Neurobiology of Disease 51 (2013) 72–81

Contents lists available at SciVerse ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

Mutant SOD1 ^{G93A} triggers mitochondrial fragmentation in spinal cord motor neurons: Neuroprotection by SIRT3 and PGC-1α

Wenjun Song, Yuting Song, Brad Kincaid, Blaise Bossy, Ella Bossy-Wetzel *

Burnett School of Biomedical Sciences, College of Medicine, University of Central Florida, Orlando, FL, USA

ARTICLE INFO

Article history:
Received 29 March 2012
Revised 8 June 2012
Accepted 3 July 2012
Available online 20 July 2012

Keywords:
Mitochondrial dynamics
Axonal trafficking
Dominant-negative DRP1
Real-time imaging
Astrocyte
Motor neuron
SIRT3
ALS
PGC-1α

ABSTRACT

Mutations in the Cu/Zn Superoxide Dismutase (SOD1) gene cause an inherited form of ALS with upper and lower motor neuron loss. The mechanism underlying mutant SOD1-mediated motor neuron degeneration remains unclear. While defects in mitochondrial dynamics contribute to neurodegeneration, including ALS, previous reports remain conflicted. Here, we report an improved technique to isolate, transfect, and culture rat spinal cord motor neurons. Using this improved system, we demonstrate that mutant SOD1^{G93A} triggers a significant decrease in mitochondrial length and an accumulation of round fragmented mitochondria. The increase of fragmented mitochondria coincides with an arrest in both anterograde and retrograde axonal transport and increased cell death. In addition, mutant SOD1^{G93A} induces a reduction in neurite length and branching that is accompanied with an abnormal accumulation of round mitochondria in growth cones. Furthermore, restoration of the mitochondrial fission and fusion balance by dominant-negative dynamin-related protein 1 (DRP1) expression rescues the mutant SOD1^{G93A}-induced defects in mitochondrial morphology, dynamics, and cell viability. Interestingly, both SIRT3 and PGC-1α protect against mitochondrial fragmentation and neuronal cell death by mutant SOD1^{G93A}. This data suggests that impairment in mitochondrial dynamics participates in ALS and restoring this defect might provide protection against mutant SOD1^{G93A}-induced neuronal injury.

Published by Elsevier Inc.

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by degeneration of upper and lower motor neurons (Boillee et al., 2006; Bruijn et al., 2004; Gurney et al., 1994; Wood-Allum and Shaw, 2010) . The vast majority of ALS cases are sporadic, whereas only approximately 10% have a familial mode of inheritance (Rosen, 1993). Previous work has focused on mutations in the SOD1 gene, which encodes Cu/Zn superoxide dismutase. This highly expressed and predominantly cytoplasmic enzyme catalyzes the conversion of superoxide to hydrogen peroxide and oxygen. However, mutant SOD1 causes motor neuron death through a toxic gain-of-function mechanism as opposed to a simple loss-of-function in superoxide scavenging activity (Boillee et al., 2006; Bruijn et al., 2004; Knott and Bossy-Wetzel, 2007; Magrane et al., 2009).

While the mechanisms of mutant SOD1-mediated motor neuron death remain unclear, multiple observations indicate that mitochondrial dysfunction plays a key role. First, abnormal mitochondrial morphology and ultrastructure with cristae vacuolization have been observed in mutant SOD1 mice (Kong and Xu, 1998; Wong et al., 1995) and ALS patient samples (Sasaki and Iwata, 1996). In addition, mutant SOD1 binds preferentially to mitochondria, impairs respiration, decreases the Ca^{2+} buffering capacity, blocks protein import, and induces apoptosis through Bcl-2 inhibition (Damiano et al., 2006; Israelson et al., 2010; Mattiazzi et al., 2002; Pedrini et al., 2010). Furthermore, substantial evidence points to a role for secondary glutamate-mediated excitotoxicity (Boillee et al., 2006; Shaw and Ince, 1997).

An important attribute of mitochondria is their ability to divide and fuse and be transported across long motor neuron axons. These processes, collectively known as mitochondrial dynamics, are critical for neuronal energy production, synaptic function, and cell survival. Proper transport and positioning of mitochondria at synapses of the neuromuscular junction are believed to play an important role in motor neuron function (Dadon-Nachum et al., 2011). Impaired mitochondrial dynamics has been proposed to trigger axonal degeneration and is consistent with the

Abbreviations: ALS, amyotrophic lateral sclerosis; BCS, bovine calf serum; BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; CMT, Charcot–Marie–Tooth; DIV, days *in vitro*; DMEM, Dulbecco's Modified Eagle Medium; DRP1, dynamin-related protein 1; DTT, dithiothreitol; EBSS, Earle's Balanced Salt Solution; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IDH, isocitrate dehydrogenase; MFN, mitofusin; NAD^+, nicotinamide adenine dinucleotide; NAM, nicotinamide; NEM, N-ethylmaleimide; OPA1, optic atrophy 1; PBS, phosphate buffered saline; Pen/Strep, penicillin–streptomycin; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; ROS, reactive oxygen species; SIRT, silent information regulator two; SMI-32, neurofilament H non-phosphorylated; SOD1, Cu/Zn superoxide dismutase; TSA, trichostatin A.

