Nox4 – RyR1 – Nox2: Regulators of micro-domain signaling in skeletal muscle

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ARTICLE INFO

Keywords:
Skeletal muscle
Calcium
NAD(P)H oxidase
Nox2
Nox4
Dystrophy
RyR1
DMD
Mdx

ABSTRACT

The ability for skeletal muscle to perform optimally can be affected by the regulation of Ca²⁺ within the triadic junctional space at rest. Reactive oxygen species impact muscle performance due to changes in oxidative stress, damage and redox regulation of signaling cascades. The interplay between ROS and Ca²⁺ signaling at the triad of skeletal muscle is therefore important to understand as it can impact the performance of healthy and diseased muscle. Here, we aimed to examine how changes in Ca²⁺ and redox signaling within the junctional space microdomain of the mouse skeletal muscle fibre alters the homeostasis of these complexes. The dystrophic mdx mouse model displays increased RyR1 Ca²⁺ leak and increased NAD(P)H Oxidase 2 ROS. These alterations make the mdx mouse an ideal model for understanding how ROS and Ca²⁺ handling impact each other. We hypothesised that elevated t-tubular Nox2 ROS increases RyR1 Ca²⁺ leak contributing to an increase in cytoplasmic Ca²⁺, which could then initiate protein degradation and impaired cellular functions such as autophagy and ER stress. We found that inhibiting Nox2 ROS did not decrease RyR1 Ca²⁺ leak observed in dystrophin-deficient skeletal muscle. Intriguingly, another NAD(P)H isoform, Nox4, is upregulated in mice unable to produce Nox2 ROS and when inhibited reduced RyR1 Ca²⁺ leak. Our findings support a model in which Nox4 ROS induces RyR1 Ca²⁺ leak and the increased junctional space [Ca²⁺] exacerbates Nox2 ROS; with the cumulative effect of disruption of downstream cellular processes that would ultimately contribute to reduced muscle or cellular performance.

1. Introduction

In skeletal muscle, Ca²⁺ release from the sarcoplasmic reticulum (SR), or Ca²⁺ store, to the contractile apparatus during excitation-contraction coupling (EC coupling) is tightly regulated [1]. Action potentials propagate down the plasma membrane into the tubular (t-) system to stimulate intracellular Ca²⁺ release channels/ryanodine receptors (RyR1) on the terminal cisternae of the SR. The t-system and SR terminal cisternae are closely apposed; these junctional membranes form a highly specialised nano-environment that is essential to the regulation of ion levels in the muscle fibre [2–4]. Impaired ability of the muscle to respond to action potentials could be the result of Ca²⁺ mishandling and oxidative stress when the fibre is at rest.

Muscle weakness in myopathies can be due to changes in the ryanodine receptor (RyR1) function resulting from a direct RyR1 genetic mutation or other modifications that have a consequential impact on the RyR1. These changes can cause muscle weakness by reducing the amount of Ca²⁺ released following excitation and by activating Ca²⁺-dependent signaling cascades (i.e. proteases and redox signaling) in the cytoplasm [5–7].

NAD(P)H oxidases (Nox) are significant contributors to a cell’s overall redox state [8,9]. Once thought to only be present in phagocytic cells, Nox isoforms have been identified in a variety of cell types and found to have specific functions based on the environment in which they are located [8,9]. Skeletal muscle is known to contain two of the Nox isoforms, Nox2 and Nox4. The canonical Nox2 complex consists of membrane-bound gp91 phox and p22 phox subunits and cytosolic subunits p67, p40 and p47 phox [8–10]. For Nox2 derived reactive oxygen species (ROS) production, all of the subunits must come together. However, in mouse models where the subunit p47 phox is rendered inactive, Nox2 ROS is unable to be produced [11–14]. Nox4, whose biological role in skeletal muscle is not well known [9], is believed to act as an oxygen sensor [15,16]. Unlike other Nox isoforms, Nox4 is constitutively active and has one membrane-bound subunit, p22 phox [9,16,17]. In striated muscle, Nox4 is located at the surface membrane as well as at the SR [9,15]. Within the SR it has been identified to be predominantly expressed at the terminal cisternae [15]. Interestingly, Nox4 has been suggested to affect RyR1 function, potentially via nitrosylation [15]. Given the close association of Nox4 to the RyR1, Nox4 ROS could alter RyR1 function; however, a clear link

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https://doi.org/10.1016/j.redox.2020.101557

Received 11 March 2020; Received in revised form 10 April 2020; Accepted 23 April 2020

Available online 26 May 2020

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Reactive oxygen species; specifically NAD(P)H oxidases located on the t-tubule are suggested to have a role in many muscle diseases with early pathological involvement in inflammation, decreased muscle function and alterations in Ca\textsuperscript{2+} handling [13,18–20]. Lack of a membrane stabilisation protein, dystrophin, can cause an initial stage of muscle degeneration induced by a disruption of Ca\textsuperscript{2+} regulation in the muscle [21–24]. The \textit{mdx} mouse, a model of dystrophin deficiency and muscle pathology, is known to have deregulated Ca\textsuperscript{2+} handling and harmful levels of ROS production [21,23–30]. In addition, RyR1 displays an enhanced Ca\textsuperscript{2+} leak due to hyper-nitrosylation [31,32]. This model provides an ideal platform for examining the interplay between Ca\textsuperscript{2+} and ROS within a cellular micro-domain. How ROS can affect Ca\textsuperscript{2+} signalling and how Ca\textsuperscript{2+} can also then propagate ROS in a reciprocal manner is slowly becoming clear; however, there is still a paucity of knowledge as to functional consequences [19,22,24,25].

