiss, Oberkochen, Germany) (Sato et al., 2012; Ohta et al., 2019).

Quantitative analysis of muscle and spinal cord sections

For the quantitative analysis of myofiber size, about 300 myofibers from three HE-stained quadriceps muscle sections per mouse (n=5 each) in each size category (<20 μm, 20-30 μm, 30-40 μm, >40 μm) were analyzed. For the quantitative analysis of HIF-1α-positive muscle fibers, about 900 myofibers from two quadriceps muscle sections stained with anti-HIF-1α antibody per mouse (n=5 each) were analyzed. For the analysis of α-motor neurons stained with both anti-ChAT and anti-NeuN antibodies, and HIF-1α-positive neurons (>20 μm) stained with both anti-HIF-1α and anti-NeuN antibodies, two lumbar spinal cord sections per mouse (n=5 each) were analyzed. For the semi-quantitative evaluation of immunoreactivity for GFAP and Iba-1 in the ventral horns, two lumbar cord sections stained with GFAP or Iba-1 per mouse (n=5 each) were analyzed using Image J (Ohta et al., 2011; Ohta et al., 2016; Ohta et al., 2019). For the evaluation of

neurodegeneration and apoptosis in the lumbar spinal cord using the Fluoro-Jade C (FJC) Ready-to-Dilute Staining Kit (Biosensis, Thebarton, South Australia) and the TACS 2TdT DAB in situ Apoptosis Detection Kit (Trevigen, Gaithersburg, MD) for terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining, two lumbar cord sections per mouse (n=5 each) were analyzed. All sections were analyzed by an investigator blinded to the treatment conditions.

Western blot (WB) analysis

The quadriceps muscles and lumbar spinal cord (L4-5) were removed from WT and G93A mice at 126 days. The samples were sonicated in ice-cold lysis buffer containing 50 mM Tris-HCl (pH 7.2), 250 mM NaCl, 1 % NP-40, and Complete Mini Protease Inhibitor Cocktail (Roche, Basel, Switzerland) (Sato et al., 2012). The lysate was centrifuged at 12,000 g for 20 min at 4 ℃, the supernatant was collected, and the protein concentration was determined by the Lowrv assay (Bio-Rad, Hercules, CA, USA). Twenty micrograms of the total protein extract was loaded onto a 12 % polyacrylamide gel, separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and transferred to a polyvinylidene fluoride membrane (Milipore, MA, USA). After being washed with PBS containing 5 % skimmed milk and 0.1 % Tween 20, the membranes were incubated with primary antibody overnight at 4 ℃ and subsequently with corresponding peroxidase-conjugated secondary antibody (Amersham Biosciences, Buckinghamshire, UK).

Immunodetection was performed with an enhanced chemiluminescent substrate (Pierce, Rockford, IL) and the signals were quantified with a luminoimage analyzer (ImageQuant LAS 500, GE Healthcare, WI, USA). The following primary antibodies were used: mouse anti-HIF-1α antibody (1: 100; Novus Biological, CO, USA), and rabbit anti-actin antibody (1: 1000; Abcam, MA, USA).

Statistical analysis

Data were analyzed in SPSS v.22.0 (IBM Corporation, Armonk, NY, USA) and expressed as means ± SD. Statistical comparisons of clinical scores (BW and rotarod score), and histological data between two groups were performed using an unpaired *t*-test. Statistical comparisons of BRET signals of in vivo imaging and histological data between three groups were evaluated using one-way ANOVA, followed by a Tukey-Kramer post hoc comparison for normally distributed data, and using a Kruskal-Wallis, followed by a Dunn's post hoc comparison for non-normally distributed data. Kaplan-Meier survival analysis and the log-rank test were used for survival. Statistical significance was set at $p<0.05$.

Results

Clinical course of G93A mice

The mean survival time of G93A mice treated with DMOG (150±6.7 days, n=10) was significantly longer than G93A mice treated with vehicle (146±5.8 days, n=10) (Fig. 1A, *p=0.03). G93A mice with vehicle showed a progressive decrease in BW and rotarod score from 98 days of age, which were not modified by DMOG treatment (Fig. 1B, C).

