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GUANABENZ DELAYS THE ONSET OF DISEASE SYMPTOMS, EXTENDS LIFESPAN, IMPROVES MOTOR PERFORMANCE AND ATTENUATES MOTOR NEURON LOSS IN THE SOD1 G93A MOUSE MODEL OF AMYOTROPHIC LATERAL SCLEROSIS

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Abstract—Amyotrophic lateral sclerosis (ALS) is a relentlessly progressive neurodegenerative disease characterized by the loss of motor neurons in the motor cortex, brain stem and spinal cord. Currently, there is no cure for this lethal disease. Although the mechanism underlying neuronal cell death in ALS remains elusive, growing evidence supports a crucial role of endoplasmic reticulum (ER) stress in the pathogenesis of ALS. Recent reports show that guanabenz, a novel inhibitor of eukaryotic initiation factor 2α (eIF2α) dephosphorylation, possesses anti-prion properties, attenuates ER stress and reduces paralysis and neurodegeneration in mTDP-43 *Caenorhabditis elegans* and *Danio rerio* models of ALS. However, the therapeutic potential of guanabenz for the treatment of ALS has not yet been assessed in a mouse model of ALS. In the present study, guanabenz was administered to a widely used mouse model of ALS expressing copper zinc superoxide dismutase-1 (SOD1) with a glycine to alanine mutation at position 93 (G93A). The results showed that the administration of guanabenz significantly extended the lifespan, delayed the onset of disease symptoms, improved motor performance and attenuated motor neuron loss in female SOD1 G93A mice. Moreover, western blotting results revealed that guanabenz dramatically increased the levels of phosphorylated-eIF2α (P-eIF2α) protein, without affecting total eIF2α protein levels. The results also revealed a significant decrease in the levels of the ER chaperone glucose-regulated protein 78 (BiP/Grp78) and markers of another two ER stress pathways, activating transcription factor 6α (ATF6α) and inositol-requiring enzyme 1 (IRE1). In addition, guanabenz increased the protein levels of anti-apoptotic B cell lymphoma/lewkmia-2 (Bcl-2), and down-regulated the pro-apoptotic protein levels of C/EBP homologous protein (CHOP), Bcl-2-associated X protein (BAX) and cytochrome C in SOD1 G93A mice. Our findings indicate that guanabenz may represent a novel therapeutic candidate for the treatment of ALS, a lethal human disease with an underlying mechanism involving the attenuation of ER stress and mitochondrial stress via prolonging eIF2α phosphorylation.

Key words: neurodegeneration, ALS, guanabenz, ER stress, P-eIF2α.

Amyotrophic lateral sclerosis (ALS) is a rapid progressive neurodegenerative disease characterized by the selective loss of motor neurons in the motor cortex, brainstem and spinal cord (reviewed by Bendotti and Carri, 2004). Though the incidence of ALS is low, at 2.08 cases per 100,000 in Europe (Chio et al., 2013), it is a lethal disease with an average course of 3–5 years, for which there is currently no cure. The only FDA-approved medication riluzole shows efficacy in the treatment of ALS with a marginal effect, and it may prolong median tracheostomy-free survival by 2–3 months in patients younger than 75 years with definite or probable ALS who have had the disease for less than 5 years and who have a forced vital capacity (FVC) of greater than 60% (Pandya et al., 2013). Nevertheless, the efforts to find new medications to treat ALS have never ceased.

The majority cases of ALS are sporadic (sALS), and approximately 10% of the remaining cases are familial (fALS). Approximately 20 genetic mutations have been found to be associated with fALS, and mutations in the *copper zinc superoxide dismutase-1 (SOD1)* gene account for approximately 20% of all fALS cases (Andersen and Al-Chalabi, 2011). Animal models developed based on the knowledge of gene mutations related to ALS, such as SOD1 glycine to alanine mutation at position 93 (G93A) mice, provide a useful tool for studying the disease and can be used in trials of new medications for treating ALS.

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Abbreviations: ALS, amyotrophic lateral sclerosis; ATF4, activating transcription factor 4; ATF6, activating transcription factor 6; BAX, Bcl-2-associated X protein; Bcl-2, B cell lymphoma/lewkmia-2; BiP/Grp78, glucose-regulated protein 78; CHOP, C/EBP homologous protein; eIF2α, eukaryotic initiation factor-2α; ER, endoplasmic reticulum; G93A, glycine to alanine at position 93; IRE1, inositol-requiring enzyme 1; NeuN, neuronal nuclei; P-eIF2α, phosphorylated-eIF2α; PERK, double-stranded RNA-activated protein kinase-like ER kinase; SOD1, copper zinc superoxide dismutase-1; UPR, unfolded protein response; WT, wild-type.