Here we describe ROS and Ca\textsuperscript{2+} interactions within the triadic cleft micro-domain of skeletal muscle. We show that the commonly examined t-tubular Nox2 ROS does not promote RyR1 leak; instead, Nox2 ROS production is likely exacerbated by Ca\textsuperscript{2+} leak in the junctional cleft. For the first time, we show that Nox4 has increased expression in skeletal muscle that is unable to produce Nox2 ROS. We also show that pharmacological and genetic inhibition of Nox4 reduced RyR1 Ca\textsuperscript{2+} leak and that Nox4\textsuperscript{-/-} skeletal muscle displays less nitrosylation of the RyR1 compared to WT. Our data suggest that Nox4 dependent nitrosylation of RyR1 exacerbates SR Ca\textsuperscript{2+} leak.

### 2. Materials and methods

#### 2.1. Animal models

WT (C57BL/6J, Stock No:000664), \textit{mdx} (C57BL/10ScSn-Dmd\textsuperscript{mdx}; J, Stock No:001801), \textit{p47}\textsuperscript{-/-} (B6(Cg)-Ncf1m1/J, Stock No:004742) and Nox4\textsuperscript{-/-} (B6.129-Nox4tm1Kkr/J, Stock No:022,996) mice were purchased from JAX and maintained in colonies. \textit{p47}\textsuperscript{-/-} mice were generated via the insertion of a neo cassette that interrupts exon 7, the exon known for gene function [14]. The interruption of exon 7 results in a non-functional \textit{p47} phox protein, yet the protein remains detectable.

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**Fig. 1.** T-system Ca\textsuperscript{2+} measurements show presence of a large RyR leak in \textit{mdx}, \textit{p47}\textsuperscript{-/-}/\textit{mdx} and \textit{p47}\textsuperscript{-/-} mice. A,B, Representative trace of WT and \textit{mdx} mice t-sys uptake at 200 nM [Ca\textsuperscript{2+}]\textsubscript{cyto} with and without tetracaine. Vertical grey bars indicate solution exchange artifacts during confocal acquisition. C-F, Steady-state (Ca\textsuperscript{2+})\textsubscript{t-sys} was determined for a broad range of resting [Ca\textsuperscript{2+}]\textsubscript{cyto} (28–1300 nM) in age-matched mice; separation in the two curves (with and without tetracaine) is due to RyR Ca\textsuperscript{2+} leak (see Methods Fig. S1). Closed circles represent control, open circles with the addition of 1 mM tetracaine.
The Nox4−/− mouse was generated using a neo cassette that replaces exon 4 within the Nox4 gene, resulting in a loss of Nox4 gene and protein expression [33,34]. Creation of the p47−/−/mdx mice has been described previously [13].

### 2.2. Muscle preparation for single EDL fibre imaging

All experimental methods using rodents were approved by IACUC at Baylor College of Medicine. Male mice at 4 – 6 weeks of age were euthanised via isoflurane overdose and cervical dislocation. The extensor digitorum longus (EDL) muscle were rapidly excised from the animals and placed in a Petri dish under paraffin oil above a layer of Sylgard. Rhod-5N salt was trapped in the sealed t-system as originally described by Lamb et al. (1995) [35]. Briefly, small bundles of fibres were isolated using fine forceps and exposed to a Na+-based physiological solution (external solution) containing (mM): Rhod 5N 2.5, CaCl2, 2.5; NaCl, 132; MgCl2, 1; KCl, 3.3; HEPES, 20 and the pH was adjusted to 7.4 with NaOH. The dye was allowed more than 10 min to diffuse into the t-system from the surrounding bubble of solution containing fluorescent dye. After this equilibration period, individual fibres that had been exposed to the dye solution were isolated from the bundle and mechanically skinned. After skinnning, the fibre was transferred to an experimental chamber containing a K+-based internal solution which allowed the sealed t-system to generate a normal resting membrane potential (Lamb & Stephenson, 1990[36]; 1994[37]). The solution contained (mM): Mg2+,1 (added as MgO); HDTA, 49; EGTA, 1; HEPES, 10; K+, 103–106 (added as KOH); Na+, 36 (from ATP and CP); ATP, 8; creatine phosphate, 10; sucrose 103–107 and N-Benzyl-p-toluene-sulfonamide (BTS), 0.05 with pH adjusted (with KOH) to 7.1.

### 2.3. RyR1 Ca2+ leak measurements

Under resting control conditions Ca2+ leaks from the RyR1 and is extruded from the intracellular junctional space to the extracellular environment via the plasma membrane Ca2+ ATPase (PMCA) and the Sodium/Ca2+ exchanger (NCX) (Fig. S1A). Tetracaine is used to inhibit
the RyR1 in order to prevent the local leak of Ca\(^{2+}\) into the diffusionally restricted junctional space [3,31]. Subsequently, the uptake of Ca\(^{2+}\) by PMCA and NCX corresponds to diffusion of Ca\(^{2+}\) from the bulk cytoplasm and not from the RyR1 leak (Fig. S1B). Any separation between the control and tetracaine curves (Fig. 1D–F) is an indication of RyR1 Ca\(^{2+}\) leak.

### 2.4. Confocal imaging

Mounted skinned fibres were imaged using a Zeiss 780 inverted confocal microscope equipped with a Zeiss 1.2NA 40× Plan-Apochromat water immersion objective. Rhod-5N was excited with a 561 nm laser diode. For tracking Ca\(^{2+}\) movements across the t-system membrane, images were continuously recorded in xyt mode with an aspect ratio of 512 × 256, with the long aspect of the image parallel with that of the fibre. Temporal resolution of imaging in this mode where the fluorescence signal was measured from within the borders of the fibre was 0.3 s/frame.

