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The Role of Nox-Mediated Oxidation in the Regulation of Cytoskeletal Dynamics

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Abstract

Nox generated ROS, particularly those derived from Nox1, Nox2 and Nox4, have emerged as important regulators of the actin cytoskeleton and cytoskeleton-supported cell functions, such as migration and adhesion. The effects of Nox-derived ROS on cytoskeletal remodeling may be largely attributed to the ability of ROS to directly modify proteins that constitute or are associated with the cytoskeleton. Additionally, Nox-derived ROS may participate in signaling pathways governing cytoskeletal remodeling. In addition to these more extensively studied signaling pathways involving Nox-derived ROS, there also exist redox sensitive pathways for which the source of ROS is unclear. ROS from as of yet undetermined sources play a role in modifying, and thus regulating, the activity of several proteins critical for remodeling of the actin cytoskeleton. In this review we discuss ROS sensitive targets that are likely to affect cytoskeletal dynamics, as well as the potential involvement of Nox proteins.

Keywords

Cytoskeleton; NADPH oxidase; ROS; oxidation; signaling

CYTOSKELETON-RELATED STRUCTURES AND NADPH OXIDASES

The cytoskeleton is a network of filaments that helps cells maintain their shape and internal organization, as well as their mechanical properties. It is made up of three kinds of protein filaments: microtubules (about 25nm external diameter), intermediate filaments (about 10nm external diameter) and actin filaments (8nm external diameter) Fig. (1). Cytoskeletal dynamics enable cells to carry out essential functions such as migration, attachment, spreading and proliferation. Another important role of the cytoskeleton is to assist the movement of proteins, cargo-containing vesicles and organelles inside the cell. This is achieved by the association of structural proteins of the cytoskeleton with motor proteins such as kinesins, dyneins and myosins, which utilize ATP to generate movement. As expected of such energetically costly processes, cytoskeletal dynamics are highly regulated to suit the requirements of the cell as a whole. In this regard, there has been increasing

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

interest in the regulation of cytoskeletal dynamics by reactive oxygen species, since many of the proteins participating in these processes are particularly sensitive to redox regulation [1].

NADPH oxidases are a family of membrane-associated multisubunit enzymes that represent a major source of reactive oxygen species (ROS) in diverse systems [2–5]. Activation of members of the Nox family, which include Nox1-5 and Duox1/2, leads to production of $O_2^{\bullet -}$ and H_2O_2. These are released extracellularly and can reach the intracellular compartment by either simple diffusion, as in the case of H_2O_2, or via membrane-enclosed vesicles, in the case of superoxide [6]. Nox-derived ROS have been shown to modulate intracellular signaling pathways in a paracrine, autocrine, and even intracrine manner [7–10]. Although their activities have been linked to multiple disease states, today it is well accepted that under physiological conditions they are required for normal cell function. Indeed, NADPH oxidase-produced ROS participate in host defense [11, 12], cardiac repair [13], and cell differentiation [14–17].

Nox-based enzymes share a similar structure, containing at least six transmembrane domains and cytosolic flavin adenine dinucleotide (FAD) and NADPH binding domains [6]. Most of the NADPH oxidase complexes consist of a distinguishing catalytic subunit, and five variable regulatory phox subunits. The first member of this family to be identified and the best-studied is the Nox2-based NADPH oxidase, which mediates the respiratory burst of neutrophils [18]. This enzyme's catalytic subunit is gp91phox (Nox2), which contains one FAD and two hemes, and catalyzes NADPH-dependent reduction of O_2 to form $O_2^{\bullet -}$ [19, 20]. Nox proteins form a complex in the membrane with a stabilizing factor, p22phox [21–23] and, with the exception of Nox4 which is constitutively active, are dormant in resting cells [6]. A number of stimuli alter the expression and activity of Nox proteins including Angiotensin II (AngII) [24], platelet derived growth factor (PDGF) [24], and transforming growth factor beta (TGF-β) [25, 26]. Upon agonist stimulation, assembly of Nox proteins with cytosolic regulatory proteins and small GTPases results in their enzymatic activity [27]. A requirement for p47phox in Nox1 activation by AngII and PDGF has been clearly established both *in vivo* and *in vitro* [28, 29]. Nox1 can also interact with two novel homologues of p47phox and p67phox known as Noxo1 and Noxa1, respectively [30, 31], as well as the small GTPase Rac [32]. In contrast, no cytosolic subunits are required for ROS generation by Nox4 [33]; however, in vascular smooth muscle cells (VSMCs) the polymerase delta interacting protein 2 (Poldip2) binds to the C-terminal tail of p22phox and increases Nox4 activity [34].