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The mechanisms underlying ALS remain elusive. They may include glutamate excitotoxicity, autophagy, apoptosis, mitochondrial dysfunction, free radical oxidative injury and immune modulation (Morren and Galvez-Jimenez, 2012). The most recent research in patients and mutant SOD1 animal models has suggested that endoplasmic reticulum (ER) stress may play an important role in the pathogenesis of neuronal degeneration in both sALS and fALS (Sasaki, 2010; Wang et al., 2011; Prell et al., 2012). ER stress is triggered by the accumulation of misfolded proteins in the ER, which induces neuronal death in ALS (Kikuchi et al., 2006). The increased levels of misfolded proteins in the ER within neurons result in activation of the unfolded protein response (UPR), which is an essential response for cellular homeostasis. Double-stranded RNA-activated protein kinase-like ER kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (IRE1) are the three key ER stress sensors that detect misfolded or unfolded proteins and then reprogram transcription and translation in a concerted manner to restore proteostasis (Sasaki, 2010). One branch of the UPR pathway functions to reduce global protein synthesis via the transient phosphorylation of eukaryotic initiation factor-2α (eIF2α) by activated PERK. This phosphorylation then upregulates the levels of activating transcription factor 4 (ATF4). If the UPR is prolonged, the pro-apoptotic transcription factor C/EBP homologous protein (CHOP) and ER-resident caspase-12 will be upregulated. The mitochondrial stress and apoptosis will subsequently be triggered (Sasaki, 2010). Phosphorylation of eIF2α (P-eIF2α) plays a cytoprotective role during ER stress when cells are sensitized following ER stress activation (Harding et al., 2000). Therefore, promoting the phosphorylation of eIF2α may attenuate ER stress and have therapeutic potential for the treatment of ALS.

Continuous phosphorylation of eIF2α may be harmful to the stressed cells because it may repress global protein synthesis, cause synaptic failure and neuronal loss in prion-diseased mice (Moreno et al., 2012). However, other reports showed that continuous phosphorylation of eIF2α induced by salubrinal, a specific inhibitor of P-eIF2α dephosphorylation (Boyce et al., 2005), protected Neuro2a cells against mutant SOD1-induced cell death, and decreased insoluble mutant SOD1 aggregates (Oh et al., 2008). Furthermore, treatment with salubrinal was shown to delay disease progression, and extend the lifespan of three different mutant SOD1 mouse models of ALS (Saxena et al., 2009). All these results indicate that eIF2α is a promising therapeutic target for the treatment of ALS.

Guanabenz, a small molecule that has been shown to act as an α2-adrenergic receptor agonist, was originally developed as an antihypertensive drug (Holmes et al., 1983). Recently, it was found that guanabenz can inhibit P-eIF2α dephosphorylation and protect wild-type ER-stressed cells from death. Another α2-adrenergic receptor agonist clonidine did not have this protective effect (Tsaytler et al., 2011). In a transgenic mouse model of prion disease, guanabenz showed therapeutic effects, prolonging the lifespan of treated animals (Tribouillard-Tanvier et al., 2008). In addition, a recent study indicated that guanabenz reduced paralysis and neurodegeneration in the mTDP-43 *Caenorhabditis elegans* and *Danio rerio* models of ALS through a mechanism involving reduction of the ER stress response (Vaccaro et al., 2013).

In the present study, we utilized the SOD1 G93A mouse model to further evaluate the therapeutic action of guanabenz against ALS in a rodent animal model.

EXPERIMENTAL PROCEDURES

Animals

Hemizygous breeding pairs of SOD1 G93A transgenic mice [Tg (SOD1-G93A)] in a B6SJL background (B6SJL-Tg) (SOD1-G93A-1Gur/J) were obtained from the Jackson Laboratory (Bar Harbor, USA). Male SOD1 G93A mice were crossed with B6SJLF1/J hybrid females as previously described (Gurney et al., 1994). Mice carrying the SOD1 G93A mutation were identified via PCR amplification of DNA extracted from the tails using a protocol provided by the Jackson Laboratory. The mice were maintained in a virus-free barrier facility with a standard 12-h light/dark cycle. Lab Diet pellets and drinking water were provided *ad libitum*. Behavioral tests were performed during the light period. All experimental protocols were approved by the Experimental Animal Ethics Committee of the Harbin Medical University in China. The number of mice used and their suffering were minimized. Previous studies have demonstrated that female mice show less variability in terms of the survival time than male mice (Shimojo et al., 2010; Feng et al., 2012) so that female mice were therefore used in this study.

