Skeletal muscle weakness due to deficiency of CuZn-superoxide dismutase is associated with loss of functional innervation

Lisa M. Larkin,1,2 Carol S. Davis,2 Catrina Sims-Robinson,3 Tatiana Y. Kostrominova,4 Holly Van Remmen,5 Arlan Richardson,5 Eva L. Feldman,3 and Susan V. Brooks1,2

Departments of 1Biomedical Engineering, 2Molecular and Integrative Physiology, and 3Neurology, University of Michigan, Ann Arbor, Michigan; 4Indiana University School of Medicine Northwest, Gary, Indiana; 5University of Texas Health Science Center San Antonio Barshop Institute, San Antonio, Texas

Submitted 22 February 2011; accepted in final form 31 August 2011

Larkin LM, Davis CS, Sims-Robinson C, Kostrominova TY, Van Remmen H, Richardson A, Feldman EL, Brooks SV. Skeletal muscle weakness due to deficiency of CuZn-superoxide dismutase is associated with loss of functional innervation. Am J Physiol Regul Integr Comp Physiol 301: R1400–R1407, 2011. First published September 7, 2011; doi:10.1152/ajpregu.00093.2011.—An association between oxidative stress and muscle atrophy and weakness in vivo is supported by elevated oxidative damage and accelerated loss of muscle mass and force with aging in CuZn-superoxide dismutase-deficient (Sod1−/−) mice. The purpose was to determine the basis for low specific force (N/cm²) of gastrocnemius muscles in Sod1−/− mice and establish the extent to which structural and functional changes in muscles of Sod1−/− mice resemble those associated with normal aging. We tested the hypothesis that muscle weakness in Sod1−/− mice is due to functionally denervated fibers by comparing forces during nerve and direct muscle stimulation. No differences were observed for wild-type mice at any age in the forces generated in response to nerve and muscle stimulation. Nerve- and muscle-stimulated forces were also not different for 4-wk-old Sod1−/− mice, whereas, for 8- and 20-mo-old mice, forces during muscle stimulation were 16 and 30% greater, respectively, than those obtained using nerve stimulation. In addition to functional evidence of denervation with aging, fiber number was not different for Sod1−/− mice at 4 wk, but 50% lower for Sod1−/− mice by 20 mo, and denervated motor end plates were prevalent in Sod1−/− mice at both 8 and 20 mo and in WT mice by 28 mo. The data suggest ongoing denervation in muscles of Sod1−/− mice that results in fiber loss and muscle atrophy. Moreover, the findings support using Sod1−/− mice to explore mechanistic links between oxidative stress and the progression of deficits in muscle structure and function.

specific force; denervation; Sod1

Address for reprint requests and other correspondence: S. V. Brooks, Associate Professor, Molecular and Integrative Physiology, Univ. of Michigan, Biomedical Science Research Bldg. (BSRB), 109 Zina Pitcher Pl., Rm. #2029, Ann Arbor, MI 48109-2200 (e-mail: svbrooks@umich.edu).

REACTIVE OXYGEN SPECIES (ROS) are by-products of normal cellular aerobic metabolism that can result in the oxidation of lipids, proteins, and nucleic acids. When ROS production exceeds antioxidant defenses, or when antioxidant mechanisms are impaired, the resulting state of oxidative stress can result in damage to cellular constituents, loss of cellular function, and cell death. ROS and oxidative stress have been implicated in the widely recognized wasting and weakness of skeletal muscle that develops with aging, commonly referred to as sarcopenia (15). Although sarcopenia undoubtedly results from complex interactions between both intrinsic and extrinsic factors, strong correlations have been reported between oxidative stress and the progression of muscle atrophy during aging (32, 34). Specifically, a mechanistic link between chronic oxidative stress in vivo and a loss of muscle mass and force is supported by studies of mice deficient in CuZn-superoxide dismutase (SOD) (Sod1−/−), a major antioxidant enzyme. Skeletal muscles from young Sod1−/− mice display elevated oxidative damage to proteins, lipids, and DNA compared with those of age-matched wild-type (WT) mice and muscle masses are significantly lower than those of WT mice as early as 6 mo of age (34). Muscle mass in the Sod1−/− mice is further reduced with age, and, by 20 mo, hindlimb muscle mass in Sod1−/− mice is nearly 50% lower than that in age-matched WT mice (34).