Depending on the cell type, Nox proteins localize to different subcellular compartments. Interestingly, Nox proteins localize to subcellular compartments associated with the cytoskeleton. For example, in VSMCs, Nox4 localizes to stress fibers in differentiated cells and focal adhesions (FAs) in proliferating cells, while Nox1 is found in caveolae [17]. p47phox has been shown to colocalize with moesin and WAVE, two proteins closely related to lamellipodia [35], and is responsible for the production of H_2O_2 required to induce actin remodeling and directed ventral lamellipodia formation in endothelial cells [36]. In the same cell type, p47phox-dependent ROS production has also been shown to mediate vascular endothelial growth factor (VEGF)-induced membrane ruffle formation through its association with WAVE1, Rac1 and p21 associated kinase (PAK)1 [37]. In addition, Nox1

enzymatic activity is stimulated by growth-promoting agonists that induce membrane ruffling, such as PDGF and AngII [12, 13, 24, 38, 39], further implicating Nox proteins in growth factor stimulated actin remodeling. There is mounting evidence that Nox proteins may mediate growth factor signaling via ROS production in endosomal compartments, or "redoxosomes" [40, 41]. The colocalization of Nox proteins with specific receptors in distinct endosomal compartments provides another regulatory mechanism for generating stimuli-specific cellular responses (see [40, 41]).

During the following sections we will first review some of the most likely targets of redox regulation within the cytoskeleton, and then we will focus on how NADPH oxidase-mediated redox regulation may impact important cellular functions supported by the cytoskeleton such as migration and cell attachment.

CYTOSKELETON AND CYTOSKELETON-ASSOCIATED PROTEINS AS TARGETS OF REDOX REGULATION

Role of Actin Oxidation in Cytoskeletal Reorganization

Conceivably, ROS may participate in the remodeling of the cytoskeleton by modification of proteins and enzymes that regulate actin dynamics, or by direct oxidization of the cytoskeleton structural filaments. It is well understood that proteins containing thiols with a low pKa (capable of rendering thiolate anions at physiological pH) are targets of oxidation. Indeed, thiolates react with H_2O_2 to form sulfenic (SOH), sulfinic (SO_2H) and sulfonic (SO_3H) acids or protein disulfides (PrSSPr). Unlike the formation of sulfonic acid or sulfinic acid (the reduction of which is dependent on sulfite reductases), sulfenic acid is reversible, and has therefore been linked to cellular signalling [42]. Oxidized thiols can also react with glutathione (GSH) to form glutathiolated disulfides (PrSSG). Glutathiolation is reversible by reduction via glutathione peroxidase, thioredoxin or peroxiredoxins. A number of proteins involved in cytoskeletal reorganization are potential targets for oxidation or glutathiolation, but only a few have been confirmed, including Src [43], Csk [44], actin [45], and a number of phosphatases (PTP-PEST, LMW-PTP and SHP-2 [46, 47]). Of these, oxidation of β-actin has been extensively studied. Indeed, in the last few years it has been recognized that H_2O_2-mediated actin oxidation regulates actin dynamics. Direct treatment of β-actin with 10–20 mM of H_2O_2 has been shown to decrease the rate of actin polymerization. Although these doses are likely to be supraphysiological, these studies helped to identify several cysteines within the actin sequence that are redox sensitive. Indeed, mass spectrometry analysis shows that the C-terminal cysteine of α-actin (Cys376) or β-actin (Cys374) can be oxidized in either G-actin or F-actin [45, 48]. This high concentration of oxidants is capable of affecting most of the parameters that determine actin polymerization such as: the time required for half-maximum assembly, the elongation rate, the critical monomer concentration for polymerization, and the binding of the actin capping protein filamin [45, 48, 49]. Another report describes that the oxidation reaction in β-actin occurs at two main redox sensitive cysteine amino acid residues, Cys272 and Cys374 [50], and can be reversed by thioredoxin or glutathione. In particular, oxidation on Cys374 has been shown to cause actin depolymerization in part due to a decreased affinity for profilin [50]. Furthermore, Cys374 has been shown to be glutathiolated [51], which leads to a reduced rate of polymerization,

