

**Oxidative stress induced dysregulation of excitation-contraction coupling
contributes to muscle weakness**

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Abstract

Background: We have previously shown that the deletion of the superoxide scavenger, CuZn superoxide dismutase (CuZnSOD) in mice (*Sod1*^{-/-} mice) results in increased oxidative stress and an accelerated loss of skeletal muscle mass and force that mirror the changes seen in old control mice. The goal of this study is to define the effect of oxidative stress and aging on muscle weakness and the EC coupling machinery in age-matched adult (8-10 months) wild-type (WT) and *Sod1*^{-/-} mice in comparison to old (25-28 months) wild-type mice.

Methods: *In-vitro* contractile assays were used to measure muscle contractile parameters. The activity of the sarcoplasmic reticulum ATPase pump (SERCA) was measured using an NADH-linked enzyme assay. Immunoblotting and immunofluorescence techniques were used to measure protein expression, and real-time reverse transcription polymerase chain reaction (RT-PCR) was used to measure gene expression.

Results: The specific force generated by the extensor digitorum longus (EDL) muscle was reduced in the *Sod1*^{-/-} and old WT mice compared to young WT mice along with significant prolongation of time to peak (TTP) force, increased half relaxation time (RT_{1/2}) and disruption of intracellular calcium handling. The maximal activity of the SERCA calcium uptake pump was significantly reduced in gastrocnemius muscle from both old WT (≈ 14%) and adult *Sod1*^{-/-} (≈ 33%) mice compared to young WT mice along with increased expression of sarcolipin, a known inhibitor of SERCA activity. Protein levels of the voltage sensor and calcium uptake channel proteins DHPRα1 and SERCA2 were significantly elevated (≈45% and ≈57%, respectively) while the ratio of calstabin, a channel stabilizing protein, to ryanodine receptor was significantly reduced (≈21%) in *Sod1*^{-/-} mice compared to young WT mice. The changes in calcium

handling were accompanied by substantially elevated levels of global protein carbonylation and lipid peroxidation.

Conclusion: Our data suggests that the muscle weakness in *Sod1*^{-/-} and old wild type mice is in part driven by ROS mediated EC-uncoupling and supports a role for reduced SERCA pump activity in **compromised muscle function**. The novel quantitative mechanistic data provided here can lead to potential therapeutic interventions of SERCA dysfunction for sarcopenia and muscle diseases.

Keywords Excitation contraction coupling, skeletal muscle, SERCA pump, Sarcolipin, Sod1, Sarcopenia

Introduction

Sarcopenia, the age related loss of muscle mass and strength [1], is a major cause of morbidity and mortality in the elderly population. While muscle atrophy contributes to weakness, the decline in muscle strength is more rapid than the atrophy, suggesting a deficit in intrinsic force-generating properties of the muscle [2]. The age-related muscle weakness independent of loss of mass is defined as dynapenia [3] and involves the excitation-contraction coupling machinery of the muscle fibers [4]. A progressive increase in cellular oxidative stress during aging has been implicated as a major contributor to sarcopenia. Accordingly, we have previously reported a direct correlation between muscle reactive oxygen species (ROS) and the muscle mass during normal aging and in muscle diseases [5].

Excitation-contraction coupling involves a sequence of events whereby action potential driven excitation of the sarcolemma results in rapid changes in cytoplasmic calcium concentration leading to activation of force-generating machinery in the sarcomere [6]. The electromechanical conduction requires activation of dihydropyridine receptors (DHPRs) in the sarcolemma and the subsequent release of calcium into the sarcoplasm from the intracellular calcium stores (sarcoplasmic reticulum, SR) through the Ca^{2+} release channels (ryanodine receptors, RyRs). The Ca^{2+} binds to regulatory proteins in the sarcomere initiating the contraction. Muscle relaxation occurs when Ca^{2+} is transported back from the sarcoplasm into the SR by the SR ATPase (SERCA) pumps. In mammalian skeletal muscle this process may dictate the rates of relaxation and a termination of a variety of Ca^{2+} dependent signaling pathways and gene transcription events that influence muscle quality and quantity [7]. Post-translational modifications of calcium release channels (RyRs) [8] and SERCA pumps [9] in the SR membrane can perturb the Ca^{2+} regulation leading to an uncoupling of excitation and contraction. These processes imply the critical importance of calcium handling in the muscle fiber as dysregulation of calcium homeostasis has been associated with reduced specific force in aging [10, 11] and conditions of increased oxidative stress [12, 13].

Our lab has previously used a mouse model of oxidative stress that was created by deleting cellular anti-oxidant enzyme Cu/Zn superoxide dismutase resulting in many features of rapid and accelerated sarcopenia [14-16]. The reduction in specific force in these mice is only partially rescued via direct

muscle stimulation that bypasses the neuromuscular junction (NMJ), suggesting a loss of functional innervation in these mice [15] but also defects within fibers. Moreover, interrogation of the function of single permeabilized fibers showed no difference between *Sod1*^{-/-} and WT mice indicating no impairment in the *Sod1*^{-/-} mice in the inherent function of the contractile machinery [17] and suggests there may be declines in the functioning of the excitation contraction machinery in CuZnSOD deficient muscle fibers. An association between functional denervation and excitation contraction uncoupling is well established in animal models and humans [18-20] and is partly explained by reduced quality and/or quantity of SR proteins. The mice lacking *Sod1* show increased oxidative damage to DNA, proteins and lipids in skeletal muscle and other tissues that is associated with muscle atrophy and functional decline. More specifically these muscles show increased 3-nitrotyrosine (3-NT) content [21], which is known to disturb SR protein function and the muscle contractile force [22].

The goal of this study was to determine whether the loss of innervation and the chronic increase in cellular oxidative stress in the *Sod1*^{-/-} mice affect the excitation-contraction apparatus in a manner similar to muscles of old WT mice. To achieve this purpose, we measured SERCA activity and *in-vitro* muscle contractile function. We also looked at the expression of key proteins involved in calcium handling. Further, as possible contributors to the muscle weakness, we also determined the quality and quantity of proteins involved in force generation. We hypothesize that, in skeletal muscles of *Sod1*^{-/-} mice, EC coupling is impaired contributing to the sarcopenia phenotype.

Material and Methods

Animals

The generation of *Sod1*^{-/-} mice is described in detail elsewhere [18, 23]. Male C57BL/6J mice were maintained under specific pathogen free conditions, housed 3-4 / cage, maintained in a 12:12 (light: dark) cycle and provided with food and water *ad libitum*. Age of the mice at the time of sacrifice was 8-10 months for young wild type (WT) and *Sod1*^{-/-} mice and 25-28 months for old WT mice (n = 10 -12 mice / group). At sacrifice, mice were euthanized via CO₂ inhalation and tissues were immediately excised and weighed. All tissues except those used for immunostaining and contractile recordings 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 the Oklahoma Medical Research Foundation (OKC, OK, USA).

Protein preparation and western blot

Muscles were ground in a mortar 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 10,000g 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 mg of total protein was applied to a 4–20% mini-PROTEAN TGX precast gel with a

4% stacking gel (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK). The separated proteins were transferred onto nitrocellulose membranes by western blotting. Membranes were probed using antibodies against calstabin, serca1, calsequestrin (abcam, Cambridge, UK), DHPR, RYR (Thermo scientific, USA), calcineurin, NFAT, calpain (Cell Signaling, Hitchin, UK) and CuZnSod (Enzo, Farmingdale NY, USA). Horseradish peroxidase conjugated anti-rabbit IgG or anti-mouse IgG (Cell Signaling, 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 (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK). All protein contents were normalized to protein levels determined by the ponceau stain.