A major factor underlying the loss of muscle with aging in humans (7, 13) and rats (9, 21, 22, 26, 40) is the loss of entire motor units. In addition, for muscles of old rats, the maximum force of fast motor units is 70% that of comparable units in adult rats, whereas the force developed by slow motor units was nearly three times the adult value (21). These data suggest that motor unit remodeling with aging occurs by selective denervation of fast type 2 fibers with some reinnervation by collateral sprouting of nerves from fibers in slow motor units (4). The occurrence of a similar process in humans is supported by the grouping of slow type 1 fibers commonly reported in the muscles of the elderly (17, 19, 28). Aging results in changes at neuromuscular junctions (NMJ) (25, 42), including a decrease in muscles of old compared with adult animals in the number of nerve terminals (1), an overall decrease in the size of the motor end plates (33), fewer motor axon contacts per end plate (33), and decreases in the number, length, and density of postsynaptic folds (35). Thus the degeneration of nerve terminals at the motor end plate may constitute an ongoing process throughout life, with highly successful reinnervation in young animals by collateral sprouting (2, 4, 38), but impaired reinnervation in old animals (25, 33, 38). Similarly, degeneration of NMJ and the resultant denervation of muscle fibers have been proposed as the mechanism underlying the accelerated loss of muscle mass in Sod1−/− mice (18).

In addition to the skeletal muscle atrophy that develops with aging, force production is diminished in excess of what can be explained by the loss of muscle mass (3). Gastrocnemius (GTN) muscles of Sod1−/− mice also demonstrate decreased specific force normalized by muscle fiber cross-sectional area (CSA) (N/cm²) compared with muscles of WT mice (18). The mechanism underlying the decreased specific force by muscles of Sod1−/− mice is not known. The purpose of this study was to determine the basis for the weakness of GTN muscles of Sod1−/− mice and to establish the extent to which the struc-
tural and morphological changes that occur in muscles of $Sod1^{-/-}$ mice resemble those associated with the ongoing denervation process associated with normal aging. Based on the observations that muscles of $Sod1^{-/-}$ mice contain a high percentage of fragmented NMJs and only a tiny percentage of postsynaptic acetylcholine receptors showing normal colocalization with presynaptic motoneuron branches (18), we hypothesized that the weakness of whole muscles in $Sod1^{-/-}$ mice was due to the presence of a large population of functionally denervated fibers. The specific hypothesis was tested that the forces generated when muscles are activated by stimulation of the nerve or by direct stimulation of the muscle would not be different for WT mice, whereas muscles of $Sod1^{-/-}$ mice that demonstrate low specific forces would generate greater forces during direct muscle stimulation compared with nerve stimulation. We also performed histological analyses of GTN muscles of $Sod1^{-/-}$ and WT mice for evidence of denervated fibers in the $Sod1^{-/-}$ mice.

**MATERIALS AND METHODS**

**Animals.** A total of 64 mice were analyzed between 1 and 22 mo of age for $Sod1^{-/-}$ mice and between 1 and 28 mo for WT mice. Sample sizes were between $N = 6$ and $N = 14$ for individual groups. The mice were obtained from the University of Texas Health Science Center at San Antonio (UTHSCSA) and have been previously described (18). In San Antonio, the mice were maintained under specific pathogen-free conditions in the heterozygous ($Sod1^{-/-}$) state and backcrossed with C57BL/6J females (Jackson Laboratory, Bar Harbor, ME) for more than 20 generations. In the colony at UTHSCSA, the median lifespans of $Sod1^{-/-}$ and WT mice are ~23 mo and ~31 mo, respectively. For the present study, $Sod1^{-/-}$ mice were acquired at ~6 or ~18 mo of age and maintained under specific pathogen-free conditions in the Unit for Laboratory Animal Medicine at the University of Michigan (UM) until they were tested at 8–10 or 20–22 mo of age. Similarly, WT mice were acquired at ~6, ~18, or ~26 mo and tested at 8–10, 20–22, or 28 mo, respectively. In addition, mice were generated in a UM facility from heterozygous breeders obtained from UTHSCSA, so that mice could be tested shortly after weaning without the risks of shipping. All procedures were approved by the University Committee on the Use and Care of Animals at UM and were in accordance with the Guide for Care and Use of Laboratory Animals (Public Health Service, 19965, National Institutes of Health Publication No. 85-23).

