Nrf2 Deficiency Promotes Apoptosis and Impairs Pax7/MyoD Expression in Aging Skeletal Muscle Cells

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Abstract

Skeletal muscle redox homoeostasis is transcriptionally regulated by nuclear erythroid-2-p45-related factor-2 (Nrf2). We recently demonstrated that age-associated stress impairs Nrf2-ARE (antioxidant response element) transcriptional signaling. Here, we hypothesize that age-dependent decline or genetic ablation of Nrf2 leads to accelerated apoptosis and skeletal muscle degeneration. Under basal-physiological conditions, disruption of Nrf2 significantly down regulates antioxidants and causes oxidative stress. Surprisingly, Nrf2-*null* mice had enhanced antioxidant capacity identical to wild-type (WT) upon acute endurance exercise stress (AEES), suggesting activation of Nrf2-independent mechanisms (i.e. PGC1α) against oxidative stress. Analysis of pro-survival pathways under the basal state reveals decreased Akt levels, while pp53, a repressor of Akt, was increased in Nrf2-*null* versus WT mice. Upon AEES, Akt and p-Akt levels were significantly ($p<0.001$) increased (>10 fold) along with profound down regulation of pp53 ($p<0.01$) in Nrf2-*null* versus WT skeletal muscle, indicating the onset of pro-survival mechanisms to compensate the loss of Nrf2 signaling. However, we found a decreased stem cell population (Pax7) and MyoD expression (differentiation) along with profound activation of ubiquitin and apoptotic pathways in Nrf2-*null* versus WT mice upon AEES, suggesting that compensatory pro-survival mechanisms failed to overcome the programed cell death and degeneration in skeletal muscle. Further, the impaired regeneration was sustained in Nrf2-null vs. WT mice after 1 week of

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post-AEES recovery. In an age-associated oxidative stress condition, ablation of Nrf2 results in induction of apoptosis and impaired muscle regeneration.

Introduction

About 40% of mammalian body mass is composed of skeletal muscle. With aging, healthy individuals exhibit 20-30% reduction in muscle mass (1). A decline in mass and function of skeletal muscle due to aging contributes to age-related musculo-skeletal diseases such as sarcopenia (muscle fiber atrophy), impaired regeneration of satellite cells, pathologic remodeling of synaptic neuronal structures, decreased protein synthesis, oxidative stress, mitochondrial dysfunction and impaired energy production (2). Skeletal muscle myocytes are dynamic cells that respond to a variety of stresses by inducing compensatory mechanisms (2-7). One of the primary stresses of skeletal muscle is oxidative stress; the response to this stress, including an antioxidant defense response, is critically regulated by nuclear erythroid-2-p45-related factor-2 (Nrf2) (8, 9).

Regulation of Nrf2 signaling is believed to preserve redox homeostasis and protect the structure and function of skeletal muscle. Recently we demonstrated that while disruption of Nrf2 has a minimal role on the mouse skeletal muscle antioxidant defense system at a young (2 months) age, Nrf2 disruption promotes oxidative stress and impairs antioxidant mechanisms upon aging (8, 9). In general, Nrf2 signaling is dependent on its activation in response to reactive oxygen species (ROS) generation, and toxic and electrophilic stresses (10-14). Excessive production of reactive oxygen and nitrogen species (ROS/RNS) cause oxidative or nitrosative stress, which are key signals for the onset of several musculoskeletal diseases (15, 16). Upon aging, the magnitude of generation and accumulation of ROS is significantly higher in aged Nrf2-*null* mice compared to WT controls. This increased oxidative stress is accompanied by a striking decline of several antioxidants due to low levels and poor activation of cellular defense mechanisms (8, 9). However, it is unknown whether Nrf2-independent pathways are activated as compensatory measure in response to aging, endurance exercise and oxidative stress.

It has been previously shown that apoptotic events are directly linked with the cellular aging of post mitotic tissues such as brain, heart and skeletal muscle (17-30). A decline in the number of viable, fat-free myocytes results in skeletal muscle dysfunction. We have shown that induction of pro-apoptotic (BAD, BAX) and apoptotic (ASK1, AIF caspase-3 and 9) signals in aged Nrf2-*null* mice are due to increased generation/accumulation of ROS/RNS and glutathione (GSH) depletion (9). Further, in Nrf2-*null* mice age-associated oxidative stress and apoptotic events are accompanied by increased ubiquitination of a wide range of proteins and lipid peroxidation (4-hydroxy nonenol, 4-HNE) (9). Increased ROS/RNS generation and prolonged oxidative/nitrosative stress contribute to damage and dysfunction of organelles and cells in several age-associated diseases in humans (4, 15, 31-36). However, it is currently unknown whether Nrf2 is required for proliferation and differentiation of myoblasts undergoing severe oxidative stress.

Typically, in response to chronic oxidative stress and skeletal muscle injury, it is expected that factors for regeneration (satellite cells) and differentiation (MyoD lineage) to be

activated to overcome the loss of myoblasts. The role of Nrf2 in such conditions has not been defined. Skeletal muscle progenitor stem cells express cell context-specific genes involved in tissue regeneration (37). Embryonic skeletal muscle requires paired-box transcriptional factors (Pax3 and Pax7) and canonical myogenic regulatory factors (MRFs; Myf-5, MyoD, myogenin and Mrf4) for its development (38). During regeneration of skeletal muscle, Pax7 and MyoD expressed in quiescent and activated satellite stem cells specifically determines and regulates cell lineage during skeletal muscle regeneration (39-42). However, it is currently unknown if the satellite cell pool is affected by aging and/or loss of Nrf2. Thus, using a genetic approach, we explored whether Nrf2 plays a critical role in skeletal muscle damage or repair in response to endurance stress on aging. We demonstrate that Nrf2 is an important regulator of redox state and determines stem cell lineage during the regeneration of aging skeletal muscle.

2. Materials and Methods

2.1 Animals

Nrf2-deficient (*Nrf2- null*) mice with a hybrid background were originally generated by Itoh and colleagues (43) and generously provided by Dr. Li Wang (University of Utah, Salt Lake City, UT). They were backcrossed with the C57/BL6 mice to the fifth generation. Nrf2-*null* mice and their littermate controls, *Nrf2*+/+ (wild-type) used in this study were obtained from intercrossing Nrf2+/- mice. Genotyping of mice was performed by PCR amplification of genomic DNA using the following primers: Nrf2 forward: 5-GCCTGAGAGCTGTAGGCCC-3, Nrf2 reverse: 5-GGAATGGAAAATAGCTCCTGCC-3 and Nrf2 mutant: 5-GGGTTTTCCCAGTCACGAC-3. The mice were housed under controlled temperature, humidity and a 12-hour light/dark cycle, and fed with a standard rodent diet and water *ad libitum*. Experimental protocols conducted on the mice were approved by IACUC at the University of Utah in accordance with the standards established by the U.S. Animal Welfare Act.

2.2 Antibodies and Reagents

The following antibodies and reagents were used: NQO-1 (ab34173), GAPDH (ab9485), lamin-B1 (ab16048), catalase (219010, Calbiochem, Merck GaA, Germany), CA, USA), SOD-1 (Enzo-CAD-SOD-100), SOD-2 (ab13534), GPX1 (ab22604), GSR (ab16801), PGC1α (ab54481), I-kBα (ab32518), I-kBβ (ab32518), Nrf1 (Kap-Tf125, Stressgen), AKT (CS#9272), pGSK3β (CS#9322), pp53 (CS#9282), BAX (CS#2772), BAD (CS#9292), ASK1 (CS#3762), PAX7 (ab34360), MyoD (ab64159), caspase-3 (CS#9662), caspase-9 (CS#9504), cleaved PARP (CS#9544s) and ubiquitin-ab (Enzo-BML-PW0150). Secondary antibodies conjugated with horseradish peroxidase IgG (Rabbit and Mouse/PI-1000 & PI-2000, Vector labs, Burlingame, USA) were used. A Bio-Rad Protein Assay (500-0006, Bio-Rad, Hercules, CA) was used to determine protein levels in skeletal muscle tissue extracts. Fluorescent probes – DCFDA, DHE and MitoSox Red to detect ROS (Molecular Probes/Invitrogen Corp). All reagents and primers for RNA extraction and real-time RT-PCR quantification were purchased from Qiagen Inc., Valencia, CA.