Drug treatments

Female SOD1 G93A mice ($n = 30$) were randomly divided into guanabenz-treated and vehicle control groups; each animal in the treatment group had a littermate in the vehicle group. Beginning at 40 days of age, the SOD1 G93A mice were treated with either vehicle (5% glucose) or guanabenz (G110, Sigma–Aldrich, St. Louis, USA) at a dose of 4 mg/kg through i.p. injection every other day (Tribouillard-Tanvier et al., 2008) until the endpoint of the experiment (see below).

Behavioral assessment and analysis

The onset of disease was defined as the time point when tremors and shaking limbs were first observed upon suspending a mouse briefly in the air by its tail (Feng et al., 2012). The disease endpoint was marked as the mouse being unable to right itself within 30 s after being placed on its side (both sides tested) (Ludolph et al., 2007). The mice were weighed every other day beginning at 40 days of age. Rotarod performance was evaluated by measuring the retention time on a rotating rod (Harbin Lock Factory, Harbin, China) every four days. Specifically, motor performance in the rotarod test (16 rpm) was measured for each mouse starting at

70 days of age and after 1 week of training. Three trials were performed for each animal, and the longest time spent on the rod (180 s max) was recorded as the retention time (Azzouz et al., 2004; Feng et al., 2008).

Immunohistochemistry

Guanabenz- and vehicle-treated mice were euthanized at 130 days of age, when motor deficits were apparent. The mice were anesthetized and transcardially perfused with phosphate-buffered saline (PBS) for 10 min, followed by 4% paraformaldehyde in 0.1% phosphate buffer (PB) for 30 min. The spinal cord was dissected and post-fixed with 4% paraformaldehyde. The lumbar spinal cord was paraffin embedded and sectioned at a thickness of 8 μm. To quantify the number of motor neurons, every 13th section was selected, with a total of 30 sections being collected for each animal. The spinal cord sections were immunostained with an antibody recognizing the neuron-specific protein, neuronal nuclei (NeuN) (ZhongShan JinQiao, Beijing, China), followed by quantification of the number of neurons under a Leica microscope (Leica, Wetzlar, Germany). In each mouse (three mice per group), large motor neurons (NeuN immunoreactive) with a clear nucleus and nucleolus were counted in the ventral horn of the lumbar spinal cord (six sections per mouse). The number of motor neurons was determined by an experienced pathologist who was blinded to the sample groups.

Western blotting

The spinal cords of 130-day-old mice were dissected and homogenized in RIPA buffer (1% NP-40, 0.1% SDS, 50 mM Tris–Cl [pH 7.4] and 150 mM NaCl) containing a protease inhibitor (GE Healthcare, Waukesha, WI, USA) and a phosphatase inhibitor cocktail (Roche, IN, USA). Aliquots of 40 μg of total protein from the samples were separated via electrophoresis on 10% SDS–polyacrylamide gels (PAGE), followed by blotting onto nitrocellulose membranes. The membranes were incubated with various primary antibodies, including anti-tubulin (1:200, Santa Cruz Biotechnology), anti-glucose-regulated protein 78 (BiP/Grp78) (1:200, Santa Cruz Biotechnology), anti-P-eIF2α (1:1000, Cell Signaling Technologies), anti-eIF2α (1:1000, Cell Signaling Technologies), activating transcription factor 4 (ATF4) (1:200, Bioss Company), anti-CHOP (1:1000, Cell Signaling Technologies), ATF6α (1:200, Bioss company), IRE1 (1:200, Bioss Company), B cell lymphoma/lewkmia-2 (Bcl-2) (1:1000, Zhongshan Jinqiao Biotechnology), Bcl-2-associated X protein (BAX) (1:1000, Zhongshan Jinqiao Biotechnology), cytochrome C (1:200, Bioss Company) and β-actin (1:1000, Zhongshan Jinqiao Biotechnology) overnight at 4 °C. The membranes were then incubated with secondary antibodies (1:10,000; Rockland Immunochemicals, Gilbertsville, PA, USA) conjugated with IRDye800DX fluorescence at room temperature for 2 h. The membranes were rinsed 3 × 10 min with 0.1% Tween 20 in phosphate-buffered solution (PBS-T) after incubation with antibody. Images were then captured using the Odyssey Infrared Imaging System (Li-COR Biotechnology). Protein bands were quantified by using Image J.