instability of F-actin filaments, and enhancement of ATPase activity *in vitro* [52, 53]. As expected, actin-myosin-mediated contraction is decreased after treatment of permeabilized rabbit Psoas muscle fibers with 50 mM H_2O_2, as is actomyosin enzyme activity [54]. On the other hand, low concentrations of oxidants may have the opposite effect on actin dynamics. Treatment of P388D1 cells with 1–5 mM H_2O_2 increases stress fiber formation [55]. Moreover, mild oxidizing conditions that favor actin-actin disulfide bond formation induce a greater rate and extent of actin polymerization. Further evidence that oxidants promote actin polymerization is that treatment of migrating endothelial cells with the flavin-containing oxidase inhibitor DPI or the $O_2^{\bullet-}$ and peroxinitrite scavenger manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin (MnTMPyP) inhibits actin monomer incorporation at the barbed end [56]. Interestingly, modification of endogenous levels of glutaredoxin 1, with no addition of exogenous oxidants, increases actin glutathionylation and impaires its polymerization in neutrophils. Many of these pathways could be sensitive to low output enzymes such as NADPH oxidases, although more research is required to define the role of Nox-produced ROS in the described mechanism.

Microtubules Oxidation

Microtubules are filamentous structures composed of a single type of globular protein, called tubulin. They are involved in cell division, organization of intracellular structures, intracellular transport, and ciliary and flagellar motility. Microtubules are formed by a dimer consisting of two closely related 55-kd polypeptides, α-tubulin and β-tubulin. In addition, a third type of tubulin (γ-tubulin) is specifically localized to the centrosome. When intracellular conditions favor assembly, tubulin heterodimers assemble into linear protofilaments, and later into microtubules. Though apparently stable, microtubular structures have an intrinsic in stability, which is normally recognized as a *dynamic equilibrium, or steady state.* Posttranslational modifications within tubulin seem to govern the degree of microtubule stability; these have been extensively studied, and the reader can find pertinent information in the literature [57, 58].

Recently, it has been recognized that cysteine oxidation may also contribute to the variety of posttranslational modifications found in tubulin. Indeed, it has been reported that oxidation of tubulin cysteines by peroxynitrite, 2-[(1-methylpropyl)dithio]-1H-imidazole or hypothiocyanous acid inhibits microtubule polymerization [59–61]. Since cysteine residues are not part of the protein domains responsible for the dimerization and polymerization of tubulin [62], more studies are needed to determine if this is a direct effect on filament stabilization, or an effect on the binding of proteins that regulate the polymerization process; in addition, more work is needed to determine the endogenous sources of ROS that may mediate these oxidations *in vivo.*

Intermediate Filament Oxidation

There are an increasing number of oxidative modifications of intermediate filaments reported in the literature, which we have summarized in Table 1. However, how these modifications impact cytoskeletal dynamics and cytoskeleton-supported functions have yet to be studied.

GTPases as ROS Effectors

The Rho family of small GTPases is composed of GTP-binding proteins belonging to the Ras superfamily. There are 20 members in this family, the most studied of which are RhoA, Rac and Cdc42. The Rho GTPases act as molecular switches to control signal transduction by cycling between a GTP-bound state (active form) and a GDP-bound state (inactive form). When GTPases are active, they can bind to and signal through downstream effectors to regulate diverse cellular responses including cell spreading, adhesion, polarity and migration. The Rho GTPase activation cycle is tightly regulated by guanine exchange factors (GEFs), which promote the exchange of GDP for GTP to activate the GTPase; conversely, GTPase-activating proteins (GAPs) catalyze the GTPase-dependent hydrolysis of GTP to GDP, thus turning off the G protein's activity. Additionally, another group of regulatory proteins known as guanine nucleotide dissociation inhibitors (GDIs) prevent the nucleotide exchange and bind to cytosolic GTPases, thereby protecting them from degradation [63, 64]. By controlling cytoskeletal dynamics, Rho GTPases play a pivotal role in physiological functions and pathological processes.

Rho GTPases can be either upstream regulators of ROS producing enzymes, or downstream effectors; in this section we will focus on their role as downstream effectors of ROS. In this regard, both ROS and reactive nitrogen species (RNS) can regulate the activity of GTPases by inducing the release of GDP. This redox-mediated regulation of Rho GTPases is specifically driven by $O_2^{\bullet-}$ and nitrogen dioxide ($^{\bullet}NO_2$), which induce reversible oxidation of a cysteine residue (Cys18 in Rac and Cys20 in RhoA) located within the conserved sequence GXXXXGK(S/T)C in the phosphoryl-binding loop (p-loop) of several GTPases including RhoA, Rac and Cdc42 [65]. This cysteine oxidation disrupts the interaction between the GTPase and GDP, leaving the GTPase in a nucleotide free state. After the dissociation of GDP, if this oxidation is reduced back in an environment with a GTP:GDP of ~10:1 (as in the cytoplasm of the cell) it is more likely that the GTPase loads GTP and becomes active. Notably, this activation of GTPases by redox occurs in the absence of GEFs, suggesting that the redox-mediated nucleotide exchange occurs in parallel to classical enzymatic GEF activity.