**Contractile measurements.** In all cases, mice were anesthetized with initial intraperitoneal injections of avertin (tribromoethanol, 250 mg/kg) with supplemental injections given to maintain an adequate level of anesthesia during all procedures. Isometric contractile properties for GTN muscles were measured in situ. In anesthetized mice, the whole GTN muscle was isolated from surrounding muscle and connective tissue using great care not to damage the nerve and/or blood vessels during the dissection. A 4–0 silk suture was tied around the distal tendon, and the tendon was severed. The animal was then placed on a temperature-controlled platform warmed to maintain body temperature at 37°C. The hindlimb was securely tied to a fixed post with 4–0 monofilament nylon suture at the knee, and the foot was clamped to the platform. The distal tendon of the GTN muscle was then tied to the lever arm of a servomotor (model 305B; Aurora Scientific). A continual drip of saline warmed to 37°C was administered to the GTN muscle to maintain its temperature. The muscle was initially activated by stimulation of the tibial nerve using a bipolar platinum wire electrode. The voltage of single 0.2-ms stimulation pulses was adjusted to give a maximum isometric twitch. Subsequently, muscle length was adjusted to the optimal length ($L_o$) at which twitch force was maximal. With the muscle held at $L_o$, 300-ms trains of stimulus pulses were applied at increasing stimulation frequency until the maximum isometric tetanic force ($P_t$) was achieved. Subsequently, the same procedure was repeated, but rather than activating the muscle via the tibial nerve, a cuff electrode was placed around the proximal and distal ends of the muscle for stimulation.

After all force measurements, muscles were removed, and deeply anesthetized mice were killed by administration of a pneumothorax. GTN muscles were trimmed of their tendons, blotted, and weighed. Muscle fiber length was calculated by multiplying $L_o$ by 0.45 (5). Total fiber CSA was calculated by dividing the muscle mass (mg) by the product of muscle fiber length (mm) and the density of mammalian skeletal muscle, 1.06 g/cm². Specific $P_t$ (N/cm²) was calculated by dividing $P_t$ by total fiber CSA for each muscle. Immediately after muscle mass was measured, muscles were coated in tissue freezing medium (Triangle Biomedical Sciences, Durham, NC), frozen in isopentane cooled by dry ice, and stored at −80°C until needed.

**Muscle histological analysis.** Samples were sectioned at −20°C through the mid belly with a thickness of 12 µm, and fluorescent immunohistochemical staining was initiated the same day. For analysis of fiber number and fiber CSAs, fibers were identified by staining for myosin heavy chain and laminin to visualize fiber outlines. Frozen sections were rinsed with phosphate-buffered saline (PBS) to remove the freezing medium and then permeabilized in 0.2% Triton X-100 in PBS for 5 min. Sections were incubated overnight in blocking solution (Vector M.O.M. Mouse IgG Blocking Reagent) in a sealed chamber at 4°C and then soaked in M.O.M. diluent (Vector) for 5 min. A second overnight incubation of the muscle sections was performed in the sealed chamber at 4°C with primary antibodies for type 1 myosin (mouse IgM; Alexis A4.840), type 2 myosin (mouse IgG; Thermo MS-1236-S), and laminin (rabbit IgG; Sigma L9393) in M.O.M. diluent. Fluorescent labeling was completed by incubating sections in 1:1:1:300 solutions of secondary antibodies in M.O.M. diluent (Vector) for 30 min in the dark at room temperature. Secondary Alexa Fluor antibodies, all from Invitrogen, were goat anti-mouse IgM (A-21426), goat anti-mouse IgG (A-21120), and goat anti-rabbit IgG (A-11008). Sections were mounted with Prolong Gold Mounting Medium without 4',6-diamidino-2-phenylindole and imaged within 24 h on an Olympus BX-51 microscope. Adobe Photoshop CS4 was used to merge microscope images, and entire cross sections were then analyzed by an observer blinded to the identity of the sample.

