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Disruption of Nrf2/ARE signaling impairs antioxidant mechanisms and promotes cell degradation pathways in aged skeletal muscle

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ABSTRACT

Age-associated decline in antioxidant potential and accumulation of reactive oxygen/nitrogen species are primary causes for multiple health problems, including muscular dystrophy and sarcopenia. The role of the nuclear erythroid-2-p45-related factor-2 (Nrf2) signaling has been implicated in antioxidant gene regulation. Here, we investigated the loss-of-function mechanisms for age-dependent regulation of Nrf2/ARE (Antioxidant Response Element) signaling in skeletal muscle (SM). Under basal physiological conditions, disruption of Nrf2 showed minimal effects on antioxidant defenses in young (2 months) Nrf2−/− mice. Interestingly, mRNA and protein levels of NADH Quinone Oxidase-1 were dramatically (*$P<0.001$) decreased in Nrf2−/− SM when compared to WT at 2 months of age, suggesting central regulation of NQO1 occurs through Nrf2. Subsequent analysis of the Nrf2-dependent transcription and translation showed that the aged mice (>24 months) had a significant increase in ROS along with a decrease in glutathione (GSH) levels and impaired antioxidants in Nrf2−/− when compared to WT SM. Further, disruption of Nrf2 appears to induce oxidative stress (increased ROS, HNE-positive proteins), ubiquitination and pro-apoptotic signals in the aged SM of Nrf2−/− mice. These results indicate a direct role for Nrf2/ARE signaling on impairment of antioxidants, which contribute to muscle degradation pathways upon aging. Our findings conclude that though the loss of Nrf2 is not amenable at younger age; it could severely affect the SM defenses upon aging. Thus, Nrf2 signaling might be a potential therapeutic target to protect the SM from age-dependent accumulation of ROS by rescuing redox homeostasis to prevent age-related muscle disorders such as sarcopenia and myopathy.

1. Introduction

An increase in the aged population is a major public health concern in the United States and across the world as well [1–3]. Age-dependent decrease in antioxidant mechanisms and profound accumulation of reactive oxygen/nitrogen species (ROS/RNS) are causally linked to various health problems, such as muscular dystrophy, sarcopenia, cancer, diabetes, cardiovascular and neurodegenerative diseases [4–8]. Major myocyte and muscular disorders in the elderly such as skeletal muscle weakness, sarcopenia, muscular dystrophy, and myopathy are ascribed to increased oxidative stress [9–15], but the underlying molecular mechanisms remain poorly defined.

Transcription factors that regulate antioxidant genes have been recently studied in humans and mouse models of disease [16–18]. One such factor, known as Nrf2, is regarded as a master regulator of antioxidant transcription [16,19–23]. Nrf2 binds to the antioxidant response element (ARE) in the promoter of target antioxidant genes and tightly regulates its transcription [24]. Though numerous studies have demonstrated a key role for Nrf2 in various disease models [17,22,25–30], its primary function in the context of aging skeletal muscle remains poorly understood. A recent study has reported a significant decrease of Nrf2 nuclear expression in elderly humans who lead a less-physically active lifestyle [31], while physically active humans exhibited improved Nrf2 function. Another study in rats has shown that aging is associated with impaired hepatic Nrf2 levels and GSH

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depletion [32]. These conditions were strongly coupled with significant down-regulation of gene and protein levels for GCLC and GCLM, subunits of GCL, the rate limiting enzyme for biosynthesis of GSH [32]. Kensler et al. [33] have observed similarities between older wild-type and Nrf2−/− mice suggesting age-dependent impairment of Nrf2-ARE signaling. However, the role of Nrf2/ARE signaling within the context of aging has not been investigated in the skeletal muscle.

Glutathione (GSH) is a ubiquitous small molecular thiol antioxidant whose biosynthesis is tightly controlled by its rate limiting enzyme γ-glutamyl cysteine ligase (γGCL), which is transcriptionally regulated by Nrf2 [34–36]. Abrogation or deficiency of Nrf2 is implicated in the impairment of GSH production and thereby alters the intracellular redox state [16,17,34,36]. In general, young animal and human SM tissues exhibit a homeostatic redox state by maintaining a balance between oxidants and antioxidants. On the other hand, excess production and accumulation of ROS in an aged condition leads to chronic pathological complications including loss of healthy myocytes, accumulation of oxidized proteins/lipids, DNA damage, etc. Skeletal muscle myocytes are enriched with mitochondria (accounts for 1/3 of the total weight of myocytes), which are one of the primary sources for ROS generation and therefore increase the susceptibility to age-dependent oxidative damage.

Aging promotes ROS/RNS accumulation in various tissues including skeletal muscle (SM), which contributes to chronic oxidative stress and muscle disorders [37–42]. Recent studies have observed that aging results in impaired function of Nrf2 in human skeletal muscle, vascular endothelial/smooth muscle cells of non-human primate (*Macaca mulatta*) and rat kidney [31,43,44]. We hypothesize that Nrf2-deficiency will lead to deregulation of skeletal muscle cytoprotective mechanisms upon age-induced oxidative stress. In this study, our primary goals are to investigate whether the Nrf2 pathway is an essential component of SM redox homeostasis and to assess whether there is age-dependent regulation of Nrf2/ARE-driven antioxidant signaling in SM. Understanding the molecular mechanisms for muscular dysfunction and pathology, that are linked with aging, will enhance our ability to design potential therapeutic strategies aimed at reversing age-related complications. Using age matched (~2 and >24 month old) WT and Nrf2−/− mice, we investigated the mechanisms for antioxidant regulation and cell degeneration in the skeletal muscle.

2. Materials and methods

2.1. Animals

Nrf2−/− mice were originally generated by Itoh et al. [20]. We obtained the Nrf2-deficient (*Nrf2*−/−) mice with a hybrid background from Dr. Li Wang (University of Utah, Salt Lake City, UT). They

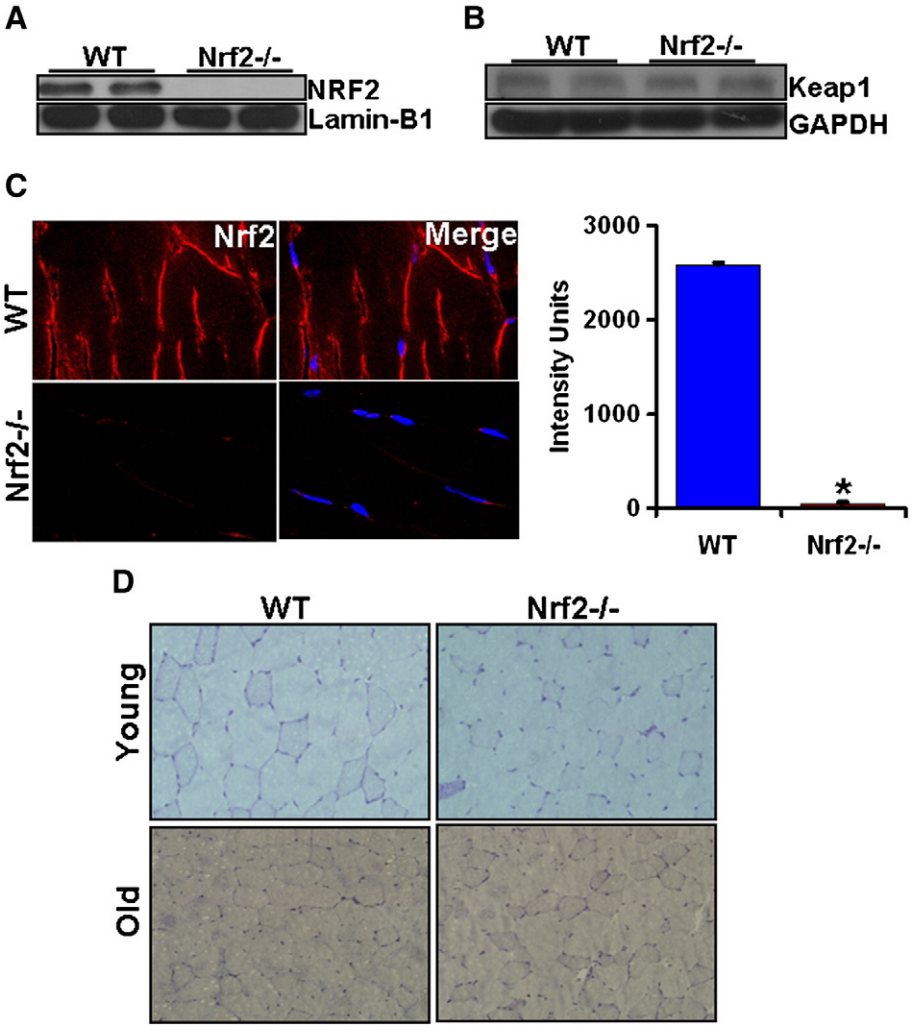

Fig. 1. Protein levels for Nrf2 and Keap1 in mice skeletal muscle (SM). Western blots showing protein expression for Nrf2 (A) and Keap1 (B) in 2 months old WT and Nrf2−/− mice SM. (C) Immunofluorescence analysis using anti-Nrf2-ab in SM sections from WT and Nrf2−/− mice confirmed Nrf2 expression in WT and abrogation in the KO. (D) Histological analysis (hematoxylin staining) of SM from young and old WT/Nrf2−/− mice. Myocytes from Nrf2−/− mice were relatively smaller than that of WT at both ages. Within the WT mice, myocytes from old had reduced size when compared to young mice. Data are mean ± SD for $n = 4–6$/group. *$P<0.001$.