* Corresponding author at: Burnett School of Biomedical Sciences, College of Medicine, 4000 Central Florida Blvd, Building 20, Orlando, FL 32816, USA. Fax: +1 407 823 0956.
E-mail address: ebossywe@mail.ucf.edu (E. Bossy-Wetzel).
Available online on ScienceDirect (www.sciencedirect.com).

0969-9961/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.nbd.2012.07.004

"dying back" hypothesis of neuronal projections as an initiating event in ALS pathogenesis (Dadon-Nachum et al., 2011).

Fast axonal transport of mitochondria is mediated by the kinesin family and the dynactin/dynein motor complex. The fact that mutations in KIF5A, KIF1Bβ, and Dynactin-1 cause motor neuron degeneration further underscores the importance of this process in motor neuron functionality (Puls et al., 2003; Reid et al., 2002; Zhao et al., 2001). In addition, impairment of mitochondrial fission and fusion balance has been linked to transport defects and neurodegeneration (Chan, 2006; Knott et al., 2008). Dynamin-related protein 1 (DRP1) mediates mitochondrial fission while mitofusin 1 (MFN1), mitofusin 2 (MFN2), and optic atrophy 1 (OPA1) mediate fusion (Hoppins et al., 2007; Legros et al., 2002; Meeusen et al., 2006; Song et al., 2009). Also, MFN2 mutations cause Charcot–Marie–Tooth type-2A (CMT-2A), a neurodegenerative disorder characterized by motor neuron degeneration (Zuchner et al., 2004). These observations suggest that there is a connection between the mitochondrial fission and fusion machinery and the molecular motors that mediate axonal trafficking. Finally, excessive mitochondrial fission is induced by a wide variety of neurotoxins, *i.e.* nitrosative/oxidative stress, and is functionally implicated in neuronal injury and cell death (Barsoum et al., 2006; Liot et al., 2009; Yuan et al., 2007).

Recently, several studies suggested possible defects in mitochondrial dynamics in models of ALS (De Vos et al., 2007; Magrane et al., 2009, 2012). The first report identified a selective decrease in anterograde mitochondrial transport in "pure" motor neuron and cortical neuronal cultures isolated from mutant SOD1 transgenic rats (De Vos et al., 2007). To visualize mitochondria, the authors used MitoTracker Red, a fluorescent mitochondrial membrane potential sensitive dye, which is not incorporated into mitochondria that lack a membrane potential. Therefore it stains predominantly mitochondria with intact membrane potential, perhaps limiting the interpretation of the results. Additionally, this fluorescent probe tends to lack specificity to label only mitochondria, and has the potential to be neurotoxic. The second report demonstrated that a decrease in both anterograde and retrograde transport increased mitochondrial fragmentation and cell death in NSC-34 cells expressing mutant SOD1 targeted to the mitochondrial intermembrane space (Magrane et al., 2009). However, this study was done only in a cell line and not primary motor neurons. The most recent report implicating defective mitochondrial dynamics in ALS was performed using "pure" motor neuron cultures, lacking astrocytic support (Magrane et al., 2012). The study identified mitochondrial defects in anterograde transport, but not retrograde transport. Although mitochondria were visualized by transfecting MitoDendra, which has no reported toxicity, potential problems might be found in the lack of astrocytic support.

To reconcile the apparent contradicting observations and to improve the experimental models, we co-expressed DsRed2-Mito, a red fluorescent protein which exhibits no toxicity in primary neurons, to visualize mitochondria. Additionally, we co-cultured motor neurons on top of a spinal cord astrocyte monolayer. This key modification makes this experimental system of greater physiological relevance compared to cell lines and "pure" neuronal culture models.

