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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