Interestingly, RhoA and RhoB possess an additional cysteine residue (Cys16 in RhoA, GXXXCGK(S/T)C) that is associated with distinct redox properties compared to the ones present in Rac and Cdc42. Under high reduction potentials, as in the cell cytoplasm where physiological concentrations of GSH are present, H_2O_2 induces oxidation of cysteines in the p-loop of RhoA, potentially enhancing the rate of nucleotide dissociation and activation. Indeed, Aghajanian *et al.* have shown that H_2O_2-dependent RhoA activation and stress fiber formation is abolished by NAC or the mutation of the Cys16 and Cys20 of RhoA [66].

However, the oxidation state of a second cysteine can negatively regulate RhoA. In this case, $^{\bullet}NO_2$ leads to thiyl radical formation at Cys20, which promotes intramolecular disulfide bond formation between Cys16 and Cys20, thereby obstructing nucleotide binding and inactivating RhoA. This disulfide bridge is not reversible by reduction in the cell cytoplasm, resulting in an irreversible inactivation of RhoA. This inactive form of RhoA is also unable to interact with the GEF Vav2 in *in vitro* studies [66, 67]. The inactivation of

RhoA by this mechanism has also been observed after exposure to highly reactive compounds that cross-link vicinal thiol groups such as phenylarsine oxide (PAO) and platinated-chemotherapeutic agents such as cisplatin (Cis-Diamminedichloroplatinum (II)) or oxaliplatin ((trans-R,R)1,2-Diamino-cyclo-hexaneoxalato-platinum (II)) [68, 69].

Thus, the activation/inactivation of RhoA will depend on the ROS/RNS levels and redox state of the cell: RhoA activation occurs under elevated ROS/RNS in a high reducing potential environment. In this case, oxidation of Cys20 releases GDP, until the redox potential is restored and Cys20 is reduced, allowing RhoA to bind GTP; however, during higher ROS/RNS (oxidative stress, chemotherapeutic drugs or radical quenching) the oxidation allows the formation of a disulfide bridge between Cys16–Cys20, thus blocking the nucleotide binding pocket and leading to an irreversible inactivation of RhoA.

A different level of regulation of GTPases by ROS is by indirect modulation of GEFs and GAPs. Since the activity of multiple GEFs/GAPs are modulated by phosphorylation/dephosphorylation cycles, the enzymatic activity of their upstream kinases and phosphatases or the translocation of GEFs/GAPs to a specific subcellular location may represent another opportunity for redox regulation of GTPase activity. The net value of such regulation will depend on the balance between kinase/phosphatase activities after a specific stimulus. In this respect, ROS-dependent regulation of protein tyrosine phosphatases (PTPs) has been broadly studied [70, 71]. It is well-established that ROS induces a reversible (or even irreversible) oxidation of redox-sensitive cysteine residues located in or near the active site of PTPs, resulting in changes to the tertiary structure of the catalytic domain that inhibit or inactivate phosphatase activity [71–74]. In this context, it has been shown that ROS can inactivate the low-molecular weight protein tyrosine phosphatase (LMW-PTP), thereby increasing the phosphorylation and activation of its target p190Rho-GAP. Consequently, RhoA is inactivated, promoting Rac-induced formation of dorsal ruffles and cell spreading [75].

Non-Muscle Myosin Heavy Chain Oxidation

Myosins are motor proteins that hydrolyze ATP in order to move along actin filaments. There are at least 20 members of this family, one of which, the non-muscle myosin heavy chain type II (NMII), plays an important role in cytokinesis, endocytosis, cell shape and cell migration [76–79]. There are at least three different NMII isoforms in humans termed NMIIA (MyH9), NMIIB (MyH10) and NMIIC (MyH14). In particular, NMIIA is highly expressed in platelets, lymphocytes and neutrophil granulocytes. One of NMIIA's most notable functions is to participate in the disengagement of integrin lymphocyte function-associated antigen (LFA)-1 at the rear end to allow tail retraction during T lymphocyte polarization [80, 81]. Fiaschi and collaborators have demonstrated that NMIIA is a target of ROS generated by integrin engagement during cell adhesion [82]. It is notable that the same group previously described that Nox4 produces ROS after integrin engagement (see integrin section for more details). They also showed that after cell spreading, oxidized NMIIA can interact with β-actin, and that this interaction is abolished using an antioxidant. Since functionality of myosin relies on its association with β-actin, these results suggest that NMIIA oxidation is integral for its contractile and structural role during cell migration.