Statistical analysis

The data are presented as the mean ± standard error of the mean (SEM). Graphs were designed with GraphPad Prism 5.0 (GraphPad Software, CA, USA). Statistical analysis of the probability of survival was performed with the Kaplan–Meier life test. The log-rank test was used to analyze disease onset and survival data. One-way ANOVA and post hoc analysis were conducted with SPSS 22.0 for the statistical comparisons. The level of significance was set at $p < 0.05$.

RESULTS

Guanabenz delayed disease onset, extended lifespan, improved motor performance and delayed body weight loss in SOD1 G93A mice

To assess whether guanabenz could alter disease onset in SOD1 G93A mice, female SOD1 G93A mice were treated with 4 mg/kg guanabenz every other day, beginning at 40 days of age until the endpoint of the experiment. The cumulative probability of the onset of symptoms plotted against the age of the animals showed a shift to the right for the guanabenz-treated group (Fig. 1A). The mean time of the onset of motor deficits was found to be 104.5 ± 2.0 days for the vehicle group and 116.9 ± 2.4 days for the guanabenz group ($p < 0.01$).

Next, we examined the effects of drug treatment on the survival time of SOD1 G93A mice and found that the curve of the survival time was shifted to the right in the guanabenz-treated group (Fig. 1B). The results showed that the mean survival time was 132.2 ± 4.0 days for the vehicle group and 150.7 ± 4.7 days for the guanabenz group ($p < 0.05$).

Rotarod performance was measured to determine whether guanabenz can delay the motor impairment of SOD1 G93A mice. The retention time on the rotating rod was greater in the guanabenz group (Fig. 1C). Overall, the performance curves showed that the rotarod performance of the guanabenz group was better than that of the vehicle group.

In addition, the body weight reduction typically observed in SOD1 G93A mice was delayed by guanabenz treatment (Fig. 1D).

Guanabenz treatment decreased motor neuron loss in the lumbar spinal cord of SOD1 G93A mice

To assess whether guanabenz delays motor neuron loss, the number of motor neurons in the spinal cord was evaluated at 130 days of age, a time point when the mice displayed severe motor dysfunction. Vehicle-treated mice showed extensive loss of motor neurons in the anterior horns of the lumbar spinal cord compared with their wild-type (WT) littermates (group means: 7.0 ± 0.58 and 24.3 ± 1.4, respectively) (Fig. 2A, C). In contrast, the motor neurons in the ventral horns of the guanabenz-treated mice were remarkably well