Analysis of F₂-isoprostanes

Levels of F₂-isoprostanes in quadriceps muscle were measured as described previously [24]. **Hind limb muscle is routinely studied for changes during aging due to the increased response in this compartment. Quadriceps muscles were specifically chosen for this assay due to the amount of muscle tissue required for this assay (200mg).** Briefly, 200 mg of tissue was homogenized in 10 ml of ice-cold Folch solution (CHCl₃: 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 3000g for 5 min at 4 °C. The aqueous layer was discarded while the organic layer was secured and evaporated to dryness under N₂ at 37 °C. Esterified F₂-isoprostanes were measured using gas chromatography–mass spectrometry. The level of F₂-isoprostanes in muscle tissues was expressed as nanograms of 8-Iso-PGF_{2α}, per gram of muscle tissue.

Real-Time PCR

Total RNA was extracted from the gastrocnemius muscles of the young and old WT and Sod1^{-/-} mice using TRI reagent (Life Technologies, Grand Island, NY, USA). RNA purity and yield were determined by measuring the absorbance at 260 and 280 nm. cDNA was prepared from 1 mg of the total RNA using iScript™ cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA). 2.5 ng of cDNA samples were amplified using primers for DHPR1α, Calstabin, Calsequestrin, SERCA1, SERCA2, Sarcolipin and 18S along with fast SYBR

green master mix (Applied Biosystems, Grand Island, NY, USA). The data were analyzed using the $\Delta\Delta Ct$ method.

Contractile Measurements

Contractility measurements were performed on isolated extensor digitorum longus (EDL) muscle using a 1200A in vitro test system (Aurora Scientific Inc.). **The hind limb EDL muscle was chosen for analysis due to the well-documented response of this muscle to oxidative stress related to aging and other muscle diseases.** Muscles were individually tied to a model 300C servomotor (Aurora Scientific Inc.) and fixed within a water bath containing an oxygenated (95 % O₂, 5 % CO₂) Krebs-Ringer solution (in mM: 137 NaCl, 5 KCl, 1 MgSO₄, 1 NaH₂PO₄, 24 NaHCO₃, 2 CaCl₂) maintained at 32 °C. Computer controlled stimulation was applied through a model 701C stimulator (Aurora Scientific Inc.) via flanking platinum plate field stimulus electrodes at supramaximal voltage, 0.2 ms pulse width, and at optimum length for twitch force production (i.e., L₀). The times required to reach maximal **force** from baseline (time to peak; TTP) and to achieve 50% relaxation from the peak (half relaxation time; RT_{1/2}) were measured for twitch and tetanic contractions. Force frequency curves were generated with stimulation frequencies between 1 and 300 Hz, while the fatigue protocol consisted of repeated 300-ms trains of 150 Hz stimuli, every 5s for a total of 400 s. All data were recorded and analyzed using commercial software (DMC and DMA, Aurora Scientific). Specific force (N/cm²) was measured using muscle length and weight as described elsewhere [25].

The methods and instrumentation use to measure the contractility of the lumbrical muscles were very similar to those used to measure EDL contractility and have been described in detail previously [26]. Notable differences were that force-frequency and fatigue responses were not determined for the lumbrical muscles, and experiments were performed in a custom-built chamber with a quartz floor to allow excitation of the fura-2 and mag-fura-2 fluorescent dyes with ultraviolet light. Experimental temperature was 25 °C and acquisition and analysis were performed using custom software developed in LabVIEW (National Instruments). Because the lumbrical muscle is too small to weight accurately,

cross-sectional area was estimated by measuring maximum muscle diameters using a calibrated reticule in the microscope eyepiece and assuming a circular cross-section [26].

SERCA activity

The measurement of SERCA ATPase activity was performed at 37°C using a spectrophotometric assay as described elsewhere [27]. **The gastrocnemius muscle, that has been shown to be particularly responsive to age and oxidative stress related changes in mass and force generation, was used for this analysis** i. Briefly, muscle samples (15–20 mg) were diluted 1:10 (wt/vol) and manually homogenized in ice-cold homogenizing buffer (pH 7.5) containing (in mM) 250 sucrose, 5 HEPES, 0.2 PMSF, and 0.2% sodium azide (NaN₃). Ca²⁺-dependent Ca²⁺-ATPase activity was measured in the assay buffer containing 100 mM KCl, 20 mM HEPES, pH 7.0, 10 mM MgCl₂, 10 mM NaN₃, 10 mM phosphoenolpyruvate, 1 mM EGTA, 5 mM ATP, 18 U/ml of both lactate dehydrogenase and pyruvate kinase, and 1 mM Ca²⁺ ionophore A-23187 (C-7522; Sigma). The reaction mixture was divided into 10 aliquots of 300 µl and mixed with CaCl₂ to generate 10 different Ca²⁺ concentrations, ranging between 7.6 and 5 pCa units. The reaction was started by adding 0.3 mM NADH. Basal activity was determined in the presence of 40 µM of the Ca²⁺-ATPase inhibitor cyclopiazonic acid (C-1530, Sigma) in dimethyl sulfoxide.

Calpain activity

Because SERCA dysfunction can perturb cytosolic calcium levels leading to activation of calcium-dependent proteases, we measured the calpain activity in the gastrocnemius muscle homogenates as previously described [28] with slight modifications. Briefly, 100 µg of protein was added to a buffer containing: 50 mM HEPES, 100 mM NaCl, 0.1% CHAPS, 10 mM DTT, 1 mM EDTA, 10% glycerol, pH: 7.4, and a fluorogenic substrate (Suc-LLVY-AMC) cleaved by calpain. Immediately after addition of the substrate, a baseline fluorescent measurement was performed using a spectrofluorometer (excitation frequency: 355 nm, emission frequency: 460 nm). The measurement was repeated after 60 min of incubation at 37°C. AMC standard were used to create a calibration curve and activity was quantified as nanomoles of AMC generated per minute per milligram of tissue homogenate protein. The difference

between AMC generation from incubation of homogenates with Suc-LLVY-AMC in the presence and absence of calpain inhibitor III was taken as an index of calpain activity.

Intracellular calcium in whole muscles

Intracellular calcium levels were monitored in lumbrical muscles from WT and *Sod1*^{-/-} mice (age 8-10 months) using calcium-sensitive fluorescent dyes loaded by incubation in their acetoxymethyl ester (AM) form. Pluronic F-127 (0.01 % w/v; Thermo Fisher) was used as a dispersing agent. **The simple architecture (essentially a parallel fibers arrangement) and small size of lumbrical muscles facilitated loading of fluorescence dyes and exchange of gases and metabolites across minimal diffusion distance.** Calcium levels were recorded under three conditions: at rest, following a 5s maximum tetanic contraction, and in response to twitch stimuli. Experiments to determine resting and post-tetanic calcium levels were performed in the same series of lumbrical muscles by loading the highly-sensitive calcium indicators fura-2AM (16.4 μ M) and fluo-4AM (15 μ M) simultaneously for 30min at 25°C. To determine resting levels, fura-2 fluorescence was excited at 340 nm and 375 nm (bandwidth 1.25 nm). The excitation source was a 75W xenon lamp and wavelengths were selected using a diffraction grating monochromator (PTI, model Delta RAM). Fluorescence responses were passed through a 510nm emission filter (bandwidth 40 nm) before being quantified using a photomultiplier tube (Hamamatsu model R1527) in photon counting mode. After subtracting corresponding pre-loading backgrounds, the ratio of the fura-2 fluorescence intensities (340/375) was formed and taken as an indication of the resting calcium level.