In vivo imaging of HIF-1α in G93A mice

In WT and G93A mice at 84 days of age (pre-symptomatic stage), HIF-1α-specific BRET signals were observed in both the spinal cord and muscles of lower limbs (Fig. 2A-C, arrowheads and arrows). Moreover, the BRET signals of G93A mice were stronger than WT mice in both areas (Fig. 2J, not significant). In G93A mice with vehicle at 105 days of age (early symptomatic stage), the HIF-1α-specific BRET signals of both the spinal cord and muscles of lower limbs decreased compared with 84 days (Fig. 2D, E, J, *p<0.05 for lower limb muscles of G93A mice with vehicle) with a further decrease at 126 days (end stage) (Fig. 2G, H, J, *p<0.05, **p<0.01). DMOG treatment, which was initiated at 98 days of age, ameliorated the decreases in HIF-1α signals of both the spinal cord and muscles of lower limbs at 105 and 126 days (Fig. 2F, I, J, arrowheads and arrows, *p<0.05).

Myofiber size in quadriceps muscles of G93A mice

Compared with WT mice, HE staining of quadriceps muscles already showed significant neurogenic myofiber atrophy in G93A mice with vehicle at pre-symptomatic 84 days of age (Fig. 3A, B, I, *p<0.05, **p<0.01), which progressed at 105 days (Fig. 3C, D, J, **p<0.01) with a further emphasis at 126 days (Fig. 3F, G, K, *p<0.05, **p<0.01). DMOG treatment did not change myofiber atrophy at 105 days of age (Fig. 3E, J), but significantly ameliorated this condition at 126 days of age compared with vehicle (Fig. 3H, K, *p<0.05, **p<0.01).

Expression of HIF-1α in quadriceps muscles of G93A mice

In quadriceps muscles of WT mice, weak immunoreactivity of HIF-1α was detected in the endomysium, but not in myofibers (Fig. 4A, C, F). In contrast, G93A mice with vehicle showed strong HIF-1α expression in myofibers, especially small myofibers (<20 μm diameter) at 84 days of age (Fig. 4B, I-K), and showed a progressive increase at 105 and 126 days (Fig. 4D, G, I-K, not significant). DMOG treatment significantly increased the HIF-1α-positive percentage in all fiber sizes, including small and middle fiber sizes, at 126 days of age (Fig. 4H-K, *p<0.05, **p<0.01). WB analysis showed that DMOG treatment remarkably enhanced HIF-1α expression in quadriceps muscle of G93A mice at 126 days (Fig. 4L).

Motor neuron degeneration in the lumbar cord of G93A mice

Compared with WT mice, the number of α-motor neurons expressing both ChAT and NeuN was significantly lower in G93A mice at 84 days of age (Fig. 5A, B, I, arrowheads, **p<0.01). This progressed at 105 days (Fig. 5C, D, arrowheads, **p<0.01) and was further emphasized at 126 days (Fig. 5F, G, I, **p<0.01). DMOG treatment significantly ameliorated α-motor neuron loss at 126 days of age (Fig. 5E, H, I, arrowheads, *p<0.05).

Expression of HIF-1α in neurons of the lumbar cord of G93A mice

In G93A mice at 84 days, HIF-1α was expressed in large neurons (size > 20 μm) of the ventral horn of the lumbar cord (Fig. 6A, arrowheads). The number of HIF-1α-positive neurons decreased in G93A mice with vehicle at 105 days of age compared with 84 days (Fig. 6A, C, arrowheads, not significant) with a further emphasis at 126 days (Fig. 6A, C, arrowheads, **p<0.01). DMOG treatment significantly ameliorated the loss of HIF-1α-positive neurons at 105 and 126 days, and the number of HIF-1α-positive neurons was larger than vehicle at 126 days (Fig. 6B, C, arrowheads, *p<0.05). WB analysis showed that DMOG treatment remarkably enhanced HIF-1α expression in the lumbar spinal cord of G93A mice at 126 days (Fig. 6D).

Gliosis, neurodegeneration and apoptosis in the lumbar cord of G93A mice

Astrogliosis was enhanced in the lumbar ventral horns of G93A mice with vehicle at both 105 and 126 days of age compared with 84 days of age (Fig. 7A, B, **$p<0.01$), which was significantly ameliorated by DMOG treatment at both 105 and 126 days (Fig. 7A, B, *$p<0.05$). Microgliosis was also enhanced in the lumbar ventral horns of G93A mice with vehicle at 105 days of age (Fig. 7D, E, not significant), further emphasized at 126 days (Fig. 7D, E, **$p<0.01$), and was significantly ameliorated by DMOG treatment at 126 days (Fig. 7D, E, **$p<0.01$). Double immunofluorescent analysis showed partial HIF-1α expression in both astrocytes and microglia of the lumbar cord of G93A mice (Fig. 7C, F, arrowheads). Neurodegeneration and apoptosis, which were presented by FJC and TUNEL positive cells, respectively, were enhanced in the lumbar ventral horns of G93A mice with vehicle at 126 days of age, which was significantly ameliorated by DMOG treatment (Fig. 8A, B, *$p<0.05$, **$p<0.01$).