were backcrossed with the C57/BL6 mice to the fifth generation in our laboratory. *Nrf2*−/− (knockout) male mice and their littermate controls, *Nrf2*+/+ (wild-type) used in this study were obtained from intercrossing Nrf2+/− (heterogenous) mice. The mice were genotyped for Nrf2 expression by PCR amplification of genomic DNA using the following primers: Nrf2 forward: 5-GCCTGAGAGCTGTAGGCCC-3, Nrf2 reverse: 5-GGAATGGAAAATAGCTCCTGCC-3, Nrf2 mutant: 5-GGGTTTTCCCAGTCACGAC-3. WT and Nrf2−/− mice were housed under controlled temperature, humidity and a 12-hour light/dark cycle. They were fed with a standard rodent diet and water *ad libitum*. At indicated time points (2 and >24 months of age), age matched WT and Nrf2−/− male mice were sacrificed and the skeletal muscle was isolated. All 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

We have used the following antibodies and reagents: Nrf2-ab (SC-722, Santa Cruz Bio, SC, USA), Keap-1 (10503-2-AP, Proteantech, Chicago, USA), NQO-1 (ab34173), GAPDH (ab9485), lamin-B1 (ab16048), Catalase (219010, Calbiochem, Merck GaA, Germany), γ-GCS (RB-1697-P1, Labvision/Neomarkers, CA, USA), SOD-1 (Enzo-CAD-SOD-100), caspase-3, caspase-9 (Cell Signaling Tech-9662/9504) and ubiquitin-ab (Enzo-BML-PW0150). Secondary antibodies conjugated with horseradish peroxidase IgG (Rabbit and Mouse/PI-1000 and PI-2000, Vector labs, Burlingame, USA) were used. Radical detecting EPR probes—CMH (NOX-2.1), TEMPOL (705748, Sigma-Aldrich, St. Louis, MO, USA), EPR grade water (NOX-7.7.1), Krebs-HEPES buffer (NOX-7.6.1), DETC (NOX-10.1), DF (NOX-9.1), glass capillary tubes (NOX-G.3.1) and critoseal (NOX-A. 3.1-VP) were purchased from Noxygen Diagnostics, Germany. Bio-Rad Protein Assay (500-0006, Bio-Rad, Hercules, CA) was used to determine protein levels in skeletal muscle tissue extracts. All reagents and primers for RNA extraction and real-time RT-PCR quantification were purchased from Qiagen Inc., Valencia, CA.

2.3. Preparation of tissues/samples for analysis

Young (2 months) and old (>24 months) wild type (WT) and Nrf2−/− mice (n=6–8/group) were sacrificed. The skeletal muscle was then excised and processed/frozen for subsequent analysis [45,46]. For EPR analysis, 10 mg portions of muscle were immediately washed and processed in EPR buffer and analyzed soon after. For

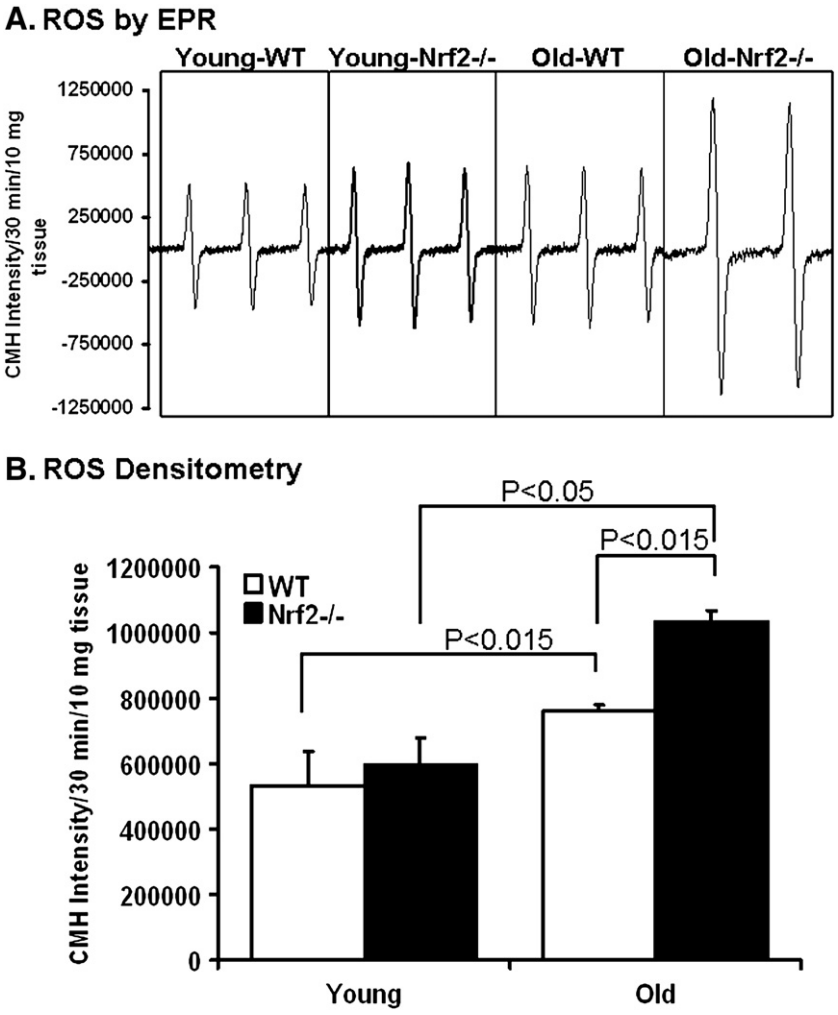

Fig. 2. Effect of Nrf2 and age on ROS levels determined by electron paramagnetic resonance (EPR) spectroscopy. SM tissues were processed in EPR buffer and incubated with CMH (spin-trap) to form adducts with ROS (superoxide and hydroxide) and measured in EPR. Increasing trend, but statistically non-significant, in ROS levels observed in Nrf2−/− SM when compared to WT in young mice (A-B). The ROS levels were significantly (P<0.015) increased in Nrf2−/− when compared to WT at >24 months of age. Data are mean ± SD for n=4–6/group. C. Detection of ROS using redox sensitive fluorescent probes: Sections from frozen SM of WT and Nrf2−/− mice at 2 and 24 months of age were treated with DCFH-DA (hydroperoxides) and DHE (superoxide) were imaged using confocal microscopy and quantified by automated image analysis using the Simple PCI 6 Imaging Software. Data indicate similar trend in the ROS levels as shown in EPR results. When compared to young, the old WT and Nrf2−/− SM showing increased DCF and DHE fluorescence suggesting age-dependent increase. At both ages, Nrf2−/− SM had significantly increased ROS when compared to WT. Data are mean ± SD for n=3/group. *P<0.01.

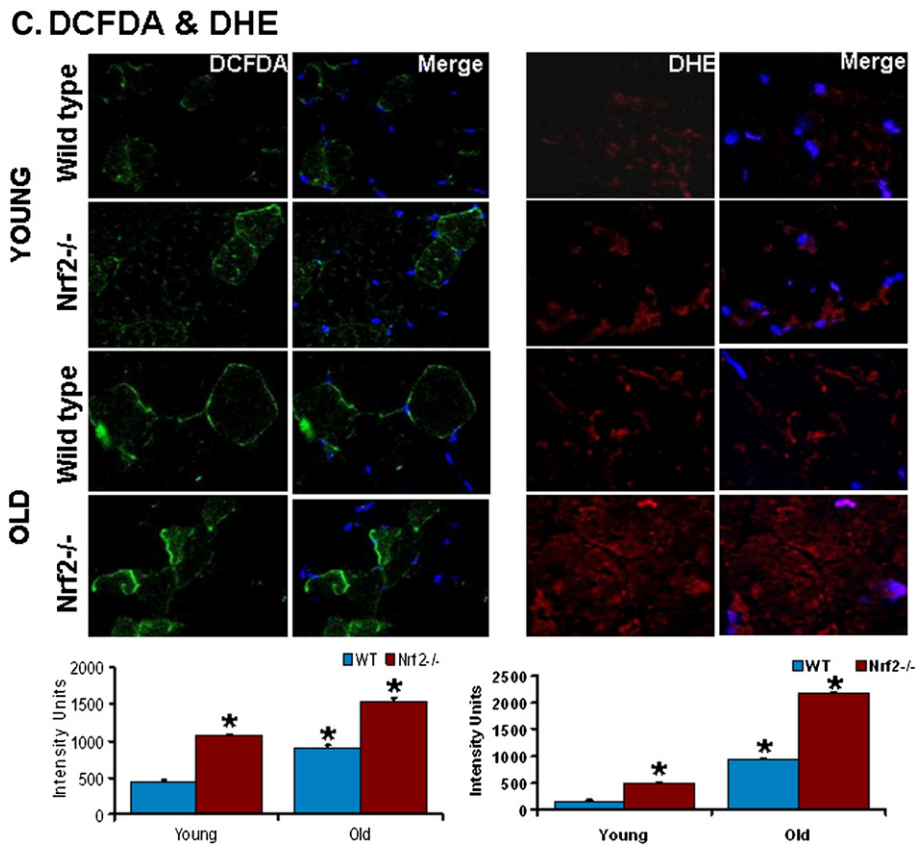

Fig. 2 (*continued*).

glutathione measurements, a portion of SM was weighed (~20 mg), washed and homogenized with MES buffer. A 10 µl aliquot was saved for protein determination and metaphosphoric acid (MPA) extracts were stored at −80 °C until the analysis.