**Immunofluorescence staining.** With a neural cell adhesion molecule (NCAM)-specific antibody was performed to detect the presence of nerves and denervated skeletal muscle fibers. Although this antibody produces faint background staining in the connective tissue surrounding innervated muscle fibers, it specifically recognizes and brightly stains nerves and plasma membranes of denervated skeletal muscle fibers. Frozen sections were fixed with ice-cold methanol for 10 min and rinsed three times with PBS. Sections were blocked for 30 min at room temperature with PBS-0.05% Tween 20 (PBST) containing 20% calf serum (PBST-S) and then incubated overnight at 4°C with rabbit anti-NCAM (Chemicon International, Temecula, CA) primary antibody diluted in PBST-S. Following three washes in PBST, a 1-h room temperature incubation with Cy3-conjugated anti-rabbit antibody (Jackson Immunoresearch Laboratory) diluted in PBST-S was used for visualization of NCAM. After three washes in PBST, the sections were mounted with Prolong Gold Mounting Medium without 4',6-diamidino-2-phenylindole and examined and photographed. After NCAM fluorescent images were acquired, coverslips were removed, and mounting medium was washed from the sections by incubation with five changes of PBST for 5 min each. Sections were blocked for 30 min with PBST-S at room temperature and incubated overnight at 4°C with rabbit anti-laminin (Chemicon International, Temecula, CA) primary antibody diluted in PBST-S. Following three washes in PBST, sections were incubated at room temperature for 1 h with Cy2-conjugated anti-rabbit antibody (Jackson Immunoresearch Laboratory) in PBST-S for visualization of laminin. Following a final
three washes in PBST, the sections were mounted with Prolong Gold
Mounting Medium, and the same areas of the sections that were
previously examined for NCAM were photographed following
laminin immunostaining.

NMJ immunohistochemistry. Muscles were processed for acetyl-
cholinesterase immunohistochemistry, as previously described (39).

Muscles were sectioned longitudinally, with a thickness of 14 μm
and mounted on SuperFrost glass slides. Sections were heated on a slide
warmer for 10 min (55°C) and hydrated in distilled water (dH2O) for
5 min. After incubating in 20% sodium sulfate for 3 min, the sections
were rinsed in dH2O, and, subsequently, a solution containing the
esterase substrate 5-bromoindoxyl acetate, which stains motor end
plates blue, was applied for 60 min at 37°C. The composition of this
acetylcholinesterase solution was 4 mg 5-bromoindoxyl acetate, 0.3
ml ethanol, 63 mg potassium ferrocyanide, 50 mg potassium ferricya-

Fig. 1. Body mass (A) and gastrocnemius (GTN) muscle mass (B and C) are
shown for wild-type (WT) and CuZnSOD-deficient (Sod1−/−) mice of varying
ages. Body masses are expressed in g, and muscle masses are expressed both
in mg (B) and as a percentage of body mass (C). Values are means ± 1 SE.
Sample sizes are N = 8, 14, 11, and 8 for WT mice at 1, 8, 20, and 28 mo,
respectively, and N = 6, 10, and 7 for Sod1−/− mice at 1, 8, and 20 mo,
respectively. Significant difference from *WT value at the same age, **8-mo-
old mice of the same genotype: P < 0.05.