2.3 Acute endurance exercise stress (AEES) and preparation of tissues/samples for analysis

Wild type (WT, C57/Bl6/SJ) and Nrf2-*null* mice >23 months of age (n = 6/group) were subjected to acute endurance exercise on a treadmill for 2 weeks [∼90-120 minutes per day; 20-30m/min; 12° angle]. The duration of exercise was optimized based on the endurance of 23 months old mice. Mice performed run on a treadmill (Columbus Instruments, Columbus, Ohio) calibrated for angle and speed. Endurance capacity was determined in a group of non-trained mice (n 10/group) to identify a maximal running endurance capacity. Mice were started at a speed of 20m/min for first 10 minutes and every 2 min the treadmill speed was increased by 1.0 m/min and at an angle of 12° (not plain). In general, a soft brush is attached to the treadmill via the back door in order to encourage the animals to run when they stop. Mice were considered exhausted when they could no longer move forward from the back of the lane despite the manual prompting using the soft brush prodding. Mice that were reluctant to run on the treadmill, despite receiving stimuli, a tickle on the hind feet using brush were not included in the study. This protocol was followed to induce severe oxidative stress as opposed to moderate training [treadmill running at 10-15m/min speed; 0° angle (plain) for 45 minutes per day] that activates compensatory cytoprotective mechanisms such as Nrf2-antioxidant signaling (8). After exercise, mice were sacrificed and the skeletal muscles (gastrocnemius) were processed/frozen (−80°C) for subsequent analysis (44, 45). For histology, 10 mg portions of muscle were immediately washed and processed in EPR buffer and analyzed. A portion (10-20 mg) of the skeletal muscle tissue was placed into RNA-*later* solution and stored in −80°C for RNA extraction.

2.4 Detection of ROS using fluorescent probes (DCFDA, DHE and MitoSox Red)

Superoxide and peroxides in the skeletal muscle of old (>23 months) WT and Nrf2-*null* mice were determined immediately after last exercise (on day 14 of AEES) using DHE/H_2DCFDA fluorescence (D11347/C6827, Invitrogen Corp. USA) by following our previous reported protocols (46). Cell-permeable non-fluorescent DHE is oxidized to fluorescent 2-hydroxyethidium, which can be measured by appropriate excitation/emission filters for rhodamine. In the presence of superoxide (O_2^-.), oxidized ethidium intercalates with DNA, staining the nucleus a bright red fluorescence. H_2DCFDA oxidized to a fluorescent DCF by hydrogen peroxide is detected by fluorescence microscopy using appropriate excitation/emission filters as reported previously (47). Briefly, 5-μm-thick transverse sections of cryo-fixed skeletal muscle tissues on clean glass slides were covered with the probe solution containing 10 μM of DHE/H_2DCFDA and incubated in a light-protected chamber at 37°C for 30 min. MitoSox Red was used to determine the ROS generation in mitochondria. After washing with 1× PBS thrice, sections were fixed and mounted using DAPI containing Vector-Flouromount-G. Fluorescent images of the DCF and DHE were obtained using a FV-1000 laser scanning confocal microscope (Olympus Inc.). Fluorescence intensity was quantified by automated image analysis using Simple PCI 6 Imaging software (Hamamatsu Corporation, Sewickley, PA).

2.5 Gene expression analysis by quantitative real time PCR

Harvested skeletal muscle tissues were washed with RNase free PBS and stored in RNA*later* reagent. About 20 mg of skeletal muscle tissue from 24 month old WT and Nrf2-*null* mice (N=4-6) was used to extract RNA using Qiagen RNA isolation kits, following the manufacturer's instructions. The quality and quantity of the RNA was analyzed using a Nano drop-analyzer. To synthesize cDNA, the reverse transcription reaction was performed on 2.0 μg of RNA using a Qiagen Reverse Transcription Kit (205311) as per manufacturer's instructions. For qPCR analysis, 100 ng of cDNA template, 10 μL of SYBR Green Master Mix (Qiagen-204054) and respective Qiagen primer sets for NQO-1 (QT00094367), catalase (QT01058106), G6PD (QT00120750), GCLC (QT QT00130543), Gclm (QT00174300), Gpx1 (QT01195936), Sod-1 (QT01762719), Sod-2 (QT00161707), Gsr (QT01758232), TRXN1 (QT01060297), Gstα (QT0098987), Gst-mu (QT00121191), Pax7 (QT00147728) and MyoD1 (QT00101983) were used and analyzed in a Roche-Light Cycler. Copy numbers of cDNA targets were quantified using Ct values. Respective gene expression levels were calculated by normalizing to the level of the housekeeping gene Arbp1 (QT00249375) or GAPDH (QT01658692).

2.6 Skeletal muscle tissue homogenization and immunoblotting

Skeletal muscle cytosolic proteins from WT and Nrf2-null mice under sedentary or AEES conditions were prepared by homogenizing the tissues using cytosolic buffer (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.5 mM $MgCl_2$, with freshly prepared 1 mM dithiothreitol, 0.1 mM phenyl methylsulfonyl fluoride (PMSF) and 1% Triton-X100, pH 7.9), followed by centrifugation at 5000 rpm for 5-6 minutes. Cytosolic contaminants in the nuclear fraction were removed by washing with 4 volumes of cytosol buffer. Nuclear fractions were dissolved in nuclear extraction buffer (NEB; 20 mM HEPES, 420 mM NaCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 25% glycerol and 1 mM dithiothreitol, 0.5 mM PMSF, pH 7.9), incubated on ice with mild shaking and centrifuged at 8200rpm for 10 minutes. Then the proteins were quantified using Bio-Rad Bradford reagent. Protein samples for immunoblots were prepared in 4× Laemmli buffer with 5% freshly added β-mercaptoethanol and boiled for 5 minutes. About 30-40 μg of cytosolic proteins were separated on 10 or 12% SDS-PAGE and transferred to PVDF membranes. The membranes were treated with antibodies to catalase, SOD-1, GSR, G6PD, NQO-1, ubiquitin, γ-GCS, caspase-3/9, PAX7, MYOD1, GAPDH and lamin-B1. Horseradish peroxidase IgG (rabbit and mouse) conjugated secondary antibodies were used for chemiluminescence detection of specific proteins.

2.7 Immunoprecipitation and in-gel AKT kinase assay

AKT kinase activity in skeletal muscle was determined using an Akt/PKB protein kinase kit (Cat#9840, Cell-Signaling). Proteins (~100-200 μg) extracted from WT and Nrf2-*null* skeletal muscle were immunoprecipated by incubating with agarose beads containing anti-Akt-antibody overnight (12 – 14 hrs) at 4°C and gentle mixing. Next, antigen-antibody complexes bound to the immunosorbant beads was purified by repeated washing with lysis buffer (1× TBS) containing 0.1% Tween 20 (TBS/T), and the pellet was used for kinase assay. Precipitated AKT from WT and Nrf2-null skeletal muscles was treated with 10 μl of

10× kinase buffer (#9802), 2 μl of 10 mM ATP (#9804), 1 μl of Akt/PKB protein kinase (10 units/μl) and sterile water to make up to 100 μl. This reaction mixture was incubated for 30 or 60 minutes at 30°C and terminated by adding 25 μL 3× SDS sample buffer, vortex and centrifuged for 30 seconds at $14,000 \times G$. Then the phosphorylated GSK3α/β (Ser21/9) was detected by immunoblotting and quantified using Image-J.