2.4. Histology of skeletal muscle

Histochemistry analysis was performed as described by Song et al. Cross-sections of skeletal muscle were cut (5 µm thick) in cryostat (−20 °C), placed on pre-coated slides, and air dried for 30 min. For assessment of cross-sectional area, geometry, and extracellular space, the sections were stained with 2–5 drops of hematoxylin and incubated for 5 min at room temperature to highlight nuclei and mitochondria. Stained sections were washed with PBS, air dried and mounted with cytoseal (Richard-Allan Scientific, MI, USA).

2.5. Skeletal muscle glutathione (GSH) redox state

Redox state of glutathione (GSH/GSSG) was determined in skeletal muscle tissue extracts from WT and Nrf2−/− mice at 2 and >24 months of age. Briefly, skeletal muscle tissue extracts were prepared in MES buffer and centrifuged at 5000 rpm for 5 min at 4 °C. Proteins were measured using 10 µl aliquot of the supernatants and the remaining samples were mixed with equal volumes of 10% MPA (239275, Sigma) to precipitate proteins. TEAM (Tri-ethanolamine) reagent was added to a known volume of the MPA extracts, followed by 2-vinyl pyridine (for GSSG analysis). The GSSG standards were treated similarly to prepare a standard graph. Kinetic readings for GSH-reductase recycling assay were recorded as per the manufacturer's instructions (703002, Cayman Chemicals, Ann Arbor, USA) using a plate reader (Bio-Tek, FLx-800) [30,47,48].

2.6. Electron Paramagnetic Resonance (EPR) spectroscopy-based ROS determination

To assess the effect of Nrf2-deficiency and aging on ROS formation, we measured the CMH (spin trap) signals in young and old SM tissues using EPR. In brief, mice were injected (intraperitoneally) with heparin (4 U/g b.w.) and sacrificed by CO_2 inhalation. A portion of the SM was cut into small pieces, placed in a clean 24-well culture plate containing 500 µL HEPES (20 µM, pH 7.4), washed twice, and immersed in 300 µL of HEPES buffer. Ten milligrams of processed SM tissue was incubated with 200 µL of 500 µM CMH (1-hydroxy-3-methoxy-carbonyl-2, 2,5,5-tetramethyl pyrrolidine) at 37 °C for 30 min in a CO_2 incubator. The ROS released by the SM reacts with CMH and forms a stable nitroxide radical, which can be measured using EPR. After 30 min of incubation, 50 µl of the reaction mixture was measured in a glass capillary using an EMX-ESR spectrometer (Bruker Instruments, Germany) as reported previously [30,49].

2.7. Detection of ROS using fluorescent probes (DCFDA/DHE)

Generation of superoxide and peroxides in the SM of young (2 months) and old (>24 months) mice was determined using DHE/H_2DCFDA (D11347/C6827, Invitrogen Corp. USA) fluorescence by following our previous report [30]. The cell-permeable non-fluorescent DHE is oxidized to fluorescent 2-hydroxyethidium, which is then detected by using the excitation/emission filters appropriate for rhodamine. In the presence of superoxide ($O_2^{-}\cdot$), oxidized ethidium intercalates with DNA, staining the nucleus a bright red fluorescence. Hydrogen peroxide oxidizes H_2DCFDA to a fluorescent DCF which can be detected by a fluorescent microscope using appropriate excitation/emission filters for fluorescence as reported previously [50]. Briefly, 5-µm-thick transverse

sections of (optimal cutting temperature/OCT) cryo-fixed skeletal muscle (SM) tissues on clean glass slides were appropriately covered with the probe solution containing DHE/H_2DCFDA (10 μM) and were incubated in a light-protected chamber at 37 °C for 30 min and washed thoroughly with 1× PBS thrice, 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 the Simple PCI 6 Imaging Software (Hamamatsu Corporation, Sewickley, PA).

2.8. Skeletal muscle tissue homogenization and immunoblotting

Skeletal muscles were harvested from 2 and >24 months old WT and Nrf2−/− animals and flash frozen in liquid nitrogen [30]. Cytosolic fractions from the SM were prepared by homogenizing the tissue 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 min. The nuclear pellet was washed with 4 volumes of homogenizing buffer to remove cytosolic contaminants. Nuclear proteins were prepared 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). Samples in the NEB were incubated on ice with mild shaking and centrifuged at 8200 rpm for 10 min. Then the proteins were quantified using Bradford reagent (Bio-rad). Protein samples for Western blots were prepared in 4× Laemmli buffer with 5% freshly added β-mercapto-ethanol and boiled for 5 min. Thirty micrograms of cytosolic proteins were separated on 10 or 12% SDS-PAGE and transferred to PVDF membranes. The membranes were treated with appropriate antibodies such as Nrf2, Keap1, catalase, SOD-1, GSR, G6PD, NQO-1, ubiquitin, γ-GCS, caspase-3/9, GAPDH and lamin-B1. Horseradish peroxidase IgG (rabbit and mouse) conjugated secondary antibodies were used for chemiluminescence detection of protein signals.

2.9. Gene expression analysis by quantitative real time PCR

Skeletal muscle tissues were harvested and washed with RNase free PBS and stored in RNA*later* reagent. RNA was extracted from ~20 mg of SM tissue of 2 and 24 month old WT and Nrf2−/− mice (N = 4–6) using RNA extraction kits (Qiagen #74106), following the manufacturer's instructions. The quantity and quality of the RNA was analyzed using a bioanalyzer. To synthesize cDNA, the reverse transcription reaction was performed on 2.5 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), Nrf2 (QT00095270), Sod-1 (QT01762719) and Gsr (QT01758232) were used and analyzed in a Roche-Light Cycler. Quantification of copy numbers of cDNA targets were performed using Ct values. Fold changes for respective gene expression were calculated by normalizing to the level of the housekeeping gene Arbp1 (QT00249375) or GAPDH (QT01658692).

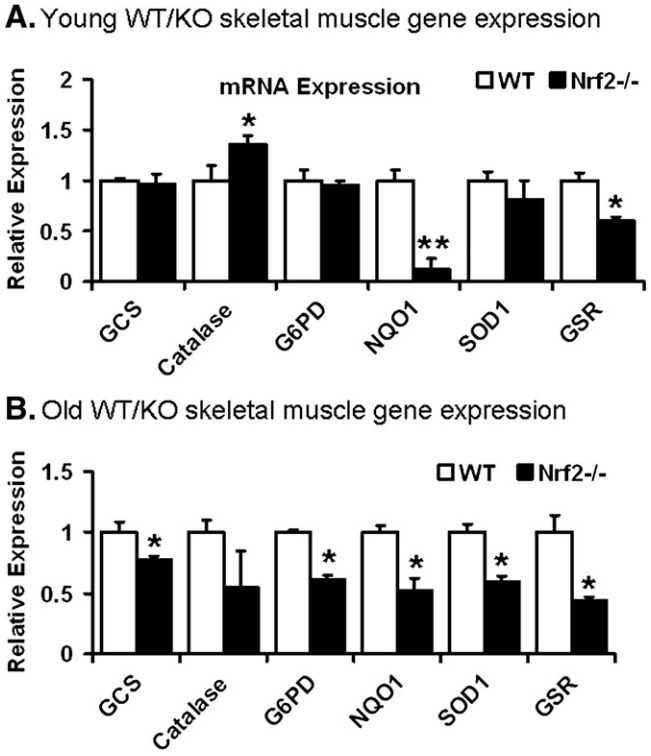

Fig. 3. RNA levels for SM from young and old WT/Nrf2−/− mice. Quantitative PCR analyses were performed using respective cDNA and Qiagen primer sets. (A) Gene expression for young mice- the RNA levels for Gcs, G6pd, Sod1 were unaltered between the WT and Nrf2−/−, but Nqo1 and Gsr were dramatically downregulated in Nrf2−/−. Catalase RNA was higher in the Nrf2−/− when compare to WT. (B) Gene expression for old mice—the RNA levels for most of the antioxidants (Gcs, G6pd, Sod1 and Gsr) except catalase were significantly down regulated in the Nrf2−/− when compared to WT at 24 months of age. The changes in the transcript levels reflect on the respective protein expression. The mRNA expression for catalase was down regulated significantly (P<0.05) in Nrf2−/− when compare to WT at 2 and 24 months of age. Data are mean ± SD for n = 4–6/group. *P<0.05; **P<0.01.

