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Research Paper

Neuron specific reduction in CuZnSOD is not sufficient to initiate a full sarcopenia phenotype

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ABSTRACT

Our previous studies showed that adult (8 month) mice lacking CuZn-superoxide dismutase (CuZnSOD, *Sod1*KO mice) have neuromuscular changes resulting in dramatic accelerated muscle atrophy and weakness that mimics age-related sarcopenia. We have further shown that loss of CuZnSOD targeted to skeletal muscle alone results in only mild weakness and no muscle atrophy. In this study, we targeted deletion of CuZnSOD specifically to neurons (n*Sod1*KO mice) and determined the effect on muscle mass and weakness. The n*Sod1*KO mice show a significant loss of CuZnSOD activity and protein level in brain and spinal cord but not in muscle tissue. The masses of the gastrocnemius, tibialis anterior and extensor digitorum longus (EDL) muscles were not reduced in n*Sod1*KO compared to wild type mice, even at 20 months of age, although the quadriceps and soleus muscles showed small but statistically significant reductions in mass in the n*Sod1*KO mice. Maximum isometric specific force was reduced by 8–10% in the gastrocnemius and EDL muscle of n*Sod1*KO mice, while soleus was not affected. Muscle mitochondrial ROS generation and oxidative stress measured by levels of reactive oxygen/nitrogen species (RONS) regulatory enzymes, protein nitration and F_2-isoprostane levels were not increased in muscle from the n*Sod1*KO mice. Although we did not find evidence of denervation in the n*Sod1*KO mice, neuromuscular junction morphology was altered and the expression of genes associated with denervation acetylcholine receptor subunit alpha (AChRα), the transcription factor, Runx1 and GADD45α) was increased, supporting a role for neuronal loss of CuZnSOD initiating alterations at the neuromuscular junction. These results and our previous studies support the concept that CuZnSOD deficits in either the motor neuron or muscle alone are not sufficient to initiate a full sarcopenic phenotype and that deficits in both tissues are required to recapitulate the loss of muscle observed in *Sod1*KO mice.

Introduction

Our previous studies have provided significant insight into potential mechanisms of sarcopenia using mice that lack CuZn-superoxide dismutase (CuZnSOD), i.e., *Sod1*KO mice, as a model of age-related muscle atrophy. Although indistinguishable from wild type mice at birth, by 5–8 months of age, gastrocnemius muscles of *Sod1*KO mice display significant reductions in mass and function that progress through adulthood, such that by 20 months of age *Sod1*KO mice resemble 30-month-old wild type mice [1–3]. In addition, both *Sod1*KO mice and old wild type mice exhibit profound alterations in neuromuscular innervation in conjunction with the initiation of skeletal muscle atrophy [2]. Post-synaptic endplates are severely disrupted, the number of acetylcholine receptors (AChRs) is reduced and the receptors are significantly fragmented. Finally, mitochondrial function is reduced and

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mitochondrial ROS generation is elevated in both adult *Sod1*KO mice and old wild type mice. Overall, our findings in the *Sod1*KO mice point to the significance of neuromuscular interaction in maintenance of mitochondrial function and muscle mass and suggest that disruption of the neuromuscular junction (NMJ) might be a trigger for decline in muscle mass and function during aging.

Because muscle and motor neurons are integrally related by structure and function, the question has always arisen as to the relative roles of these two tissues in the mechanism responsible for muscle atrophy. Similar questions have been raised in the field of ALS and other neuromuscular diseases in which muscle atrophy and weakness are a predominant phenotype. The observation that *Sod1*KO mice replicate the sarcopenia phenotype observed in old wild type mice provides us with the ability to use genetic approaches to dissect out the role of the motor neuron and muscle in sarcopenia and to begin identifying key pathways in these tissues that are critical in the loss of muscle mass and function. Our first study showed that replacing CuZnSOD specifically in the motor neurons in *Sod1*KO mice (n*Sod1*-Tg/*Sod1*KO mice) prevented muscle atrophy and weakness, as well as the NMJ degeneration associated with loss of mass and function. These data suggested that motor neuron deficits resulting from oxidized redox status are a key initiating event in sarcopenia in the *Sod1*KO mice [4]. In contrast, deleting *Sod1* specifically in skeletal muscle (m*Sod1*KO mice) had no effect on either muscle atrophy or NMJ degeneration [5], demonstrating that loss of *Sod1* in muscle alone is not sufficient to generate atrophy. Based on our experiments with the *n*Sod1-Tg/*Sod1*KO and m*Sod1*KO mice, we speculated that the loss of *Sod1* in the motor neuron was the critical feature contributing to the sarcopenia observed in the whole body *Sod1*KO mice. Thus, we hypothesized that neuronal specific *Sod1*KO (n*Sod1*KO) mice would recapitulate the phenotype of the *Sod1*KO mice. To test this hypothesis, we generated a neuronal specific knockout mice using our *Sod1*-floxed mice crossed to transgenic mice expressing Cre recombinase driven by the nestin promoter (nestin-Cre transgenic mice) and compared n*Sod1*KO and wild type mice for muscle atrophy and weakness, as well as a number of parameters that we had previously measured in the *Sod1*KO mice, including muscle properties such as fiber diameter and myonuclear domain, oxidative stress and markers of altered redox, changes in acetyl choline receptor morphology and markers of denervation. Contrary to our hypothesis, our results show that loss of neuronal CuZnSOD was not sufficient to induce muscle atrophy and weakness that is observed in the *Sod1*KO mice.