### 2.5. Image analysis for Ca\(^{2+}\) measurements

The t-system (t) rhod-5N fluorescence (\(F(t)\)) was collected by continuous xyt imaging during multiple internal solution changes. At the end of the experiment each fibre was exposed to ionophore (20 μM) and 5 mM Ca\(^{2+}\), followed by 0 Ca\(^{2+}\) to obtain the fluorescence maximum (\(F_{\text{max}}\)) and minimum (\(F_{\text{min}}\)), respectively. These values were used in conjunction with the previously determined \(K_{\text{d}}\) of Rhod-5N in the t-system of 0.8 mM [3,38,39] to determine \([\text{Ca}^{2+}]_{\text{t-sys}}\) with the relationship:

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[\text{Ca}^{2+}]_{\text{t-sys}}(t) = \frac{K_{\text{d, Ca}}}{F(t) - F_{\text{min}}}/(F_{\text{max}} - F(t)).
\]

### 2.6. Western blotting

A whole muscle homogenate was prepared using flash-frozen EDLs as described by Murphy et al. 2006 [6]. Briefly, muscle (8–12 mg), was weighed and homogenised on ice (1:20 w/v) in a K-EGTA solution containing (mM): 103 K+ (from KOH), 50 EGTA, 90 HEPES, 1 Mg\(^{2+}\) (from MgO), 8 total ATP, 10 creatine phosphate, pH 7.10) with a protease inhibitor cocktail (PIC; Complete; Roche). Immediately following this, the homogenate was diluted to 1:50 with the K-EGTA and PIC solution and then 1:1 with 2x SDS Laemml buffer. A small amount of homogenate from all samples was mixed to generate a suitable sample for 4 point calibration curves, spanning 3–60 μg of total muscle wet weight [40–44]. Samples were electrophoresed for 30 min at 200 V using 4–15% or 10% stain free tris/glycine gels and transferred overnight at 4 °C and 30 V. PVDF membranes (BioRad) were blocked in 5% skim milk in TBST or 3% BSA in PBST; primary antibodies were probed in 1% BSA in PBST. Chemiluminescent images were acquired on a BioRad ChemiDoc Touch using BioRad Clarity and Max ECL reagents. Images were analysed using BioRad Image lab software.

Stain-free tris/glycine gels (BioRad) were utilised in order to assess total protein loading and normalisation for Western blot analysis. The use of stain-free total protein technology reduces the possibility of genotype and age-dependent changes of housekeeping proteins such as GAPDH and actin [40–49]. Western blot figures are displayed with protein of interest representative blots and myosin heavy chain (MHC) as a visual indicator of protein loaded. Analysis utilised total protein from the stain-free gels.

For studies analysing the RyR1, 4–12% bis/tris gels were utilised. These gels were not stain-free and were post-stained with Coomassie blue for myosin heavy chain (MHC) density. Total RyR1 was then normalized to MHC for protein loading differences. See Supplementary Materials Fig. 2.

### 2.7. Antibodies

Antibodies used in this paper and the working concentrations are provided in Supplementary Table 1.

2.8. S-Nitrosylation biotin switch assay

Whole muscle, EDL lysates were processed via the biotin switch method using the S-nitrosylated Protein Detection Kit (Cayman Chemical). Biotin switch samples were run on a 4–12% Bis-Tris gel and transferred to PVDF. The membrane was blocked in 3% BSA in PBS-Tween, and the secondary HRP was used to detect total nitrosylation. The membrane was then stripped and re-probed for RyR1 total protein levels. The nitrosylation of the band at the MW of RyR1 was then expressed relative to the total RyR1 protein density in the sample.

### 2.9. Carboxylation assay

Whole muscle EDL lysates were derivatized according to the manufacturer’s directions in order to determine levels of carboxylation (EMD Millipore, Oxyblot Kit). After derivatization and neutralization, samples were run on a 4–12% Bis-Tris gel and transferred to PVDF overnight. The membranes were blocked in 1% BSA in PBS-Tween and probed using anti-DNP and secondary antibodies provided in the kit. For analysis of total sample carboxylation whole lanes were used and expressed relative to total protein (MHC).

### 2.10. Force measurement

EDL muscles were dissected, placed in a physiological saline solution containing (mM): NaCl 120, KCl 4, CaCl\(_2\) 2, MgSO\(_4\) 1, KH\(_2\)PO\(_4\) 1, NaHCO\(_3\) 25, glucose 10 and continuously gassed with 95% O\(_2\)–5% CO\(_2\). Muscle length and voltage were adjusted to elicit maximum twitch force. Force-frequency characteristics were measured at 1, 5, 10, 20, 40, 80, 120, 150, 200 and 300 Hz every 1 min. Fatigue was performed at 70 Hz tetani for 5 min, followed by a recovery protocol consisting of a 30 Hz and 100 Hz tetani every minute for 10 min. At the end of the contractile protocol, muscle length and weight were determined to assess absolute forces expressed as N/cm [2].

### 2.11. Isolation of single, intact enzymatically dissociated FDB fibres

Flexor digitorum brevis (FDB) muscles were placed in a dish containing DMEM media supplemented with 1% penicillin/streptomycin (Gibco) and 4 mg/ml of collagenase (Sigma). After 1–2 hrs at 37 °C, the muscles were triturated with decreasing bore sizes of fire-polished pipettes to yield single fibres. Cultures were maintained at 37 °C in DMEM with 1% pen/strep and 10% FBS for a maximum of 48 h.