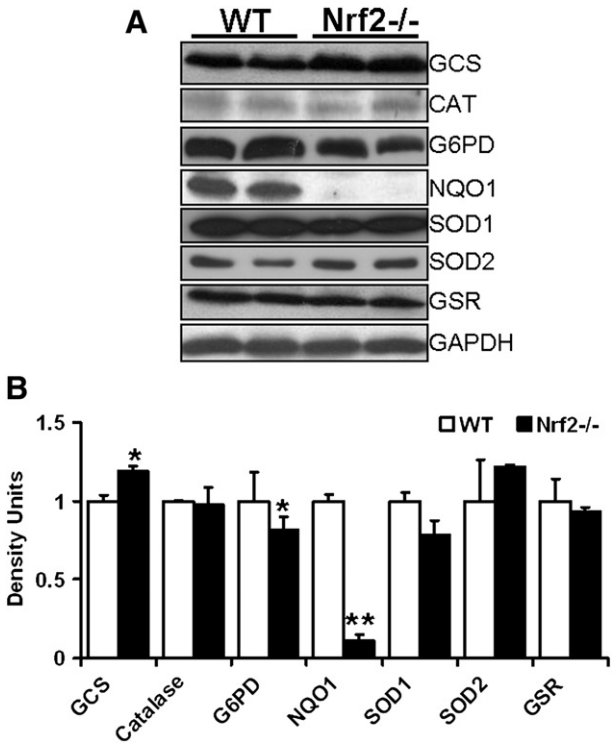

Fig. 4. Antioxidant protein expression for young mice: Western blots showing protein expression for antioxidant enzymes in WT and Nrf2−/− mice at 2 months of age. Abrogation of Nrf2 does not affect on the levels of catalase, G6PD, SOD1, SOD2 and GSR, but NQO1 protein levels were significantly decreased (>5 fold down regulation) in Nrf2−/− when compared to age matched WT mice. Immunoblots of GCS showed a minimal (10%) increase in Nrf2−/− when compared to WT, indicating the presence of Nrf2 independent mechanisms for its regulation. Data are mean ± SD for n = 4–6/group. *P<0.05; **P<0.01.

2.10. Immunofluorescence analysis (IF) of antioxidant enzymes

Frozen skeletal muscle (OCT) sections at 5 μm thickness from the WT and Nrf2−/− mice at 2 and 24 months old were immunostained with respective primary antibodies as reported previously [30,47]. The SM tissue sections were primarily fixed in 4.0% paraformaldehyde for 10 min, permeabilized with 0.25% Triton X-100 in PBS containing 0.01% Tween-20 for 5 min, and blocked for 60 min with 5% goat serum in 0.01% PBST. Incubation with the appropriate primary antibody diluted in 0.01% PBST containing 1% BSA was carried out over night at 4 °C. After three (5 min each) washes with PBST, the sections were incubated with appropriate secondary anti-mouse Alexa-Fluor 488-conjugated or anti-Rabbit Alexa-Fluor 647-conjugated antibodies (1:500 dilution) for 1 hour at room temperature. After three washes (5 min 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 of specific localization and expression of NEF2 (1:200), GSR (1:200), catalase (1:300), G6PD (1:150), NQO1 (1:400), and GCS (1:200). Fluorescent intensity was quantified by automated image analysis using the Simple PCI 6 Imaging Software (Hamamatsu Corporation, Sewickley, PA).

2.11. Statistical analysis

All data were statistically analyzed using Student *t*-test and *P*-values less than 0.05 were considered statistically significant. Data are presented as mean ± standard deviation. For each parameter, the WT was compared to a corresponding Nrf2−/− group of the respective age. No comparisons were made between the young vs. old groups.

3. Results

3.1. Nrf2 and Keap1 proteins are conserved in the mouse skeletal muscle

The Nrf2-Keap1 pathway is one of the critical regulators of intracellular redox homeostasis in multiple cells and organs [33,51]. Western blots from skeletal muscle (SM) homogenates confirm the expression of Nrf2 and Keap1 (Fig. 1A–B) in WT and its abrogation in Nrf2−/− mice. Comparable expression of Keap1 protein and transcript (data not shown) levels were observed in the SM of WT and Nrf2−/− mice, suggesting abrogation of Nrf2 did not influence Keap1 protein levels. Further, immunofluorescence analysis confirms Nrf2 expression in adult WT, while Nrf2−/− mice had no signals for anti-Nrf2-ab. (Fig. 1C). Next, we performed histological analysis (hematoxylin staining) of frozen skeletal muscle sections from young (2 months) and old (24 months) WT and Nrf2−/− mice (Fig. 1D). When compared to WT, the size of the myocytes from Nrf2−/− were smaller (30%), but there was no evidence of intercellular space at 2 months of age. Interestingly, the old SM of WT and Nrf2−/− exhibit atrophy and derangement of nuclei was noted. However, the old Nrf2−/− SM showed an increased number of myocytes exhibiting atrophy when compared to the age matched WT. These results indicate that loss of Nrf2 might be a contributing factor for age-associated myocyte degeneration.

3.2. Under unstressed/physiological setting, the loss of Nrf2 has minimal effect on skeletal muscle redox state in young mice

The skeletal muscle (SM) generates relatively more ROS, through mitochondrial activity and NADPH oxidase(s), than other tissues [52–55]. To combat accumulation of ROS, the myocytes have evolved with a potential defense system that includes non-protein thiols such as glutathione and enzymatic antioxidants. To investigate whether the loss of Nrf2 can impair GSH synthesis and induce oxidative stress in SM tissue, we measured the reduced/oxidized forms of glutathione (GSH/GSSG) in 2 month old mice. Under basal physiological state and younger age, GSH levels and the redox ratio were unaltered in Nrf2−/− SM when compared with age matched WT mice (data not shown). However, significant ($P<0.05$) decrease in GSH content (1.0 ± 0.08 vs. 0.6 ± 0.1; WT vs. Nrf2−/−) and GSH/GSSG ratio (30.5 ± 3.05 vs.20.0 ± 2.0; WT vs. Nrf2−/−) were recorded in Nrf2−/− when compared to SM of WT mice at 24 months of age. These results indicate that age-associated decline in antioxidant function is accelerated due to abrogation of Nrf2. Maintaining a balance between antioxidant and pro-oxidant levels is critical for the intracellular redox homeostasis. We hypothesize that disruption of Nrf2, a master regulator of antioxidant transcription, will impair antioxidant defense mechanisms in SM under basal settings. To understand the free radical load in the SM, we performed radical-spin trap analysis using EPR spectroscopy. The EPR signals for CMH (superoxide + hydroxyl) were indistinguishable in the young WT and Nrf2−/− mice (Fig. 2A–B). Upon aging, there was a significant increase in ROS levels of Nrf2−/− SM when compared to age matched WT mice (Fig. 2). We further confirmed the levels of ROS using redox sensitive fluorescent probes (DCFH-DA/DHE) of WT and Nrf2−/− mice at young and old ages. The fluorescence analysis revealed increased ROS (superoxide and hydrogen peroxide) levels in the old WT and Nrf2−/− mice when compared to young mice, suggesting the accumulation of ROS with aging (Fig. 2C). An accumulation of ROS along with GSH depletion was evident in the Nrf2−/− mice SM. Thus, abrogation of Nrf2 impairs defense mechanisms and promotes oxidative stress in SM upon aging. Taken