Fig. 2. Maximum isometric specific force (specific P0) values are shown for
GTN muscles of WT and CuZnSOD-deficient (Sod1−/−) mice developed when
the muscles were activated using nerve stimulation (A) or direct muscle
stimulation (B). Specific P0 is expressed in N/cm². C: difference between the
forces measured using these two methods of stimulation. Values are expressed
as the percent increase in force developed with direct muscle stimulation
compared with the force generated in response to nerve stimulation. Values are
means ± 1 SE. Significant difference from *WT value at the same age,
**8-mo-old mice of the same genotype, and ***20-mo-old mice of the same
genotype: P < 0.05.
nide, and 30 ml 1 × Trizma hydrochloride solution (Sigma Aldrich, St. Louis, MO). After rinsing in dH2O followed by PBS (0.1 M, pH 7.2) for 5 min, endogenous peroxidase activity was blocked with 0.5% hydrogen peroxide for 10 min and rinsed in PBS. Nonspecific adherence of antisera was reduced by rinsing with PBS containing 0.1% Triton X-100, 1% normal goat serum, and 2% nonfat dry milk for 10 min. To detect nerve fibers, an antibody for neurofilament (Millipore, Billerica, MA), a major component of the axonal cytoskeleton, diluted in PBS containing 0.1% Triton X-100, was applied overnight in a humidified chamber at 22°C. Sections were incubated with goat anti-rabbit (Vectastain Kit, Vector Laboratories, Burlingame, CA) diluted in PBS containing 0.1% Triton X-100 for 1 h at 22°C. Following a PBS rinse, Vectastain ABC reagent (Vector Laboratories) containing avidin DH and biotinylated enzyme, was applied for 30 min. The chromogen was developed in 3,3′-diaminobenzidine solution (Vector Laboratories) for 30 s. The sections were rinsed in PBS, dehydrated with a series of ethanol and xylene rinses, and cover-slipped with di-n-butyl-phthalate in xylene (DPX, Electron Microscopy Sciences, Hatfield, PA). Images were captured using a Spot-RT camera (Diagnostic Instruments, Sterling Heights, MI) attached to a Nikon Microphot-FXA microscope at 1000X.

To detect nerve fibers, an antibody for neurofilament (Millipore, Billerica, MA), a major component of the axonal cytoskeleton, diluted in PBS containing 0.1% Triton X-100, was applied overnight in a humidified chamber at 22°C. Sections were incubated with goat anti-rabbit (Vectastain Kit, Vector Laboratories, Burlingame, CA) diluted in PBS containing 0.1% Triton X-100 for 1 h at 22°C. Following a PBS rinse, Vectastain ABC reagent (Vector Laboratories) containing avidin DH and biotinylated enzyme, was applied for 30 min. The chromogen was developed in 3,3′-diaminobenzidine solution (Vector Laboratories) for 30 s. The sections were rinsed in PBS, dehydrated with a series of ethanol and xylene rinses, and cover-slipped with di-n-butyl-phthalate in xylene (DPX, Electron Microscopy Sciences, Hatfield, PA). Images were captured using a Spot-RT camera (Diagnostic Instruments, Sterling Heights, MI) attached to a Nikon Microphot-FXA microscope at 1000X.

RESULTS

For young mice (1 mo), no differences between Sod1−/− and WT mice were observed for body mass (Fig. 1A) or GTN muscle mass, expressed either in absolute terms (Fig. 1B) or relative to body mass (Fig. 1C). For older mice, consistent with previous studies (18), body masses were ~20% lower for Sod1−/− than WT mice (Fig. 1A). The lower body masses were observed for both 8- and 20-mo-old mice, with no effect of age for either group. Measurements of muscle mass showed substantial atrophy by 8 mo of age for GTN muscles of Sod1−/− mice that worsens by 20 mo (Fig. 1B). At 8 mo, GTN muscle masses were 50% lower for Sod1−/− compared with WT mice and decreased over 20% between 8 and 20 mo. The decrease in mass observed between 8 and 20 mo for GTN muscles of Sod1−/− mice was similar to the loss in mass for muscles of WT mice by 28 mo of age (Fig. 1B). When muscle mass was normalized by body mass, age-associated atrophy was observed for GTN muscles of both Sod1−/− and WT mice. Despite the significantly smaller body masses for the Sod1−/−
mice, muscle mass normalized for body mass still showed a 25% decrease between 8 and 20 mo, which compared with a smaller, but statistically significant, 13% decrease for the WT mice between 8 and 28 mo (Fig. 1C).