In the presence of contractile activity, fura-2 becomes less reliable as a calcium indicator because its excitation and emission spectra overlap those of NAD(P)H [29-31] The time-course of the return of calcium to resting level following a long tetanic contraction was therefore monitored by recording the fluorescence response of fluo-4, which is not contaminated by contraction-induced NAD(P)H fluctuations. Fluo-4 fluorescence was excited at 493 nm (bandwidth 1.25nm) and passed through a 535nm emission filter (bandwidth 40nm) to a photomultiplier tube in photon-counting mode, sampling at 100s⁻¹. A line was fitted to the fluo-4 response beginning 60s before the 5s tetanic contraction and

ending 420s after the initiation of contraction, but excluding a 180s segment that began with the contraction. Thus the fitted line served to establish the decline in fluo-4 fluorescence over time due to dye leakage, extrusion, bleaching, etc. The raw fluo-4 response was divided by the fitted line to produce a “detrended” response, which was then shifted such that the average value of the 60 s pre-stimulus recording (i.e. the resting level) was zero. The time required for the modified fluo-4 response to decay to within 50% and 25% of the resting level were measured relative to the level reached 1.5 s after cessation of tetanic stimulation. The 1.5s post-contraction delay was incorporated to allow time for the calcium concentration and its rate of change to reach levels that could be reported by fluo-4 with minimal distortion [32].

Experiments to determine the dynamic calcium responses to twitch stimuli were performed on a separate series of lumbrical muscles by loading the low-affinity calcium indicator mag-fura-2AM (10 μ M) for 30min at 25°C. Mag-fura-2 fluorescence was excited at 344nm and 375nm (bandwidth 10 nm) and responses were passed through a 510nm emission filter (bandwidth 40 nm) before entering a photomultiplier tube operating in analog mode, which results in higher time-resolution and reduced noise when compared with photon counting. The experimental sequence consisted of first recording twitch force and mag-fura-2 fluorescence during excitation at 344 nm, waiting 20 s, and then recording twitch force and mag-fura-2 fluorescence during excitation at 375 nm. The alternating sequence was repeated until a total of 16 responses had been recorded at each excitation wavelength. Pre-loading backgrounds were then subtracted from all fluorescence records and the 16 responses at each excitation wavelength were averaged. Finally, the ratio of the averaged mag-fura-2 fluorescence intensities was formed (344/375) and taken as an undistorted representation of the rapid intracellular changes in calcium concentration that give rise to a twitch contraction [33]. All fluorescent dyes were obtained from Thermo Fisher. Additional details on the techniques and apparatus used to measure lumbrical muscle contractile properties and fluorescence have been described previously [26].

Protein carbonylation

Protein oxidation in tissues was determined by the level of protein carbonyls using a modification of the original method described by Ahn et al.[34]. **Gastrocnemius muscles were utilized due to large body of literature on the response of these muscles to age and Sod1 deficiency.** In brief, fresh tissue was homogenized in degassed 20mM sodium phosphate buffer, pH 6.0, containing 0.5mM MgCl₂, 1mM EDTA, and protease cocktail inhibitors (500μM AEBSF, HCl, 150nM aprotinin, 0.5 mM EDTA, disodium salt, and 1μM leupeptin hemisulfate). For the cytoplasmic fraction, the homogenate was centrifuged at 100,000g at 4°C for 1h and the supernatant was saved for further processing. One percent streptomycin sulfate was added to remove nucleic acids. The protein samples were then bubbled with nitrogen for 15s at 25kPa followed by treatment with 0.3M guanidine HCl for partial unfolding of the proteins in the sample. The hydrazine reagent, fluorescein-5-thiosemicarbazide (FTC, Molecular Probes, Eugene, OR) (1mM) was added and incubated for 2h at 37°C in the dark. The excess unreacted FTC was removed by precipitation with an equal volume of 20% TCA followed by washing 4 times with ethanol/ethyl acetate (1/1) (v/v). Equal amounts of protein were loaded on a 12% SDS-PAGE to resolve the FTC-labeled proteins. A fluorescence scan of the gel was used to measure the amount of bound FTC, i.e., the amount of protein carbonyls. The gel was stained with Coomassie blue, and the protein concentration was determined. The carbonyl content of the protein samples was expressed as the ratio of FTC fluorescence (carbonyls) to Coomassie blue absorption (protein concentration).

Myosin Heavy Chains (MHC) quantification and MHC isoform composition

Gastrocnemius muscles were homogenized in high salt lysis buffer consisting of 0.3M NaCl, 0.1M NaH₃PO₄, 0.05M Na₂PO₄, 0.01M Na₄P₂O₇, 1 mM MgCl₂, 10 mM EDTA and 1 mM DTT (pH 6.5) and protease inhibitors. Tissue lysates were subsequently centrifuged at 16,000 g for 3 min at 4°C. Supernatant was collected and 0.6 μg protein was loaded/lane into a 10% SD-PAGE and run at 200 V for 50 min at 4°C. The gel was stained with Coomassie blue and the optical densities of the bands corresponding to MHC and actin were quantified.

For MHC isoforms composition, cryosections of gastrocnemius muscles were stained with monoclonal anti-myosin heavy chain antibodies as described elsewhere [35]. All images were taken with Nikon eclipse ni confocal microscope and analyzed with Nikon elements c image acquisition software.

Statistical Analysis

Data are presented as mean \pm SEM and the comparisons among the three groups were performed by one-way analysis of variance (ANOVA). Data were analyzed using Sigma plot 12 and p values of less than 0.05 were considered statistically significant.

Results

The absence of CuZnSOD protein was confirmed in the gastrocnemius muscles of the *Sod1*^{-/-} mice (Figure 1). This is previously reported to have no effect on the other major anti-oxidant enzymes in the muscle fiber [36].

Body weights and muscle weights

The adult *Sod1*^{-/-} mice show significantly smaller body mass ($p < 0.001$) than age matched young WT and the old WT mice (Figure 1a). The gastrocnemius muscles from the old WT and *Sod1*^{-/-} mice showed reduced mass when compared to young WT mice, which remained reduced when normalized to body weight. The absolute muscle weights of the quadriceps, TA and EDL were significantly reduced in the old WT and *Sod1*^{-/-} mice as compared to young WT mice. However, these differences were limited to old WT mice when normalized to body weights (Supplementary Table 1).

Contractile function

We have previously shown that the gastrocnemius muscle of the *Sod1*^{-/-} mice shows significant weakness in comparison with the age-matched controls [37], which is due in part to disruption of neuromuscular junctions [36, 38, 39]. Here we measured the *in-vitro* contractile properties of EDL and lumbrical muscles focusing on the parameters of calcium regulation. In agreement with our previously reported findings we found a significant decline in specific force in the EDL muscles of old WT mice ($\approx 26\%$, $p < 0.05$) and *Sod1*^{-/-} mice ($\approx 24\%$, $p < 0.05$) when compared to young WT mice (Table 1). The twitch TPT and the $RT_{1/2}$ were not significantly different in the EDL muscles from old WT mice and *Sod1*^{-/-} mice when compared to young WT mice. However, we report a significant prolongation of TPT and the $RT_{1/2}$ in the lumbrical muscles of *Sod1*^{-/-} mice when compared to young WT mice (Table 1). Further, we assessed the calcium homeostasis and the fiber type profile by measuring the tetanic forces during a series of stimulus trains of increasing frequency. A significant downward shift in the force frequency curve between 100 and 300 Hz was observed in old WT and *Sod1*^{-/-} mice EDL (Figure 2a). Fused tetanus was achieved at a lower stimulation frequency resulting in a leftward shift of the frequency for curve for muscles of old WT mice.

Intracellular calcium transients

Performance of cellular calcium release and sequestration functions were also assessed under more physiological conditions in intact lumbrical muscles (Figure 2b, c). The large, rapid calcium transients associated with twitch contractions were reduced in amplitude and took longer to decay in *Sod1*^{-/-} compared with WT mice (Figure 3a). No difference was detected between lumbrical muscles from *Sod1*^{-/-} and WT mice in either the resting calcium level or the slow return to resting level following a long tetanic contraction (Figure 3b).