### 2.12. DCF ROS measurements

Intact FDB fibres were plated on 96 well plates using ECM (Sigma). Fibres were loaded with 5 μM DCFH-DA (Molecular Probes) at 37 °C. After de-esterification fibres were imaged on a Sutter Lambda DG-4 Ultra-high-speed wavelength switcher was used to excite DCF (480 nm) emission intensity was collected at 510 nm on a charge-coupled device (CCD) Camera (CoolSNAP MYO, Photometrics) attached to an Axio Observer (Zeiss) inverted microscope at 0.1 Hz. Baseline measurements were collected for 50 s and maximal oxidation elicited via the addition of 1 mM H\(_2\)O\(_2\) at the end of imaging. Linear curves were fitted to the data at 15–40 s of baseline and the final 40 s of maximal response to H\(_2\)O\(_2\) to calculate the slopes. The values of the slopes were then used to create a ratio of base over max in order to determine the oxidative state of the fibre.

### 2.13. Statistical analysis

Data is presented as mean ± SEM. A one-way ANOVA was performed for statistical analysis with Dunnett’s post-hoc multiple comparison test.
comparisons to WT control unless otherwise stated. Statistical analysis was performed with Graph Pad Prism.

3. Results

3.1. Genetic inhibition of Nox2 ROS does not abolish RyR1 leak in mdx or WT mice

For initial experiments, we utilised the mdx mouse due to the perturbations in Ca²⁺ and ROS handling presented in this model. Use of the dystrophin-deficient mouse allowed us to assess micro-domain Ca²⁺ regulation in the presence or absence of different Nox isoforms in order to investigate the influence of redox changes on the RyR1.

*Mdx* mice are reported to have RyR1 Ca²⁺ leak at the pathologically critical degeneration/regeneration timepoint [31,32]. In Fig. 1A–D, we show the presence of RyR1 Ca²⁺ leak in the early period of the dystrophic *mdx* mouse where the muscle undergoes cycles of degeneration and regeneration; this leak was consistent with previous reporting [32]. We wished to examine the level of RyR1 leak in dystrophic mice that could not generate Nox2 ROS (p47⁻/−/mdx); with the hypothesis that the increased level of Nox2 ROS observed in the *mdx* mouse at 19 days was contributing to leak of Ca²⁺ from the RyR1 [19,20,29]. We have previously shown that p47⁻/−/mdx mice display improved muscle pathology and improvement in force production [13,50]. We targeted the same 4–6 week time period used in measurements of the *mdx* mouse for analysis of RyR1 Ca²⁺ leak in p47⁻/−/mdx mice. A pronounced

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**Fig. 3.** Basal redox state was assessed in WT and Nox4⁻/−/ deficient mice using DCFH-DA in enzymatically isolated intact FDB fibres. A. A representative trace of DCF fluorescent activity in WT and Nox4⁻/−/ mice. Linear curves were fit to the data with lines indicated in green showing slopes fit to the baseline data (15–40s), lines in red display slopes fit to the maximum response of the curve (80–120s). B. The ratio of base slope over max slope was calculated as an indication of the oxidative capacity of the respective fibres. No significant difference (p = 0.497, nested t-test) was found in the basal oxidative capacity of the Nox4 ROS deficient mice suggesting a similar redox state to that of WT. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

T.R. Cully and G.G. Rodney

Redox Biology 36 (2020) 101557
RyR1 leak was present in both p47−/−/mdx (Fig. 1E), and in dystrophin positive p47−/− control mice (Fig. 1F) despite the improvement in pathology previously reported in this model [13,50,51]. To pharmacologically block Nox2 ROS production, we applied the gp91ds peptide during RyR1 leak experiments in WT, mdx and p47−/− mice. We observed a non-specific inhibition of the RyR1 with the peptide (data not shown), rendering it inappropriate for use in our skinned fibre studies.

3.2. Eliminating Nox2 ROS elevates Nox4 protein levels

EDL whole muscle homogenates from WT, mdx, p47−/− and p47−/−/mdx were probed via Western blot to determine the expression levels of redox proteins Nox4 and p22 phox. In p47−/− and p47−/−/mdx mice, which are unable to produce Nox2 ROS, Nox4 expression increased significantly (p = 0.001) (Fig. 2A). Nox4 has been suggested to be constitutively active with its only other accessory subunit being p22.
phox [15,17,52]. Therefore, for higher amounts of Nox4 ROS production, increased Nox4 expression is necessary [53]. Mdx and p47−/−/mdx mice were found to have significantly increased p22 phox levels compared to WT (Fig. 2B). The increase in p22 phox in mdx mice is inconsistent with previous reporting [29]; however, in our circumstances whole, non-fractionated tissue homogenate was used, and mice were at least ten days older.

To examine if elimination of Nox4 induced a reciprocal increase in Nox2 complex proteins, p47 phox and Nox2 expression levels were examined in Nox4−/− EDL lysates. No change was present in the expression levels of p47 phox or Nox2 in the Nox4 ROS deficient animals (Fig. 2C-D). However, this does not necessarily indicate that there would be no change in the ROS producing activity of these proteins. Note that the presence of p47 phox protein in the p47−/− mouse was created, there is protein present but it is not active. The phosphorylation levels of a specific phosphoserine site, pSer370 in p47 phox were assessed via western blotting. Expression of active. The phosphorylation levels of a specific phosphoserine site, Ser370 in p47 phox was found to have significantly increased p22 phox levels compared to WT. The increase in p22 phox in mdx mice is inconsistent with previous reporting [29]; however, in our circumstances whole, non-fractionated tissue homogenate was used, and mice were at least ten days older.

3.3. Basal ROS production is no different in Nox4 ROS deficient mice

Basal redox state was assessed in WT and Nox4−/− deficient mice using DCFH-DA in intact FDB fibres. Fig. 3A shows a representative trace of DCF fluorescent activity in WT and Nox4−/− mice. The ratio of base slope over max slope was calculated as an indication of the oxidative capacity of the individual fibres (Fig. 3B). No significant difference was found (p = 0.497, nested t-test) in the basal oxidative capacity of the Nox4 ROS deficient mice suggesting a similar redox state within the bulk cytoplasm to that of WT.