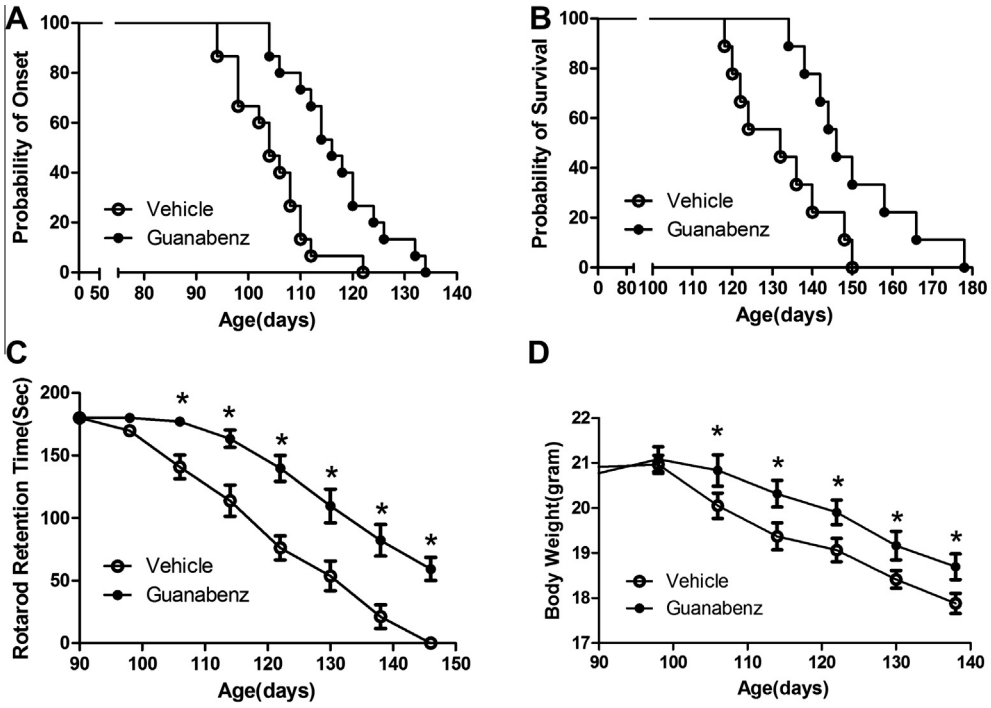

Fig. 1. Effects of guanabenz in SOD1 G93A mice. Beginning at 40 days of age, either vehicle (5% glucose) or guanabenz (4 mg/kg) was administered to female SOD1 G93A mice through i.p. injection every other day, as described in the Experimental procedures. (A) Guanabenz delayed the onset of disease symptoms in SOD1 G93A mice. The cumulative probability of the onset of symptoms is plotted against the age of the mutant mice. (B) Guanabenz prolonged the survival of SOD1 G93A mice. The cumulative probability of death plotted against the age of the mutant mice is shown. (C) Guanabenz improved the motor performance in the SOD1 G93A mice. Rotarod performance was significantly improved in the guanabenz-treated mice compared with that of the vehicle-treated mice. (D) Guanabenz treatment delayed body weight loss compared with vehicle treatment.

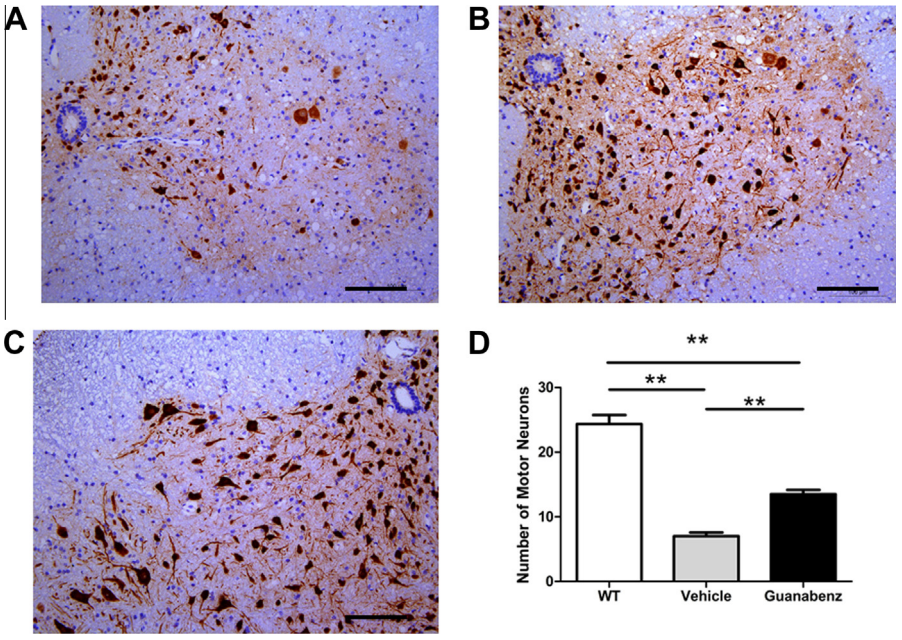

Fig. 2. Guanabenz reduced the death of motor neurons in the spinal cord of the SOD1 G93A mice. The animals were euthanized at 130 days of age, and the lumbar spinal cord was removed. Motor neurons in the spinal cord were stained with the anti-NeuN antibody. Representative images of the vehicle-treated control mice (A), guanabenz-treated mice (B) and WT littermates (C) are shown. (D) The number of motor neurons was determined. The vehicle group (7.0 ± 0.58) showed an extensive ($p < 0.01$) loss of motor neurons in the anterior horns of the spinal cord lumbar region compared with the WT group (24.3 ± 1.4). Motor neurons were remarkably preserved in the guanabenz-treated group (13.5 ± 0.67). $^{**}p < 0.01$. Scale bar = 100 μm.

preserved (13.5 ± 0.67) (Fig. 2B); however, the number of motor neurons in the guanabenz group remained significantly lower than in WT mice.