SERCA activity and regulator proteins

Reduced activity of SR Ca^{2+} -ATPase (SERCA) has previously been reported in skeletal muscle during aging and conditions of increased oxidative stress [40, 41]. We hypothesized that the *Sod1*^{-/-} mice would show reduced SERCA pump activity in a manner similar to old WT mice. Measurement of SR Ca^{2+} -ATPase activity was determined by measuring the decrease in NADH absorbance at 340nm in gastrocnemius homogenates from young WT, old WT and *Sod1*^{-/-} mice. The maximal rate of change in NADH

absorbance was significantly reduced by $\approx 14\%$ ($p = 0.004$) and $\approx 33\%$ ($p < 0.001$) in the old WT and *Sod1*^{-/-} mice respectively, when compared to young WT (Figure 4a). The SERCA pump dysfunction cannot be explained by altered SERCA expression since WB analysis failed to detect any reduction in protein levels of SERCA1 and SERCA2 isoforms (Figure 4b). The protein expression of SERCA1, the most abundant SERCA isoform was unchanged in the gastrocnemius muscles of the three groups while SERCA 2 expression was upregulated in *Sod1*^{-/-} mice ($\approx 57\%$, $p < 0.05$) when compared to young WT mice (Figure 4b).

Sarcolipin, a small membrane protein, inhibits SERCA by reducing the apparent Ca^{2+} affinity of the pump [42]. We hypothesized that an elevated level of sarcolipin could account for compromised SERCA function. Although we could not detect sarcolipin protein expression in the soleus and red gastrocnemius muscles of the young WT mice, we observed a high level of SLN protein expression in muscle from old WT and *Sod1*^{-/-} mice (Figure 4c). Similar changes were observed in mRNA expression in the old WT (≈ 5 fold increase, $p = 0.003$) and the *Sod1*^{-/-} mice (≈ 500 fold increase, $p = 0.004$) (Figure 4c). These results suggest that the sarcolipin may contribute to the impaired SERCA function in the old WT and the *Sod1*^{-/-} mice. Indeed, the overexpression of sarcolipin is associated with reduced SERCA function and impaired muscle force in the rat soleus [43].

Voltage sensors and SR calcium handling proteins

The reduction in SERCA pump activity and force-generating capacity implicate potential alterations in the quantity and/or quality of proteins involved in voltage sensing and calcium handling. To determine whether the deficiency of *Sod1* can affect these proteins in a manner similar to aging phenotype, we measured the expression levels of proteins involved in voltage sensing and SR calcium handling in the gastrocnemius muscle of young WT, old WT and *Sod1*^{-/-} mice. We hypothesized that the expression of SR calcium binding and release units will be affected by the loss of *Sod1*. As shown in Figure 5, there is a reduced expression of calstabin relative to RyR expression ($\approx 21\%$, $p < 0.05$) in the *Sod1*^{-/-} mice when compared to young WT mice. Calstabin is the major stabilizing unit of the calcium release channels, RyRs, and its dissociation and reduced relative expression from RyR have been implicated in impaired

muscle quality [44]. There was no effect of age and *Sod1* deficiency on the RyR protein expression. Further, calsequestrin which binds Ca^{2+} in the SR lumen is increased in the old WT ($\approx 45\%$, $p = 0.009$) but not in *Sod1*^{-/-} mice ($p = 0.902$) when compared to young WT mice. The protein level of the voltage sensor α -1 subunit of the dihydropyridine receptor (DHPR) was upregulated in *Sod1*^{-/-} mice ($\approx 45\%$, $p < 0.05$), while α -2 subunit protein expression was unchanged.

Force generating machinery and its regulators

Preferential loss of **MHC**, the major molecular motor of the myofibril, is shown to be associated with age-related muscle atrophy [45]. In accordance with this previous report, we found a selective loss of **MHC** in the gastrocnemius muscles of the old WT mice ($\approx 14\%$, $p < 0.05$) which is recapitulated in the *Sod1*^{-/-} mice ($\approx 17\%$, $p < 0.05$) when compared to young WT mice (Figure 6a). Since the activation of calpain or calcium dependent cysteine protease is the major contributor of sarcomeric myosin degradation [46] we measured calpain activity. Cleavage of α -spectrin to signature fragment of 145kDa correlates well with the calpain activity [47]. Accordingly, with the **MHC** loss, we observed an increased expression of calpain-specific breakdown product of α -spectrin in the gastrocnemius muscles of old WT (≈ 6 fold, $p < 0.05$) and *Sod1*^{-/-} mice (≈ 9 fold, $p < 0.05$) when compared to young WT mice (Figure 6a). We next assessed calpain activity in the muscle homogenates using fluorogenic substrate and found higher activity in the *Sod1*^{-/-} mice ($\approx 22\%$, $p < 0.01$) when compared to young WT mice although old WT mice did not show significant difference from young WT mice (Figure 6b). The calpain protein showed an increased level, albeit not statistically significant in the old WT ($p = 0.446$) and *Sod1*^{-/-} mice ($p = 0.431$) when compared with young WT mice.

Post-translational modifications

We next assessed the markers of oxidative damage in the old WT and *Sod1*^{-/-} mice. F_2 -isoprostane and protein carbonyl levels were significantly higher in old WT mice (F_2 -isoprostanes; $\approx 42\%$, $p < 0.05$, proteins carbonyls; $\approx 19\%$, $p < 0.05$) and in the *Sod1*^{-/-} mice (F_2 -isoprostanes; $\approx 112\%$, $p < 0.05$, protein carbonyls; $\approx 28\%$, $p < 0.05$) while protein S-nitrosylation was only increased in the *Sod1*^{-/-} mice ($\approx 136\%$, $p < 0.05$) when compared to young WT mice. Considering the vulnerability of EC coupling proteins to the

oxidative damage [48], we postulate that the oxidative stress is a significant contributor to the EC uncoupling in the old WT and *Sod1*^{-/-} mice. (Figure 7a,b,c)

Calcium signaling proteins

Cytosolic Ca²⁺ is an important contributor to skeletal muscle plasticity by activating specific signaling pathways. Accordingly, we looked at the protein expression of calmodulin/calcineurin and NFAT proteins. No significant difference was found in the old WT and *Sod1*^{-/-} mice when compared to young WT mice (Figure 8a, b). NFAT proteins control muscle fiber type distribution which is also unchanged among the three experimental groups (Figure 8d). However, DSCR1, an inhibitor of calcineurin was significantly elevated in the *Sod1*^{-/-} mice when compared to young WT mice (Figure 8c). These findings, nevertheless do not exclude changes in enzyme activities of these proteins.

Discussion

Aging is associated with a decline in muscle force generation, which is explained by reduced muscle quantity and quality. Despite many reports of age related loss of muscle quality, the mechanism (s) between oxidative stress and muscle quality remain to be elucidated. We have previously recapitulated many features of the aging phenotype in our *Sod1*^{-/-} mouse model of sarcopenia, including denervation, atrophy and reduced muscle specific force [38, 49]. The reduction in specific force is independent of muscle size and neuronal stimulation [49] and relates to a deficit in the intrinsic force-generating properties of the muscle. These findings prompted us to look at components of sarcolemmal excitation, SR calcium handling and the muscle contraction. The results from this study support and extend the sarcopenia phenotype of *Sod1*^{-/-} mice and more importantly provide clues to the underlying mechanisms of sarcopenia in humans. We report that the disruption of excitation-contraction coupling contributes to impaired force generation in the mouse model of *Sod1* deficiency. Briefly, we found a significant reduction in SERCA activity as well as reduced expression of proteins involved in calcium release and force generation. Another potential factor involved in EC-uncoupling in *Sod1*^{-/-} mice is oxidative damage to proteins involved in the contractile response. Here we found an increase in markers of **global** protein carbonylation and nitration as well as markers of lipid peroxidation (F₂-isoprostanes). Together, the loss of quantity and quality of EC-coupling machinery in *Sod1*^{-/-} mice is in agreement with impaired force generation we report in our study.