Nox4 deficient mice displayed no changes in force production or fatigue recovery. Force frequency, fatigue and recovery from fatigue were assessed in 4–6 week old C57Bl6 WT mice and Nox4 deficient mice. Recovery was performed at 30 Hz and 100 Hz to observe potential effects on low-frequency recovery due to ROS [54]. No change in specific force was observed or any alteration in the rate of fatigue or recovery (Fig. S3). Of note, both genotypes did not recover force at 30 Hz to the degree seen at 100 Hz.

3.4. Calcium handling proteins are not altered in Nox4 ROS deficient muscle

To examine the potential interplay between calcium flux and ROS production, we quantified the levels of critical calcium handling proteins. STIM1S, “short” isofrom of STIM1, is an SR calcium sensor involved in the process of store-operated calcium entry (SOCE) which has previously been shown to be upregulated in the mdx mouse at 3 months of age [27]. SOCE can locally raise Ca2+ levels in the junctional space and the cytoplasm in response to SR Ca2+ reduction [55–58]. We found no statistical differences in the levels of STIM1S [59,60] in either p47−/−, or Nox4−/− mice compared to WT (Fig. 4A). Interestingly, we observed a significant increase in STIM1S expression in only the p47−/−/mdx mouse compared to WT (p = 0.0073) (Fig. S4). Calsequestrin 1, an important SR Ca2+ buffering protein, is not statistically different in p47−/−, Nox4−/− and p47−/−/mdx mice at 4 weeks of age (Fig. 4B). In addition, the Sarco/endoplasmic reticulum ATPase 1 (SERCA1), responsible for the uptake of Ca2+ from the cytoplasm to the SR, is not different in p47−/−, Nox4−/− and p47−/−/mdx mice (Fig. 4C).

Therefore an increase in the uptake rate by the SERCA pump is unlikely.

3.5. RyR1 leak is reduced with pharmacological or genetic inhibition of Nox4

In Fig. 1, RyR1 Ca2+ leak was present in the p47−/−/mdx mouse despite a reported improvement in pathology [13,50]. Due to the increase of Nox4 expression in the Nox2 ROS deficient mice (Fig. 2) and

Fig. 5. Lack of Nox4 ROS production via genetic knock out or pharmacological inhibition results in lessening of RyR1 leak. A, Representative trace of Nox4−/−RyR1 leak. B, RyR1 leak is not present in the Nox4−/− at a variety of [Ca2+]cyto. C,D, Nox4 ROS inhibition with GKT137831 reduces leak. N = number of animals, n = number of fibres.
the location of Nox4 near the RyR1 [9,15], we sought to elucidate if genetic knockout or pharmacological inhibition of Nox4 would result in a decrease of RyR1 Ca\(^{2+}\) leak. We examined RyR1 Ca\(^{2+}\) leak in 4–6 week old Nox4 male knockout mice. We found that RyR1 leak is minimal in the Nox4 knock out mice (Fig. 5A-B). Pharmacological inhibition of Nox4 with GKT137831 (150 nM, 6–10min) reduced RyR1 Ca\(^{2+}\) leak in WT and p47\(^{-/-}\) muscle across all [Ca\(^{2+}\)]\(_{c}\) (Fig. 5C-D).

Taken together, our data supports a model in which Nox4 ROS promotes RyR1 leak.

3.6. S-Nitrosylation levels and RyR1 phosphorylation are altered in Nox4 ROS deficient muscle

In order to assess whether Nox4 promotes S-nitrosylation of the RyR1, we performed a biotin switch assay using a commercially available kit to assess bulk nitrosylation of the channel. Intriguingly, RyR1 total protein was found to be upregulated in Nox4\(^{-/-}\) and p47\(^{-/-}\) and p47\(^{-/-}\)/mdx mice (p = 0.0001 and 0.01 respectively) (Fig. 6A, p47\(^{-/-}\)/mdx not shown). As we used whole, non-fractionated muscle homogenates we sought to determine potential contributors to the increased RyR1 outside of skeletal muscle itself. Circulating macrophages express RyR1 [61]; therefore, we probed for CD68 as a marker of macrophage infiltration to rule out any contribution of macrophage RyR1 to the total RyR1 protein levels in the skeletal muscle. CD68 was found to not be different in the Nox4\(^{-/-}\) mouse (Fig. S6).

S-nitrosylation was found to be increased relative to WT (p = 0.05) in the p47\(^{-/-}\) and decreased in the Nox4\(^{-/-}\) (p = 0.02) (Fig. 6B). S-nitrosylation of the RyR1 was also found not to be increased in the p47\(^{-/-}\)/mdx (Fig. S5C). Phosphorylation of S2808 of RyR, a site known for regulation of RyR1 Ca\(^{2+}\) leak in cardiac and skeletal muscle [62], was found to be decreased (p = 0.04) in the Nox4\(^{-/-}\) mice (Fig. 6C).

