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REGULATION OF NADPH OXIDASES IN SKELETAL MUSCLE

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Abstract

The only known function of NAD(P)H oxidases is to produce reactive oxygen species (ROS). Skeletal muscles express three isoforms of NAD(P)H oxidases (Nox1, Nox2, and Nox4) that have been identified as critical modulators of redox homeostasis. Nox2 acts as the main source of skeletal muscle ROS during contractions, participates insulin signaling and glucose transport, and mediates the myocyte response to osmotic stress. Nox2 and Nox4 contribute to skeletal muscle abnormalities elicited by angiotensin II, muscular dystrophy, heart failure, and high fat diet. Our review addresses the expression and regulation of NAD(P)H oxidases with emphasis on aspects that are relevant to skeletal muscle. We also summarize: i) the most widely used NAD(P)H oxidases activity assays and inhibitors, and ii) studies that have defined Nox enzymes as protagonists of skeletal muscle redox homeostasis in a variety of health and disease conditions.

Keywords

reactive oxygen species; oxidative stress; exercise; muscle contraction; antioxidants; muscle weakness

Introduction

The history of redox signaling in skeletal muscle biology and NAD(P)H oxidases are inextricably intertwined. The production of reactive oxygen species (ROS) has long been recognized as a critical component of skeletal muscle cell biology in health and disease (reviewed in [1–3]). Early studies identified extracellular superoxide ($O_2^{\cdot-}$) release from isolated skeletal muscle at rest and during fatiguing contractions [4]. In hindsight, these findings were consistent with the presence and activation of NAD(P)H oxidases, which were not fully explored because mitochondria have been considered the most relevant ROS source in muscle (see review by Powers & Jackson [5]). Ten years after the discovery of extracellular superoxide release by muscle, Javesghani et al [6] reported the presence and molecular characterization of NAD(P)H oxidase in skeletal muscle. In recent years,

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NAD(P)H oxidases have emerged as the main (or initial) source of ROS in skeletal muscle cells. Our main goal in this review article is to provide an overview of the expression and regulation of NAD(P)H oxidases, highlighting key aspects relevant to skeletal muscle biology. We have also summarized studies that have defined NAD(P)H oxidases as protagonists of skeletal muscle redox homeostasis in health and disease.

NAD(P)H oxidases in skeletal muscle cells

The homologues Nox1, 2, and 4 are expressed in skeletal muscle cells in culture [7–11]. Nox homologue abundance in C2C12 muscle cells, based on mRNA data, is as follows Nox4 > Nox2 > Nox1 [8, 10]. Although Nox1 is upregulated by myostatin in differentiating C2C12 myoblasts [12], a physiological role for Nox1 has not been identified in skeletal muscle. The Nox2 homologues Duox1 and 2 are also expressed in C2C12 muscle cells, but very little is known about their physiological relevance beyond a recent report implicating a role in myogenesis [13]. Another important aspect to consider is the substrate of Nox enzymes. In non-muscle cells, Nox enzymes preferentially utilize NADPH over NADH [14]. However, NADH appears to elicit a three-to-five fold higher Nox activity than NADPH in adult skeletal muscle, which is consistent with NADH as the primary substrate [6]. Based on the above, we will focus our review on Nox2 and Nox4 regulation, and use the term NAD(P)H to consider both substrates.

Nox2

The functionally active Nox2 is a multimeric enzyme that catalyzes the conversion of O_2 to superoxide. The enzyme was first discovered in phagocytes, and the assembled phagocytic oxidase (*phox*) consists of Nox2 (gp91^{phox}), p22^{phox}, p47^{phox}, p67^{phox}, p40^{phox}, and Rac (reviewed in [14]). The subunits Nox2 and p22^{phox} form the redox core *flavocytochrome b558* responsible for electron transfer and superoxide production [15–17]. The other subunits organize and assemble the functional complex and regulate its activity. All Nox2-related subunits are present in skeletal muscle [6, 18], and the active enzyme complex in muscle cells appears to be similar to that found in other cell types (Fig. 1).

Nox2 subunit composition and localization

Nox2 is a 58 kDa protein, but can appear as bands of higher molecular weight in Western Blots due to glycosylation [14]. Interestingly, a new splice variant of Nox2 (Nox2β, ~30 kDa) has been identified in macrophages [19]. The Nox2β splice variant does not seem to be present in cardiomyocytes [19], and it is unclear whether it is expressed in skeletal muscle. In conventional Western Blots performed by our group [20], we have not detected the 30 kDa splice variant in mouse diaphragm. Thus, the Nox2β isoform requires further investigation, and we will refer to the conventional Nox2 isoform in this review.

In skeletal muscle cells, Nox2 and p22^{phox} are present in membrane-enriched protein fractions and co-localize with membrane proteins [6, 18]. These findings are consistent with Nox2 and p22^{phox} being sarcolemmal proteins. Immunohistochemistry and triad/t-tubule fractionation experiments also suggest the presence of Nox2 and p22^{phox} in t-tubules (invaginations of the membrane in skeletal muscle cells) [21, 22]. However, it appears that

Nox2 is not present in the sarcoplasmic reticulum [21, 22]. Based on its localization, Nox2 produces superoxide outside the cell that can be enzymatically converted, by extracellular superoxide dismutase [23], into hydrogen peroxide (H_2O_2). Hydrogen peroxide readily crosses membranes and will exert its effects in the intracellular compartment [24]. This notion is supported by recent data in isolated skeletal muscle fibers [25].