Intracellular calcium transients associated with twitch contractions reach high levels at their peak ($\approx 20\mu\text{M}$) and undergo rapid changes throughout [50]. As such, only low-affinity calcium sensors such as mag-fura-2 are capable of reporting calcium responses to twitch stimulation without saturation or kinetic delay [33]. The time-course of the twitch transient as reported by low-affinity dyes consists of a large, very rapid increase due to release of calcium from the SR followed by an equally large, less rapid fall to resting level. The fall in cytoplasmic calcium is due primarily to rapid binding to troponin and parvalbumin, with little contributing from the SR pump [50, 51]. The finding that the peak of the twitch calcium transient was reduced by 20% in muscles from the *Sod1*^{-/-} mice with no change in width (FDHM) is similar to that reported in fibers from *mdx* mice [52]. Modeling simulations by those authors

suggested that the result is consistent with a reduction in calcium release coupled with a reduced binding of calcium by parvalbumin, which could also contribute to the 35% slowing of fall time observed in the present study. A number of mechanisms including reduced RyR expression and/or its coupling with DHPR and calstabin can lead to reduced calcium release. However, we did not observe any significant reduction in the RyR expression in the two groups, which is consistent with previous findings in studies of aging rodents [53, 54]. The long half-life (5-8 days) of the RyR is further increased ($\approx 25\%$) with aging [55], **theoretically** making it susceptible to post-translational modifications. Accordingly, oxidative damage to RyR can result in irreversible inactivation of the channel, leading to defective Ca^{2+} release and force generation [53, 56]. The increased level of biomarkers of oxidative damage to both lipids and proteins are compatible with potential damage to the RyR resulting in muscle deficits. Further, the reduced relative expression of the RyR stabilizing unit, calstabin observed in *Sod1^{-/-}* mice can also contribute to reduced E-C coupling and Ca^{2+} release in a manner similar to models of cardiac diseases [57] and over-training [44]. In fact oxidative damage to calstabin has been linked to its dissociation and reduced relative expression to the RyR, resulting in calcium leak [54]. Muscle aging can also result in changes in DHPR subunits and their coupling with the RyR leading to defective calcium release upon stimulation [58]. We only found significant increase in the α -2 subunit of the DHPR in the *Sod1^{-/-}* mice while the α -1 subunit was unchanged, but this observation, nevertheless does not exclude physical dissociation between DHPR and RyR at the triad junction.

Calcium clearance from the sarcoplasm dictates half relaxation time and is primarily regulated by SERCA pumps in the SR membrane. The significant reduction in SERCA pump activity of *Sod1^{-/-}* mice mimics the reduction in conditions of increased oxidative stress such as aging [59] and chronic diseases [60]. In adult skeletal muscle two SERCA isoforms predominate, namely SERCA1 and SERCA2, with the former being most abundant in fast-twitch mouse gastrocnemius muscle. However, our measures of SERCA protein content showed no change in SERCA1 while SERCA2 protein is significantly upregulated in the *Sod1^{-/-}* mice. Hence, the mechanism(s) underlying reduced SERCA activity do not involve changes in SERCA pump expression. It is possible that post-translational modification(s) of SERCA could compromise its function. Like the RyR, SERCA has a long half-life of ≈ 14 -17 days, making it potentially

susceptible to oxidative damage [55]. Previous studies have demonstrated an age related nitrosylation of SERCA proteins [59] which is most prominent in SERCA2 proteins. Here we report an increase in the global protein nitration in the *Sod1*^{-/-} mice which is consistent with the hypothesis that nitrosylation of SERCA may contribute to the reduced SERCA activity. Another possible mechanism underlying reduced SERCA activity is inhibition by sarcolipin, which is a SERCA pump inhibitor [42]. Upregulation of sarcolipin protein and/or mRNA expression has been associated with SERCA dysfunction, Ca²⁺ dysregulation and myopathies [61-63]. Furthermore, studies in mouse models of muscular dystrophy suggest that its overexpression may be directly proportional to disease severity [63]. We found high expression of sarcolipin mRNA in the gastrocnemius muscles of old WT and *Sod1*^{-/-} mice but not in young WT mice. These findings are mirrored at the protein level in the soleus and red gastrocnemius muscles. Sarcolipin overexpression has been shown to result in reduced SERCA activity and contractile impairment in rodent skeletal muscle [43]. Thus, it is probable that the increased expression of sarcolipin in aged and *Sod1*^{-/-} mouse muscle contributes to the reduced SERCA activity in these **models which in turn reduces the SR Ca²⁺ reserves, resulting in reduced Ca²⁺ release upon stimulation and subsequent muscle functional deficit.**

Elevated oxidative stress may also induce EC uncoupling by affecting the SR calcium buffering proteins. Although *Sod1*^{-/-} mice do not show any significant change in calsequestrin 1 and parvalbumin protein levels, we cannot exclude phosphorylation and/or oxidative damage to them in cellular redox environment.

The preferential loss of molecular motor protein **MHC** in the *Sod1*^{-/-} mice recapitulates the age related loss of **MHC** as reported elsewhere [45, 64]. This selective reduction of **MHC** may lead to a decrease in number of active cross bridges involved in force generation, contributing to muscle weakness. The **selective reduction of MHC** is not unprecedented and is previously reported in conditions of increased oxidative stress including muscle disease [65] and disuse [66]. Myosin and other cytoskeletal proteins are mainly cleaved by calpains, the calcium-activated cysteine proteases [67]. Accordingly, we report an increased calpain activation in *Sod1*^{-/-} mice as assessed by an increase in calpain-dependent cleavage products of α -2 spectrin and fluorogenic substrate.

In summary, this study provides strong support for the coupling between increased oxidative stress and disruption of cellular excitation contraction machinery in mouse skeletal muscle. We report that the muscle weakness in the *Sod1*^{-/-} mice is in part caused by ROS-mediated EC uncoupling which results in reduced quality and/or quantity of machinery involved in SR calcium handling and force generation. The novel quantitative mechanistic data provided here can lead to potential therapeutic interventions of SERCA dysfunction for sarcopenia and muscle diseases.

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Conflict of Interest

Rizwan Qaisar, Shylesh Bhaskaran, Pavithra Premkumar, Rojina Ranjit, Kavithalakshmi Satara Natarajan, Bumsoo Ahn, Kaitlyn Riddle, Dennis R Clafin, Arlan Richardson, Susan V. Brooks and Holly Van Remmen declare that they have no conflict of interest.

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Figure legends

Fig 1 Characteristics of mice used in the study.