Discussion

The present study showed, for the first time, enhanced hypoxia stress in both the spinal cord and muscles of lower limbs of G93A mice at the pre-symptomatic stage by in vivo BRET HIF-1α signals, which decreased as the disease progressed (Fig. 1, 2). DMOG treatment ameliorated the decrease of BRET and immunofluorescent HIF-1α signals and the degeneration of both spinal motor neurons and myofibers in

lower limbs (Fig. 2-6, 8), as well as gliosis and apoptosis in the spinal cord (Fig. 7, 8), and was accompanied by prolonged survival (Fig. 1).

Hypoxia stress and an impaired response to hypoxia are related to the pathogenesis of ALS (Tankersley et al., 2007; Vanacore et al., 2010; Zhang et al., 2011; Kim et al., 2013). We previously reported reduced blood flow and increased glucose utilization in the spinal cord of G93A mice from a pre-symptomatic stage (Miyazaki et al., 2012), suggesting that the lack of oxygen and flow-metabolism uncoupling induce motor neuron death in ALS (Tankersley et al., 2007; Kim et al., 2013). HIF-1α is a key transcription factor that maintains oxygen homeostasis (Huang et al., 1996; Semenza, 1998), and impaired HIF-1α function and a pathway for hypoxia stress progress ALS pathogenesis (Moreau et al., 2011; Xu et al., 2011; Zhang et al., 2011; Nagara, et al., 2013). We also previously showed the dysregulation of HIF-1α downstream proteins, vascular endothelial growth factor (VEGF) and erythropoietin (EPO) in the lumbar cord of symptomatic G93A mice (Sato et al., 2012). The activity and expression level of HIF-1α decreased in normal elderly mice compared to young mice (Frenkel-Denkberg, et al., 1999; Rivard, et al., 2000; Rohrbach, et al., 2005). The present study showed that BRET and immunofluorescent HIF-1α signals decreased in the spinal cords of ALS model mice, even at the pre-symptomatic stage (Fig. 2, 5-7), suggesting that the protective mechanism with HIF-1α began to be impaired from the pre-symptomatic stage. Electron paramagnetic resonance (EPR) oximetry, ^{19}F and blood oxygen level-dependent (BOLD) contrast MRIs,

which evaluate the oxygen level, can also be used to analyze hypoxia stress of ALS mice in the future (Jiang et al., 2013; Zhou et al., 2015; Desmet et al., 2018).

Skeletal muscles of ALS are involved in ALS pathogenesis including oxidative stress (Ohta et al., 2019), but hypoxic stress has not been investigated in ALS skeletal muscles, although the HIF-1α pathway plays an important role in muscle activity (Mason et al., 2004; Mason and Johnson, 2007). The present study is the first to show activated BRET and immunofluorescent HIF-1α expression in the HE-stained small myofibers of degenerated lower limbs in ALS model mice (Fig. 2-4), suggesting the pathological role of hypoxia stress in the degeneration of ALS skeletal muscles. HE staining is usually used for the evaluation of muscle degeneration of ALS mice, as in the present study, whereas nicotinamide adenine dinucleotide (NADH) and modified Gomori trichrome staining are rarely used (Lehmann., 2018; Zhang et al., 2018).

DMOG, which is an inhibitor of PHD, upregulates the stability of HIF-1α (Milkiewicz et al., 2004; Sinha et al., 2017). DMOG treatment has neuroprotective effects on cerebral ischemia by increasing HIF-1α expression (Nagel et al., 2011; Ogle et al., 2012; Selvan et al., 2017; Yang et al., 2018), and also has anti-inflammatory effects (Cummins et al., 2008; Yang et al., 2018). In the present study, DMOG treatment ameliorated the decrease of HIF-1α expression in both spinal motor neurons and muscles of lower limbs (Fig. 2, 4, 6), myofiber and motor neuron degeneration (Fig. 3, 5, 8), gliosis (Fig. 7) and apoptosis (Fig. 8) of ALS model mice. Furthermore, DMOG treatment significantly prolonged survival in ALS model mice

(Fig. 1A). In vivo imaging and immunofluorescent analysis showed a decrease in HIF-1α expression in both the spinal cord and lower limbs muscles of G93A mice even at the pre-symptomatic stage, suggesting that DMOG treatment, when started at the disease onset, could not fully improve motor performance and myofiber degeneration at an early stage of the disease, but improved survival and disease degeneration at a late stage of the disease in G93A mice. This suggests that the improvement of hypoxia stress by HIF-1α enhancement due to DMOG may show a clinical benefit in ALS patients, since the improvement of oxidative stress by edaravone shows a therapeutic benefit in ALS patients (Abe et al., 1997; Abe et al., 2017; Ohta et al., 2019).