2.8 Immunofluorescence analysis (IF) of PAX7 expression

Frozen skeletal muscle in OCT medium were sectioned at 5 μm thickness from WT and Nrf2-*null* mice > 23 months old and immunostained with PAX7 primary antibodies as previously described (46, 48). Briefly, skeletal muscle tissue sections were fixed in 4.0% paraformaldehyde for 10 minutes, permeabilized with 0.25% Triton X-100 in PBS containing 0.01% Tween-20 for 5 min, and blocked with 5% goat serum in 0.01% PBST for 60 min. Primary antibody diluted in 0.01% PBST containing 1% BSA was added to the tissue sections and incubated overnight at 4°C. After 3×5 minutes washes with PBST, the sections were incubated with secondary anti-mouse Alexa-Fluor 488-conjugated or anti-Rabbit Alexa-Fluor 647-conjugated antibodies (1:1000 dilution) for 1 hour at room temperature. After three washes (5 minutes each) with 1× PBST, the sections were mounted with DAPI containing mounting medium (Vector Shield) and imaged using a FV-1000 Confocal (Olympus Inc.) microscope at a magnification of 60× for visualization and specific localization of PAX7 (1:200). Intensity of the PAX fluorescence was quantified by automated image analysis using the Simple PCI 6 Imaging Software (Hamamatsu Corporation, Sewickley, PA).

2.9 Statistical analysis

Data are expressed as mean ± standard deviation. All data were statistically analyzed using a Student's t-test, and P-values 0.05 were considered statistically significant. For each parameter, the WT-sedentary group was compared to corresponding Nrf2-null mice. Additional comparisons also were made between the sedentary vs. AEES groups.

Results

Abrogation of Nrf2 exacerbates oxidative stress induced by acute endurance exercise stress (AEES) in WT and Nrf2-*null* mice

Wild type (WT) and Nrf2-*null* mice >23 months old were subjected to AEES for 2 weeks and the skeletal muscles (gastrocnemius) were used to evaluate the role of Nrf2 and AEES on oxidative stress. First, we analyzed cytoplasmic and mitochondrial-dependent reactive oxygen species (ROS) using DCFHDA, DHE and MitoSox Red fluorescent probes. In the cytoplasm, the ROS levels were similar among the skeletal muscle of WT and Nrf2-*null* mice (Fig. 1A). Further, AEES induced excess ROS generation in WT and Nrf2-*null* mice skeletal muscle indicating oxidative stress. Importantly, a much greater increase in ROS was observed in Nrf2-*null* when compared to WT following AEES. Co-localization of DHE and MitoSOX Red was noted in mice that underwent AEES in relation to sedentary group, indicating that mitochondria might be a major source of ROS in response to AEES (Fig. 1B).

Nrf2 knockout significantly alters the transcriptional bio-signature of antioxidants in response to AEES

Nrf2 is a master transcriptional regulator of several antioxidant and cytoprotective genes (13, 14, 43, 46, 49-52). To evaluate the molecular pathways involved in AEES-mediated oxidative stress in skeletal muscle myocytes, we analyzed transcript levels of Nrf2-dependent antioxidant genes. Real-time qPCR analysis using Qiagen primer sets for the targets of Nrf2 showed significant differences between the WT and Nrf2-*null* mice (Fig. 2). Under a basal state, most of the antioxidant genes (Nqo1, G6pd, catalase, Gpx1, Gclc, Gsr, Txn1 Gst-α, and Gst-μ) were significantly down regulated in Nrf2-*null* skeletal muscle when compared to that of WT at >23 months of age. Our previous findings indicated that the levels of antioxidant enzymes were comparable between WT and Nrf2-*null* mice at younger (2 months) age (9). Previously, we demonstrated that acute exercise stress for 2 consecutive days resulted in significant induction of Nrf2-dependent antioxidant genes in WT while Nrf2-*null* mouse hearts exhibited down regulation of these genes (8). Thus, we assessed the effect of AEES on transcriptional regulation of the antioxidants in skeletal muscle. Interestingly, in WT some of the antioxidant (Nqo1, catalase, Gsr, G6pd and Gst-μ) genes were upregulated significantly (p<0.05) after AEES in comparison to sedentary controls. In contrast, Nrf2-*null* showed either decrease (Nqo1, Gclm, Gst-α and Gst-μ) or blunted (Gclc and Txn1) transcription of most antioxidants in response to AEES. However, a few of the antioxidant genes (G6pd, catalase and Gpx1) were significantly upregulated in response to AEES when compared to sedentary Nrf2-*null* cohorts, suggesting Nrf2-independent mechanisms.

Ablation of Nrf2 and impaired transcriptional regulation of antioxidant genes results in diminished defense mechanisms

To verify whether abrogation of Nrf2 and AEES-mediated oxidative stress evokes an antioxidant protein response in skeletal muscle, we performed immunoblotting analyses for major antioxidant enzymes in WT and Nrf2-*null* sedentary or AEES mice. Under basal state, protein expressions of NQO1, catalase, G6PD, SOD1 and GPX1 were significantly decreased in Nrf2-*null* in relation to WT (Fig. 3A-B). Upon AEES, while protein levels for catalase increased, G6PD, GPX1, NQO1 and SOD1 were comparatively decreased in WT mice suggesting differential translational regulation of the antioxidant enzymes. Interestingly, most of the antioxidants (catalase, G6PD, SOD1 and GPX1) were significantly increased in Nrf2-*null* after AEES when compared to Nrf2-*null* sedentary mice. Thus, it appears that AEES induces the antioxidants through activating the antioxidant transcription machinery in skeletal muscle, as we have previously reported in the heart (12). However, this acute response is not sufficient to overcome the excess ROS generation and chronic oxidative stress (Fig. 1).

PGC1α is activated in response to acute endurance exercise stress in skeletal muscle

Next, we assessed whether alternative pathways to Nrf2 were activated in response to AEES. Since PGC1α and NF-kB have been proposed to mediate stress response induction of antioxidants (53-55), we determined their expression by immunoblotting. PGC1α is a master regulator of mitochondrial biogenesis (56-58). Under basal condition, Nrf2-*null*

skeletal muscle had lower PGC1α protein in comparison that of WT mice. Interestingly, PGC1α protein levels were significantly increased in WT and Nrf2-*null* skeletal muscle in response to AEES (Fig. 4A). These results suggest that activation of PGC1α might be a primary response in skeletal muscle during AEES. Further, as shown in Fig 4B, significant activation of IkBα and IkBβ, negative regulators of NF-kB, in WT and Nrf2-*null* was evident in response to AEES. These observations indicate that NF-kB is not activated by either AEES or Nrf2 abrogation.

AKT pro-survival and p53 pathways are activated in response to AEES

Next, we analyzed whether, in response to PGC1α activation, pro-survival mechanisms protect myocytes from oxidative stress caused by AEES and/or abrogation of Nrf2. Here, we probed the skeletal muscle proteins from WT and Nrf2-*null* sedentary and AEES mice for AKT, pGSK3 and pp53 (Fig. 5B). Under basal conditions, both transcript and protein levels for AKT were significantly lower in Nrf2-*null* in comparison to WT skeletal muscle (Fig. 5A-B). Upon AEES, WT and Nrf2-*null* skeletal muscle significantly increased protein levels for AKT. In response to AEES, increased AKT was associated with profound upregulation of phosphorylated GSK3β, a direct downstream substrate for AKT. In-gel kinase assays for AKT function indicated activation of AKT in response to AEES (Fig. 5C). Surprisingly, after AEES the AKT activity was markedly higher in Nrf2-*null* compared to WT mice. These results indicate excessive activation of pro-survival pathways under Nrf2 abrogation. In agreement with the decreased AKT levels in Nrf2-null skeletal muscle at rest, profound activation of pp53 was observed. Upon AEES, pp53 protein levels were significantly increased in WT when compared to sedentary mice. However, after AEES pp53 levels were drastically decreased in Nrf2-*null* mice suggesting possible diminution of cell cycle mechanisms.

Apoptotic pathways override the AKT/pro-survival mechanisms in Nrf2-*null* mice

Activation of AKT/pro-survival mechanisms is known to protect cells from apoptosis (59-62). Since pp53 levels were also dramatically decreased in Nrf2-*null* skeletal muscle following AEES, we next analyzed whether apoptotic pathways were activated under Nrf2 abrogation due to AEES-induced oxidative stress. Immunoblot analyses for BAX, BAD, ASK1, caspase-3/9 and cleaved-PARP were performed in skeletal muscle of WT and Nrf2-*null* mice before or after AEES (Fig. 6A-D). Under basal conditions, apoptotic markers (BAD, BAX, ASK1, caspse3/9 and cleaved-PARP) were significantly increased in Nrf2-*null* compared to WT mice. Upon AEES, most of the apoptotic markers were also substantially increased in Nrf2-*null* compared to WT mice. Although a moderate increase of apoptotic markers was observed in WT in response to AEES, these parameters were lower when compared to Nrf2-*null* mice. These observations indicate that activation of pro-survival mechanisms in response to AEES in Nrf2-*null* mice is inadequate to suppress the induction of apoptotic pathways.