While Nox2 localization has been defined, the exact subcellular location of Nox2 subunits remains unclear. The protein Rac1 is present in multiple compartments and regulates several cellular functions. Cytosolic Rac1, which translocates to the cell membrane upon activation [26], appears to be the most relevant for Nox2 activation in non-phagocytic cells [27–29]. To date, there is no evidence to suggest that a different process takes place in skeletal muscle. The subunits $p67^{phox}$ and $p40^{phox}$ are also mainly cytosolic and translocate to the cell membrane upon Nox2 activation [14, 24, 30]. Overall, the cytosolic localization of Rac1, $p67^{phox}$, and $p40^{phox}$ conforms to the traditional view of Nox2 regulation and signaling [14, 30].

The subunit $p47^{phox}$ is required for Nox2 activity [31]. In non-skeletal muscle cells, $p47^{phox}$ is a cytosolic subunit [14, 30]. However, the localization of $p47^{phox}$ in skeletal muscle is less clear. Specifically, $p47^{phox}$ has been detected in membrane-enriched, but not cytosolic fractions of the diaphragm [6]. Immunohistochemistry and co-localization experiments also support the notion that endogenous $p47^{phox}$ is localized at (or very near) the sarcolemma and t-tubules in limb muscles [18, 25, 32] and diaphragm [6]. Other studies have identified increases in sarcolemmal $p47^{phox}$ with stimulation, which is consistent with membrane translocation. These findings pose a challenge to our understanding of Nox2 regulation in skeletal muscle and how $p47^{phox}$ participates in this process, as will be discussed below. Regardless, it is clear that $p47^{phox}$ is involved in the activation and regulation of Nox2 activity in skeletal muscle.

Activation and regulation

In several cell types, phosphorylation of $p47^{phox}$ or $p67^{phox}$, and activation of Rac1 regulate the production of superoxide by Nox2 [27, 33–36]. Two of these processes, phosphorylation of $p47^{phox}$ and Rac1 activation, have been reported in skeletal muscle [20, 37, 38]. These post-translational events lead to translocation and binding of cytosolic subunits to the cell membrane and $Nox2/p22^{phox}$ to form a functionally active complex. For example, activation of Nox2 by muscle contraction is associated with an apparent translocation of $p40^{phox}$ and $p67^{phox}$ to the cell membrane [18]. In the traditional view of Nox2 regulation, $p47^{phox}$ is considered the organizer component that forms a complex with all cytosolic subunits and governs their translocation to the cell membrane [14, 30]. Nox2 contains a $p47^{phox}$ binding motif [39–41]. Considering the potential localization of $p47^{phox}$ in skeletal muscle cells in the membrane versus the cytosol, it is unclear how the protein serves as organizer of cytosolic subunits. The apparent inconsistency between skeletal muscle and other cell types presents an issue that must be resolved for a better understanding of Nox2 regulation in skeletal muscle. One possibility is that $p47^{phox}$, which binds to cytoskeletal proteins [42], co-localizes predominantly near the sarcolemma with proteins of the costamere in skeletal

muscle. Such localization would permit p47^{phox} to function as organizer of cytosolic subunit translocation.

p47^{phox}

A discussion on p47^{phox} structure and function is paramount in understanding Nox2 signaling in skeletal muscle. P47^{phox} is a 390 amino acid protein that consists of one PX domain with phospholipid binding properties, two SH3 domains, one auto-inhibitory region, and a proline-rich region [33, 43, 44]. In the inactive state, the auto-inhibitory and proline-rich regions interact with the PX and SH3 domains. This conformation prevents p47^{phox} from binding to Nox2 membrane and cytosolic subunits (Fig. 2) [33, 43, 45, 46]. Conversely, serine phosphorylation exposes the SH3 domain and PX region, which allows membrane-binding and docking to Nox2 and p22^{phox} [33, 47, 48]. Hence, serine phosphorylation of p47^{phox} is required for Nox2-dependent ROS production and has been used as an indicator of enzyme activation. Serine phosphorylation of p47^{phox} is elevated in skeletal muscles in mouse models of Duchene muscular dystrophy and heart failure [20, 37], and *in vitro* exposure to a mediator of inflammatory signaling [38]. These are conditions where p47^{phox} is required for elevated Nox activity and ROS emission.

The above evidence suggests that serine-threonine kinases and protein phosphatases play a major role on p47^{phox} phosphorylation and Nox2 regulation in skeletal muscle. Serine-threonine kinases known to be involved in phosphorylation of p47^{phox} include PKC α, β, δ, and ζ (reviewed in [33]), mitogen-activated protein (MAP) kinases p38 [49, 50] and ERK-1/2 [50–52], JNK-1 [52], PI3K [53–55], AKT [55–57], and p21-activated kinase 1 [58]. Not all kinases mentioned interact directly with p47^{phox}. Some exert their effects via intermediate kinases. The tyrosine kinase Src promotes p47^{phox} serine phosphorylation in skeletal and vascular smooth muscle cells [37, 59], which must be mediated by intermediary serine-threonine kinases sensitive to Src. However, protein kinases do not always activate Nox2 [33]. In cardiomyocytes, p21-activated kinase 1 acts as a negative regulator of Nox2 [60]. These findings suggest cell-type specificity of kinases involved in p47^{phox} phosphorylation. Tyrosine phosphorylation of p47^{phox} and high concentration of arachidonic acid have also been identified as regulatory mechanisms of Nox2 activation in non-muscle cells [61, 62]. However, it is unclear whether tyrosine phosphorylation or arachidonic acid participate in Nox2 regulation in skeletal muscle.

Less is known about specific phosphatases that regulate the phosphorylation status of p47^{phox}. P47^{phox} plays a required role on Nox2 activation during repetitive muscle contractions and deactivation during recovery [25]. Nox2 deactivation likely requires p47^{phox} dephosphorylation. Thus, it is reasonable to speculate that p47^{phox} protein phosphatases have a major impact on Nox2 regulation and redox homeostasis during skeletal muscle contractions and rest. Pharmacologic inhibitor studies in non-muscle cells suggest that protein phosphatases type 1 and 2A regulate p47^{phox} dephosphorylation [63–65], but these have yet to be confirmed with genetic manipulations (knockdown, knockout, and overexpression of dominant-negative or constitutively active constructs).

