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Original Contribution

Advanced age-related denervation and fiber-type grouping in skeletal muscle of SOD1 knockout mice

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ABSTRACT

In this study skeletal muscles from 1.5- and 10-month-old Cu/Zn superoxide dismutase (SOD1) homozygous knockout (*JLSod1*$^{-/-}$) mice obtained from The Jackson Laboratory (C57Bl6/129SvEv background) were compared with muscles from age- and sex-matched heterozygous (*JLSod1*$^{+/-}$) littermates. The results of this study were compared with previously published data on two different strains of *Sod1*$^{-/-}$ mice: one from Dr. Epstein's laboratory (*ELSod1*$^{-/-}$; C57Bl6 background) and the other from Cephalon, Inc. (*CSod1*$^{-/-}$; 129/CD-1 background). Grouping of succinate dehydrogenase-positive fibers characterized muscles of *Sod1*$^{-/-}$ mice from all three strains. The 10-month-old *Sod1*$^{-/-}$ *C* and *JL* mice displayed pronounced denervation of the gastrocnemius muscle, whereas the *ELSod1*$^{-/-}$ mice displayed a small degree of denervation at this age, but developed accelerated age-related denervation later on. Denervation markers were up-regulated in skeletal muscle of 10-month-old *JLSod1*$^{-/-}$ mice. This study is the first to show that metallothionein mRNA and protein expression was up-regulated in the skeletal muscle of 10-month-old *JLSod1*$^{-/-}$ mice and was mostly localized to the small atrophic muscle fibers. In conclusion, all three strains of *Sod1*$^{-/-}$ mice develop accelerated age-related muscle denervation, but the genetic background has significant influence on the progress of denervation.

One of the hallmarks of the normal aging process of organisms as diverse as flies, worms, rats, mice, and humans is a progressive denervation and atrophy of skeletal muscle fibers [reviewed in 1]. The time of onset and rate of progression of muscle denervation are highly dependent on the species and even the strains used for the studies. In humans, this process could progress faster or slower in different individuals, depending on the genetic makeup and the lifestyle [reviewed in 2,3]. Denervation, atrophy, and loss of muscle performance are significantly attenuated in master athletes, but still, these processes cannot be completely stopped [4,5]. Even regular exercise and calorie restriction, which are known to slow down denervation and atrophy of skeletal muscle in the elderly, do not prevent the aging process [reviewed in 1,6].

Although significant progress has been achieved in aging research in recent years, the process of age-related denervation is complex and its mechanisms are not completely understood. One of the leading theories of aging suggests that oxidative stress and intracellular accumulation of reactive oxygen species leading to oxidative damage of DNA, proteins, and lipids have a critical role in the advancement of aging [7]. Superoxide dismutase (SOD) is one of the critically important antioxidants; it converts superoxide free radicals ($O_2^{\bullet-}$) into hydrogen peroxide and O_2. In mammals, three forms of SOD exist: primarily cytoplasmic Cu/Zn SOD (SOD1), mitochondrial Mn SOD (SOD2), and extracellular SOD (SOD3) [reviewed in 8]. Although SOD2 knockout mice do not survive past day 12 after birth [reviewed in 8], neonatal SOD1 knockout (*Sod1*$^{-/-}$) mice do not show gross abnormalities and these mice live to about 25 months of age, after which the majority of the mice die from liver cancer [9]. The median life span of *Sod1*$^{-/-}$ mice is 23 months [10].

Three independently developed strains of *Sod1*$^{-/-}$ mice generated on different genetic backgrounds have been reported [11–13]. *ELSod1*$^{-/-}$ mice were produced by Dr. Epstein's laboratory on the C57Bl6 background by the deletion of most of exon 3 and all of exon 4 of the *Sod1* gene [12]. Male 10- to 12-month-old *ELSod1*$^{-/-}$ mice display ~30% smaller body and ~40% smaller gastrocnemius (GTN) and anterior tibialis (ATB) muscle mass, smaller muscle cross-sectional areas, and pronounced grouping of succinate dehydrogenase (SDH)-positive fibers compared with WT mice [14]. Female *ELSod1*$^{-/-}$ mice at 3–6 months of age have ~13% smaller total body weight and ~21% smaller muscle mass [10]. At 11 months of age they have ~16% smaller total body weight and ~37% smaller muscle mass. At 19–24 months of age female *ELSod1*$^{-/-}$ mice have only ~50% of muscle mass compared to their WT littermates of the same