Cortactin Oxidation

Cortactin is a nucleation-promoting factor that binds F-actin at the cell periphery of most cell types [83]. Its activity can be modulated by phosphorylation at tyrosine residues by kinases activated downstream of cell adhesion receptors such as integrins and cadherin [84–86]. After activation, cortactin induces rearrangement of the cortical actin by binding the Arp2/3 complex, facilitating actin branching and promoting the formation of structures such as lamellipodia and invadopodia during cell migration, or by facilitating endocytosis [83].

In addition to the canonical activation, there are also several non-receptor tyrosine kinases that can phosphorylate cortactin such as: Src, Abl, Syk and Fer [87–90]. Of these, Fer kinase has been linked to cortactin phosphorylation after integrin activation [90]. In this case, mouse embryonic fibroblast (MEF) adhesion on fibronectin induces Fer and cortactin phosphorylation, which is inhibited by pre-treatment with N-acetyl cysteine (NAC), the ROS scavenger Tiron or DPI [90]. Treatment with exogenous H_2O_2 induces both Fer and cortactin phosphorylation [90, 91], and cortactin phosphorylation is blunted in MEFs derived from mice harboring a catalytically inactive form of Fer ($fer^{DR/DR}$) [90]. The remaining cortactin phosphorylation in $fer^{DR/DR}$ MEFs [90] was abrogated after the treatment with the Src family (Src/Yes/Fyn) kinase inhibitor PP2, suggesting that Src also acts in parallel with Fer during the phosphorylation of cortactin [90]. It is also noteworthy that Src directly binds cortactin through a disulfide bond formed between the Cys185 in the SH2 domain of Src and the Cys112/246 in the repeats domain of cortactin. This interaction is independent of tyrosine phosphorylation of cortactin, but is required for Src-mediated cortactin phosphorylation and cell migration [92].

Additionally, it has been shown that the mechanism by which ROS induces Fer activation is not direct, as autophosphorylation of Fer or GST-cortactin phosphorylation is not observed *in vitro* after H_2O_2 treatment of immunoprecipitated Fer [90]. Taken together, these results suggest that ROS can indirectly increase Fer phosphorylation levels by the inactivation of a phosphatase, and at the same time directly mediate the binding of Src to cortactin. In both cases the result is an increase in phospho-cortactin leading to actin reorganization.

Furthermore, it has been shown that p47phox binds to cortactin [93], an association that suggests new possible levels of regulation. First, the subunit p47phox can have a regulatory effect on cortactin that may not be dependent on ROS. Accordingly, in recent years Patel *et al* have shown that the effect of p47phox on cytoskeletal remodeling seems to be independent of Nox2 in hearts isolated from both p47phox and Nox2 KO mice subjected to transverse aortic constriction-induced pressure overload and heart failure. In this case, after mechanical stress the hearts from p47phox KO animals exhibited an upregulation of N-cadherin and β-catenin, but the cortactin/N-cadherin interaction was abolished, leading to impaired actin filament rearrangement [94]. In contrast, Nox2 did not interact with cortactin, and Nox2 KO hearts were protected against biomechanical stress-induced remodeling [94]. Similarly, the p47phoxcortactin interaction may modulate the trafficking and localization of the subunits p47phox/p67phox/p40phox to mediate NADPH oxidase activation at a particular subcellular location; it may also be possible that cortactin modulates Nox2 activity by influencing the signaling pathways implicated in the activation of p47phox [93, 95, 96].

Histone Deacetylase (HDAC) Oxidation

HDACs are enzymes that remove acetyl groups from N-acetyl lysine residues within histones and other proteins such as tubulin, cortactin, Smad7 and HSP90. Class IIa HDACs include HDAC4, HDAC5, HDAC7, HDAC9 and a truncated splice variant of HDAC9 known as myocyte enhancer factor (MEF)2-interacting transcriptional repressor (MITR) or HDAC-related protein (HDRP). They are distinguished from the other HDAC classes because they shuttle between the nucleus and the cytoplasm by a redox-dependent mechanism. One of the most studied effects of HDAC nuclear-cytoplasm export is the activation of gene transcription observed during hypertrophy, development and the inflammatory response [97]. In this context, HDAC4 and HDAC5 are in the nucleus under basal conditions and accumulate in the cytoplasm after pro-hypertrophic stimuli such as phenylephrine (PE) and Endothelin-1 (ET-1). In cardiomyocytes, the nuclear export of HDAC4 is independent of phosphorylation and completely driven by Nox4 [98]. Although the mechanisms of redox-induced nuclear export are still unknown, it has been proposed that the formation of a disulfide bond between the Cys667 and Cys669 in HDAC leads to a conformational change that exposes the nuclear export signal [99]. In the case of HDAC5, its nuclear export also seems to be regulated by redox, since it is abrogated by the antioxidant NAC or overexpression of the disulfide oxido-reductase (thioredoxin-1) [99–102]. However, in skeletal muscle fibers, the export of HDAC5 to the cytoplasm in response to stretch has been shown to be dependent on Nox2 (Fig. 2) [100]. Whether the oxidation of HDAC5 is a direct consequence of O2-derived H_2O_2 diffusion to the nucleus, or Nox2-dependent changes in other redox enzymes is unclear and requires further study.