Materials and methods

Generation of neuron-specific Sod1-knockout (nSod1KO) mice

Details of the generation of the *Sod1*$^{flox/flox}$ mice was reported in our earlier publication [5]. To generate n*Sod1*KO mice, *Sod1*$^{flox/flox}$ mice were bred with a mouse strain that expresses Cre recombinase under the control of the nestin promoter [(B6.Cg (SJL)-Tg(Nes-cre)1Kln/J)] that we obtained from Jackson laboratory (Bar Harbor, ME, USA). Mice were maintained on a 12-h dark/light cycle and provided with food and water ad libitum. At sacrifice, mice were euthanized by CO_2 inhalation and tissues were immediately excised and weighed. All the tissues, except those used for immunostaining, were snap frozen and stored at −80 °C. All animal protocols were consistent with The Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at Oklahoma Medical Research Foundation (OKC, OK, USA).

Measurement of CuZnSOD and MnSOD activity

CuZnSOD and MnSOD activity in brain, spinal cord and muscles were determined using native gels as described [7].

CuZnSOD immunoblot analysis

Equal amounts of protein from brain, spinal cord, gastrocnemius and quadriceps muscle were resolved by SDS-PAGE and transferred to PVDF membrane. The membranes were blocked and probed with CuZnSOD (Enzo Life Sciences, Inc., Farmingdale, NY, USA) and GAPDH (Sigma, St. Louis, MO, USA) antibodies overnight at 4 °C. Membranes were washed extensively and incubated with secondary antibodies linked to horseradish peroxidase (Santa Cruz Biotechnology, Dallas, TX, USA). Proteins were visualized using enhanced chemiluminescence reagent and signal intensities were quantified using ImageJ 1.45b software (developed by Wayne Rasband, National Institute of Health, Bethesda, MD).

Western blotting of RONS proteins in skeletal muscle

Muscles were ground in a motor and pestle under liquid nitrogen and frozen muscle powder was placed into RIPA buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, and protease inhibitors. Samples were homogenized on ice and centrifuged at 10,000*g* for 10 min at 4 °C. Protein content of samples was determined using the bicinchoninic acid method (Sigma-Aldrich, Poole, UK). For assessment of specific proteins in muscle, 20 μg of total protein was applied to a 4–20% mini-PROTEAN TGX precast gel with a 4% stacking gel (Biorad Laboratories Ltd., Hemel Hempstead, UK). The separated proteins were transferred onto nitrocellulose membranes by western blotting. Membranes were probed using antibodies against MnSOD (SOD2), (Stressgen Inc., UK), eNOS, iNOS, PRXV, and GAPDH (Abcam, Cambridge, UK). Horseradish peroxidase conjugated anti-rabbit IgG or anti-mouse IgG (Cell Signalling, Hitchin, UK) was used as secondary antibody. Peroxidase activity was detected using an ECL Plus substrate (Amersham International Cardiff, UK), and band intensities were analyzed using Quantity One Software (Biorad Laboratories Ltd., Hemel Hempstead, UK). The specificity of the bands was identified in comparison with a sample that had not been exposed to the primary antibody and the molecular weight was determined by using molecular weight markers. All protein contents were normalized to the GAPDH content of the same sample.

Analysis of the 3-nitrotyrosine (3-NT) content of muscle proteins

Total cellular protein was isolated and 20 μg was separated by SDS-PAGE followed by western blotting as describe above. The separated proteins were transferred onto polyvinylidene difluoride (PVDF) membranes. The content of 3-NT was analyzed by using a rabbit monoclonal antibody (Cell Biolabs, San Diego, USA), as per the manufacturer's instructions. Bands were visualized and densitometric quantification was undertaken using Quantity One Software (Biorad Laboratories., Hemel Hempstead, UK).

Analysis of F_2-isoprostanes

Levels of F_2-isoprostanes in quadriceps muscle was measured as described [6,8]. Briefly, 200 mg of tissue was homogenized in 10 ml of ice-cold Folch solution ($CHCl_3$:MeOH, 2:1) containing butylated hydroxytoluene (BHT). The mixture was incubated at room temperature for 30 min. 2 ml of 0.9% NaCl was added and mixed well. The homogenate was centrifuged at 3000*g* for 5 min at 4 °C. The aqueous layer was discarded while the organic layer was secured and evaporated to dryness under N_2 at 37 °C.