Caloric restriction delays aging and age-related diseases including neurodegeneration (Anderson et al., 2009; Colman et al., 2009; Qin et al., 2006; Sohal and Weindruch, 1996; Someya et al., 2007; Weindruch and Walford, 1982). In recent years the sirtuins, a group of NAD^+-dependent deacetylases, have been found to mediate the protective effects of caloric restriction (Cohen et al., 2004; Donmez and Guarente, 2010; Finkel et al., 2009; Haigis and Sinclair, 2010; Howitz et al., 2003; Lin et al., 2000; Someya et al., 2010). SIRT3, the majority of which resides in the mitochondria, plays an important role in the cellular ROS defense and mitochondrial energy metabolism, and provides protection against aging-related hearing loss *in vivo* and excitotoxic insults in cultured neurons *in vitro* (Bell and Guarente, 2011; Qiu et al., 2010; Shi et al., 2005; Someya et al., 2010; Tao et al., 2010). Moreover, SIRT3 has been shown to be regulated by PGC-1α which stimulates mitochondrial biogenesis and is associated with ROS suppression and neuroprotection (Giralt et al., 2011; Kong et al., 2010; St-Pierre et al., 2006; Wu et al., 1999). Whether SIRT3 can restore defects in mitochondrial dynamics and protect against mutant $SOD1^{G93A}$ has never been tested.

Here, we report that mutant $SOD1^{G93A}$ causes mitochondrial fragmentation and inhibits both anterograde and retrograde mitochondrial transport in primary spinal cord motor neurons co-cultured with spinal cord astrocytes. In addition, we demonstrate that mutant $SOD1^{G93A}$-mediated mitochondrial changes are associated with impaired motor neuron development and reduced dendritic sprouting. Furthermore, restoring the fission and fusion balance with dominant-negative $DRP1^{K38A}$ expression rescued the neurons from $SOD1^{G93A}$-induced mitochondrial fragmentation and cell death. In addition, SIRT3 and PGC-1α expression prevented mitochondrial fragmentation and cell death by mutant $SOD1^{G93A}$. In summary, our data provides an updated model of the effects of mutant $SOD1^{G93A}$ on mitochondrial morphology and transport in motor neurons.

Experimental procedures

Reagents and plasmids

The pDsRed2-Mito vector was obtained from Clontech. The pcDNA3.1-$SOD1^{WT}$ and -$SOD1^{G93A}$ vectors were obtained from Dr. Alvaro G. Estevez (University of Central Florida, Orlando, FL, USA). The pcDNA3-$DRP1^{K38A}$ vector was from Dr. Alexander M. van der Bliek (David Geffen School of Medicine at UCLA, Los Angeles, CA, USA). The pcDNA3.1-SIRT3-HA vector was provided by Dr. Eric M. Verdin (Gladstone Institute of Virology and Immunology at UCSF, San Francisco, CA, USA). The pcDNA4-myc-PGC-1α (Addgene plasmid 10974) was obtained from Dr. Toren Finkel (Center for Molecular Medicine, NIH, Bethesda, MD, USA) through Addgene. The pβ—actin-Map2c/EGFP was from Dr. Doll (Novartis Institute for Biomedical Research, Basel, Switzerland). All plasmids were purified using the Endotoxin-free Marligen Maxiprep Kit (Diagnostic technology, Belrose, Australia). The detergents, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), were purchased from Omega Scientific (Tarzana, CA, USA) and G-biosciences (Maryland Heights, MO, USA), respectively. Poly-L-lysine, BSA, DNase I, Opti-Prep, Phenol Red, Penicillin–streptomycin (Pen/Strep), nicotinamide (NAM), N-ethylmaleimide (NEM), trichostatin A (TSA), cytosine arabinoside, formaldehyde, glutamine, $MgCl_2$, pyruvate and F-12 HAM were all obtained from Sigma-Aldrich (St. Louis, MO, USA). Phenol-free Dulbecco's Modified Eagle Medium (DMEM) with high glucose and bovine calf serum (BCS) were obtained from Thermo Scientific (Rockford, IL, USA). Trypsin (2.5%, 10×), L-15, GlutaMAX, Earle's Balanced Salt Solution (EBSS), Lipofectamine 2000, Hoechst 33342, neurobasal medium, and Alexa 488-conjugated goat anti-mouse secondary antibodies were purchased Life Technologies (Grand Island, NY, USA). Protease inhibitor cocktails were purchased from Roche (Indianapolis, IN, USA). EDTA was purchased from EMD (Billerica, MA, USA). Dithiothreitol (DTT) was purchased from GE Healthcare (Piscataway, NJ, USA). Amaxa Nucleofector kit for rat neurons was purchased from Lonza (Basel, Switzerland). Mouse monoclonal anti-rat p75 antibody MC-192 and mouse monoclonal anti neurofilament H non-phosphorylated (SMI-32) was from Abcam (Cambridge, MA, USA). Rat anti-mouse IgG1 microbeads and MACS separation columns were obtained from Miltenyi Biotec (Auburn, CA, USA). SIRT2/3 Fluorimetric Drug Discovery Kit was purchased from Enzo Life Sciences (Farmingdale, NY, USA).

Mice and rats

$SOD1^{G93A}$ transgenic mice (B6.Cg-Tg (SOD1-G93A) 1Gur/J) were purchased from Jackson Laboratory. Timed-pregnant Sprague Dawley rats were purchased from Charles River. All experiments were