Overexpression of p47^{phox} *per se* is typically considered insufficient to heighten Nox2-dependent ROS production. These considerations are based mainly on studies of NAD(P)H

oxidases in vascular biology. For instance, overexpression of $p47^{phox}$ in endothelial cells does not increase Nox2-dependent ROS emission [49]. However, these effects are cell-type specific. In murine microglia, overexpression of $p47^{phox}$ heightened Nox2 activity [66]. The effects of $p47^{phox}$ overexpression on Nox activity in skeletal muscle cells have not been defined. Preliminary studies from our group suggest increased Nox activity with overexpression of $p47^{phox}$ (data not shown). Importantly, sustained and pathophysiological elevation of skeletal muscle Nox2 activity and ROS in diseases states is associated with upregulation of $p47^{phox}$ mRNA and protein. The transcription factors HBP1, Ets-1, STAT1/3, and NF-kB regulate $p47^{phox}$ expression [67, 68]. Signaling pathways that modulate these transcriptional factors are, therefore, likely to influence Nox2 expression in skeletal muscle.

Rac1

Rac1 (Ras-related C3 botulinum toxin substrate 1) is a small G-protein that, once activated, facilitates translocation of cytosolic subunits to the membrane and the interaction between $p67^{phox}$ and Nox2 that are required for enzyme activity [27, 69]. In fact, targeting Rac1 to the cell membrane is sufficient to induce Nox activity [26]. Rac1 activity is determined by the GDP-bound (inactive) and GTP-bound (active) states [28, 70, 71]. Rac1 activity is increased in skeletal muscle of *mdx* mice and participates in the pathophysiology of Duchenne Muscular Dystrophy via its effects on Nox2 signaling [37]. The transition from Rac1-GDP (inactive) to Rac1-GTP (active) is modulated by GDP Dissociation Inhibitors (GDI) and guanine nucleotide exchange factors (GEF). There are several GEF in cells, but Tiam1 [72], Trio [73], P-Rex2 [74], and obscurin [75] appear to be particularly relevant for skeletal muscle. P-Rex2 is expressed predominantly in skeletal muscle [74]. Obscurin interacts with titin, a mechanosensitive sarcomeric protein. This makes obscurin an attractive candidate for mechano-dependent activation of Rac1 and Nox2, which occurs during skeletal muscle stretch [76] and contraction [25]. Rac1 is inactivated by its intrinsic GTPase activity, which is stimulated by GTPase-activated proteins (GAP) [27, 28, 70]. In this regard, p190 Rac-GAP can inhibit Nox2 activity and superoxide production in neutrophils [77]. However, the role of specific GAPs on regulation of skeletal muscle Nox2 remains to be defined.

Nox4

The Nox4 isoform was first discovered in the kidney and is homologous (39%) to Nox2 [14, 48, 78]. Nox4 is highly expressed in tumor cells [79], being considered an oncoprotein [80]. Nox4 can produce both $O_2^{\cdot -}$ and H_2O_2 [81–83], but the predominant ROS produced by skeletal muscle Nox4 remains unknown. The production of H_2O_2 depends on the presence of a histidine residue within an extra-cytosolic loop, which is 28 amino acids longer in Nox4 than Nox2 [84]. Nox4 can appear as two bands on Western Blots (75–80 kDa and 67 kDa), which may reflect glycosylation or splice variants [14]. In skeletal muscle homogenates, we have found higher levels of the 67 kDa band in diaphragm than other limb muscles [85]. These findings suggest that Nox4 abundance may vary based on muscle fiber type composition or recruitment pattern.

Nox4 subunit composition and localization

Nox4 requires binding to $p22^{phox}$ for ROS production in a cell-free system, but is independent from other Nox subunits [30, 78, 83]. Nox4 contains a mitochondria localization sequence in its N-terminal region [86]. Accordingly, Nox4 is present in cardiac and limb muscle mitochondria [18, 86]. Nox4 is also present in skeletal muscle sarcoplasmic reticulum [8]. Considering that Nox4 is constitutively active and requires $p22^{phox}$ for ROS production [82, 87], we speculate that $p22^{phox}$ is also present in the sarcoplasmic reticulum.

Nox4 regulation and activation

The traditional view is that Nox4 is constitutively active and, therefore, transcriptionally regulated [30, 78]. The expression of Nox4 is induced by cytokines, transforming growth-factor β, and angiotensin II [68, 88]. The following conditions and diseases are associated with increased Nox4 mRNA or protein levels in skeletal muscle: heart failure (unpublished results), cancer [89], and aging sarcopenia [90]. Transcription factors that have been involved in regulation of Nox4 expression in various cell types include Nrf2 [91], NF-κB [92], HIF-1α [93], STAT1/3 [92], E2F [94], c-Jun [95], AP-1 [96], and SMAD3 [96]. The mRNA expression of Nox4 is also regulated by histone deacetylases (HDAC) [97]. In human endothelial cells, pharmacological inhibition of HDACs or knockdown of HDAC3 decreases Nox4 transcription [95]. In C2C12 myotubes, knockdown of the deacetylase sirtuin 1 (SIRT1) increases Nox4 protein levels, suggesting that SIRT1 is a negative regulator of Nox4 expression [11]. Lastly, the interaction among Hic5, heat shock protein 27, and Cbl-c regulate Nox4 expression post-transcription/translation through the ubiquitin-proteasome system [98].