The redox dependency of HDAC shuttling has implications for cardiac hypertrophy. When HDAC is in the nucleus it suppresses the activity of pro-hypertrophic transcription factors and recruitment of epigenetic regulators such as histone methyl-transferases and class I HDAC [99, 103]. Moreover, the inhibition of nuclear export has been shown to prevent cardiomyocyte hypertrophy, suggesting an important role for the cytoplasmic localization of HDAC in the hypertrophic response [98, 102–104]. Besides the effect of HDAC on gene transcription after its trafficking to the cytoplasm, little is known about the direct effect of HDAC shuttling on cell morphology and cytoskeletal dynamics during hypertrophy. Because the oxidation of HDAC4 does not affect the deacetylase activity of the enzyme, and considering that other HDACs belonging to class IIb that are normally present in the cytoplasm affect the acetylation status of cytoskeletal proteins such as tubulin, actin, cortactin, cofilin and myosin, it is likely that redox-controlled cytosolic shuttling of HDAC4 and HDAC5 also play a role in cytoskeletal rearrangement.

Acetylation/deacetylation of cytoskeletal components play a pivotal role in a plethora of cell processes. For example, HDAC3, HDAC4 and the acetylase PCAF have been shown to localize at cardiac sarcomeres and play a role in regulating myofilament contractile activity by regulating α- and β-myosin heavy chain (MHC) activity [105, 106]. The same group showed that the levels of myosin acetylation increased progressively after hypertrophy, indicating that a balance between acetylases/deacetylases may be essential for the contractile performance of the heart [106]. Moreover, HDAC8, which has been accepted as a cytosolic marker of smooth muscle cell differentiation, can also associate with α-smooth muscle actin

and may regulate the contractile capacity of smooth muscle cells [107]. Similarly, α-tubulin acetylation at the C-terminus affects the binding of microtubule-associated proteins (MAPs) or motors [108], and HDAC6 can deacetylate α-tubulin and cortactin to regulate microtubule-dependent cell movement.

Integrin Activation as a Redox Sensitive Process

Integrins are transmembrane receptors that mediate cell-cell and cell-extracellular matrix interactions. They are heterodimers composed of α and β subunits that transduce signaling in the cell to control diverse processes such as cell transcription, cell cycle, cell shape, endocytosis and motility. There are about 18 α and 8 β subunits, which are differentially expressed in diverse cell types and combine in specific α/β pairs that recognize and interact with specific ligands to elicit precise cellular responses.

ROS have emerged as important mediators of both integrin function and integrin-triggered signal transduction. For example, the platelet $\alpha_{2b}\beta_3$ integrin contains several unpaired cysteine residues, the redox status of which can modulate the activation of this integrin [109, 110]. It has also been shown that reducing agents, such as dithiothreitol (DTT), can induce activation of $\alpha_{2b}\beta_3$ integrin [111]. This observation was corroborated with *in vitro* assays which demonstrate that DTT reduces two disulfide bonds in the cysteine rich domain of the integrin; this reduction induces a conformational change on both α_{2b} and β_3 subunits, thereby opening the integrin RGD and fibrinogen binding sites [109]. The $\alpha_{2b}\beta_3$ integrins are the primary mediators of platelet aggregation. Accordingly, a “reducing extracellular environment” (produced by application of DTT) activates integrins, and consequently increases platelet aggregation (activation of fibrinogen receptors), as opposed to higher concentrations of H_2O_2 which inhibit platelet aggregation by inducing an “oxidizing extracellular environment” [111–113]. Nevertheless, it is still not clear how lower concentrations of H_2O_2 induce platelet aggregation.