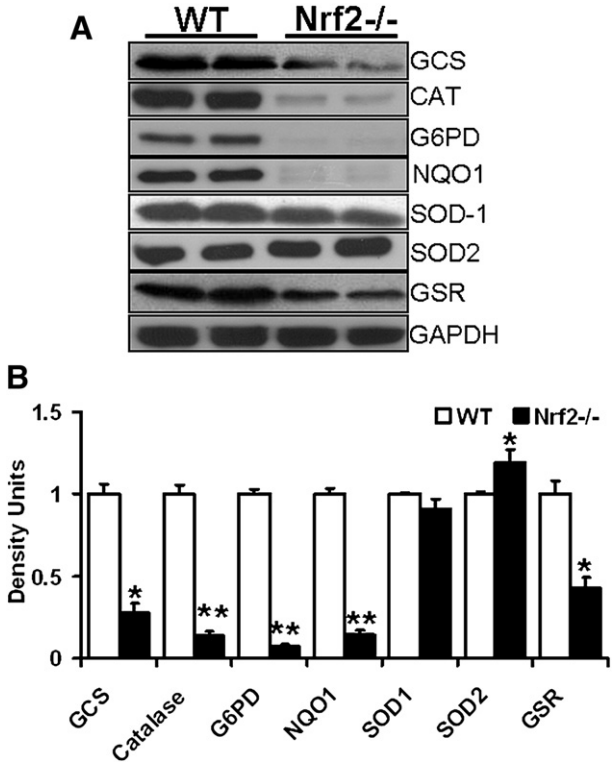

Fig. 5. Antioxidant protein profile for old mice: Western blots showing protein expression for antioxidant enzymes in WT and Nrf2−/− mice at 24 months of age. Abrogation of Nrf2 significantly affects the protein levels of most of the antioxidants (GCS, catalase, G6PD, GSR and NQO1) on aging. All these proteins were dramatically decreased in aged-Nrf2−/− when compared to the age matched WT mice. However, the level of SOD1 was unaltered, but the SOD2 protein was increased in Nrf2−/− on aging. Age dependent decrease in transcript levels for antioxidants in Nrf2−/− mice correspond well with the protein levels. Data are mean ± SD for $n = 4$–6/group. $^{*}P<0.05$; $^{**}P<0.01$.

together, the loss of Nrf2 is not deleterious to the skeletal muscle under basal-physiological or unstressed states.

3.3. NQO1 (but not other antioxidants) mRNA and protein expression are decreased in young Nrf2−/− mouse SM

NAD(P)H-quinone oxidoreductase-1 (NQO1) is one of the major phase-II antioxidant enzymes that catalyzes the 2-electron reduction of quinones and thereby quenches toxicity [56]. To determine the effects of Nrf2 abrogation on major antioxidant gene expression, we performed q-PCR analysis in young (2 months of age) WT and Nrf2−/−mice. Results showed significant ($P<0.001$) down regulation of Nqo1 mRNA in Nrf2−/− when compared to WT mice SM at both age points (Fig. 3A–B). Next, we analyzed NQO1 protein levels by Western blotting and observed a 5 fold decrease in Nrf2−/− when compared with age-matched WT mice (Figs. 4–5). These results suggest that disruption of Nrf2 is necessary and sufficient to down regulate Nqo1 transcription and thereby decrease its protein levels. Though the transcription of NQO1 is induced by various xenobiotics, it has been recently demonstrated that Nrf2 tightly regulates Nqo1 expression. Next, we observed a moderate, but not significant, increase in some of the antioxidants (namely G6PD and GCS) in Nrf2−/− when compared to WT mice at 2 months of age. However, the transcript and protein levels for other antioxidants such as catalase, GSR and SOD-1 were similar between the WT and Nrf2−/− groups, suggesting that loss of Nrf2 has a minimal role on antioxidant regulation/function under basal physiological state and/or young age.

3.4. Sustained abrogation of Nrf2 down-regulates transcription of ARE-containing antioxidant genes upon aging

We hypothesized that abrogation of Nrf2 might inhibit transcriptional activation of downstream targets including critical antioxidant pathways associated with glutathione homeostasis in the SM. To test this hypothesis, we performed qPCR analysis using RNA isolated from SM of 24 month old WT and Nrf2−/− mice. We selected major antioxidant genes under the control of Nrf2/ARE. Expression of catalase, G6pdx, Gsr, Gcs, Sod-1 and Nqo1 (Fig. 3B) was significantly lower in the Nrf2−/− compared with WT mouse SM, suggesting a lack of

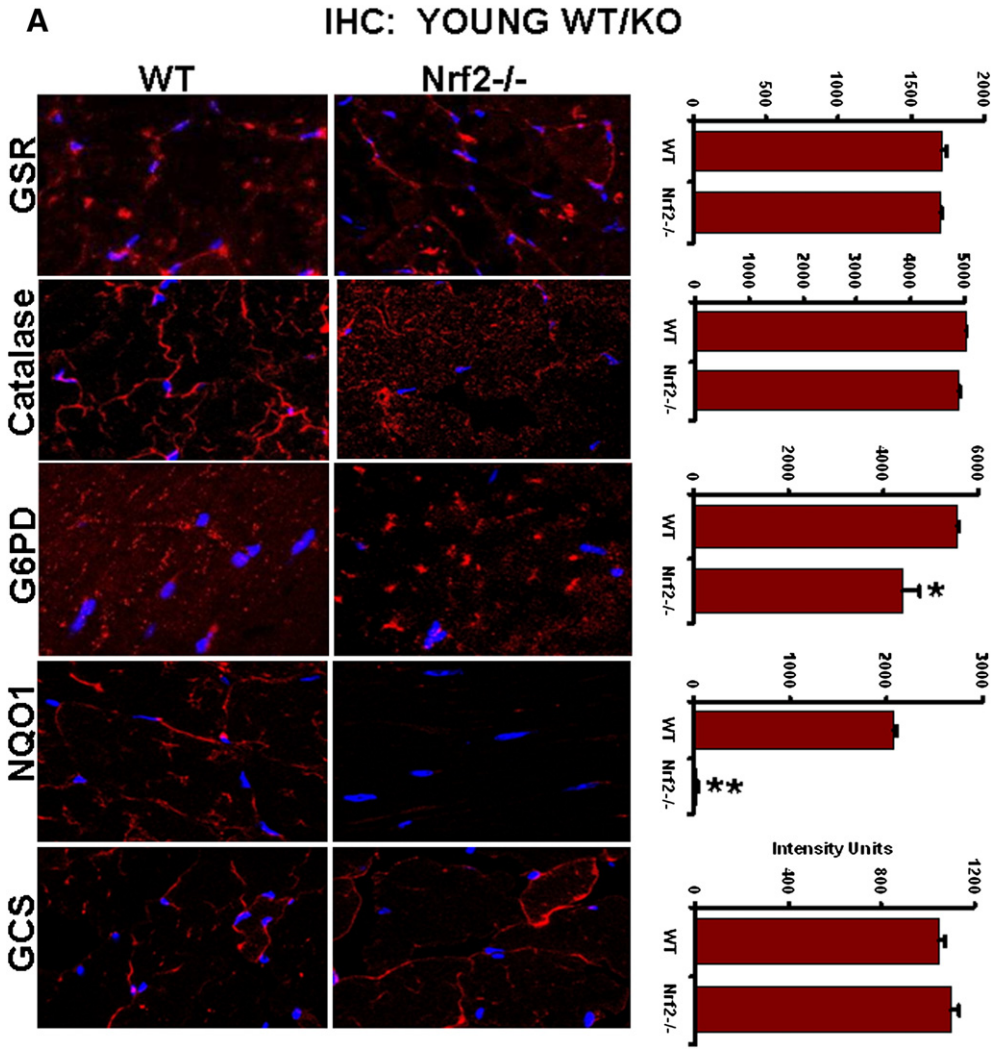

Fig. 6. Immunofluorescence analyses of antioxidant enzymes. Immunofluorescence analysis using anti-GSR (1: 200), anti-catalase-ab (1:400), anti-G6PD (1:200), anti-NQO1 (1:400) and anti-GCS (1:200) antibodies showing their expression in WT and Nrf2−/− of young (A) and old (B) skeletal muscle sections. In the young mouse, the levels of GSR, catalase, GCS were comparable between the groups, but the NQO1 and G6PD were significantly decreased in Nrf2−/− when compared to the WT. Upon aging, all these antioxidant enzymes were significantly decreased in Nrf2−/− when compared to age-matched WT mice. Data are mean ± SD for $n = 3$–4/group. $^{*}P<0.05$; $^{**}P<0.01$. Blue: nucleus (DAPI); red: antioxidant and pink: merge of blue and red indicating nuclear localization of Nrf2.