The rapid increase in Nox4-dependent ROS stimulated by agonists has challenged the notion that ROS production by Nox4 is regulated solely at the transcriptional level. Myotubes exposed to IGF-I (15 min; [10]), adipocytes exposed to insulin (5 min, [99]), cerebral microvascular endothelial cells exposed to TNF-α (1–3 hr., [100]), and mesangial cells exposed to angiotensin II or arachidonic acid (5 min, [88]) show heightened ROS or ROS-mediated signaling that is inhibited by knockdown of Nox4. Some modulators of Nox4 activity include Poldip2 (polymerase [DNA-directed] delta-interacting protein 2), Tks5 (tyrosine kinase substrate 5), Hsp70 (heat shock protein 70), and possibly Rac1 [14, 30, 101]. Poldip2, which is highly expressed in skeletal muscle [102], interacts with $p22^{phox}$ and Nox4 in vascular smooth muscle cells. In this setting, Poldip2 serves as a stabilizer of the Nox4 and $p22^{phox}$ complex and regulator of enzyme activity [30, 102]. Similarly, Tks5 has emerged as a possible regulatory subunit that is required for Nox4 dependent ROS production in certain cell types, e.g., melanoma [103]. Tks5, which has a structure closely related to $p47^{phox}$, is expressed in myoblasts and interacts with dystroglycan [103, 104]. Nox4 contains a binding sequence for $p47^{phox}$ within the B loop [41, 105]. Thus, the identification of Tks5 as a regulator of ROS production by Nox4 raises the possibility that Tks5 modulates Nox4 activity in skeletal muscle cells. Another potential post-translational regulator of Nox4 activity is Hsp70. In vascular smooth muscle cells, Hsp70 functions as a chaperone that negatively regulates Nox4 activity and expression. The acute inhibitory effect of Hsp70 on Nox4 appears to be due to membrane translocation of cytosolic Hsp70 [106, 107], whereas chronic inhibitory effects involve pathways that result in Nox4 ubiquitination

and degradation [108]. Rac1 has also been implicated in Nox4 regulation [14, 69, 88, 109]. Specifically, Nox4-dependent increase in ROS stimulated by angiotensin II and arachidonic acid were inhibited by Rac1 knockdown or expression of dominant-negative Rac1 [88]. In kidney cells, Nox4 interacts with toll-like receptor 4 and mediates ROS signaling in response to lipopolysaccharide [110]. This raises the interesting possibility that abnormalities induced by TLR4 signaling in skeletal muscle are in fact mediated by Nox4, e.g., rheumatoid arthritis [111], mechanical ventilation [112], and endotoxemia [113].

The constitutive activity of Nox4 is an important aspect to take into account regarding ROS production in skeletal muscle cells. For instance, NADH serves as substrate for Nox4 and elevation in the $NADH/NAD^+$ ratio, which occur during high-intensity muscle contractions or hypoxia, could heighten ROS production. Indeed, Nox4-dependent ROS are important for skeletal muscle response to hypoxia (see below and ref. [8]). Nevertheless, it remains to be established whether changes in $NADH/NAD^+$ ratio affect Nox4-dependent ROS production.

Nox activity assays

The lack of specific measurements of Nox activity has been a problem in the field. Methods currently available present technical difficulties and limitations that have diminished the enthusiasm of skeletal muscle biologists to investigate NAD(P)H oxidases. The techniques used more frequently such as lucigenin-enhanced chemiluminescence, cytochrome C reduction, and p47-roGFP are discussed below.

Lucigenin-enhanced chemiluminescence is a method that has typically been used for measurement of Nox activity. Lucigenin, which is actually a fluorescent chloride indicator, reacts with superoxide to produce light. The lucigenin signal stimulated by addition of NAD(P)H to the reaction buffer is considered a measurement of Nox-derived superoxide. A conceptually similar approach involves the use of NAD(P)H consumption by tissue homogenates or membrane fractions. The lucigenin and NAD(P)H consumption assays have been used to assess Nox activity in skeletal muscle, e.g., [6, 20, 114–116]. However, a recent study from Prof. Ralf Brandes' group shows that the assay is not specific to Nox activity and, in several tissues and cell types, the signal was unchanged with a triple Nox1, Nox2, and Nox4 knockout [117]. Based on these findings, the authors suggested that assays based on lucigenin and NADPH consumption are invalid [117].

A derivation of the lucigenin-based assay involves measurement of cytochrome c reduction by superoxide. The assay has been widely employed to measure superoxide in skeletal muscle preparations [4, 118, 119], and was adapted to determine Nox activity in skeletal muscle homogenates. In this setting, the signal that is induced by NADH and inhibited by superoxide dismutase is considered a measure of Nox activity [114, 120]. However, the specificity of the cytochrome c reduction assay for Nox activity has yet to be verified in skeletal muscle. At present, the recommendation by experts in the biology of NAD(P)H oxidase is "…*to measure ROS formation in intact tissue rather than boosting their signal with NADPH or using homogenates when addressing ROS production to Noxes*" [117].

A promising approach to study $p47^{phox}$-dependent Nox activity in live tissue and cells has been developed recently [25]. Specifically, the group led by Dr. George Rodney fused the redox sensitive probe roGFP with $p47^{phox}$ (p47-roGFP). When expressed in cells, p47-roGFP was localized to cellular compartments where Nox2 is present, seemingly sarcolemma and t-tubules as discussed above. The probe has several advantages that include sensitivity to Nox-dependent ROS production in skeletal muscle, ratiometric fluorescence that makes it independent of differences in content or expression levels, rapid response time, and reversibility. The limitation of p47-roGFP is that overexpression of $p47^{phox}$ may induce Nox activation in certain cell types. The probe also rescues Nox activity in $p47^{phox}$ deficient cells, which are frequently used to test the role of Nox(s) on ROS production. However, the advent of p47-roGFP has already provided insights into Nox activation during skeletal muscle contraction and stretch [25]. P47-roGFP can now be used to define signaling pathways leading to Nox activation and its role in several physiological and pathological settings.