The mechanisms of ROS-mediated integrin activation have been studied in more detail for the integrin β_3 subunit. This protein contains four epidermal growth factor (EGF)-like domains that each harbor a disulfide bond (Cys437-Cys457 in EGF-1, Cys473-Cys503 in EGF-2, Cys523-Cys544 in EGF-3, and Cys560-Cys583 in EGF-4). Mutagenesis of these cysteine residues differentially regulates $\alpha_{2b}\beta_3$ and $\alpha_v\beta_3$ integrins. Further *in silico* analyses suggest that Cys560 within EGF-4 of the β_3 subunit reacts with one of the cysteines in the Cys567–Cys581 bonded pair, thus producing a disulfide exchange reaction leading to integrin activation [114]. The Cys560–Cys583 bond is integrin-specific, and the naturally occurring mutation C560R was identified as the cause for a bleeding disorder associated with a constitutively active state of integrin $\alpha_{2b}\beta_3$ [115].

Redox-mediated integrin activation has also been described for the α_4 subunit in Jurkat cells [116] and for $\alpha_7\beta_1$ in VSMCs [117], which increases cell adhesion on fibronectin or laminin respectively. Moreover, in the case of $\alpha_7\beta_1$ they also showed that there is a Nox4-dependent cysteine oxidation during early stage adhesion that could be responsible for concomitant integrin activation [117]. Another result suggests that the cysteine rich region of integrins can change the affinity of the receptors for their substrates and also help constrain the activation of integrins, since the replacement of the cysteine rich region of the β_2 integrin

with the corresponding region of β_1 (β_2NV1) allows the formation of a constitutively active form of $\alpha_L\beta_2$NV1 [118, 119].

Integrins are not only a target for ROS modification, but can also produce ROS following their engagement after cell adhesion. This ROS production has been shown to be derived from NADPH oxidases, 5-LOX and mitochondria. Fibroblast adhesion results from integrin-dependent ROS production, mainly by 5-LOX and mitochondria [120, 121]. Further assays show that adhesion on fibronectin results in ROS release from the mitochondria during early adhesion (first 10 min) and a more robust 5-LOX-derived ROS production during late adhesion (after 45 min) [47]. It is well known that integrins can activate Rac, and at the same time Rac activity regulates Nox1, Nox2 and Nox3. In addition, other proteins such as Hic-5, TRF4, IQGAP and WAVE can target Nox4 or Nox regulatory subunits (Poldip2 and p22phox) to integrin sites to induce localized ROS production [122, 123]. However, the exact mechanisms by which integrins activate Nox complexes and the regulatory feedback loop by which ROS regulate integrin activity are not fully understood. Until now, it has only been shown that $\alpha_2\beta_1$ integrin adhesion to type IV collagen induces Nox1 activation in the human adenocarcinoma cell line (Caco-2) [90, 124].

Nox Proteins in Cell Migration and Adhesion

The study of platelet derived growth factor-induced signaling and migration has shed light on the role of ROS in cell migration. Since the seminal study by Sundaresan *et al.* [125] showing that incubation of cells with catalase prevents PDGF-induced signaling, the important role of Nox enzymes in cellular signaling under normal physiological conditions has become well accepted. With respect to cellular migration, it has been shown that Nox1 participates in endothelial cell (EC) migration during angiogenesis, as Nox1 silencing decreases EC migration and tube-like structure formation through the inhibition of PPARα, a regulator of NF-κB [126]. Similarly, VSMCs from Nox1 deficient mice display inhibited PDGF or bFGF-induced migration [127, 128], while PDGF-induced migration is increased in VSMCs from Nox1 transgenic mice [13]. Additionally, studies have implicated Nox1 activity in the response to agonists that stimulate growth in cell culture such as AngII or LDL [129, 130] and in vascular pathologies with migratory components such as restenosis following balloon injury [131]. Indeed, neointimal formation following femoral artery injury is significantly reduced in Nox1 knockout mice [13].

The role of Nox proteins in migration is neither agonist nor cell type restricted. Phenylephrine- and VEGF-induced VSMC migration are prevented by catalase treatment and the antioxidants N-acetyl cysteine (NAC) and pyrrolidine dithiocarbamate, respectively [132, 133]. Meng *et al.* [134] found a role for ROS in IGF-1 induced VSMC migration that is derived from a Rac-dependent oxidase (presumably Nox1) as well as Nox4. Haurani *et al.* [135] showed that overexpression of Nox4 impaired AngII-induced migration in fibroblasts. In addition, Nox1 and Nox4 are required for a full migratory response to PDGF [13, 34]. Thrombin-stimulated migration is blocked by DPI and the putative NADPH oxidase inhibitor apocynin, implicating NADPH oxidase-derived ROS in this response as well [136]. Similarly, PDGF-induced migration of VSMCs is attenuated by the Nox inhibitor VAS2870

[137]. Endothelial cell migration is also reduced by antioxidant treatment [56] and inhibition of the NADPH oxidases [138].