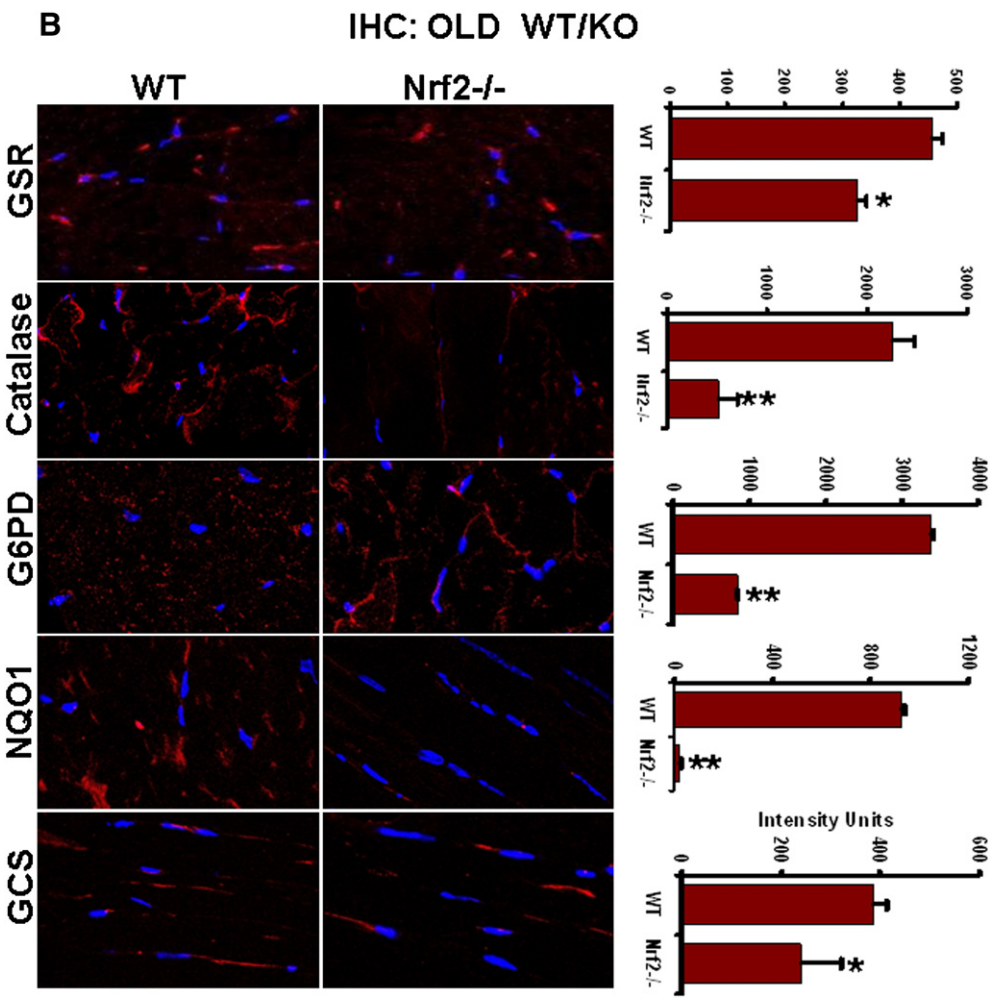

Fig. 6 (*continued*).

compensation against age-induced ROS accumulation. However, at 2 months of age, the WT and Nrf2−/− mice had indistinguishable levels of transcript expression for most of these genes (except Nqo1 and Gsr) (Fig. 3A), indicating that age-dependent oxidative stress could severely compromise antioxidant mechanisms under Nrf2-deficient conditions.

3.5. Depletion of major antioxidant enzymes in the SM of Nrf2-deficient mice upon aging

To characterize whether Nrf2-deficiency, in part, is responsible for the decreased GSH levels upon aging in the mouse skeletal muscle, we measured protein expression for major targets of Nrf2 and the enzymes that are involved in GSH metabolism and redox homeostasis using Western blots. At 2 months of age, there appeared to be comparable levels of protein expression for most of the antioxidants (GCS, CAT, SOD1, SOD2, GSR) among WT and Nrf2−/− mice (Fig.4). However, many of the antioxidants including catalase (1.0 vs. 0.15 in old), NQO1 (1.0 vs 0.15 in old), G6PD (1.0 vs. 0.12 in old), GCS (1.0 vs. 0.25) and GSR (1.0 vs. 0.45 in old) were dramatically ($P<0.001$) decreased in the old Nrf2−/− when compared to age-matched WT mice SM (Fig. 5). Overall, Western blot analysis revealed significant depletion of antioxidants (GCS, NQO1, SOD1, catalase, G6PD and GSR) in Nrf2−/− mice, while WT mice exhibited compensatory antioxidant responses to the aging changes. Further, immunofluorescence analysis revealed identical changes in the expression of antioxidant enzymes. Though GSR, catalase, and GCS were unaltered, G6PD and NQO1 were significantly decreased in the Nrf2−/− when compared to WT mice at 2 months of age (Fig. 6A). In the aging SM, all the antioxidants (GSR, catalase, G6PD, NQO1 and GCS) were dramatically decreased in the Nrf2−/− when compared to age-matched WT mice (Fig. 6B). These results confirm the protein blots (Figs. 4–5) and suggest that the effect of aging is exacerbated in the absence of Nrf2. The observed lower antioxidant enzyme levels could be attributed to (i) increased utilization of these antioxidants and (ii) absence of inducible antioxidant mechanisms via Nrf2 to combat age-associated ROS accumulation.

3.6. Abrogation of Nrf2 enhances/activates apoptotic pathways in aged mice

Apoptosis, a well known mechanism for programmed cell degradation, is associated with aging. To understand possible mechanisms linked to Nrf2-deficiency and subsequent oxidative stress in SM, we analyzed key markers of apoptotic pathways. Western blots for caspase-3 and caspase-9 indicated no changes between WT and Nrf2−/− mice at 2 months of age (Fig. 7A). Notably, these levels were significantly increased in Nrf2−/− when compared to WT at 24 months of age along with other apoptotic markers (AIF, ASK1, BAX, BAD) (Fig. 7B), suggesting prominent activation of apoptosis due to the loss of Nrf2 and/or aging.

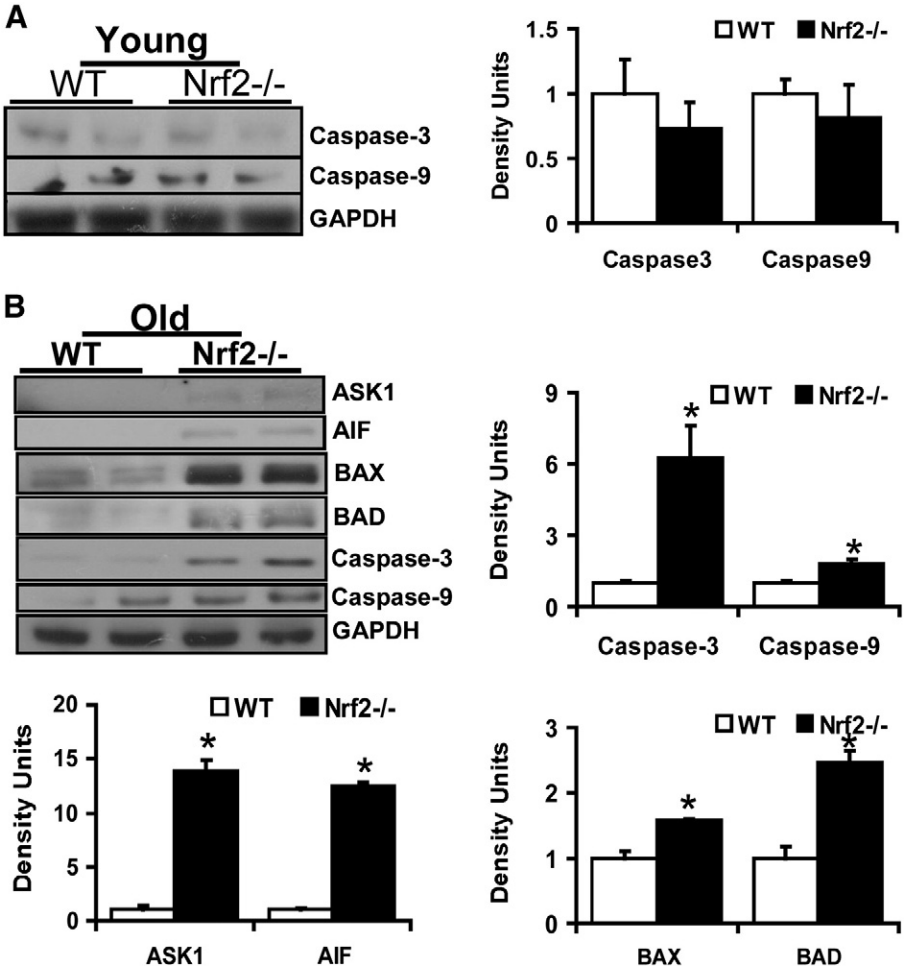

Fig. 7. Effect of age on Nrf2 dependent oxidative stress and apoptotic pathways. Western blots showing caspase-3, caspase-9, ASK1, AIF, BAX and BAD expression in WT and Nrf2−/− mice SM at young and old ages. (A) Caspase-3 and 9 levels were indistinguishable between WT and Nrf2−/− mice at 2 months of age. (B) Protein expression of most of the apoptotic markers (casp-3/9, AIF, ASK1, BAX, BAD) were significantly increased in Nrf2−/− when compared to WT mice at 24 months of age. Activation of apoptotic pathway is evident in the aged Nrf2−/− mice. Data are mean ± SD for $n = 4$–6/group. *$P<0.01$.