AEES induced oxidative stress is associated with increased oxidation and ubiquitination of proteins

Chronic oxidative stress promotes ubiquitination of proteins (35, 63, 64). Degradation and subsequent removal of oxidatively modified or misfolded proteins are facilitated through the ubiquitin-proteasome systems (UPS). We examined whether Nrf2 abrogation or age-dependent increases in ROS results in oxidative modification and/or ubiquitination of skeletal muscle proteins. We determined the level of oxidation of skeletal muscle by measuring 4-hydroxynonenol (4-HNE) content in aged WT and Nrf2-*null* mice under sedentary or AEES conditions. There was a significant increase in 4-HNE positive proteins observed in Nrf2-*null* relative to WT mice under basal conditions. Robust induction of 4-HNE signals for a wide range of proteins (from 15 to 250 kDa) in old WT mice occurred in response to AEES. Upon AEES, Nrf2-*null* mice exhibited additional increase in 4-HNE positive proteins when compared to sedentary-Nrf2-*null* mice (Fig. 7A). Under resting state, ubiquitin-protein conjugates were significantly higher in Nrf2-*null* compared to WT skeletal muscle. Upon AEES, both WT and Nrf2-*null* mice had increased levels of ubiquitinated skeletal muscle proteins and the magnitude of ubiquitination was much greater in Nrf2-*null* compared to WT skeletal muscle (Fig. 7B). These results suggest that abrogation of Nrf2, coupled with induction of oxidative stress, could accelerate the age-associated augmentation of protein degradation pathways that contribute to skeletal muscle loss.

Nrf2 abrogation and AEES-induced oxidative stress mediated apoptosis impairs muscle regeneration and stem cell lineage

Regenerative capacity of skeletal muscle in response to oxidative stress or aging might require activation of quiescent muscle precursor cells (65-67). Activation of satellite cells is essential for regeneration, maintenance and repair of adult skeletal muscle damage due to aging and/or chronic stress states. Therefore, we analyzed the protein expression for PAX7 (stem cell marker) and MyoD (muscle differentiation factor) by immunoblotting. Under basal conditions, both the PAX7 and MyoD1 protein levels were significantly higher in Nrf2-*null* compared to WT skeletal muscle. However, after AEES, WT mice had increased PAX7 and MyoD1, but these levels were dramatically reduced in Nrf2-*null* mice (Fig. 8A). Next, we assessed the PAX7 population of skeletal muscle cells using immunofluorescence followed by microscopic examination (Fig. 8B). Under basal conditions, the number of Pax7 cells was higher in Nrf2-*null* than WT mice. In response to AEES, the Pax7 positive cell number significantly increased in WT, but decreased in Nrf2-*null* mice (Fig. 8B-C). Moreover, regenerative capacity was significantly decreased in Nrf2-*null* compared to WT mice. Although both the WT and Nrf2-*null* mice exhibited impaired regenerative capacity after AEES, the magnitude of decrease was significantly lower in Nrf2-*null* relative to WT mice (Fig. 8B-C). These results indicate that AEES abrogation of Nrf2 might be a confounding factor contributing to age-associated degeneration of myocytes. These results suggest that WT and Nrf2-*null* mice respond differently to AEES, and this could be related to the differences in the degree of oxidative stress and compensatory defense mechanisms in these genotypes.

Sustained impairment of skeletal muscle regeneration in Nrf2-*null* mice after post-AEES recovery

Since it is evident that AEES induced oxidative stress impaired the regeneration (PAX7) and differentiation (MyoD1) in aging skeletal muscle of Nrf2 deficient, we next sought to determine whether recovery from AEES normalize these regulatory markers. In the post-AEES recovery experiments, we determined transcript (Fig. 9A-B) and protein levels for PAX7 and MyoD1 (9C). We observed that AEES induced downregulation mRNA levels of PAX7 and MyoD1 were prolonged even after 1 week of recovery from AEES (Fig. 9A-B). However, the protein levels of PAX7 and MyoD1 in WT mice showed a trend of normalization in response to recovery from AEES (compare lane 1 vs 3; Fig. 9C). Notably, down regulation of these proteins were sustained in Nrf2-null mice versus WT (compare lane 3 vs lane 4; Fig. 9C) following recovery from AEES. Overall, these data suggest that abrogation of Nrf2 results in a delayed regeneration of the skeletal muscle.

Discussion

Oxidative stress associated pathological processes in the skeletal muscles of aged humans has been an important research focus for decades. While Nrf2-Keap1 pathway is one of the critical regulators of intracellular redox homeostasis in skeletal muscle (9, 68, 69), the age-dependent effect of Nrf2 signaling in skeletal muscle is poorly understood. We recently demonstrated that either ablation or age-dependent decline of Nrf2 is coupled with increased oxidative stress and accelerated skeletal muscle degeneration through induction of apoptotic and ubiquitin mediated protein degradation pathways (9). However, the molecular cross talk between Nrf2-signaling and muscle differentiation or stem cell lineage has not been investigated previously in the context of acute stress/aging-mediated oxidative stress. In the present investigation, we demonstrate that (i) abrogation of Nrf2 induces oxidative stress in association with impaired antioxidant defense mechanisms upon aging; (ii) acute endurance exercise stress (AEES) in aged mice differentially regulate the transcription and translation of antioxidants; (iii) PGC1α-dependent mechanisms are activated in response to AEES and (iv) apoptotic signaling overrides PGC1α and AKT dependent cytoprotection/pro-survival associated with impaired stem cell activation and muscle differentiation in Nrf2-*null* mice. We also demonstrate that abrogation of Nrf2 in combination with oxidative stress aggravates age-associated muscle degeneration and impairs stem cell-based regenerative mechanisms.

Stress-mediated activation of PGC1α is an alternate mechanism to Nrf2 signaling, but it is not sufficient to protect myocytes from aging or oxidative stress-associated injury and apoptosis

Since the discovery of Nrf2 as a potential transcriptional regulator of antioxidant genes, it has been demonstrated that other transcription factors including AP1, PGC1α and NF-kB are also major redox responding factors responsible for transcription of antioxidant genes (53-55). In particular, it has been proposed that PGC1α is critical for the biogenesis of mitochondria and skeletal muscle function (57, 58). In this study, augmentation of PGC1α was observed in both WT and Nrf2-*null* mice in response to AEES, suggesting this factor responds to AEES independent of Nrf2. Furthermore, it is conceivable that either gain or loss of Nrf2 may not have a direct role on PGC1α function. The role of NF-kB-mediated

transcription of antioxidant genes in the context of aging muscle regeneration under forced stress or Nrf2 ablation is not known. Here, we demonstrate that NFkB is not directly involved in antioxidant transcription as indicated by the activation of IkBα and IkBβ, repressors of NFkB in response to severe oxidative stress. The magnitude of increase in IkBα and IkBβ was higher in Nrf2-*null* mice upon AEES, indicating inhibition of NFkB-dependent cytoprotective mechanisms in skeletal muscles. Taken together, these results demonstrate that PGC1α, at least in part, is an alternative mechanism to Nrf2 signaling in response to severe oxidative stress, aging and AEES.