Guanabenz significantly increased P-eIF2α, without affecting total eIF2α and ATF4, decreased BiP, CHOP, ATF6α and IRE1 expression

To identify the underlying mechanism, we investigated the effect of guanabenz on the expression of multiple ER stress markers, including BiP, P-eIF2α, eIF2α, ATF4, CHOP, ATF6α and IRE1 via western blotting. Increase of P-eIF2α, without an effect on total eIF2α, was observed in the guanabenz group. Decrease of the ER chaperone BiP and pro-apoptotic protein CHOP was detected. In addition, we found that guanabenz decreased protein levels of ATF6α and IRE1, markers for another two pathways of ER stress (Fig. 3).

Guanabenz significantly increased the expression of Bcl-2, but down-regulated the levels of BAX and cytochrome C

To detect the effect of guanabenz on mitochondrial stress, we tested the markers of mitochondrial stress. The results showed that guanabenz increased the protein levels of Bcl-2, but down-regulated the protein levels of BAX and cytochrome C in SOD1 G93A mice (Fig. 4).

DISCUSSION

ALS is an adult-onset, rapid progressive motor neuron disease and is currently incurable. The mechanisms underlying pathogenesis of ALS are still not fully understood. Diverse agents have been tested in rodent models of ALS or in clinical trials targeting neuronal glutamate excitotoxicity (riluzole, gabapentin, and topiramate), autophagy (lithium), apoptosis (minocycline, pentoxifylline), mitochondrial dysfunction (creatine, dexpramipexole), free radical oxidative injury (vitamin E, coenzyme Q10) and immune modulation (celecoxib, thalidomide) (reviewed by Morren and Galvez-Jimenez, 2012). Unfortunately, only riluzole has been approved by the FDA for the treatment of ALS, and this drug has only a marginal effect, prolonging the patient's lifespan for approximately 2–3 months (Miller et al., 2012). Thus, there is still an urgent need to find more successful medications for the treatment of ALS.

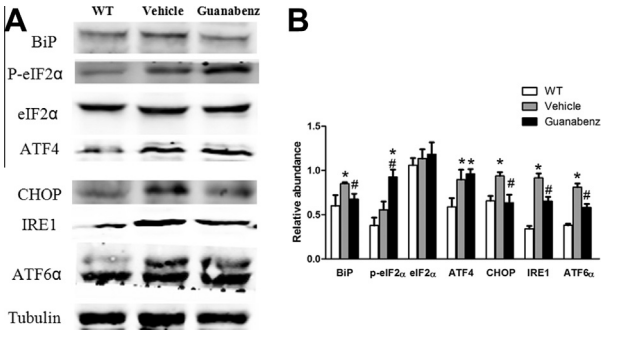

Fig. 3. Guanabenz affected multiple ER stress markers, including BiP, P-eIF2α, ATF6α, IRE1 and CHOP, in SOD1 G93A mice. (A) Representative results from the western blot analysis of spinal cord homogenates from the WT, vehicle and guanabenz groups. (B) The levels of BiP, P-eIF2α, eIF2α, ATF4, CHOP, ATF6α and IRE1 were quantified. The results are depicted as mean ± SEM (n = 3). $^{*}p < 0.05$ compared with the WT group and $^{\#}p < 0.05$ when compared with the vehicle control group.