3.7. Age-dependent oxidative stress increases ubiquitination of proteins in Nrf2−/− mice

In general, degradation of oxidatively modified or misfolded proteins occurs through ubiquitin-proteasome systems (UPS). Oxidative stress promotes ubiquitin-protein conjugates and degradation of several proteins and enzymes [57–59]. We tested whether the increased ROS and oxidative stress would trigger protein degradation in Nrf2-abrogated SM. Analysis of protein blots using mono- and polyclonal anti-ubiquitin-ab revealed no difference in ubiquitin-protein conjugates between WT and Nrf2−/− groups at young age (data not shown), whereas the aged Nrf2−/− SM showed a significant increase of ubiquitin-protein conjugates when compared to WT upon aging (>24 months) (Fig. 8A). Next, to assess the effect of Nrf2 and aging on lipid peroxidation, we have measured the skeletal muscle 4-HNE (lipid peroxidation marker) content in young and old WT/Nrf2−/− mice. There were no significant differences observed between the WT and Nrf2−/− at young age, but a dramatic increase in HNE-positive proteins was seen in Nrf2−/− when compared to WT at old age (Fig. 8B). Significant increase in HNE-signals for a wide range of proteins (from 10 kDa to 250 kDa) in the Nrf2−/− mouse SM when compared to WT was evident. These results suggest that abrogation of Nrf2 and age-associated augmentation of oxidative stress are associated with increased ubiquitination, which in turn results in protein degradation.

4. Discussion

Our investigation has identified Nrf2 as a critical factor that hastens age-associated oxidative stress, ubiquitination and apoptosis in skeletal muscles. The age-dependent effect of Nrf2 signaling in skeletal muscle has been only partially understood. Oxidative stress and associated pathogenic mechanisms in aged humans received ample attention in the context of muscular function/dysfunction (Fig. 9). However, the Nrf2-dependent antioxidant function and protein degradation/apoptosis in SM has not been previously investigated in the context of aging. Our current findings reveal that abrogation of Nrf2 (i) has a minimal role on SM redox state in younger age, (ii) result in myocyte atrophy with aging, (iii) impairs transcription of antioxidants upon aging, (iv) increases oxidative stress and (v) activates ubiquitination and apoptotic signals upon aging. Our results demonstrate that abrogation of Nrf2 contributes to age-associated oxidative stress and impairs cytoprotective mechanisms.

4.1. Disruption of Nrf2 has a minimal role on skeletal muscle antioxidant mechanism at younger age

The majority of Nrf2 is activated upon changes in the redox state due to ROS generation, and both toxic and electrophilic stress [60]. Coordinated regulation of Nrf2 signaling is believed to preserve the redox state and protect skeletal muscle structure and function.

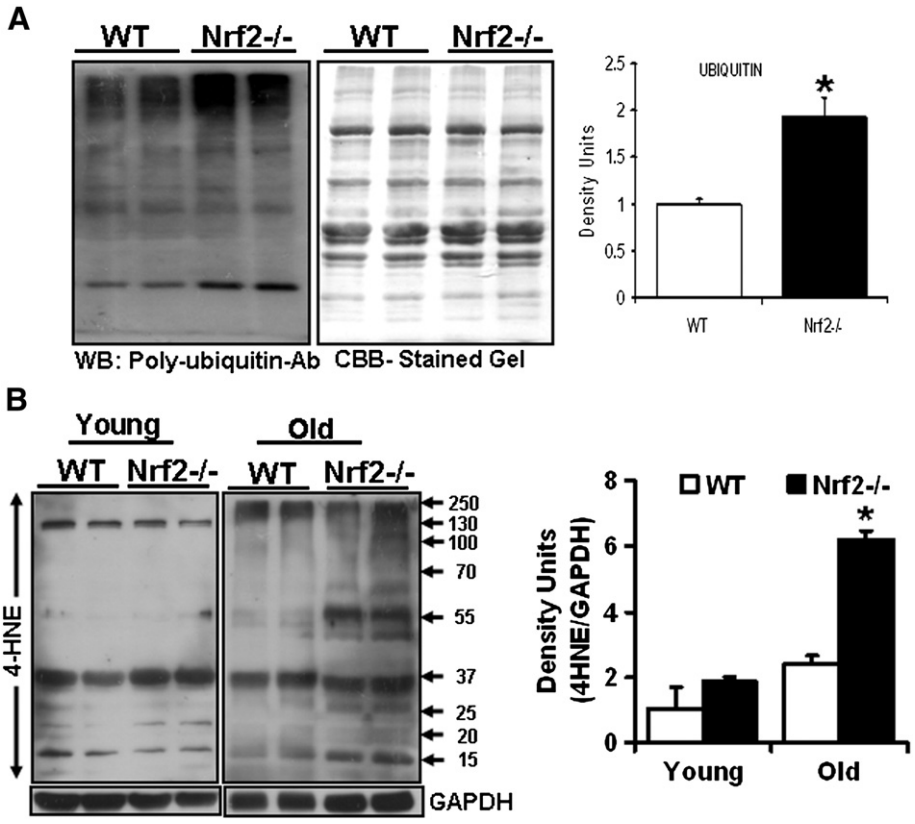

Fig. 8. Oxidative stress induced lipid peroxidation and ubiquitination in Nrf2−/− mice. (A) Western blots probed for mono-/poly-ubiquitination-ab revealing increased ubiquitination of proteins in aged Nrf2−/− mice. Densitometry analysis showing ~2 fold increase in poly-ubiquitinated proteins in aged Nrf2−/− when compared to age matched WT mice. (B) Western blots probed for anti-HNE-ab showing comparable signals in skeletal muscles of WT and Nrf2−/− at 2 months of age. In the old mice, significant increases of HNE-modified proteins are evident in Nrf2−/− SM when compared to age-matched WT ($n=4$/group; $^*P<0.01$).

Certainly, we observed identical mRNA and protein levels of most of the antioxidants and normal redox state in SM of age-matched WT and Nrf2−/− groups (2 months). Hence the young mice had basal ROS and glutathione levels; we believe that the skeletal muscle did not develop oxidative stress despite the loss of Nrf2 in young mice. However, the transcript and protein expression for NQO1 was dramatically decreased in young Nrf2−/− mice (Figs. 3–5), but this was idle on the redox state of the SM. These findings suggest that primary regulation of NQO1 occurs through Nrf2 albeit age. Of late, few studies have documented the role of stress-induced Nrf2 in age-associated transcriptional regulation of defense systems against oxidative stress. In addition, we observed comparable levels of ROS, redox state and ubiquitination among WT and Nrf2−/− mice indicating a homeostatic, basal intracellular environment at 2 months of age. Taken together, these results provide evidence of a minimal role for Nrf2 under basal conditions including young age.

4.2. Abrogation of Nrf2 promotes oxidative stress and impairs antioxidant mechanisms upon aging

Increased production of reactive oxygen and nitrogen species (ROS/RNS) results in oxidative stress, which is implicated in the development of multiple diseases including muscular dystrophy [38,53,61]. Previous reports have documented that the loss of Nrf2 is strongly coupled with dysregulation of antioxidant pathways and progression of diseases [16–18]. A question addressed in this study is whether Nrf2−/− mice are susceptible to an age-associated trigger for ROS generation and accumulation in the skeletal muscle. Earlier investigations show evidence that aging can induce ROS generation through a variety of mechanisms including loss of potential radical scavenging antioxidants [62]. Here we demonstrate that ROS was generated in both the WT and Nrf2−/− mouse skeletal muscles upon aging, but the magnitude of ROS accumulation was significantly higher in aged Nrf2−/− mice, suggesting low abundance and poor activation of cytoprotective mechanisms. The striking decline in several antioxidant proteins (catalase, NQO1, γ-GCS, GSR, SOD-1, G6PD etc.) upon aging in Nrf2−/− mice might be attributed to an increased consumption of these antioxidant enzymes and decreased translation processes due to loss of Nrf2/ARE-dependent transcription. Further, gene expression (real time qPCR) analysis revealed significant down-regulation of several antioxidant genes in Nrf2−/− mice after aging, indicating an important transcriptional regulatory role of Nrf2 in skeletal muscle antioxidant defenses. Our results suggest that the loss of Nrf2 makes myocytes vulnerable to oxidative damage. Thus, Nrf2/ARE signaling is crucial to protect the skeletal muscle from age-dependent oxidative stress diseases.