Abbreviations: AChR, acetylcholine receptor; ATB, anterior tibialis muscle; COX, cytochrome *c* oxidase; EDL, extensor digitorum longus muscle; GPX, glutathione peroxidase; GTN, gastrocnemius muscle; MHC, myosin heavy chain; MT, metallothionein; MTF-1, metal-induced transcription factor; NCAM, neural cell adhesion molecule; PLN, plantaris muscle; ROS, reactive oxygen species; SDH, succinate dehydrogenase; SOD, superoxide dismutase; Sol, soleus muscle; Txnrd, thioredoxin reductase; WT, wild type.

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age [10]. Muscles of male 10- to 12- month-old *ELSod1*$^{-/-}$ mice have a small amount of atrophic denervated muscle fibers in the GTN, but not ATB, plantaris (PLN), or extensor digitorum longus (EDL) muscles [14]. Despite the lack of extensive muscle fiber denervation in GTN muscles of 8-month-old *ELSod1*$^{-/-}$ mice, they have ~35% lower specific force when stimulated through the nerve [15]. Young *ELSod1*$^{-/-}$ mice also have elevated levels of oxidatively damaged proteins, lipids, and DNA [10]. Later in life (20–25 months of age), *ELSod1*$^{-/-}$ mice develop pronounced muscle denervation, low specific force, and high levels of mitochondrial abnormalities accompanied by high levels of oxidative stress [10,15].

Sod1$^{-/-}$ mice developed at Cephalon, Inc. (*CSod1*$^{-/-}$; produced on a 129/CD-1 background by the deletion of the entire coding sequence of *Sod1* gene [11]) are characterized by peripheral neuropathy and skeletal muscle denervation [16,17]. By 6 months of age, all of the *CSod1*$^{-/-}$ mice showed a small number of denervated muscle fibers that significantly increased by 12 months of age [16]. Despite denervation of some muscle fibers, 6-month-old *CSod1*$^{-/-}$ mice did not show signs of motor neuron deficits when stride length and running wheel performance were analyzed [11]. Fourteen-month-old *CSod1*$^{-/-}$ mice show significant motor deficits in rod running time and stride length in comparison with WT mice [16].

Although both *ELSod1*$^{-/-}$ and *CSod1*$^{-/-}$ mice show muscle fiber-type grouping and develop denervation of skeletal muscles [10,14–16], they have substantial differences in the time of the onset of denervation and the impact of denervation on the structure and function of skeletal muscles. These differences could result from the variations in the genetic backgrounds of the mice used for the generation of *Sod1*$^{-/-}$ mice. In addition, differences in the adaptive changes developed in skeletal muscles in response to the lack of SOD1, including the up-regulation of antioxidants, could be of critical importance.

Structural features and age-related changes in the muscles of *Sod1*$^{-/-}$ mice from The Jackson Laboratory (*JLSod1*$^{-/-}$; produced on the C57Bl6/129S background by Dr. Lebovitz's laboratory by the deletion of exons 1 and 2 of the *Sod1* gene [13]) have not previously been described. The purpose of our study was to investigate whether the accelerated age-related denervation accompanied by the smaller skeletal muscle mass, muscle fiber denervation, and grouping of SDH-positive fibers previously described for *ELSod1*$^{-/-}$ and *CSod1*$^{-/-}$ mice was also a characteristic feature of *JLSod1*$^{-/-}$ mice. *JLSod1*$^{+/-}$ and *JLSod1*$^{-/-}$ male and female mice were compared at 1.5 and ~10 months of age to investigate how age, sex, and lack of SOD1 activity affects the structure of skeletal muscles. We also assessed whether changes in cellular localization and/or the expression levels of several proteins known to be regulated by denervation (myogenin, MyoD, AChRα, AML1, myosin heavy chain isoforms (MHCs), and NCAM), as well as antioxidant proteins (metallothionein (MT), glutathione peroxidase 4 (GPX4), catalase, and thioredoxin reductase 1 (Txnrd1)), could have an effect on the time of onset and progression of the denervation of the muscles of *Sod1*$^{-/-}$ mice.