Nox Inhibitors

There are several Nox inhibitors available [48, 101, 121]. The compounds that have been mostly used to examine Nox in skeletal muscle are apocynin, diphenyleneiodonium (DPI), gp91dstat, and GKT-137831 [18, 25, 38, 122]. Although inhibitor studies provide useful information, the compounds commercially available are generally non-specific as discussed below.

Apocynin is a plant phenol that depends on hydrogen peroxide and peroxidases for conversion to a radical form that inhibits Nox activity. The mechanism of apocynin action appears to involve oxidation of the flavocytochrome b558 core that prevents interaction with Nox cytosolic subunits. Apocynin does not affect skeletal muscle contractile function in concentrations employed to inhibit Nox, whether used *in vitro* [38] or *in vivo* [123, 124]. However, it is important to note that, beyond being non-specific and having direct antioxidant properties in cells, apocynin can react directly with oxidant-sensitive probes and interfere with assays *in vitro* [38].

The broad-spectrum flavoprotein inhibitor DPI is often used to block ROS production by NAD(P)H oxidases. However, DPI inhibits other sources of ROS, including mitochondrial complex I [9, 125]. Because DPI is a general flavoprotein inhibitor, it can affect cell metabolism and have an unrecognized impact on function. Indeed, we have found that DPI depresses skeletal muscle force in a dose dependent manner, with diaphragm muscle force decreasing by 40% at 5 μM and 65% at 10 μM [38, 85]. Thus, the loss of force induced by DPI occurs at concentrations that are typical for studies of NAD(P)H oxidases, supporting the notion that DPI should no longer be considered for studies of Nox. Instead, new and more specific inhibitors are a better choice [48, 101, 117].

The inhibitor Nox2ds-tat (originally named gp91ds-tat) was developed based on the mapping of peptide sequences involved in the interaction of Nox2 and $p47^{phox}$ [41, 126]. The peptide sequence that most potently inhibited Nox activity in cell-free assays was then conjugated to the peptide sequence of HIV viral coats (HIV-tat) to promote cellular uptake.

Nonetheless, the HIV-tat sequence can elicit confounding effects such as increased autophagy and remodeling of the actin cytoskeleton [127, 128]. Thus, control experiments should include the scrambled peptide with the tat sequence. Other issues include cleavage of the tat sequence *en route* to tissues and development of immune response to the peptide that limits repeated exposure and long-term treatment [129]. The Nox2ds-tat peptide sequence is also present in Nox1 and Nox4 [105, 129, 130], but the compound is considered a specific Nox2 inhibitor at low concentrations [101]. In skeletal muscle, Nox2ds-tat prevents ROS production during contraction [18, 25] and in disease [37, 131].

Novel inhibitors of Nox1/4 such as GKT-137831 have been developed by the pharmaceutical industry and are currently undergoing clinical trials. The compound appears to be specific to Nox1/4 within the nano-to-micromolar range [101], and initial safety data are promising as indicated by press-releases from Genkyotex S.A. The compound GKT-137831 does not interfere with submaximal muscle function [122], yet its efficacy in inhibiting ROS in skeletal muscle is unknown.

Redox cross-talk

The formation of ROS can promote further ROS production and release, which has been termed ROS-induced ROS release – reviewed in detail by Zorodov et al. [132]. NAD(P)H oxidases and mitochondria are the primary sources of ROS in skeletal muscle, and a redox-mediated crosstalk between Nox and mitochondria can exacerbate ROS production and disrupt redox homeostasis [133–135]. Several mechanisms can account for the cross-talk between Nox and mitochondrial ROS production and release. Oxidation of mitochondrial electron transfer chain complexes or glutathione can play an important role in ROS production and release. The former leads to uncoupling of electron transfer that results in superoxide formation [136, 137]. The latter diminishes mitochondrial H_2O_2 scavenging by glutathione peroxidase and exacerbates H_2O_2 production by the pyruvate dehydrogenase complex [137, 138]. Studies in the last 15 years have shown that the cross-talk involves mitochondrial ATP-sensitive K^+ channels (mito-K_{ATP}). Nox-derived ROS can oxidize and promote the opening of mitochondrial ATP-sensitive K^+ channels (mito-K_{ATP}) [139]. Potassium influx into the matrix lowers the mitochondrial membrane potential, which causes mitochondrial swelling, opening of permeability transition pores, and elevates ROS production (Fig. 3, [133–135]). Another possibility is that Nox-derived ROS cause sarcoplasmic reticulum Ca^{2+} leak [89, 140] or extracellular Ca^{2+} entry into the sarcoplasm through ‘transient receptor potential’ channels [131, 141, 142]. These processes heighten cytosolic Ca^{2+} concentration. Mitochondria acts as buffers of elevated cytosolic Ca^{2+}, and the Ca^{2+} overload heightens mitochondrial ROS emission [143, 144]. In this model, Noxes act as ligand- or mechano-activated triggers and mitochondria are amplifiers of ROS in the cell.

The cross talk can also be initiated at the mitochondria by conditions that impair myocyte metabolism and increase mitochondrial ROS such as high fat diet, diabetes, denervation, and inactivity [145–150]. Mitochondrial ROS activate redox-sensitive kinases (e.g., PKC and Src [151, 152]) that increase Nox activity (see above) and promote upregulation of Nox2 and Nox4 [153]. Excess mitochondrial ROS can also cause RyR oxidation and Ca^{2+} release. In

non-muscle cells, Ca^{2+} accumulation triggers Nox activation [154, 155]. Overall, the cross-talk between Nox and mitochondrial ROS consists of positive feedforward and feedback loops that, in general, disrupts redox homeostasis.