Apoptotic pathways override the AKT/pro-survival mechanisms under Nrf2 diminution or ablation

Recently, we demonstrated that loss of Nrf2 with aging is associated with increased ubiquitination, lipid peroxidation and activation of apoptotic cascades in the heart (8). Several reports have indicated that oxidative stress and other pathological conditions are strongly correlated with protein degradation and increased cell death (15, 16, 34, 35, 63, 70). However, there is limited information on Nrf2 signaling in skeletal muscle regeneration with aging. Using older (>23 months) WT and Nrf2-*null* mice, we investigated the molecular pathways involved in these events. We demonstrated that loss of Nrf2 activates pro-survival pathways under AEES, as shown by induction of AKT and pGSK3β, which was accompanied by activation of pp53 dependent cell cycle pathways. This increased AKT expression after AEES may reflect compensatory activation of pro-survival pathways due to stress. Further, the AEES-induced pro-survival mechanisms were greater in Nrf2-*null* compared to WT skeletal muscle. We expected that activation of antioxidant and pro-survival pathways in response to stress might be associated with diminished apoptotic signals. Therefore, we further analyzed apoptotic pathways under basal and stressed conditions in WT and Nrf2-*null* mice. At basal conditions, Nrf2-*null* mice exhibited increased apoptosis as shown by increased BAX, BAD, Caspase-3 and cleaved PARP. These effects were further magnified with AEES in Nrf2-*null* but not WT mice suggesting that abrogation of Nrf2 increased cell death. This likely account for the significant structure alterations we observed in Nrf2-*null* skeletal muscle and perhaps functional changes.

Nrf2 signaling is necessary to protect stem cell lineage and muscle differentiation under stress conditions

Effective regeneration of skeletal muscle could occur through activation and proliferation of satellite cells (7, 67, 71). Defects in activation or a decrease in number of satellite cells results in reduced muscle regeneration. It has been reported that redox-dependent regulation is involved in self-renewal of stem cells and myogenic differentiation (72-78). Upon skeletal muscle injury, activated satellite cells rapidly enter the cell cycle and proliferate, augmenting skeletal muscle in the presence of Pax7 (78-82). Further, activated satellite cells possess the ability to induce the downstream genes such as Myf5 and MyoD, which promote differentiation into myoblasts (83, 84). Pax7 expressing satellite cells are necessary for muscle regeneration (85). We postulated that chronic oxidative stress plays a role in stem cell renewal. Our results indicate that loss of Nrf2 signaling is associated with increased oxidative stress over time that facilitates a compensatory activation of stem cells as indicated by increased Pax7 positive cells. This suggests that moderate pro-oxidative states are able to

promote expression of stem cell lineage cells even in the absence of Nrf2 signaling, indicating Pax7 can be regulated by an Nrf2-independent pathway. Interestingly, the observed increase in Pax7 and MyoD in WT skeletal muscle in response to AEES suggests that induction of moderate oxidative stress might have a key role in activation of satellite cells and muscle regeneration. However, more intense oxidative stress in Nrf2-*null* mice induced by AEES is associated with attenuation of Pax7 and MyoD expression. Interestingly, MyoD expression is tightly correlated with Pax7 expression in both Nrf2 abrogation and AEES conditions. Further, Nrf2-*null* mice displayed a delayed rescue of regeneration after withdrawal of AEES due to overwhelming oxidative stress that is a consequence of loss of Nrf2 and aging. Therefore, we suggest that Nrf2 signaling is necessary to protect stem cells by diminishing harmful oxidative stress conditions. Clearly, while mild oxidative stress favors stem cell and MyoD lineage, severe oxidative stress (Nrf2 abrogation) is detrimental to these mechanisms. Taken together, these findings implicate a role for Nrf2 in the regulation of stem cell lineage and muscle differentiation during aging.

Conclusions

In summary, we identify Nrf2 as a critical component in the transcriptional signaling of antioxidants to maintain skeletal muscle during AEES. Although Nrf2-independent pathways such as PGC1α and AKT are activated in response to AEES, this induction is insufficient to prevent subsequent onset of apoptosis in aging skeletal muscle. Furthermore, significant activation of apoptotic pathways overrides the PGC1α and AKT signaling and promotes cell protein degradation as indicated by increased ubiquitination and lipid peroxidation. In summary, suppression of survival and activation of apoptotic signals along with ubiquitination and oxidation of proteins impair skeletal muscle regeneration through decreasing Pax7 and MyoD lineage. This study supports Nrf2 as a key player in skeletal muscle protection and opens up new avenues for potential therapeutic treatment of aging skeletal muscle disorders.

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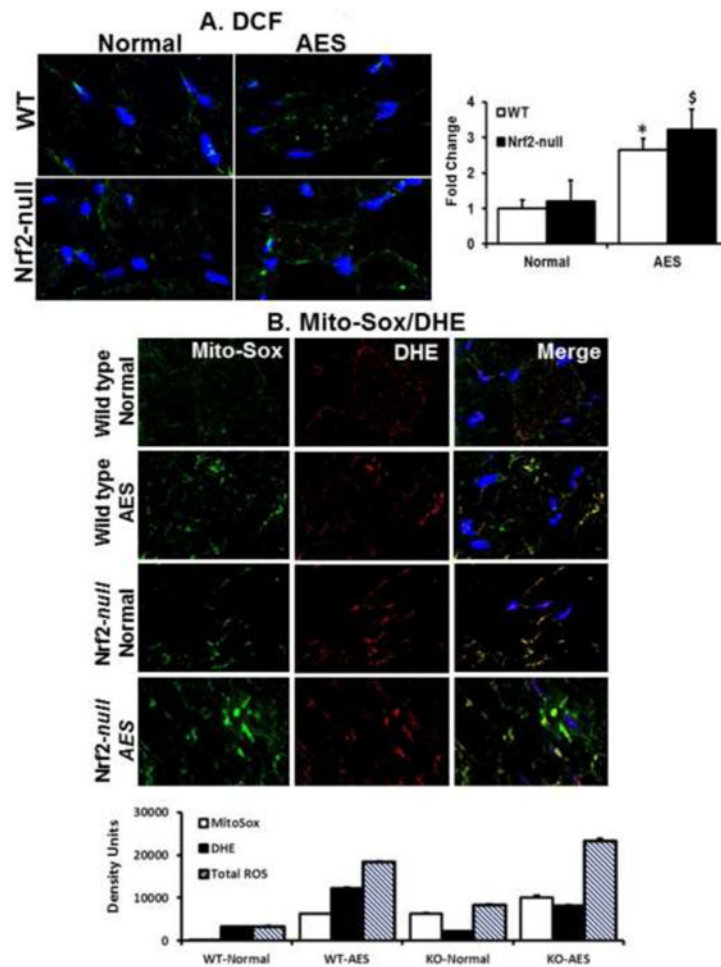
Figure 1. Aging and lack of Nrf2 increases oxidative stress in skeletal muscle

A. Determination of ROS in the skeletal muscle tissue sections using fluorescent (dihydro difluro diacetate (H2DCFDA)/dihydroethidium (DHE) probes and microscopy. Frozen tissue sections were incubated with 10μM of H2DCFDA for 30 min at 37° C. Sections were then washed with 1XPBS, mounted and analyzed by Zeiss 510 Meta confocal microscopy. Significantly increased ROS levels were seen in the WT and Nrf2-*null* mice in response to AEES compared with SED at 23 months (p<0.01), but these values were comparable between WT and Nrf2-*null* under sedentary condition.