4. Discussion

Ca\(^{2+}\) handling and signaling within any cell type is essential for normal cell function and Ca\(^{2+}\) homeostasis in both healthy and diseased tissue [63]. Reactive oxygen species are also necessary for modulating cellular functions with alterations in the balance between reactive oxygen species and reactive nitrogen species potentially causing problems within cells if scavenging of these species is insufficient [9,10,19]. In skeletal muscle disease, it has been suggested the oxidative stress from aberrant ROS production may not itself be enough to

![Fig. 6. RyR1 post-translational modifications in Nox2 and Nox4 ROS deficient mice. A, Total RyR1 expression in WT, p47\(^{-/-}\) and Nox4\(^{-/-}\) EDL whole muscle lysates. Nox4\(^{-/-}\) mice display a significant increase in total RyR1 content (p < 0.0001). B, S-nitrosylation is expressed relative to the total protein content of RyR in WT, p47\(^{-/-}\) and Nox4\(^{-/-}\) mice. S-nitrosylation is increased (p = 0.05) in the RyR leaky p47\(^{-/-}\) mice and decreased in the Nox4\(^{-/-}\) (p = 0.02). C, Phosphorylation of the RyR1 at Serine2808 is decreased in the Nox4\(^{-/-}\) muscle. Individual n values are displayed on the plots, * denotes significantly different to WT p < 0.05, ***p < 0.0001.](image-url)
drive pathological changes but rather the interplay between ROS/RNS and Ca\(^{2+}\) handling [19,64,65]. The mdx mouse model of Duchenne Muscular Dystrophy displays both aberrant Ca\(^{2+}\) handling and alteration in ROS species (particularly NAD(P)H oxidases), providing a suitable model for describing how Ca\(^{2+}\) and ROS affect each other and potentially lead to pathology [13,25,28,29,50,51].

Here, we examined the roles of two NAD(P)H oxidases in contributing to RyR1 Ca\(^{2+}\) leak, with particular focus on how one isoform, Nox4, regulates RyR1 leak and thus contributes to alterations in Ca\(^{2+}\) and ROS levels within a skeletal muscle micro-domain. While this study has been carried out in skeletal muscle, the interplay between the Nox isoforms and modulation of Ca\(^{2+}\) levels is relevant for all cell types [10].

Our experiments revealed in muscle that is unable to produce Nox2 ROS, Nox4 protein is upregulated. As Nox4 is constitutively active, increased protein expression would drive increased Nox4 ROS production [53]. Previous work on ROS has found a bell-shaped curve of ROS homeostasis in the cell [66,67]; which might explain an upregulation in Nox4 ROS in response to the loss of Nox2 ROS within a cell. The Nox4 upregulation could be a compensatory mechanism to regulate the redox state within the triadic micro-domain. Nox4 likely has a role in regulation of RyR1 leak to induce changes in other signaling cascades and also homoeosis through Nrf2 [67,68].

To date, measuring Nox4 ROS production has been difficult due to the location of Nox4 in subcellular compartments [9]. Indeed, when we examined basal ROS levels in Nox4\(^{-/-}\) mice using DCF, we were unable to establish differences to that of WT. This data suggests that when Nox4 ROS is eliminated that Nox2 ROS production does not increase, contrary to increased Nox4 expression that was seen in Nox2 ROS deficient p47\(^{−/−}\) mice. Chemical ROS probes like DCF only allow for assessment of bulk production of ROS within the cell. Much like when measuring cytoplasmic Ca\(^{2+}\), bulk measurements in the cytoplasm are unable to discern, or are obscuring, changes in and around cellular micro-domains. While the a genetically encoded biosensor, p47-rodGFP, can detect Nox2-dependent ROS it also can induce Nox activation [69]. These imaging limitations highlight the rising usefulness of developing new genetically targeted encoded ROS and Ca\(^{2+}\) biosensors that can be targeted to micro-domains within the cell [69-71].

Our results showed that in dystrophic muscle and dystrophic muscle that was unable to produce Nox2 ROS, p22 phox, a subunit required for both Nox2 and Nox4 ROS production was upregulated. The upregulation of p22 phox in dystrophic tissue could be associated with the increase in p47 phox and Nox2 ROS seen in that model [13,18]. As p22 phox is membrane-embedded, the membrane disturbances associated with dystrophic tissue could also play a role in driving increased expression. It is of interest that p22 phox is a critical subunit for both Nox2 and Nox4 ROS, with different regions of the protein associating with each isoform to drive ROS production [17]. The upregulation of Nox4 in Nox2 ROS deficient muscle could be a by-product of the upregulation of p22 phox [72].

The RyR possesses thiol groups of cysteine (Cys) residues that can be affected by reactive oxygen or nitrogen species (ROS/RNS) [20,73-76]. Both RyR1 and RyR2 have nitric oxide (NO) bound to cysteines which enables a post-translational modification (s-nitrosylation) to occur [77,78]. These post-translational protein modifications (s-nitrosylation, phosphorylation, glutathionylation, and alkylation) can lead to changes in the redox state of the RyR, leading to increased open or closed state of the channel, thus altering the function. Certain cysteines, such as Cys3635, have been identified to be more prone to nitrosylation modifications; with Cys3635 being redox-sensitive under physiological oxygen tension and nitrosylated to aid in muscle contraction [75,76,78].