The pathways discussed above have been shown in several cell types, including cardiac and smooth muscle [135]. To our knowledge, there is only indirect evidence to support the existence of a redox cross-talk in skeletal muscle. Specifically, systemic administration of angiotensin II increases Nox and mitochondrial ROS in skeletal muscle [156, 157], and $p47^{phox}$ knockout blocks the increase in skeletal muscle cytosolic superoxide induced by angiotensin II [157]. Mechanical ventilation elevates diaphragm ROS, which can be prevented by either apocynin or a mitochondria-targeted antioxidant [123, 148]. Similarly, the increase in diaphragm ROS elicited by chronic heart failure or exposure to exogenous sphingomyelinase are prevented by knockout of Nox2 subunits ($Nox2^{-/y}$ [158], $p47^{phox-/-}$ [38]) or mitochondria-targeted antioxidants [159]. Finally, repetitive contraction induces a rapid rise in cytosolic superoxide mediated by Nox, which is followed by a slower rise in mitochondrial ROS [18, 160]. Other examples of potential skeletal muscle redox cross-talk involving Nox and mitochondria include high fat diet and sepsis [124, 146, 161, 162].

Nox and skeletal muscle biology in health and disease

Contraction and E-C coupling

Skeletal muscle contraction heightens ROS production that modulates cellular homeostasis and adaptations. The original hypothesis was that mitochondria were the main source of contraction-induced ROS. However, several pieces of evidence suggest that Noxes are the main source of ROS induced by skeletal muscle contractions. During repetitive contractions, the rise in cytosolic ROS precedes and is greater than the increase in mitochondrial ROS [160, 163]. Immunohistochemistry data are consistent with membrane translocation of cytosolic subunits of Nox2 [18]. A cause and effect relationship involving Nox2 has been established with pharmacologic and genetic interventions. Specifically, contraction-induced increase in ROS was blocked or attenuated by pharmacologic agents (apocynin and Nox2*ds-tat*) [18, 25, 158, 164, 165] or knockout of Nox2 subunits [25, 166]. These data suggest that Nox2 is the main source of ROS during muscle contractions.

ROS modulate excitation-contraction coupling in skeletal muscle. Skeletal muscle depolarization activates Noxes in the t-tubule and sarcoplasmic reticulum, and ROS modulate Ca^{2+} release by the RyR1 channel [21, 22, 164]. Some studies suggest that ROS produced by Nox2 modulates excitation-contraction coupling [21, 164], whereas other studies have suggested that Nox4 is the main source of ROS affecting excitation-contraction coupling [8]. Calcium transients are similar in muscle fibers from Nox2 knockout and wild-type mice [166], and force production is normal in $p47^{phox}$ and Nox2 knockout mice during single or repetitive fatiguing contractions [20, 38, 158]. Knockdown of Nox4 lowers tetanic force production in experiments with solutions gassed with 20% O_2, 5%CO_2 [8]. In contrast, we have found that twitch and tetanic forces are similar in muscles from whole-body Nox4 knockout and wild type mice tested under standard *in vitro* conditions (95% O_2, 5% CO_2) [85]. However, adaptations that occur with embryonic deficiency of a protein might affect

redox balance in knockout animals. For instance, diaphragm from mice lacking $p47^{phox}$ have downregulation of protein levels of antioxidant enzymes SOD1 and catalase [38] and upregulation of Nox4 protein levels (B. Ahn and L. Ferreira, unpublished observations). In this regard, pharmacologic compounds are useful to resolve acute effects of Nox inhibition. Apocynin blunts depolarization-induced Ca^{2+} release in myotubes [164]. In isolated fibers, Nox2ds-tat or GKT137831 did not change Ca^{2+} transients or force during repetitive fatiguing contractions, but appeared to increase resting Ca^{2+} concentration [122]. At this stage, it is difficult to define the specific role of Noxes as modulators of E-C coupling. Data from knockout animals suggest that Nox2 or Nox4 are not required for normal E-C coupling in skeletal muscle.

Angiotensin II-induced muscle abnormalities

Angiotensin II mediated signaling has been identified as an important component of skeletal muscle abnormalities in cancer [167], muscular dystrophy [168], mechanical ventilation [169], and chronic heart failure [170, 171]. Wei et al. [172] demonstrated that angiotensin II increases ROS in skeletal muscle cells. This increase in ROS was associated with higher levels of $p47^{phox}$ and $p67^{phox}$ in the plasma membrane fraction and was blunted by apocynin. Apocynin, gp91ds-tat, or knockdown of $p47^{phox}$ inhibited responses stimulated by angiotensin II in muscle cells [172–174]. Similarly, whole-body $p47^{phox}$ knockout mice are protected from increases in skeletal muscle ROS and atrophy elicited by systemic administration of angiotensin II [157]. Therefore, Noxes are critical mediators of angiotensin II signaling in skeletal muscle cells.