Consistent with the similarity in muscle sizes for young Sod1−−/− and WT mice, force-generating capacity was also not different between the two genotypes, either when activated by stimulation through the nerve (Fig. 2A) or by directly stimulating the muscle (Fig. 2B). In contrast, by 8 mo of age, the muscles of Sod1−−/− mice generated lower forces than those of WT mice. The lower forces were not simply a consequence of the smaller muscle masses, as evidenced by the observation that, when forces were normalized by muscle CSA, specific P0 was lower for muscles of Sod1−−/− mice than for those of WT mice. When stimulated through the nerve, specific P0 for muscles of Sod1−−/− mice was ~45% lower than the value for WT mice at both 8 and 20 mo of age (Fig. 2A). For Sod1−−/− and WT mice, GTN muscles demonstrated progressive muscle weakness with increasing age. Specific P0 decreased by 35% between 1 and 20 mo for muscles of Sod1−−/− mice and by 40% between 8 and 28 mo for WT mice (Fig. 2A), although specific P0 may peak for Sod1−−/− mice between 1 and 8 mo, in which case the magnitude of the decline in force with aging is underestimated by comparing the values at 1 mo and 20 mo of age. Consistent with our hypothesis, activating GTN muscles of WT mice by direct stimulation resulted in no change in force generation compared with the force elicited using nerve stimulation. Similarly, for 1-mo-old Sod1−−/− mice, the response of the muscles to nerve and direct muscle stimulation was not different, whereas for 8- and 20-mo-old Sod1−−/− mice, direct muscle stimulation increased force above values obtained using nerve stimulation by 16 and 31%, respectively (Fig. 2C).

The lower GTN muscle masses in Sod1−−/− compared with WT mice were explained largely by a decrease in the number of muscle fibers. Total fiber counts from whole muscle cross sections (Fig. 3A) indicated ~5,000 fibers in GTN muscles of both Sod1−−/− and WT mice at 1 mo of age (Fig. 3B). For WT mice, fiber number did not change between 1 and 20 mo, whereas dramatic fiber loss of nearly 45% was observed for Sod1−−/− mice as GTN muscle mass decreased, such that fiber number was significantly lower in muscles of Sod1−−/− compared with WT mice at both 8 and 20 of age (Fig. 3B). Accompanying the 13% decrease in GTN muscle mass by 28 mo for WT mice was an 11% decrease in fiber number compared with the pooled average for the younger mice, although the decrease did not reach statistical significance. Average fiber CSAs were not different between genotypes at any age. Despite the similarity in mean CSAs for fibers from muscles of Sod1−−/− and WT mice, muscles of Sod1−−/− mice showed increased numbers of small atrophic fibers. This increase in the number of atrophic fibers was especially evident in the 20-mo group, where histograms illustrating the distributions of muscle fiber CSAs showed that nearly 40% of the fibers in GTN muscles of Sod1−−/− mice had CSAs <1,500 μm² compared with only 20% of the fibers in WT mice within this size range (Fig. 3D).

To investigate whether the small atrophic fibers observed in muscles of Sod1−−/− mice were denervated, cross sections were stained for laminin and NCAM. Immunofluorescent images are shown in Fig. 4 and demonstrate that the atrophic fibers in the muscles of Sod1−−/− mice, identified by the laminin boundaries, are largely positive for NCAM. Using NMJ immunohistochemistry, we quantified the number of motor end plates that showed colocalization of neurofilament with acetylcholinest-
erase staining as an indication of innervation of the end plate (Fig. 5). There was no difference in the number of innervated end plates for muscles of WT mice at 8 and 20 mo of age (Fig. 5B). In contrast, compared with the value obtained for muscles of 8-mo-old WT mice, there were 25–50% fewer innervated end plates in the muscles of Sod1<sup>−/−</sup> mice and even fewer in muscles of 28-mo-old WT mice (Fig. 5B).