B. Determination of the source of ROS generation using mitosox green and DHE fluorescent probes. Frozen sections of SM were stained with 10μM mitosox green and dihydroethidium (DHE) for 30 minutes and imaged using confocal microscope. Images from green and red fluorescence were merged (purple) to locate the mitochondrial source of ROS generation. The purple color is indicating ROS within the mitochondria. Both AEES and abrogation of Nrf2 showing increased ROS generation in the mitochondria of SM. Histograms showing density of the fluorescent signals from WT and Nrf2-*null* mice under sedentary and AEES conditions. The data are from n=3 mice/group and *p<0.05.
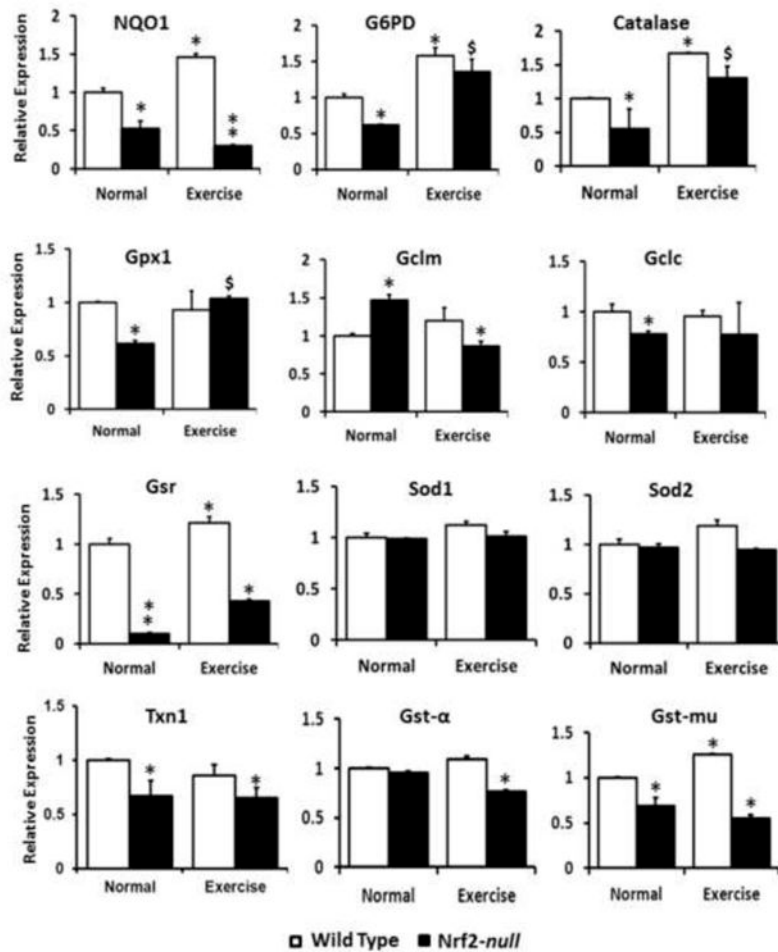
Figure 2. Abrogation of Nrf2 down regulates the bio-signature of antioxidants upon AEES
Real-time RT-PCR determinations of Nrf2 target genes in WT and Nrf2-*null* mice under
sedentary and AEES conditions at >23 months were performed using Qiagen-mouse primer
sets (n=4/gp.). Data were first normalized to Arbp1 expression and then to the corresponding
gene expression in the WT group (Delta-delta-CT method). While there is an increasing
trend in the messenger RNA (mRNA) expression for major targets of Nrf2 (i.e. catalase,
G6pd, Nqo1 and Gst-mu) were significantly upregulated in WT upon AEES, most of these
genes were down regulated in Nrf2-*null* mice under sedentary or AEES conditions. The data
are from n=3-4 mice/group. (*p<0.05 vs. WT-SED).
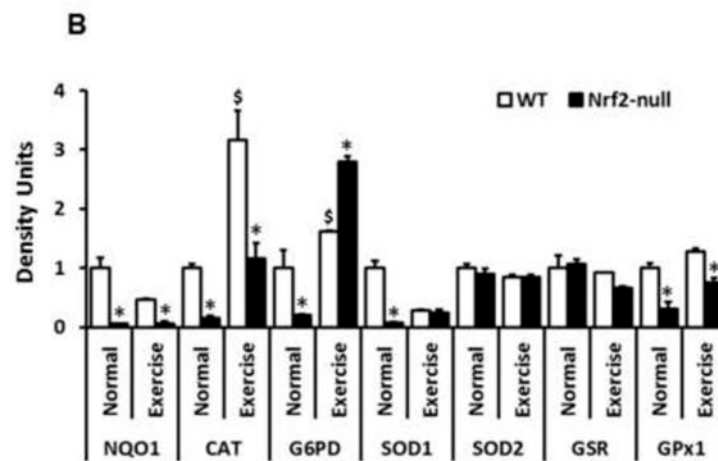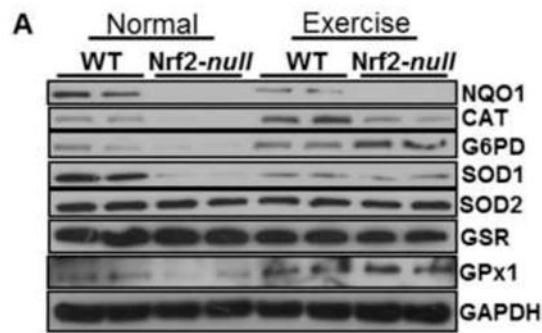
Figure 3. Abrogation of Nrf2 impairs the protein levels of antioxidants

A) Representative Immunoblotting (IB) experiments of cytosol from WT and Nrf2-*null* mice under sedentary and AEES conditions at >23 months were performed using appropriate antibodies (n=4/group.). Protein blots were probed with anti-Nqo1, catalase, glucose-6-phosphate dehydrogenase, super oxide dismutase-1/2 (Sod-1/2), glutathione reductase, glutathione peroxidase and GAPDH. Each lane indicates an individual mouse.

B) Densitometry analysis was performed as relative intensity values calculated as mean arbitrary units obtained from the IB shown in (A). Significant decreases in NQO1, CAT, G6PD, SOD1 and GPX1 in Nrf2-*null* when compared to WT were evident under sedentary state. AEES upregulates most of the antioxidant proteins, but this effect was moderate in Nrf2-*null* mice. (*p<0.05, **p< 0.01, vs. WT-SED).
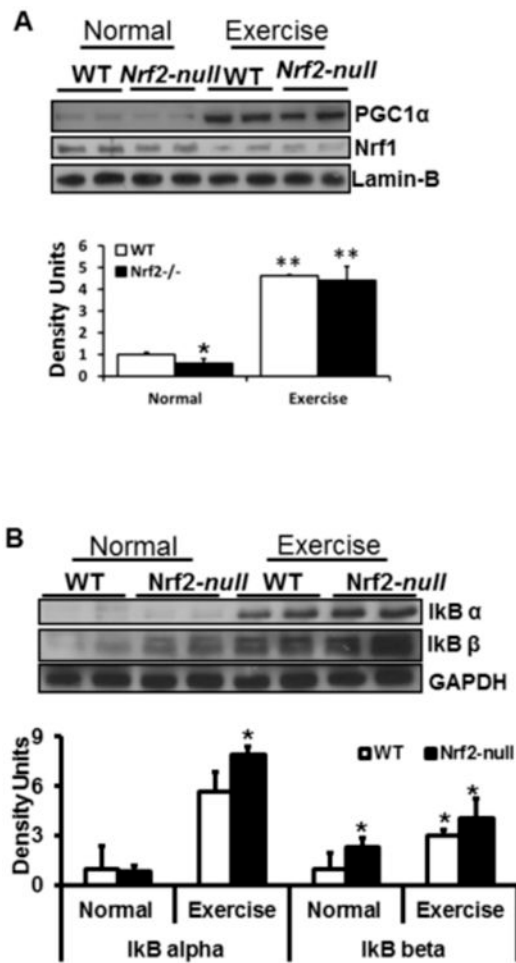
Figure 4. Proliferator–activated receptor co-activator-1-alpha (PGC1α) is activated in response to acute exercise stress in skeletal muscle