We show that S-Nitrosylation of the RyR is reduced in the Nox4\(^{-/-}\) compared to WT and modestly increased in the p47\(^{-/-}\) mouse. The decrease in nitrosylation of the RyR1 in the Nox4\(^{-/-}\) suggests that Nox4 ROS influences Ca\(^{2+}\) leak through the RyR1. Our measurements of RyR1 leak in the Nox4\(^{-/-}\) mouse showed minimal RyR1 leak in this model. The p47\(^{-/-}\) mouse showed increased Nox4 protein expression, an increase in nitrosylation of RyR1, and a corresponding increase of RyR1 leak. We also used GKT137831 as an inhibitor of Nox4 in all of our experimental mouse models; WT, mdx, p47\(^{-/-}\), p47\(^{-/-}\)/mdx. We found that RyR1 leak was reduced in mdx, p47\(^{-/-}\), p47\(^{-/-}\)/mdx with the Nox4 inhibitor (Fig. 5, Fig. S7). Reducing thiols with DTT (10 mM, 6 min) reduced RyR1 leak at 200 nM Ca\(^{2+}\) in the p47\(^{-/-}\)/mdx mouse model (Fig. S5D). Dutka et al., 2017 [79] have previously shown that DTT treatment of individual skinned muscle fibres resulted in the removal of S-Nitrosylation of troponin I and reduced Ca\(^{2+}\) sensitivity of the protein. In our experiment the p47\(^{-/-}\)/mdx is unable to produce Nox2 ROS, limiting the likely ROS source present to Nox4. Carbonylation experiments (Fig. S5A) also showed that whilst the p47\(^{-/-}\)/mdx mouse had reduced oxidation relative to the mdx mouse, carbonylation was still increased relative to the WT. We would suggest that the DTT is further reducing the oxidation of the p47\(^{-/-}\)/mdx muscle and potentially also removing any S-Nitrosylation modifications. The net result of both actions being that leak is heavily reduced in this mouse model.

The Marks lab has previously shown that PKA phosphorylation of the RyR at Serine2808 results in altered modulation of the RyR1 in skeletal muscle during heart failure [62,80]. Changes in phosphorylation of the RyR have also been shown in response to exercise-induced SR Ca\(^{2+}\) leak during training [81] and cold acclimatisation [82]. We also examined the level of phosphorylation of the RyR at Serine2808 and found a reduction in the phosphorylation of the RyR in skeletal muscle from the Nox4\(^{-/-}\) mouse. Conversely, we did not observe an increase in phosphorylation of the RyR1 in the p47\(^{-/-}\), a mouse model with leak present. Comparing the phosphorylation data to the changes observed in the nitrosylation status of the RyR1, we would suggest that the nitrosylation modifications are the initial dominant influence of Nox4 ROS on the RyR1 although alternate modifications may subsequently occur that contribute to the overall leakiness of the channel. Future work utilising mass spectrometry and proteomics would aid in providing greater detail as to specific locations and modifications.

We observed an increase in RyR1 expression in the Nox4\(^{-/-}\) mouse. In our hands, the increase in basal RyR1 is difficult to explain as RyR1 protein increases are not typically observed. One report has previously found an increase in RyR1 mRNA in a sepsis model in rats [83] although no protein analysis was performed to determine if the increased mRNA translated to increased protein. Muscle cells from a model of Central Core Rod disease have been found to have more RyR1 present in the central core rods themselves [84]. Probing of CD68 showed no changes, suggesting no immune cell infiltration or contribution to the overall increase in the RyR expression in Nox4\(^{-/-}\). The lack of immune cell involvement would suggest that the increase in RyR1 expression in Nox4\(^{-/-}\) is indeed occurring within the skeletal muscle alone, raising the question as to where the increased RyR1 channels are located. At this stage, the reason behind this increase is still undetermined.

We also examined Nox2 ROS and Nox4 ROS deficient muscle for changes in key Ca\(^{2+}\) handling proteins. STIM1, CSQ1 and SERCA1 were all found not to be different from WT. This is suggestive that the different ROS species present are not influencing compensatory expression changes in the Ca\(^{2+}\) handling proteins. While the total protein levels have not changed there is still the possibility of changes in function of the proteins due to oxidation or post-translational modifications yet this has not been assessed here.

To date, many of the studies examining the effects of ROS in skeletal muscle focus on changes to the contractile function of the fibres. ROS can alter myofilament Ca\(^{2+}\) sensitivity in addition to altering Sr Ca\(^{2+}\) release during excitation-contraction coupling [54,79,85-88]. There is also data suggesting that ROS produced during contraction can also delay recovery from fatigue [54,89]. While it may seem that ROS produced during exercise has a negative impact due to changes in the muscle's ability to produce force, studies have suggested that ROS...
regulates an adaptive response (hormesis) in the muscle [67,68]. Cheng et al. [54] used pharmacological inhibitors to target ROS from Nox2. Nox4 and the mitochondria in order to examine effects on single fibre muscle fatigue. Interestingly, they found minimal changes with the use of Nox inhibitors in muscle performance and recovery. The authors suggest that using whole muscle for these measurements could yield more consistent results. We examined force production, time to fatigue and recovery from fatigue in Nox4−/− EDL muscles. When compared to an age-matched WT mouse, we found no difference in our experimental parameters. This finding indicates that Nox4 ROS does not have a role in contributing to fatigue via changes to myofilament Ca2+ sensitivity or alterations in SR Ca2+ release upon activation.

While Nox4 did not appear to have a role in altering the force-generating properties of skeletal muscle, the functional role of Nox4 still needed to be determined. The RyR1 is known to be susceptible to S-nitrosylation, yet the mechanism behind this modification has remained unclear. We report a modest increase in S-Nitrosylation of the RyR1 in muscle from p47−/− with no alteration in the RyR1 in muscle from p47−/−/mdx. As the RyR1 SNO data is expressed as a ratio over total RyR present, the large increase in RyR present in the Nox4−/− could be contributed to the overall decrease in RyR1 SNO. However, when RyR1 SNO is expressed relative to MHC (for total protein), RyR1 SNO remains significantly decreased. Future studies would examine the exact cysteine thiols that are nitrosylated in order to provide further insight, as our studies are looking at bulk nitrosylation of the RyR1. Nox2 and Nox4 produce superoxide and H2O2, which can lead to protein carbonylation; so how might Nox ROS promote S-Nitrosylation of RyR1? Hydrogen peroxide has been shown to promote NOS dependent NO production and subsequent protein S-Nitrosylation in endothelial cells (eNOS) [90], cardiomyocytes (nNOS) [91], and skeletal myotubes (nNOS) [92]. Furthermore, H2O2 has been shown to activate nitrate reductase [93-95], an enzyme which catalyzes NAD(P)H-dependent reduction of nitrite to NO, and nitrate reductase generated NO has been shown to play a role in functional hyperemia in skeletal muscle [96]. Cellular nano-domains can respond quickly to Nox derived ROS, suggesting that Nox ROS may be exerting actions via affecting signalling targets close to the ROS source i.e. RyR1 proximity to Nox4. Thus, Nox4 derived H2O2 may promote protein carboxylation as well as increased NO production directly through increased ROS activity or through activation of nitrate reductase; with subsequent RyR1 S-Nitrosylation.