**DISCUSSION**

The major finding of the present study is that a significant number of fibers in GTN muscles of Sod1<sup>−/−</sup> mice do not contribute to force generation during nerve stimulation. The observation that direct stimulation of the muscles of Sod1<sup>−/−</sup> mice consistently increased force production over that generated using nerve stimulation suggests that a population of fibers is present in these muscles that are capable of producing force, but do not maintain a robust functional connection to the nerve. Our finding of a population of functionally denervated fibers in muscles of Sod1<sup>−/−</sup> mice is consistent with previous reports of morphological, as well as gene expression changes indicative of muscle-fiber denervation in CuZnSOD-deficient mice (18, 23). Our conclusion that muscle fibers in Sod1<sup>−/−</sup> mice undergo progressive denervation is supported by histological findings, including the presence of a population of small atrophic fibers positive for NCAM. NCAM, also known as the cluster of differentiation 56, is a glycoprotein normally expressed on the surface of neurons and skeletal muscle fibers. In innervated muscle fibers, NCAM expression is restricted to the NMJ, but, following denervation and during reinnervation, NCAM is abundantly expressed in nonsynaptic regions of the fibers (11). NCAM immunohistochemistry identified a population of myofibers in muscles of 8- and 20-mo-old Sod1<sup>−/−</sup> mice that would not be expected to respond to neural stimulation (8). Our observation of a lack of colocalization between neurofilament and acetylcholinesterase in substantial numbers of fibers in muscles of 8- and 20-mo-old Sod1<sup>−/−</sup> mice, as well as 28-mo-old WT mice, is also consistent with the presence of denervated fibers and with previous reports of large numbers of denervated motor end plates in CuZnSOD-deficient mice (18). Over three-fourths of NMJ sampled by Jang and colleagues (18) were categorized as denervated using fluorescently tagged α-bungarotoxin to visualize postsynaptic acetylcholine receptors. Although the present study identified fewer denervated end plates than Jang et al. previously reported, both studies showed dramatic effects of CuZnSOD deficiency on NMJ morphology.

During normal aging in human beings and rats, denervation of muscle fibers with limited reinnervation by collateral sprouting from neighboring motoneurons (21, 27) results in fiber loss and the accumulation of clusters of fibers of the same fiber type situated adjacent to one another (27, 29). Consistent with a similar process in the Sod1<sup>−/−</sup> mice, substantially fewer fibers were present in GTN muscles of Sod1<sup>−/−</sup> compared with WT mice in the present study by 8 mo of age, whereas fiber numbers were similar in Sod1<sup>−/−</sup> and WT mice early in life. In addition, Kostrominova (23) reported type 2a fiber grouping in muscles of Sod1<sup>−/−</sup> mice, suggesting that the denervation/reinnervation process in Sod1<sup>−/−</sup> mice may not be entirely distinct from the motor unit remodeling that occurs with normal aging in humans. While a loss of muscle fibers is the primary contributor to age-associated muscle atrophy (30), decreased CSA is also observed for the individual fibers that remain (14, 29). Similarly, an increase was observed in the present study in the number of small atrophic fibers in GTN muscles of Sod1<sup>−/−</sup> mice by 20 mo. Although denervation, fiber loss, and atrophy in Sod1<sup>−/−</sup> mice represent phenomena associated with normal aging, the state of partial innervation that exists for some muscle fibers in Sod1<sup>−/−</sup> mice, in which NMJ do not transmit action potentials but provide sufficient neural support to maintain the fiber’s ability to generate force when directly activated, appears to be unique to the high oxidative stress environment of the CuZnSOD-deficient mice. In WT mice, muscles exhibit no difference in their response to nerve and muscle stimulation throughout the lifespan, despite the presence of denervated end plates in muscles of 28-mo-old WT mice.