Insulin-signaling and glucose transport

ROS are mediators of insulin signaling and glucose transport in skeletal muscle, and Nox-derived ROS play a critical role in these processes. For instance, Espinosa et al. [175] demonstrated that insulin promotes sarcolemmal translocation of $p47^{phox}$ and increases ROS levels in cultured muscle cells. The increase in ROS stimulated by insulin was blunted by knockdown of $p47^{phox}$, suggesting a role for Nox2 on insulin signaling [175]. Consistent with this notion, Nox2 knockout mice have higher baseline levels of plasma glucose and insulin compared to wild type animals [161]. Muscle contraction and stretch stimulate glucose transport in a ROS-dependent manner [176, 177], and recent studies have indicated that Rac1 is required for contraction- and stretch-stimulated glucose uptake in skeletal muscle [76, 178]. These findings suggest Nox2-derived ROS as potential mediators of skeletal muscle glucose transport. However, the stimulating effects of ROS on muscle glucose transport are concentration-dependent. High levels of ROS impair insulin signaling and cause insulin-resistance [146]. For instance, Nox2-derived ROS mediates insulin resistance induced by angiotensin II in muscle cells [172, 174]. Skeletal muscle insulin resistance after myocardial infarction is associated with elevated Nox activity and ameliorated by systemic administration of apocynin [179]. A direct role for Nox2 in insulin resistance has also been shown with high fat diet. Specifically, abnormalities in insulin signaling and glucose transport caused by high fat diet were blunted in Nox2 knockout animals [161]. Therefore, the relationship between Nox-derived ROS and insulin signaling/glucose transport is likely a bell-shaped curve, as with muscle contraction [3]

Mechanotransduction

Emerging data suggest that ROS are important in mechano-sensing and signaling in striated muscles. This phenomenon has been termed *X-ROS* and was reviewed in detail by Ward et al. [141]. The working hypothesis is that Rac1 is associated with microtubules and activates pre-assembled Nox2 complexes upon mechanical perturbation [141]. Stretching causes pronounced increases in ROS production by muscle [25, 176, 180]. Nox2 appears to be the primary source of stretch-induced ROS. Specifically, stretch-induced increase in ROS is absent in skeletal muscle fibers that lack Nox2 or Rac1 [25, 76]. Nevertheless, Nox4 may also be involved in mechanotransduction in skeletal muscle. For instance, Ito et al [181] have shown that Nox4 knockout or pharmacological inhibition of Nox4 blunted signaling through mTOR (mammalian-target of rapamycin) and prevented overload-induced skeletal muscle hypertrophy. However, it is unclear whether Nox4 is directly involved in mechanotransduction or if the blunted hypertrophy is secondary to impaired angiogenesis in whole-body Nox4 knockout [182]. Importantly, the data from Ito et al. [182] suggest that ROS derived from Nox4 are critical mediators of biological responses of skeletal muscle tissue to mechanical stress, i.e., overload.

Diseases, muscular dystrophies, and aging

Skeletal muscle Nox subunits are upregulated and appear to contribute to skeletal muscle abnormalities in muscular dystrophy [32, 37, 183, 184], several chronic acquired diseases [20, 185], and aging sarcopenia [90]. Skeletal muscles of the *mdx* mouse model of Duchenne Muscular Dystrophy show elevated mRNA or protein levels of Nox2 subunits ($p47^{phox}$, $p67^{phox}$, Rac1, and Nox2) [32, 37, 183, 184]. The increase in Nox2 subunits is also evident in cultured primary myotubes from mdx mice [184], suggesting that a mechanism independent of infiltration of macrophages underlies the overexpression of Nox2 subunits in Duchenne Muscular Dystrophy. Recent studies suggest that Nox2 plays a causative role on the pathology of Duchenne Muscular Dystrophy. Specifically, the Nox inhibitor Nox2ds-tat and knockout of $p47^{phox}$ blunt excess ROS and skeletal muscle abnormalities in *mdx* mice [37, 184]. Heart failure also increases Nox2 subunits in limb and diaphragm muscle of mice [20, 179]. Recent studies point to Nox2 as a mediator of skeletal muscle dysfunction in heart failure [20, 179]. For instance, mice lacking $p47^{phox}$ are protected from increases in diaphragm ROS and weakness induced by heart failure [20]. Moreover, there is indirect evidence to suggest the involvement of Noxes in muscle abnormalities in other settings. Specifically, apocynin prevents increases in markers of redox imbalance, atrophy, and loss of specific force in sepsis or mechanical ventilation [123, 124]. Overall, the findings summarized above show the importance of Noxes to the pathophysiology of skeletal muscle abnormalities.

Osmotic stress

Skeletal muscle fibers are exposed to a number of shifts in intracellular and extracellular solute content during exercise, heat, and dehydration. These changes in solutes can result in osmotic stress and stimulate ROS production [186]. Exposure to a hypotonic extracellular fluid, experimentally causing muscle fibers to swell, elevates ROS and triggers Ca^{2+} sparks [187, 188]. Both the ROS signal and Ca^{2+} sparks can be inhibited by apocynin [187],

suggesting that Noxes might be involved in skeletal muscle responses to osmotic stress. However, a causal role remains to be established with genetic manipulations or specific pharmacologic inhibitors.

Conclusions

Skeletal muscle cells express several Nox isoforms. The cellular localization of Nox subunits in skeletal muscle seems distinct from other cell types. It has become clear that Noxes are critically involved in redox homeostasis in skeletal muscle cells. Nox2 is the main source of ROS during repetitive skeletal muscle contractions, while Nox4 is required for overload-induced skeletal muscle hypertrophy. In disease states, Nox2 contributes to the pathophysiology of muscular dystrophy, muscle fiber atrophy, and contractile dysfunction. However, evidence supporting the involvement of skeletal muscle cell-specific Noxes and their regulation in hypertrophy and diseases is still lacking. Overall, we are in the early stages of understanding factors and mechanisms regulating Nox expression, activation, and deactivation in skeletal muscle cells. The initial evidence suggests some level of similarity in these pathways across cell types. However, aspects unique to skeletal muscle cells are also likely to emerge. These will be relevant for rational drug design and interventions to specifically manipulate Nox expression and activity in skeletal muscle.