In Fig. 7, we propose a model of Ca2+ and ROS interplay in the triadic junctional space. This model has been generated based on the mdx mouse. Contrary to our initial hypothesis of t-tubular Nox2 ROS initiating RyR leak, we found Ca2+ leak persists in muscle models that cannot generate Nox2 ROS. The overall improvement in the phenotype of the p47−/−/mdx mouse [13,50,51] suggests that perhaps Nox2 ROS production is exacerbated in the mdx mouse by the local increase of Ca2+ within the junctional space from the RyR1 leak. This reciprocal interplay between Ca2+ and ROS is consistent with previous findings in skeletal muscle and cardiac fibres [19,25,97].

In our model, we propose a multiprostep process of Ca2+ and ROS interactions within the triadic micro-domain. Initially, Nox4 ROS drives changes to the RyR1 via S-nitrosylation modifications leading to increased Ca2+ leak (step 1). The increase in Ca2+ leak promotes a higher [Ca2+]i, and promotes enhanced Nox2 activation and ROS production (step 2). During this time in the mdx mouse model, increased Ca2+ influx from the extracellular space is also contributing to the activation of Nox2 [21,27,51] (step 3). Increased Ca2+ in the bulk cytoplasm and junctional space as well as increased Nox2 ROS and potentially increased mitochondrial ROS leads to impairment of cellular functions such as autophagy [98-101], ER stress [102-104], or mitochondrial Ca2+ accumulation [105] (step 4). As the steps in our model are occurring at rest in skeletal muscle, the alteration in calcium homeostasis and redox balance could also lead to changes in force production [88,106] or compensatory mechanisms. The disruption of these processes contributes to the pathology observed in dystrophic muscle and potentially in many other disease types. The persistent RyR1 leak could also serve to induce mitochondrial biogenesis [81] which would also alter the muscles’ capacity for performance and metabolism, driving compensatory mechanisms within the cell. These compensatory mechanisms could assist in allowing the cell to adapt, for example, during exercise, or push the cell to a pathological state. Recently it has been shown that Ca2+ from RyR1 leak can accumulate in the mitochondria [105]. In the model we propose here, the mitochondrial accumulation of Ca2+ would be occurring in the bulk cytoplasm, as a consequence of the events outlined in the triadic micro-domain.

As Nox4 is constitutively active but can be regulated in an inducible manner [107], for many cell types and situations, the precursor to alterations in Nox4 will need to be determined. Nox4 can serve as an inflammation promotion factor, with expression increasing as inflammatory markers such as Cox2, IL-1β and TNF-α are increased [108]. In vasculature cells, it appears that the circadian clock can

Fig. 7. Model of the cascade of ROS/Ca2+ interactions that occur in the skeletal muscle triadic micro-domain.1. Nox4 ROS contributes to an increase in S-nitrosylation of the RyR1, resulting in increased Ca2+ leak from the RyR1 into the junctional space. 2. Increased Ca2+ in the junctional space due to the RyR1 Ca2+ leak promotes increased Nox2 ROS activation and production. 3. Increased Ca2+ influx from the extracellular space also contributes to Nox2 activation and ROS production. 4. Increased Ca2+ in the bulk cytoplasm and the junctional space, in addition to the increased Nox2 ROS leads to alterations in autophagy, protein degradation and other cellular functions which contributes to the pathology observed in dystrophic skeletal muscle.
influence Nox4 expression with alterations in the circadian rhythm, causing ROS production [109]. There are multiple transcription factors that can alter Nox4 promoter activity; such as NFkB, SMAD2/3, TGFβ, E2F, HIF1α and Nrf2 [110]. In dystrophic muscle, the persistent degeneration/regeneration cycles lead to increased TGFβ exposure and inflammatory cell infiltrate [111]. This increase in TGFβ could, in turn, lead to increased Nox4 expression and initiate the RyR1 leak seen in the mdx mouse.

5. Conclusion

Nox4 ROS induces RyR1 Ca2+2 leak, via S-nitrosylation modifications. Although Nox2 ROS does not directly contribute to RyR1 leak, it responds reciprocally to the increased Ca2+2 in the juncational space from the RyR1 leak and to the increased Ca2+2 influx from the extra-cellular compartment. The reciprocal interplay between the Nox isoforms and Ca2+2 occurs within the triad of skeletal muscle as the plasma membrane has been peeled back during the mechanical skinning process. Our model of the cascade of ROS and Ca2+2 interactions highlights the importance of assessing these movements not only in skeletal muscle as a whole, but within and around organelle micro-domains.

Declaration of competing interest

None.

Acknowledgements

The authors would like to thank Courtney Carvasos for technical assistance. The monoclonal RyR1 antibody (34C) developed by J. Airey and J. Sutko and the SERCA antibody (CA F2-5D2) developed by Dr. Fambrough, were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD, National Institutes of Health, and maintained by the University of Iowa (Iowa City, IA, USA).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2020.101557.

Funding sources

This work was supported by the National Institutes of Health (AR061370) and The Department of Defense (MD170090).

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