In conclusion, we showed for the first time, the involvement of hypoxia stress in ALS pathology using in vivo BRET HIF-1α imaging and the therapeutic effects of DMOG treatment for ameliorating decreased HIF-1α expression and the progressed degeneration of spinal motor neurons and skeletal muscles in ALS mice. The present study suggests that in vivo BRET HIF-1α imaging can be useful for evaluating hypoxic stress in ALS mice, and that the enhancement of HIF-1α can be a therapeutic target for ALS.

Conflicts of interest

The authors disclose no potential conflicts of interest.

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Figure legends

Figure 1. Clinical analysis of G93A mice on (A) a Kaplan-Meier cumulative survival curve with vehicle (n=10) or DMOG (n=10), (B) body weight, and (C) rotarod scores. Note significant prolonged survival in G93A mice with DMOG (A, solid line) compared with G93A mice with vehicle (A, dotted line) (*p=0.03).

Figure 2. In vivo imaging of hypoxic stress marker HIF-1α in G93A mice. Note stronger HIF-1α signals in both the spinal cord and muscles of bilateral lower limbs of G93A mice compared with WT mice even at the pre-symptomatic stage (A-C, J, arrowheads and arrows, not significant) with a progressive decrease based on disease progression (D, E, G, H, J, arrows, *p<0.05, **p<0.01), and significant improvement by DMOG treatment (F, I, J, arrowheads and arrows, *p<0.05).

Figure 3. Myofiber atrophy in HE-stained quadriceps muscles of G93A mice. Note significant neurogenic myofiber atrophy in G93A mice even at the pre-symptomatic stage (A, B, I, *p<0.05, **p<0.01) with a progressive atrophy based on disease progression (C, D, F, G, J, K, *p<0.05, **p<0.01), and significant improvement by DMOG treatment at the end stage (E, H, J, K, *p<0.05, **p<0.01). Scale bar = 100 μm (A-H).

Figure 4. HIF-1α expression in quadriceps muscles of G93A mice. Note strong HIF-1α expression in myofibers, especially small myofibers (<20 μm diameter) of G93A mice (B, D, G, I-K), and significant enhancement by DMOG treatment at the end stage (E, H-K, *p<0.05, **p<0.01). Scale bar = 50 μm (H).

WB analysis of quadriceps muscle showing enhanced HIF-1α protein level of G93A mice by DMOG treatment at the end stage (L).

Figure 5. Motor neuron number loss in the lumbar cord of G93A mice. Note the progressive decrease in the number of α-motor neurons expressing both ChAT and NeuN in G93A mice based on disease progression (B, D, G, I, arrowheads, **p<0.01), and significant improvement by DMOG treatment at the end stage (E, H, I, arrowheads, *p<0.05). Scale bar = 20 μm (H).

Figure 6. HIF-1α expression in large neurons of the lumbar cord of G93A mice. Note the progressive decrease in the number of large neurons (size > 20μm) expressing HIF-1α in G93A mice based on disease progression (A, C, arrowheads), and significant improvement by DMOG treatment at the end stage (B, C, arrowheads, *p<0.05). Scale bar = 20μm (A, B). WB analysis of lumbar cord showing enhanced HIF-1α protein level of G93A mice by DMOG treatment at the end stage (D).

Figure 7. Gliosis in the lumbar cord of G93A mice. Note enhanced astrogliosis (A, B, **p<0.01) and microgliosis (D, E, **p<0.01) in G93A mice based on disease progression, and significant improvement by DMOG treatment (A, B, D, E, *p<0.05, **p<0.01). Note partial HIF-1α expression in both astrocytes and microglia of G93A mice at the end stage (C, F, arrowheads). Scale bars = 20 μm (C, F); 50 μm (A, D).

Figure 8. Neurodegeneration and apoptosis in the lumbar cord of G93A mice. Note the significant decrease in the number of FJC-positive (A, white arrows, **$p<0.01$) and TUNEL-positive (B, block arrows, *$p<0.05$) cells by DMOG treatment at the end stage. Scale bars = 20 μm (A, B).