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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 in 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.

1. 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 and 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, 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.

2. 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.

2.1. 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

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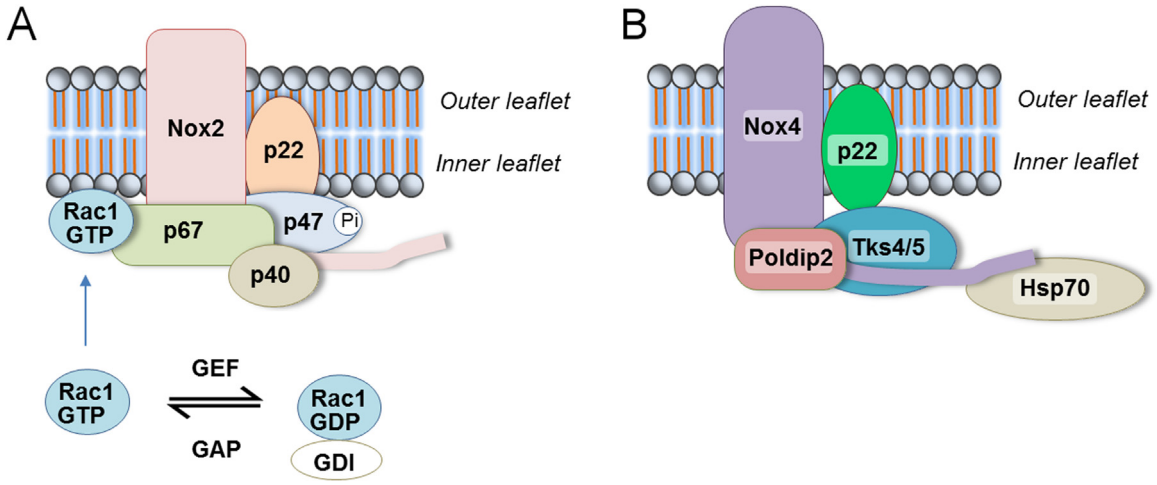

Fig. 1. Schematic diagram of Nox2 (A) and Nox4 (B) complex and associated proteins that modulate enzyme activity. GEF: Guanine exchange factors, GAP: GTPase-activated proteins, GDI: GDP-dissociation inhibitor, Tks4/5: tyrosine kinase substrate 4/5, Hsp70: heat shock protein 70; Poldip2: polymerase (DNA-directed) 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.

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).

2.1.1. 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.

2.1.2. 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.

2.1.2.1. $p47^{phox}$. 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