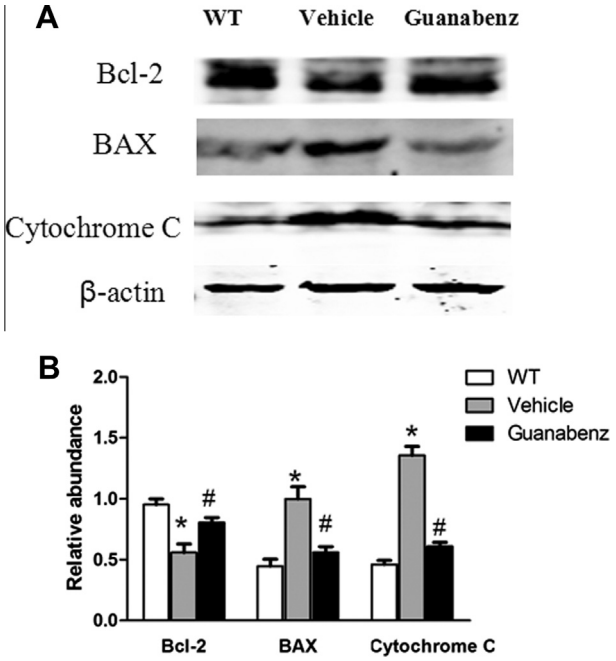

Fig. 4. Guanabenz affected mitochondrial stress markers in SOD1 G93A mice. (A) Representative results from the western blot analysis of spinal cord homogenates from the WT, vehicle and guanabenz groups. The protein levels of Bcl-2 increased while the expression of BAX and cytochrome C decreased in the guanabenz group, compared with those of the vehicle control group. (B) The levels of Bcl-2, BAX and cytochrome C were quantified. The results are depicted as mean ± SEM (n = 3). $^{*}p < 0.05$ compared with the WT group and $^{\#}p < 0.05$ when compared with the vehicle control group.

In the present study, we found that guanabenz significantly delayed the onset of disease symptoms and extended the survival of female SOD1 G93A mice. We also observed that guanabenz improved motor performance, delayed body weight loss and prolonged motor neurons survival in the lumbar spinal cord.

Growing evidence suggests ER stress plays an important role in the pathogenesis of ALS (Roussel et al., 2013). The accumulation of mutant SOD1 in the ER triggers ER stress and leads to neuronal death (Kikuchi et al., 2006). Reducing protein synthesis by phosphorylating eIF2α is a legitimate approach for reducing ER stress and may have protective effects in ALS. The stress-inducible subunit of PPP1R15A/GADD34 and the constitutive regulatory subunit of PPP1R15B are components of the protein phosphatase 1 phosphatase complexes that carry out P-eIF2α dephosphorylation (Tsaytler et al., 2011). Thus, regulation of the subunits of phosphatases to restore proteostasis may represent a viable drug target for treating ALS. Salubrinal is an

inhibitor of P-eIF2α dephosphorylation and has been shown to be protective in both cell (Oh et al., 2008) and mouse (Saxena et al., 2009) models of ALS.

Guanabenz may exert its neuroprotective effect via reducing ER stress. Our results revealed that guanabenz dramatically increased the protein levels of P-eIF2α, without affecting total eIF2α levels. In addition, guanabenz downregulated the levels of the ER chaperone BiP, ATF6α and IRE1, the markers of another two ER stress pathways. At the same time, guanabenz could increase the expression of Bcl-2, but down-regulated the expression of pro-apoptotic protein CHOP, BAX and cytochrome C in SOD1 G93A mice. Guanabenz-induced increases of P-eIF2α may attenuate motor neurons death via reducing ER stress and mitochondrial stress. The downregulation of BiP suggests that guanabenz has the ability to reduce ER stress by decreasing protein misfolding and to attenuate neuronal damage through CHOP downregulation, so that a greater number of neurons are preserved. The better motor performance observed can be explained by the presence of more preserved motor neurons. The delay of body weight loss may at least partially be explained by the decreased loss of neurons and increased ability to move in the experimental animals.

Guanabenz has its advantages for the treatment of ALS. In contrast to salubrinal, guanabenz binds only to stress-inducible PPP1R15A, which spares the constitutive activity of PPP1R15B for essential protein synthesis. Thus, guanabenz may have fewer side effects than salubrinal (Pavitt and Ron, 2012). As an anti-hypertensive drug, guanabenz has already been used in the clinic for approximately 30 years without any major side effects being reported (Holmes et al., 1983). Furthermore, guanabenz easily crosses the blood–brain barrier (Meacham et al., 1980). Taken together, our results indicate that guanabenz has the potential for the treatment of ALS and may be useful in other neurodegenerative diseases involving ER stress.

CONCLUSIONS

In summary, our results indicate that the administration of guanabenz significantly delayed the onset of disease symptoms, extended the lifespan, improved motor performance and attenuated motor neuron loss in female SOD1 G93A mice. The underlying mechanisms might be associated with the prolongation of eIF2α phosphorylation and attenuation of ER stress and mitochondrial stress. Our findings suggest a potential therapeutic role for guanabenz in the treatment of ALS.

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