(a) Mouse age, body mass and gastrocnemius muscle weights, (b) western blot analysis of CuZnSod1 expression in the gastrocnemius muscles from the young and old WT and *Sod1*^{-/-} mice. Values are expressed as Mean ± SEM. (n = 8-12 per group). ; One-way ANOVA. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$

Fig 2 Muscle contractile properties

Muscle contractile properties measured in the young and old WT and *Sod1*^{-/-} mice. (a) Force-frequency curves for EDL muscles represented as percentage of maximum force and specific force; (b) Representative force (blue trace) and fluo-4 fluorescence (red trace) responses to 5 s maximum tetanic stimulation. Arrows indicate times of initiation and termination of stimulation. Upon termination of stimulation, force immediately returned to resting level whereas the fluo-4 fluorescence remained elevated for several seconds. Relative to the level reached 1.5 s after stimulation was stopped, the times required for the fluo-4 response to decay to 50% ($t_{50\%}$) and 25% ($t_{25\%}$) were 6.4 s and 15.7 s, respectively. Fluo-4 fluorescence units are $\Delta F/F_r$, where ΔF is fluorescence measured relative to resting level, and resting level is designated F_r . (c) Representative force (blue trace) and mag-fura-2 fluorescence (red trace) responses to twitch stimulation. Arrow indicates time at which a single stimulation pulse was delivered. The mag-fura-2 response was obtained by averaging 16 individual responses to excitation at 344 nm and 16 responses to excitation at 375 nm and then forming the ratio of the two averages (344/375). The force response is the average of all 32 recordings. Values are expressed as Mean ± SEM. (n = 8-12 per group). ; One-way ANOVA. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$

Fig 3 Intracellular calcium transients

Parameters of calcium release and sequestration in the intact lumbrical muscles from the young WT and *Sod1*^{-/-} mice. ICT peak was reduced while the ICT fall time and width durations were prolonged during the large, rapid calcium transients associated with twitch contractions in *Sod1*^{-/-} compared with WT mice (a). No difference was detected between lumbrical muscles from *Sod1*^{-/-} and WT mice in either the resting calcium level or the slow return to resting level following a long tetanic contraction (b). Values are expressed as Mean ± SEM. (n = 6-9 per group). ; One-way ANOVA. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Fig 4 SERCA pump activity and protein expression

(a) SERCA Ca²⁺ – dependent ATPase activity and the maximal ATPase activity (V_{max}) in gastrocnemius muscle homogenates; (b) Western blot analysis of SERCA protein content; (c) Sarcolipin protein and mRNA expressions from the gastrocnemius muscles of young and old WT and *Sod1*^{-/-} mice. Values are expressed as Mean ± SEM. (n = 8-12 per group). ; One-way ANOVA. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$

Fig 5 Expression of other EC coupling proteins.

Western blot analysis of EC coupling protein content in gastrocnemius muscles of young and old WT and *Sod1*^{-/-} mice. Values are expressed as Mean ± SEM. (n = 8-12 per group). ; One-way ANOVA. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$

Fig 6 MHC content and calpain protease activity

(a) **MHC** content normalized to total protein content; (b) Western blot analysis of calpain content and α -2 spectrin cleavage as a measure of calpain activity; (c) calpain activity as measured by cleavage of fluorogenic substrate in the gastrocnemius muscles of young and old WT and *Sod1*^{-/-} mice. Values are expressed as Mean ± SEM (n = 8-12 per group). ; One-way ANOVA. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$

Fig 7 Markers of oxidative stress

(a) Protein carbonylation detected using avidin-fluorescein isothiocyanate (FITC) affinity staining in gastrocnemius muscle; (b) F₂-isoprostane levels in quadriceps muscle; (c) Western blot analysis of the nitrated proteins from the gastrocnemius muscles of young and old WT and *Sod1*^{-/-} mice. Values are expressed as Mean ± SEM (n = 8-12 per group). ; One-way ANOVA. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$

Fig 8 Calcium signaling and muscle fiber type composition

(a, b, c) Western blot analysis of calcium signaling proteins levels; (d) muscle fiber type composition and cross-sectional areas from the gastrocnemius muscles of young and old WT and *Sod1*^{-/-} mice. Values are expressed as Mean ± SEM (n = 8-12 per group). One-way ANOVA. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$

Supplementary Fig 1 Muscle fatigue

Time course of contractile force decline in EDL muscles during 400 seconds of fatiguing stimulations. Values are expressed as Mean ± SEM (n = 8-12 per group). ; One-way ANOVA. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$ ANOVA.

Supplementary Fig 2 Expressions of genes associated with muscle quantity and quality

mRNA expression of genes associated with muscle functional and structural phenotypes in the gastrocnemius muscles of young and old WT and *Sod1*^{-/-} mice. Values are expressed as Mean ± SEM (n = 8-12 per group). One-way ANOVA. * $P \leq 0.05$,