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Highlights

- Skeletal muscle cells express Nox1, Nox2, and Nox4 isoforms and subunits
- Nox2 and Nox4 are critical sources of skeletal muscle ROS in health and disease
- Recent studies suggest unique aspects of Nox2 and Nox4 in skeletal muscle
- Several studies suggest a cross-talk between Nox2 and mitochondrial ROS

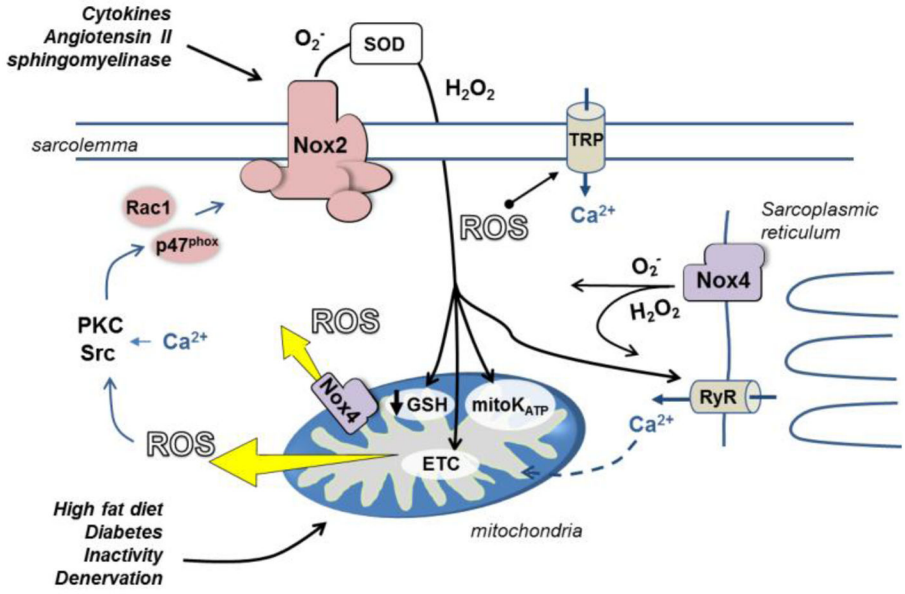

Figure 1.

Schematic diagram of Nox2 (A) and Nox4 (B) complex and associated proteins that modulate enzyme activity. GEF: Guanine exchange factors, GAP: GTPase-activated proteins, GDI: GDP-dissociation inhibitor, Tks4/5: tyrosine kinase substrate 4/5, Hsp70: heat shock protein 70; Poldip2: polymerase (DNA-directed) delta-interacting protein 2. Other heat shock proteins also interact with or modulate Nox2 and Nox4 [101, 189], but their relevance to skeletal muscle biology is less clear.

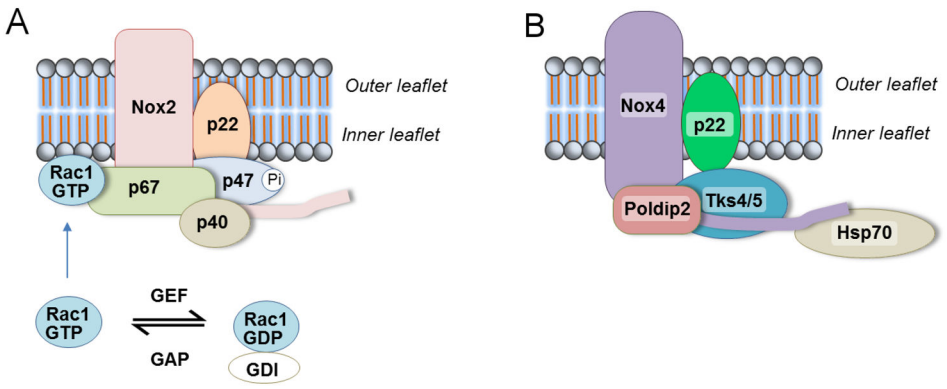

Figure 2.

Schematic diagram of conformational changes in p47^{phox} that promote binding to the cell membrane and activation of Nox subunits. * High concentration of arachidonic acid promotes a conformational change in p47^{phox} similar to that elicited by phosphorylation, without adding the phosphate group, and activates Nox. AIR: auto-inhibitory region, PRR: proline-rich region, SH3: Src-homology domain 3, PX: PX domain. Figure adapted from ref. [33, 46]

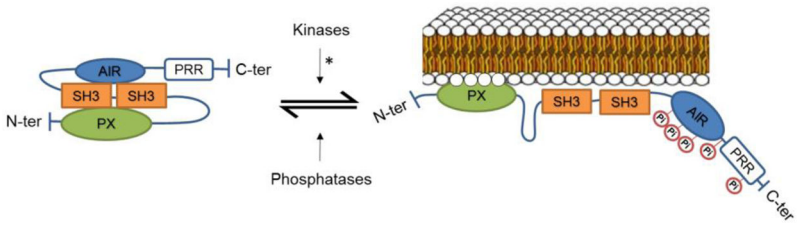

Figure 3.

Illustration of redox cross-talk between Nox and mitochondria. The cartoon includes potential sites of reactive oxygen species (ROS) production, localization of Nox2 (sarcolemma) and Nox4 (sarcoplasmic reticulum and mitochondria), and examples of factors that can trigger the redox cross-talk by acting initially on Nox2 or mitochondria. SOD: superoxide dismutase, TRP: transient receptor potential channel GSH: glutathione, mitoK_{ATP}: mitochondrial ATP-sensitive potassium channel, ETC: electron transfer chain, PKC: protein kinase C, Src: tyrosine kinase Src, RyR: Ryanodine receptor channel. The figure is based on concepts developed by Profs. Brandes [133], Daiber [134], and Dikalov [135, 190] and is adapted to skeletal muscle.