Although the present findings indicate that the lower specific forces generated with nerve stimulation by GTN muscles of Sod1<sup>−/−</sup> compared with WT mice is due, in part, to the presence of a population of fibers that were not activated, using direct stimulation to activate these functionally denervated muscle fibers did not completely eliminate the deficit. The failure of direct stimulation to abolish the weakness of GTN

---

**Fig. 5.** A: representative section is shown for GTN muscles assayed for acetylcholinesterase activity (blue) to visualize the motor end plate and costained with an antibody for neurofilament (brown) to visualize the motoneuron branches. Arrowhead indicates an end plate contacted by a nerve fiber, and a denervated end plate is indicated by the arrow. B: the number of motor end plates that are contacted by a nerve fiber was quantified and expressed as percentage of the total number of motor end plates in each section. Values are means ± 1 SE. Significant difference from *WT value at the same age. **8-mo-old mice of the same genotype, and ***20-mo-old mice of the same genotype: P < 0.05.
muscles in Sod1−/− mice indicates that, in addition to the impact of the lack of CuZnSOD to disrupt NMJ, there are also detrimental effects within individual muscle fibers. Similarly, the weakness displayed by muscles of old WT mice with direct muscle stimulation suggests that contractile deficits may also exist within GTN fibers of old WT mice, as has been previously reported for individual flexor digitorum brevis, extensor digitorum longus, and soleus muscle fibers of mice (16, 20). The 30–40% deficits in isometric specific force observed in the present study during direct stimulation of whole GTN muscles of 8- and 20-mo-old Sod1−/− mice and 20- and 28-mo-old WT mice indicate that either individual fibers within these muscles also show 30–40% deficits in force generation, or most fibers generate normal forces while many fibers generate essentially no force. Some combination of fibers with a wide range of contractile deficits may also exist. To distinguish between these possibilities requires assessment of force generation of individual fibers.

Previous studies of single skinned fibers report effects of exposure to ROS that range from force reductions (6, 12), to modifications of contractile proteins may disrupt actin-myosin interactions, thereby reducing force-generating capacity of individual muscle fibers (31).

In summary, this study provides strong support for a process of denervation in the muscles of Sod1−/− mice, resulting in profound fiber loss and muscle atrophy between 1 and 8 mo of age. In addition, the deficits in specific force generation observed in the present study with direct stimulation for GTN muscles of 8- and 20-mo-old Sod1−/− mice and 20- and 28-mo-old WT mice suggest additional effects of elevated oxidative stress on individual muscle fiber function. Overall, the findings of the present study support previous reports of a direct role for oxidative stress as an in vivo mediator of the age-associated progression of deficits in muscle structure and function (18).

**Perspectives and Significance**

The progressive disruption of NMJs, loss of innervation, and ultimate fiber loss observed in Sod1−/− mice resemble, in some ways, the motor unit remodeling and loss known to occur with normal aging in rats and humans. The factors that initiate the loss of motor units with aging are not known, although Jang et al. (18) proposed that superoxide-induced NMJ degeneration represents a mechanism of sarcopenia. While the findings of the present study support an important role for oxidative stress to promote muscle fiber denervation, neither the present study nor previous studies (18, 23) of atrophy and weakness of muscles in Sod1−/− mice address whether cellular events within muscle fibers, in motoneurons, or both contribute in a causative manner to denervation process. The rapid development of muscle atrophy and weakness between 1 and 8 mo of age in Sod1−/− mice support the utility of this model to identify mechanistic links between oxidative stress and neuromuscular dysfunction.

**ACKNOWLEDGMENTS**

We are grateful to Elizabeth Armstrong, Mark Bolinger, Adriana Lingl, Douglas Montesano, and Emily Orban for help with data collection.

**GRANTS**

This work was supported by National Institute on Aging Grant AG-020591.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**


**REFERENCES**