Materials and methods

Animals

The breeding pairs of *JLSod1*$^{-/-}$ male and *JLSod1*$^{+/-}$ female mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA; Sod1^{tm1Leb}; Stock 002972) and were bred and genotyped at Indiana University. At 1.5 and ~10 (9–12) months of age, the male and female *JLSod1*$^{-/-}$, *JLSod1*$^{+/-}$, and *Sod1*$^{+/+}$ mice, and 22-month-old female *JLSod1*$^{+/-}$ mice, were anesthetized with Avertin, after which the GTN, ATB, PLN, EDL, and soleus (Sol) muscles were dissected from each leg, frozen in isopentane (for sectioning) or in liquid nitrogen (for quantitative reverse transcription and PCR (QRT-PCR) analysis), and stored at −80 °C. No significant differences were detected in the total body weight and muscle mass of sex-matched *JLSod1*$^{+/-}$ and *JLSod1*$^{+/+}$ mice; therefore the data for both groups were combined and the combined group is referred to here as *JLSod1*$^{+/-}$ mice. There was no significant age difference between the four groups of 1.5-month-old mice (male and female *JLSod1*$^{-/-}$ and male and female *JLSod1*$^{+/-}$) or between the four groups of ~10-month-old mice that were analyzed in this study.

To obtain 15-day denervated muscles for this study at 10 months of age, the right legs of *JLSod1*$^{+/-}$ mice were denervated by a high sciatic nerve section in the hip region of the hind limb. After Avertin anesthesia, the right sciatic nerve was ligated tightly with silk in two places and the nerve was cut between the sutures. Proximal and distal nerve stumps were implanted into muscular tissue as far away from each other as possible. This procedure results in a permanent denervation of the lower hind leg. At 15 days postdenervation the mice were anesthetized with Avertin, after which the muscles were dissected from each leg and preserved for QRT-PCR analysis as described above.

After the removal of the muscles, the mice were euthanized with an overdose of Avertin, and the thorax was opened to ensure the immediate death of the mouse. All animal care and animal surgeries were performed in accordance with the *Guide for Care and Use of Laboratory Animals* (Public Health Service, 1996, NIH Publication No. 85-23); the experimental protocol was approved by the Indiana University Committee for the Use and Care of Animals.

Histochemical and immunohistochemical analyses

For the histochemical analysis, unfixed samples were placed into a TBS medium (Triangle Biological Sciences, Durham, NC, USA), frozen in cold isopentane, and stored at −80 °C until needed. Samples were sliced with a cryostat at a thickness of approximately 12 μm, adhered to Superfrost Plus microscopy slides, and used for staining. The enzymatic activities of cytochrome *c* oxidase (COX) and SDH were visualized in muscle sections as described previously [18]. The activity of SDH was used to assay the proportion of oxidative fibers containing a large number of mitochondria and the activity of COX was used to assess the functional activity of the mitochondria. High SDH activity, but low COX activity, in the fibers suggests damage to the mitochondrial electron transport system.

For immunostaining, frozen sections were fixed with ice-cold methanol for 10 min and rinsed three times with phosphate-buffered saline (PBS). For myosin immunostaining methanol fixation was omitted and the sections were air dried and boiled in PBS for 5 min as previously described [19]. Sections were blocked for 30 min with PBS–0.05% Tween 20 (PBST) containing 20% calf serum (PBST-S) at room temperature. Sections were incubated overnight at 4 °C with the primary antibodies in PBST-S. The following primary antibodies were used: rabbit anti-metallothionein obtained from LifeSpan Biosciences (Seattle, WA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA); mouse anti-slow MHC (clone A4.84), mouse anti-fast 2b MHC (clone 10F5c), and mouse anti-fast 2a MHC (clone A4.74) obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA, USA); rabbit anti-NCAM (Chemicon International, Temecula, CA, USA); rabbit anti-AML1 (Abcam, Cambridge, MA, USA); mouse anti-myogenin (clone F5D; Developmental Studies Hybridoma Bank); and rabbit anti-MyoD (Santa Cruz Biotechnology). A 1-h room temperature incubation with Cy2- or Cy3-conjugated anti-mouse or anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA, USA) was used for visualization. Sections incubated only with Cy3-conjugated anti-mouse or anti-rabbit antibody were used as negative controls. Costaining of sections with fluorescein-conjugated wheat germ agglutinin (green, WGA–fluorescein, 1 μg/ml; Molecular Probes, Eugene, OR, USA) or biotinylated WGA/AMCA streptavidin (blue,