A-B) Analysis of nuclear levels of PGC1α and Nrf1 in WT and Nrf2-*null* mice under sedentary and AEES conditions at >23 months of age. Representative Immunoblotting (IB) experiments from nuclear extracts were performed using anti-PGC1α and anti-Nrf1 and lmin-B (A), and anti-IkBα, IkBβ, and GAPDH antibodies (n=4/group.). In sedentary mice, PGC1α levels were decreased in Nrf2-*null* versus WT mice. AEES augmented the PGC1α and IkBα/β levels in both genotypes. Each lane indicates an individual mouse. Protein blots/ values represent n=4 or more mice from each group. *p<0.05; **p<0.01 between WT-SED vs. other experimental groups.
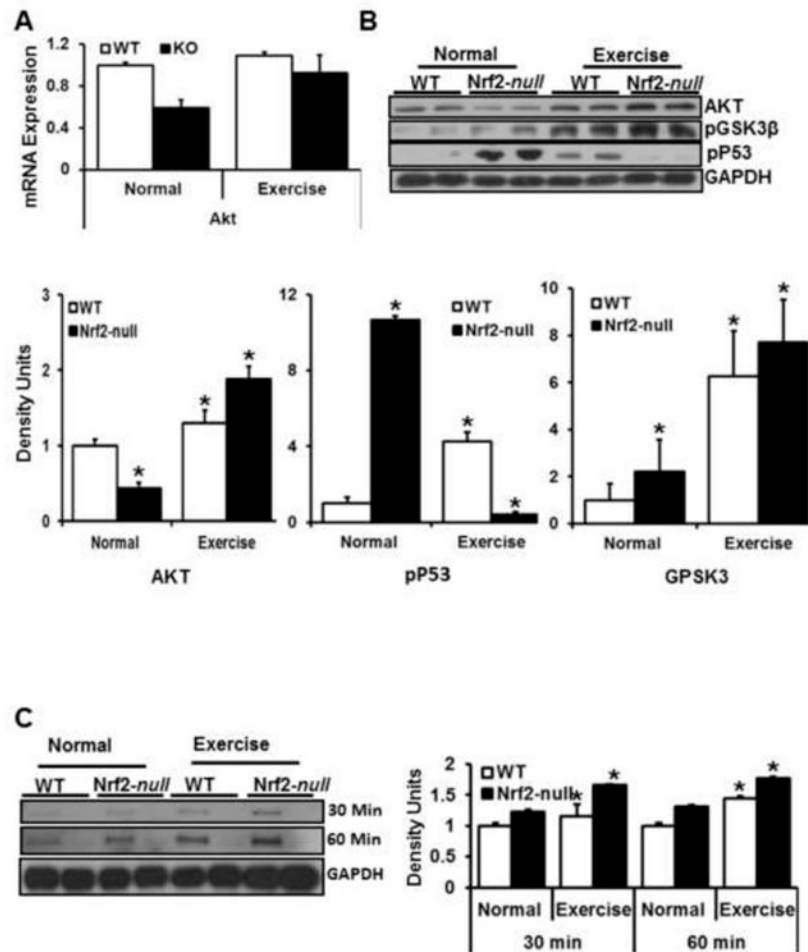
Figure 5. Acute exercise stress augments AKT pro-survival and P53 signaling in skeletal muscle
A. QPCR analysis using Qiagen primers showing down regulation of AKT in Nrf2-*null*
versus WT mice under sedentary condition. AEES upregulates AKT mRNA in Nrf2-*null*,
but has no effect on WT mice. N=3-4 mice/group; *p<0.05 vs. WT-SED.
B. Representative immunoblots (IB) and corresponding densitometry analyses for AKT,
phosphor-GSK3β and phospho-P53 in WT and Nrf2-*null* mice under sedentary and AEES
conditions. Upon AEES, pro-survival (AKT and GSK3β) mechanisms were activated along
with decreased cell cycle (phospho-P53) mechanisms in Nrf2-*null* mice.
C. AKT kinase (in-gel) assay and Immunoblotting. Representative IB from WT and Nrf2-
null mice indicating time dependent activation of AKT on phosphorylation of GSK3β.
Increased AKT kinase function is evident in Nrf2-*null* versus WT mice after AEES. N=3
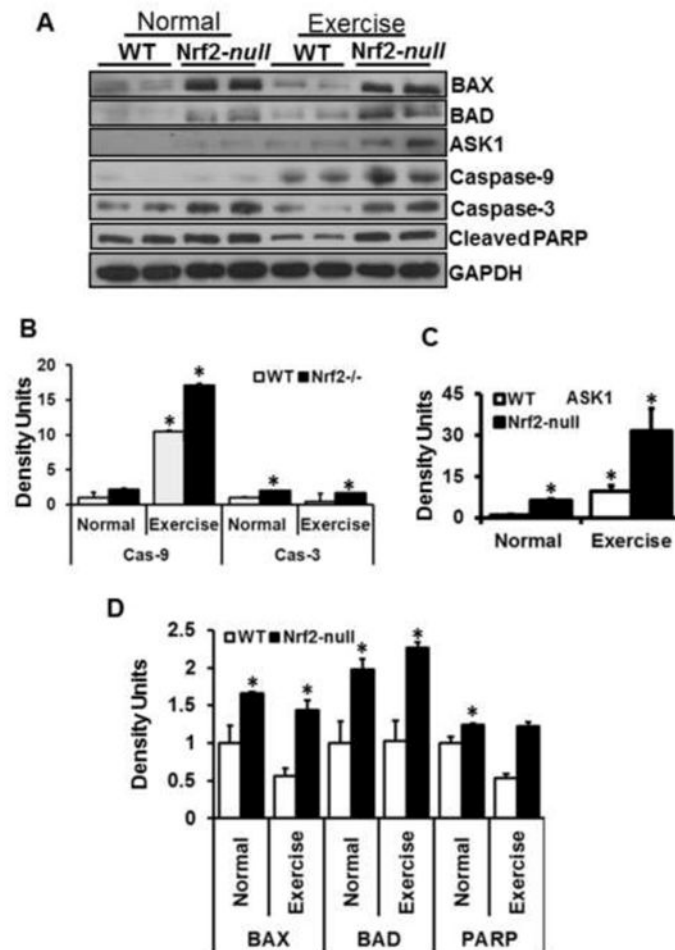mice/group; *p<0.05 vs. WT-SED.

Figure 6. Apoptosis revokes AKT/pro-survival mechanisms in Nrf2-*null* mice

A. Representative immunoblots of cytosolic protein extracts from skeletal muscles of WT and Nrf2-*null* mice under sedentary and AEES conditions. Protein blots were probed with respective antibodies as indicated. Individual lanes represent separate animals (n=4/group). Analyses of apoptotic pathway revealed significant changes (*p<0.05 vs. WT-SED) in various markers including BAX, BAD, ASK1, caspase-3, caspase-9 and cleaved PARP between WT vs. Nrf2-*null* and SED vs. AEES.

B – D. Densitometry analyses of representative protein signals were performed using image-J and expressed relative to mean values of the WT-SED group. Overall apoptotic signals were significantly (*p<0.05 – 0.01) increased in Nrf2-*null* vs. WT mice.
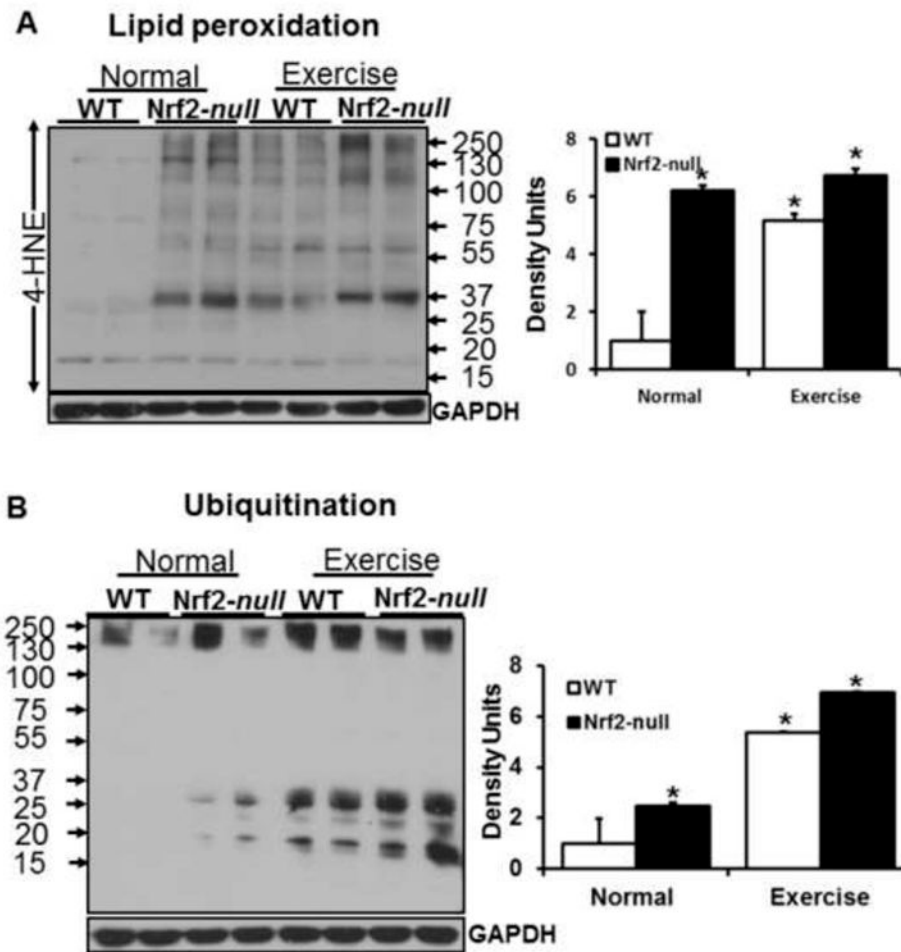
Figure 7. AEES induces oxidative stress and augments ubiquitination of proteins in Nrf2-*null* mice