Figure 1

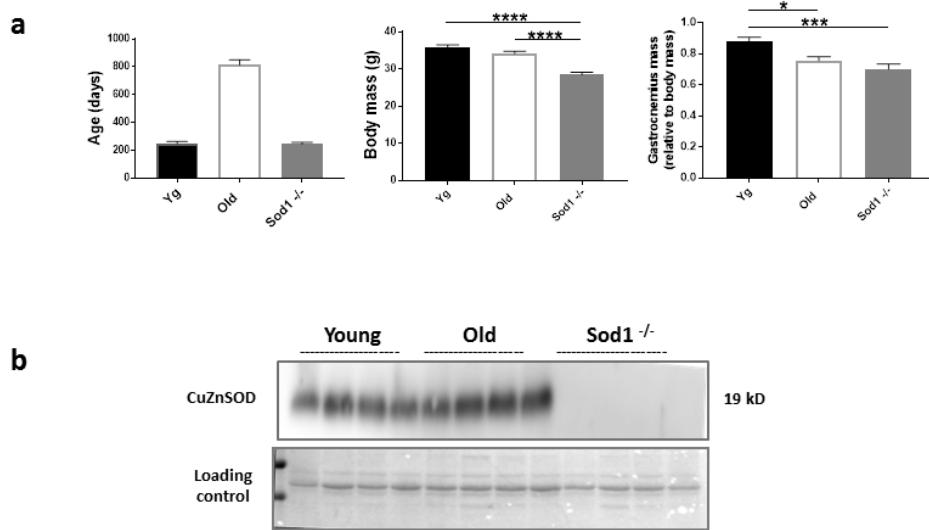


Figure 2

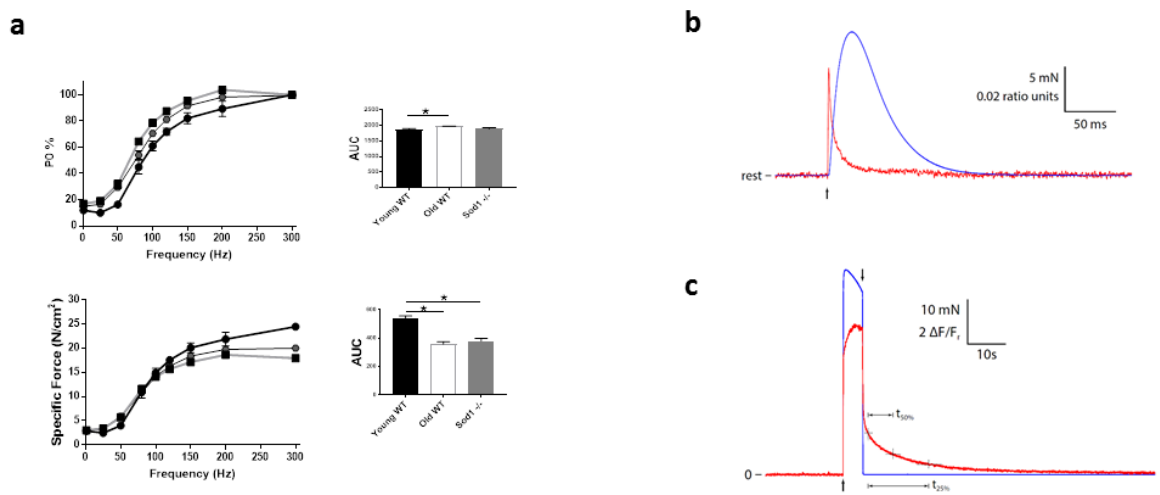


Figure 3

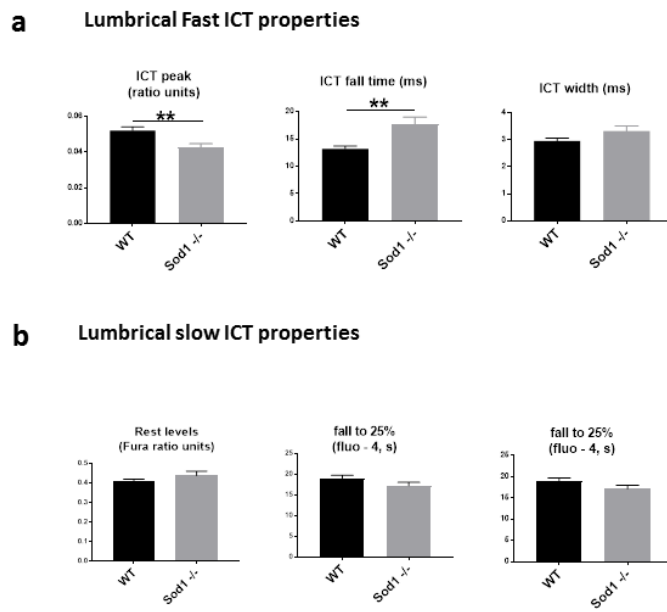


Figure 4

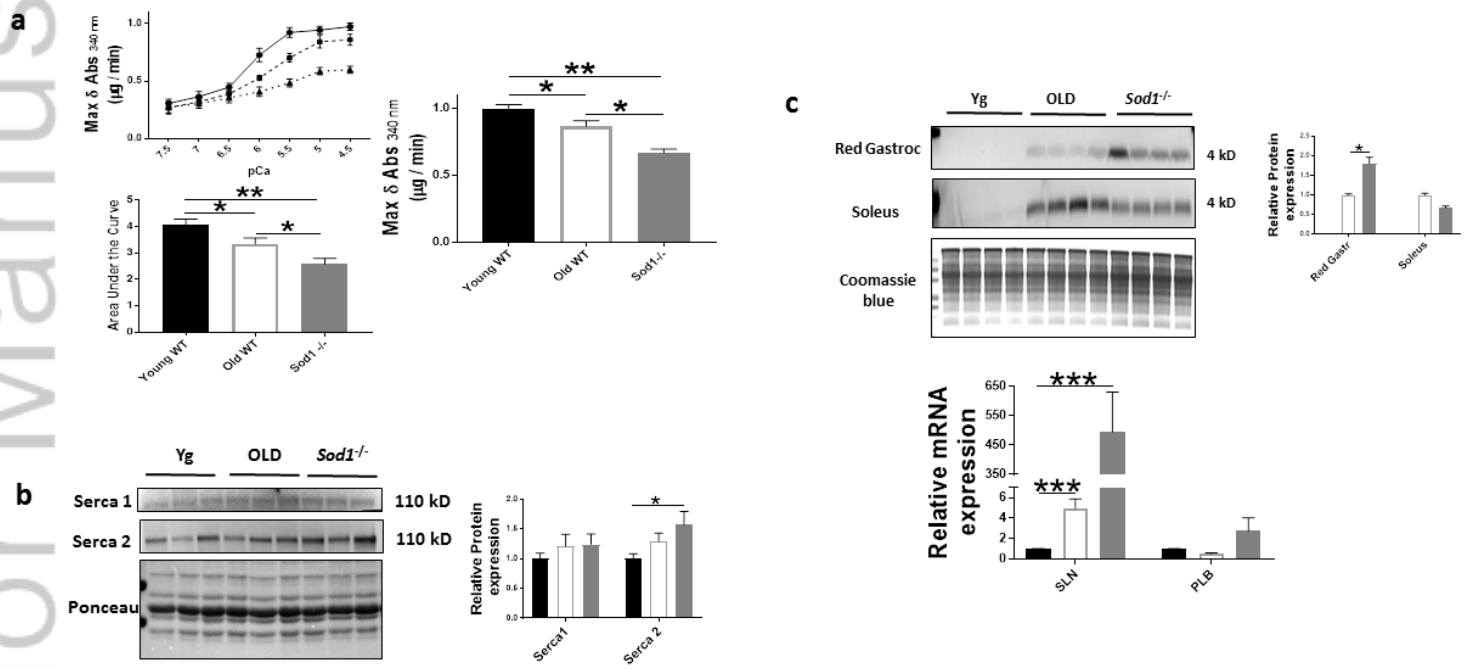


Figure 5

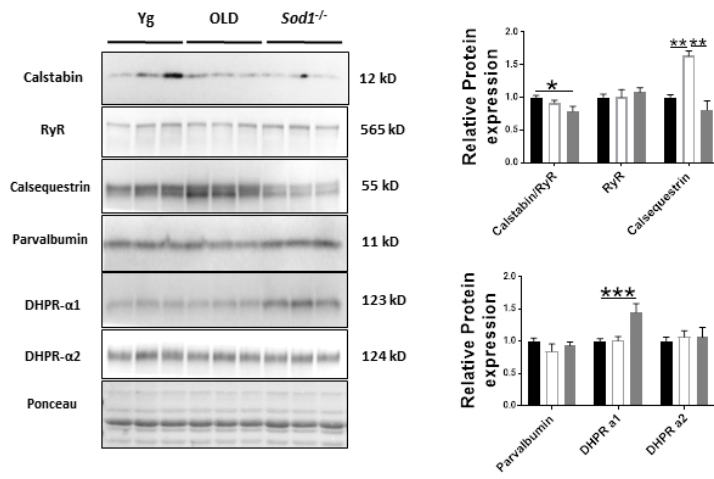


Figure 6

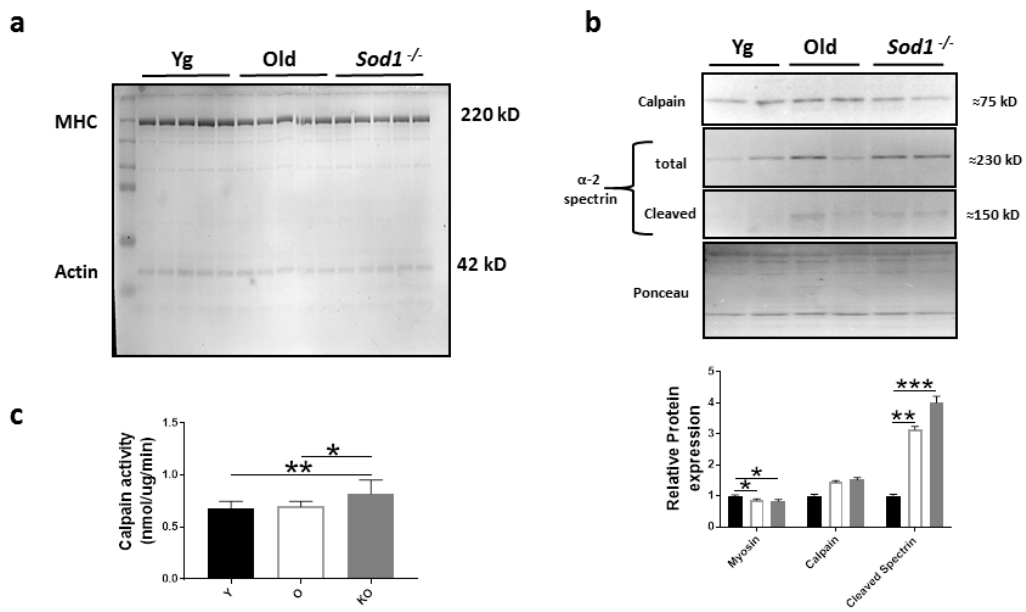


Figure 7

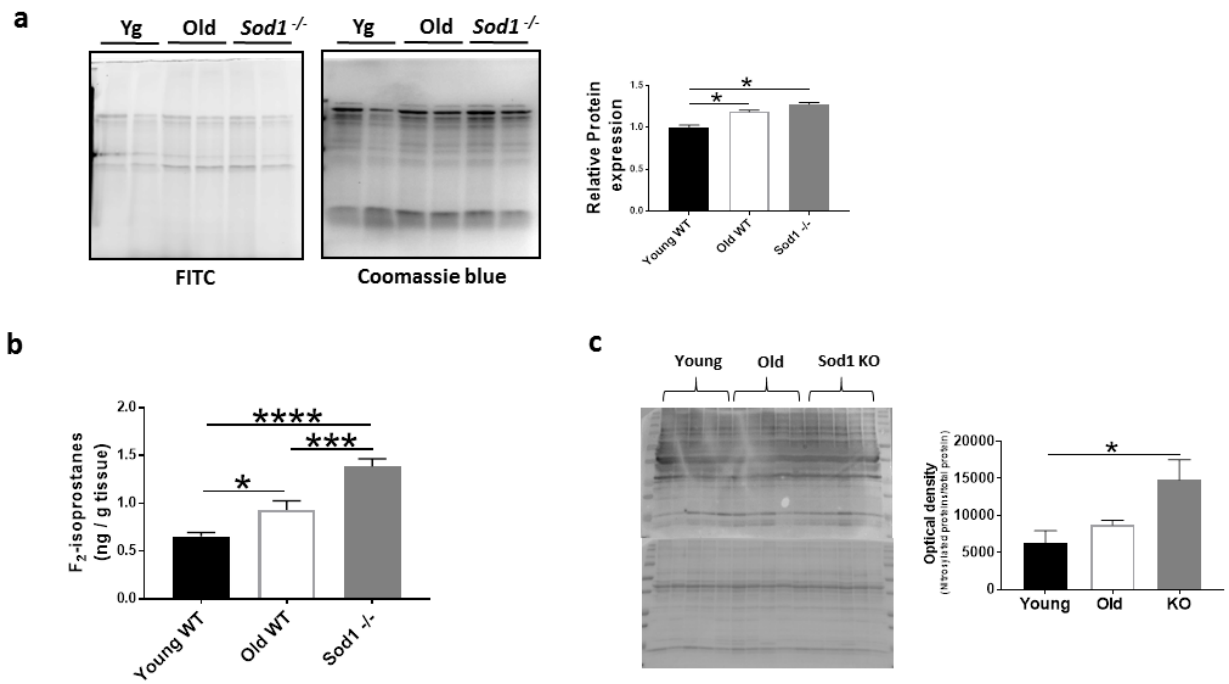
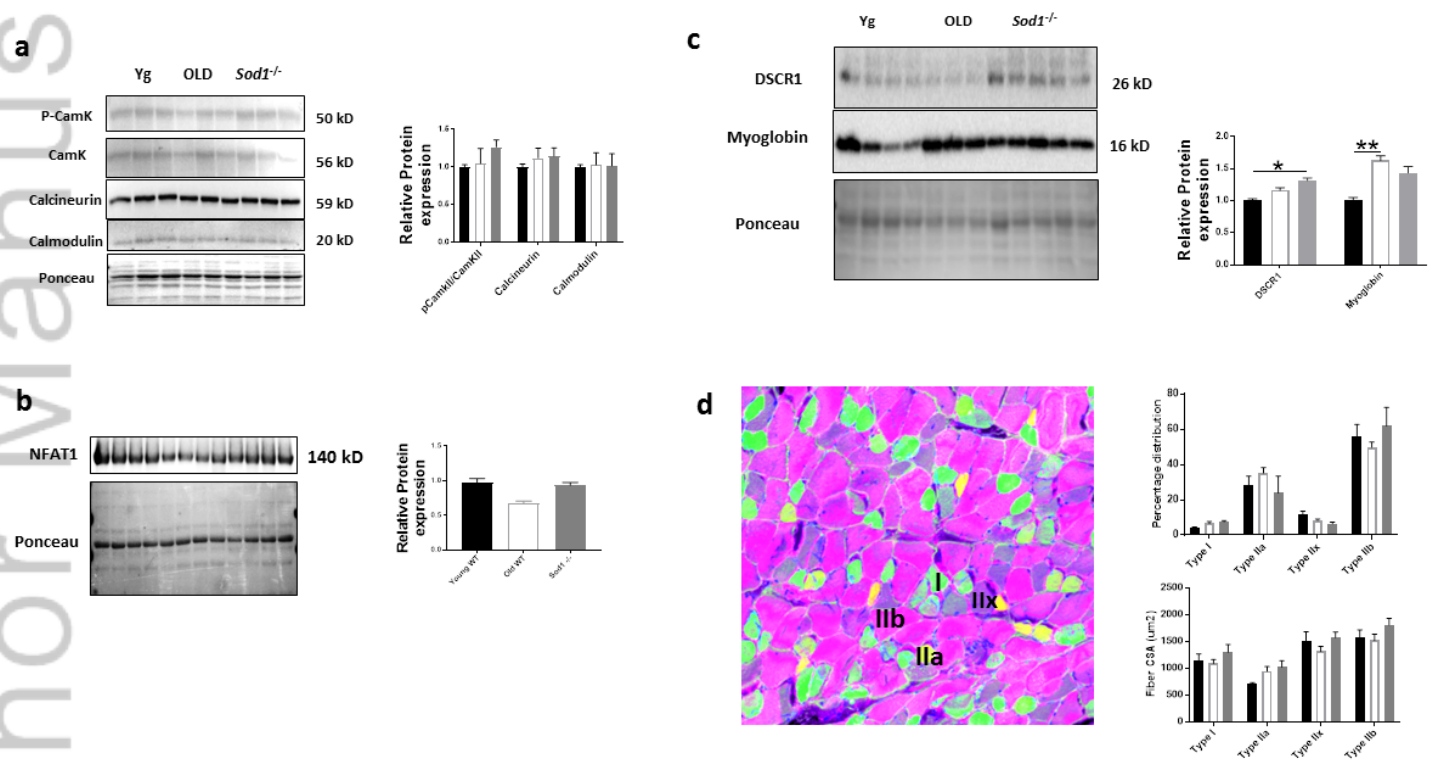


Figure 8



**Oxidative stress induced dysregulation of excitation-contraction coupling
contributes to muscle weakness**

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Table 1. Muscles contractile properties

Contractile properties from EDL and lumbricals muscles of young and old WT and *Sod1*^{-/-} mice. TTP: Time To Peak twitch force; RT_{1/2}: **Twitch** Half relaxation time; CSA: Cross Sectional Area; Pt: Absolute twitch force; Fo: Absolute tetanic force; sFo: Specific force; Values are expressed as Mean ± SEM (n = 8-15 per group). * $P \leq 0.05$, ** $P \leq 0.01$, vs. Young WT,; 1-way ANOVA.

	Young WT	Old WT	<i>Sod1</i> ^{-/-}
EDL			
Absolute Force (mN)	388.6 ± 15.3	301.4 ± 13.7*	309.4 ± 18.8*
Specific Force (N/cm ²)	24.4 ± 0.41	18.0 ± 0.75*	18.8 ± 0.49*
TTP (ms)	17.38 ± 3.11	18 ± 3.29	18.35 ± 3.2
RT _{1/2} (ms)	30.35 ± 2.06	25 ± 1.86	26.15 ± 3.73
Lumbricals			
CSA (mm ²)	0.335 ± 0.017		0.277 ± 0.018*
Pt (mN)	21.8 ± 0.9		18.4 ± 0.8**
TTP (ms)	21.1 ± 0.3		22.6 ± 0.5*
RT _{1/2} (ms)	32.7 ± 0.8		38.6 ± 1.2**
Pt /Fo	0.429 ± 0.009		0.451 ± 0.01
Fo (mN)	50.8 ± 1.8		40.7 ± 1.4**
sFo (N/cm²)	15.7 ± 1		15 ± 0.6