4.3. Deregulation of antioxidants and oxidative stress induce apoptosis in skeletal muscle of aged Nrf2−/− mice

Apoptosis is a regulated energy-dependent process of cellular self-destruction. Activation of a cascade of cysteine proteases and endonucleases such as caspases occurs at the onset of the apoptotic process [63]. It has been previously shown that apoptotic events are directly linked with cell aging [39,64–66]. Usually, aging is associated with a loss of cells in the post mitotic tissues of the brain, heart and skeletal muscle [39,63–66]. Thus, a decline in the number of viable, fat-free myocytes results in skeletal muscle dysfunction. We postulated that apoptosis is one of the mechanisms involved in the process of age-associated oxidative stress and that abrogation of

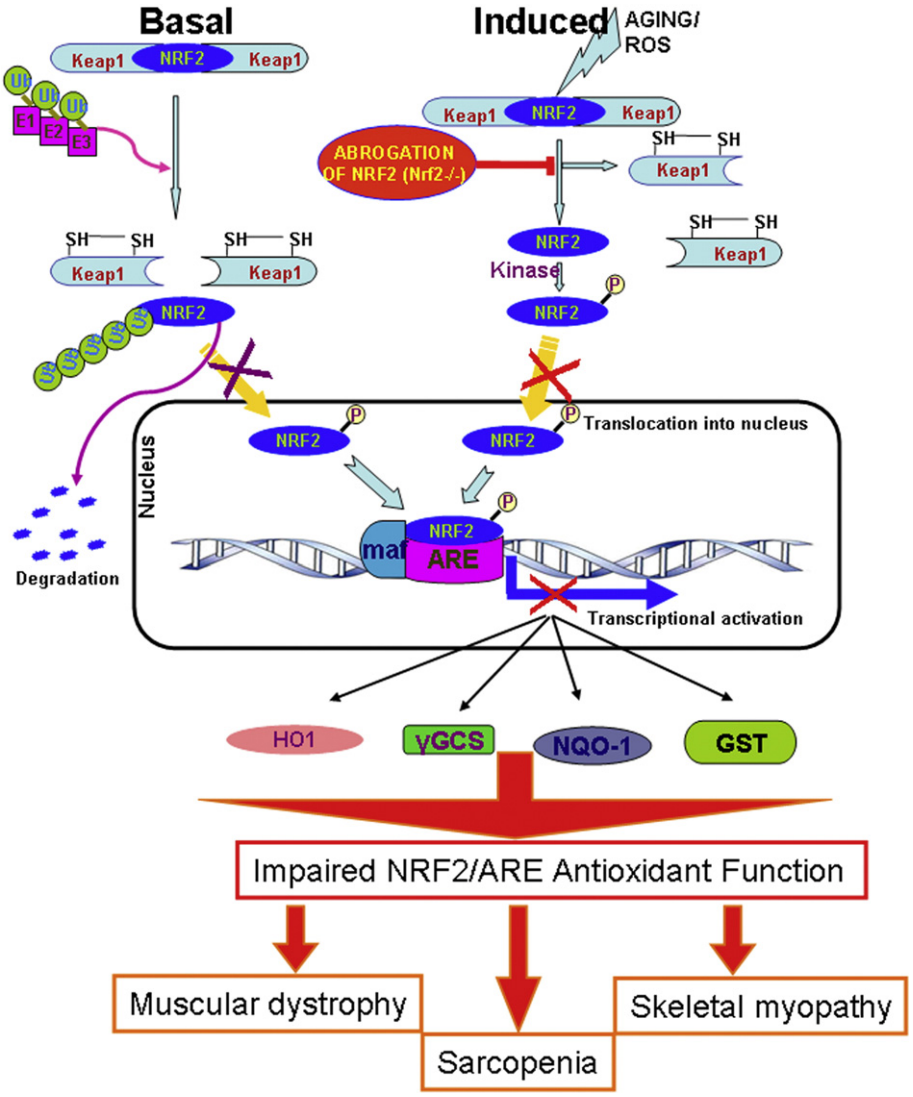

Fig. 9. Effect of Nrf2-deficiency and age induced oxidative stress on ubiquitination. Under basal state, majority of the Nrf2 is degraded by its repressor, Keap1. Upon induction, the Nrf2 dissociates from Keap1 and translocates into nucleus. Abrogation of Nrf2 leads to accumulation of ROS and chronic oxidative stress during aging.

Nrf2/ARE-antioxidant signaling may play a major role in influencing apoptosis. In the current study, activation of apoptotic (ASK1, AIF) and pro-apototic (BAD, BAX) signals induces downstream apoptotic pathways (caspase-3 and 9) in aged Nrf2−/− mice due to increased generation/accumulation of ROS/RNS and decreased reducing equivalents such as glutathione (GSH). Excess generation of ROS and/or RNS could indirectly induce apoptotic signals by altering the membrane potential, permeability pore opening and release of cytochrome-C from the mitochondria [67–69]. Therefore, aging and loss of Nrf2 might have hastened the apoptotic process in the skeletal muscles of aged Nrf2−/− mice.

4.4. Loss of Nrf2 exacerbates age-associated oxidative stress and promotes ubiquitination

Age associated oxidative stress is believed to cause a variety of biochemical and conformational modifications in macromolecules [40–42]. These changes, along with prolonged oxidative damage in organelles and cells, result in dysfunction. Numerous studies have reported that increased ROS/RNS generation and oxidative/nitrosative stress contribute to several age-associated diseases in humans [5,7,8,39,44,70,71]. Previous studies have shown that declined Nrf2 signaling is associated with increased oxidative stress in aged sedentary humans while aged active human skeletal muscle exhibited increased nuclear Nrf2 levels and subsequent activation of its major target antioxidant enzymes such as HO1 and γ-GCLC [31]. Accordingly, aged WT mice had increased ROS levels and impaired redox capability, which was further magnified in the Nrf2−/− mice due to the loss of inducible cytoprotective mechanisms. Steady state levels of phase II defense components are crucial to scavenge oxidative/nitrosative free radicals and protect cells/organs from oxidative, toxic and xenobiotic stress. In the current study, depletion of several key antioxidant scavenging enzymes promoted oxidative stress and ubiquitination in aged Nrf2−/− skeletal muscle. Further, lipid peroxidation analyses reveal that abrogation of Nrf2 is coupled with increased HNE-positive proteins on aging, which might also contribute to activation of apoptotic cascades. It has been reported that moderately high levels of 4-HNE can induce apoptosis and leads to muscular weakness [72–74]. Therefore, a steady state function of Nrf2 signaling is critical to preserve SM antioxidant defense and redox homeostasis.

4.5. Future directions and clinical impact

Our findings show that induction of a battery of major antioxidant genes at transcriptional and translational capacity is feasible through

Nrf2/ARE signaling. We speculate that activation of Nrf2-dependent pathways could be cytoprotective against a wide range of age-associated diseases of skeletal muscle in humans. In the follow-up studies, we intend to investigate whether pharmacological or non-pharmacological (exercise) manipulation could promote sustained activation of Nrf2 in aged mice. Such constitutive Nrf2 function is expected to rescue redox homeostasis and prevent age-dependent disorders in skeletal muscle. Future clinical investigations include assessing the effect of chronic regimented and spontaneous exercise (to increase adaptive response) on maintaining a steady-state function of Nrf2 and thereby achieving potential intracellular cytoprotective defense mechanisms and redox homeostasis in elderly humans. Pharmacological based activation of Nrf2 signaling will be aimed at patients with moving disabilities.

5. Conclusions

Abrogation of Nrf2 promotes an age-associated increase in ROS generation and oxidative stress in skeletal muscle. Further, disruption of Nrf2 increases ubiquitination and apoptosis upon aging. Nrf2 signaling might be a potential therapeutic target to protect the SM from well-known muscular diseases due to ROS and oxidative stress. **Innovation:** Our study provides evidence, for the first time, that abrogation of Nrf2 is coupled with increased ubiquitination and apoptosis in skeletal muscle upon aging. A constant activation/stabilization of Nrf2 signaling might be efficacious in maintaining homeostatic redox milieu and thereby ameliorating age-associated muscular disorders.

Abbreviations Used

Arbp1	acidic ribosomal phosphoprotein
ARE	antioxidant response element
CMH	1-hydroxy-3-methoxy-carbonyl-2, 2,5,5-tetramethyl pyrrolidine
DETC	diethyl dithio carbomate
DF	defroxamine
EPR	electron paramagnetic resonance
ESR	electron spin resonance
G6PD	glucose-6-phosphate dehydrogenase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GCLC	glutamyl cysteine ligase (catalytic)
GCLM	glutamyl cysteine ligase (modulatory)
GSH	reduced glutathione
Gsr	glutathione reductase
GSSG	oxidized glutathione
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Ho1	hemoxygenase-1
KCl	potassium chloride
Keap1	Kelch like associated protein-1
$MgCl_2$	magnesium chloride
MPA	meta-phosphoric acid
mRNA	messenger RNA
NaCl	sodium chloride
Nqo1	NADH-quinone oxidase
Nrf2	nuclear erythroid 2 related factor 2 (NF-E2)
Nrf2−/−	Nrf2-knockout
qPCR	quantitative real-time polymerase chain reaction
RNS	reactive nitrogen species
ROS	reactive oxygen species
SD	standard deviation
SOD1	super oxide dismutase-1
SOD2	super oxide dismutase-2
TBST	tris buffered saline-tween 20
TEAM	tri-ethanolamine
UPS	ubiquitin proteasome system
WT	wild type
γGCS	γ-glutamyl cysteine synthase

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