Oxidative stress induced lipid peroxidation and ubiquitination in the skeletal muscles of Nrf2–/– mice. (A) Immunoblots probed for anti-HNE-ab showing ∼6 fold increased signals in skeletal muscles of Nrf2-*null* versus WT at 23 months of age. After AEES, significant increases of HNE-modified proteins are evident in the SM, but these were comparable among WT and Nrf2-*null* mice (n=4/group; *p<0.05; **p<0.01 vs. WT-SED).
(B) Immunoblots probed for mono-/poly-ubiquitination-ab revealing increased ubiquitination of SM proteins in Nrf2-*null* versus WT mice. Densitometry analysis showing ∼2 fold increase in poly-ubiquitinated proteins in aged Nrf2-*null* when compared to age matched WT Mice, but AEES resulted in increased ubiquitination in both genotypes with a higher efficiency in Nrf2-*null* mice.
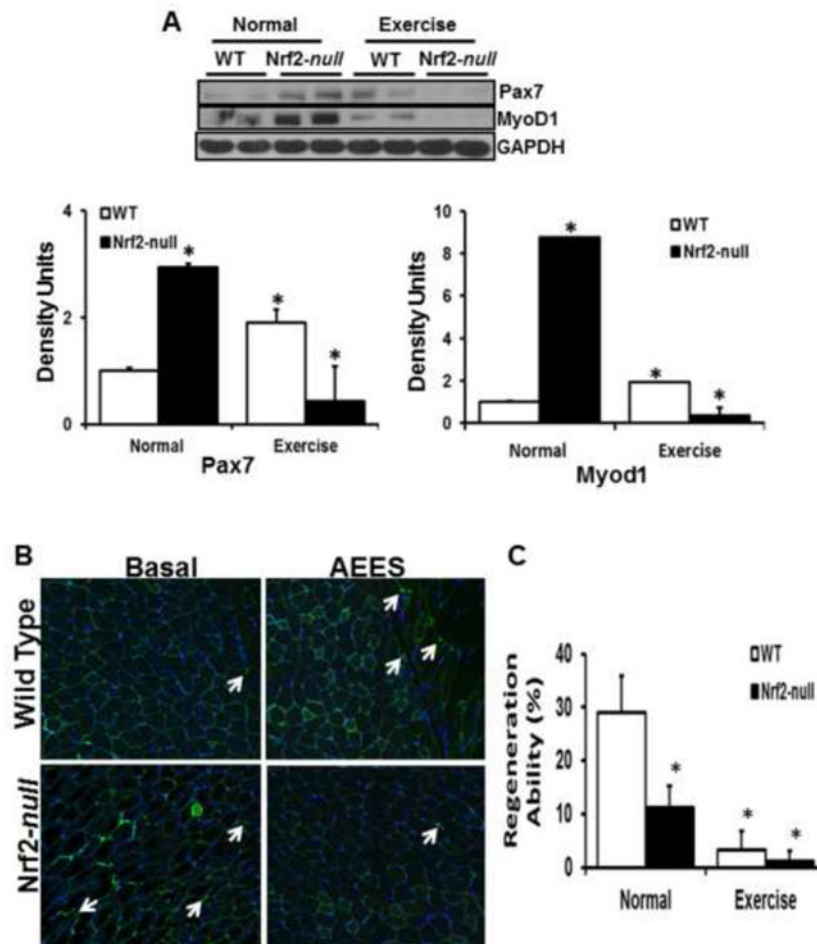
Figure 8. AEES and loss of Nrf2 diminishes stem cell lineage and muscle regeneration in Nrf2-*null* mice

Abrogation of Nrf2 and AEES modulates the expression of stem cell and muscle regeneration factors (PAX7 and MYOD). (A) Representative immunoblots showing significantly diminished PAX7 and MYOD proteins in SM of Nrf2-*null* versus WT mice in response to AEES (n=4/group; *p<0.05). (B) Immunofluorescence analyses of PAX7 positive myocytes from WT and Nrf2-*null* mice (n=3 mice/group; *p<0.05 vs. WT-SED). C. Histograms of regeneration ability for WT and Nrf2-*null* mice were determined by calculating the percent of myocytes with active nuclei. Decreased satellite cell population is evident in Nrf2-*null* in response to AEES. Green – PAX7 positive; red – laminin and blue – DAPI/nucleus.
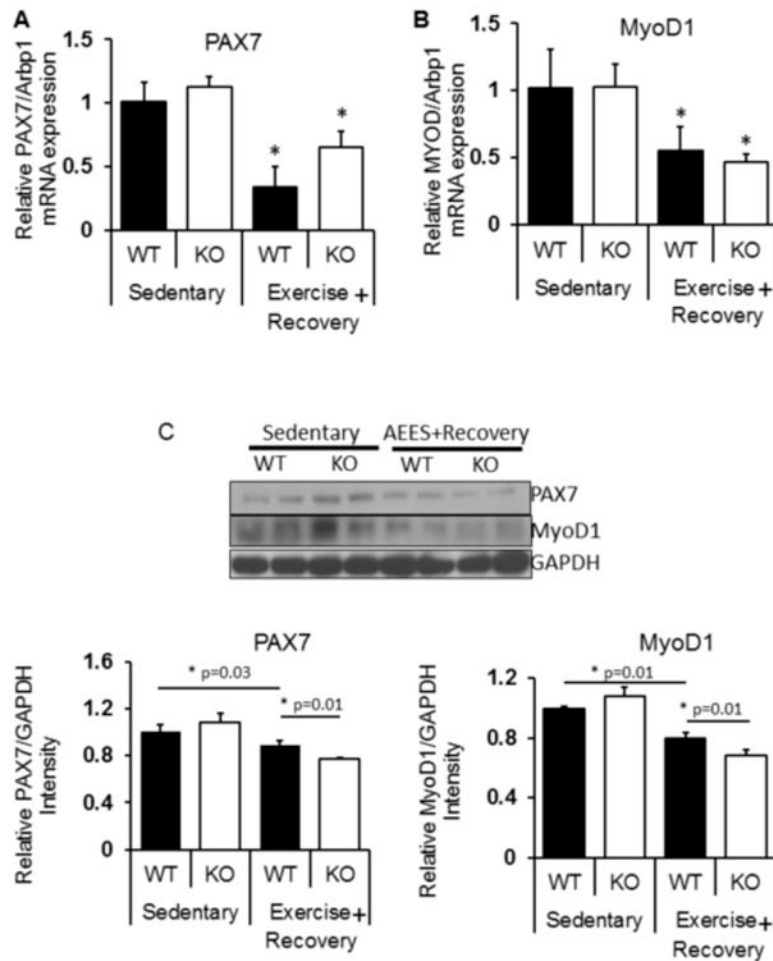
Figure 9. Sustained impairment of skeletal muscle regeneration in Nrf2-null mice after post-AEES recovery

Effect of recovery from AEES on skeletal muscle regeneration. In the post-AEES recovery experiments, we determined transcript (Fig. 9A-B) and protein levels for PAX7 and MyoD1 (9C).

(A-B) RTPCR experiments showing downregulation of mRNA levels (PAX7 and MyoD1) were prolonged even after 1 week of recovery from AEES.

(C) Representative Immunoblots for PAX7 and MyoD1 protein levels in WT mice showed a trend of normalization in response to recovery from AEES (compare lane 1 vs 3; Fig. 9C). Notably, down regulation of these proteins were sustained in Nrf2-null mice versus WT (compare lane 3 vs lane 4; Fig. 9C) following recovery from AEES. (n=4/group; *p<0.05).