Nox proteins participate at multiple levels in the remodeling of the actin cytoskeleton, a process that is integral to migration. Upon sensing a chemoattractant, the cell initiates migration via the formation of F-actin rich membrane protrusions called lamellipodia [139]. This lamellipodia formation is driven by actin reorganization, which in turn is regulated by actin modifications, actin binding proteins, and small GTPases. Actin polymerization occurs at the leading edge of the cell [140], while depolymerization predominates at the interface of the lamellipodium with the cell body (the lamella) [141]. Nox1 regulates the activity of Slingshot 1L (SSH1L), a member of a recently described family of protein phosphatases [142] that dephosphorylates and activates cofilin, an actin binding protein that is absolutely required for cell migration in VSMCs [143] (Fig. 2). Furthermore, we and others have recently shown that the mechanism of PDGF-induced SSH1L is redox sensitive and uses Nox1-derived ROS to disrupt an inhibitory complex with 14-3-3 proteins (Fig. 2) [12, 13, 144, 145]. Conversely, cofilin activity is negatively regulated by the LIM kinase (LIMK) family of serine/threonine kinases through phosphorylation of cofilin at Ser-3 [146–148]. LIMK may also be regulated by NADPH-produced ROS downstream of PAK or Src [149]. We should mention that the oxidative modification of cofilin has emerged as an important node of redox-mediated cytoskeleton regulation, as has been recently reviewed in the literature [150].

REGULATION OF CELL ATTACHMENT BY NOX PROTEINS

In addition to regulating the polymerization state of actin, Nox proteins may also support migration by regulating the adhesive forces responsible for the generation of cell traction [151, 152]. Focal adhesions (FAs), which are integrin-containing macromolecular complexes that serve as mechanical links between the actin cytoskeleton and the extracellular matrix (ECM), mediate these adhesive forces; at the same time, their dissolution in the rear allows the cell to contract and move forward. FAs are localized at the ventral surface of cells, where they concentrate and regulate numerous signaling proteins in response to integrin activation during cell adhesion [153]. Efficient cell migration involves the formation of focal complexes at the front of the cell, which are subsequently converted to FAs. It is important to note that both FAs formation and turnover are required for cell motility, such that focal adhesions must form and dissolve properly for normal migration. These processes are primarily controlled by FAK, a non-receptor tyrosine kinase that is activated by integrin receptors. FAK mediated translocation of paxillin and p130Cas to FAs enhances their formation [154], while autophosphorylation of FAK on Y397 is essential for FAK-induced FA disassembly [155].

Cell contraction, and hence movement of the cell, occurs via engagement of actin-myosin interactions. This is facilitated by the Rho signaling pathway, leading to increased Rho kinase activity and inhibition of myosin light chain phosphatase [156]. As the body of the cell moves forward, the newly formed FAs become stronger and arrive at the rear of the cell where they are disassembled, allowing their components to recycle to the leading edge of the cell for the next wave of migration [151].

One major mechanism by which ROS regulate FAs is through inactivation of protein tyrosine phosphatases (PTPs). PTPs have been shown to localize to FAs and regulate their stability [46]. Furthermore, inactivation of PTPs has also been shown to be a major mechanism by which Nox proteins regulate various signaling pathways [157]. Shinohara *et al.* [158] found that in Ras-transformed cells, Nox1-generated ROS mediate down-regulation of Rho activity via oxidative inactivation of the low molecular weight protein tyrosine phosphatase (LMW-PTP). Similarly, ROS production induced by integrin mediated cell attachment also inhibits LMW-PTP, thus preventing inactivation of focal adhesion associated protein FAK [121]. Another PTP, PTP-PEST, has been strongly associated with FA turnover, and like other phosphatases, is known to be inactivated by ROS [46]. Overexpression or ablation of PTP-PEST results in impaired migration, suggesting that tight regulation of PTP-PEST is necessary for efficient migration [159–161]. In PTP-PEST overexpressing fibroblasts, p130Cas phosphorylation and motility are inhibited [160]. Similarly, PTP-PEST null fibroblasts exhibit impaired motility, but in this case exhibit hyperphosphorylation of p130Cas, paxillin and FAK, and also display an enhanced rate of spreading and an increase in the size and number of FAs [159].

Another important cytoskeletal protein that stabilizes FAs and F-actin networks is cortactin [162]. Cortactin localizes to specialized microdomains at the focal adhesion/stress fiber interface and cooperates with p190RhoGAP in the control of FA turnover [163]. Knockdown of cortactin causes a reduction of FA formation in fibrosarcoma cells [164]. A role in cell migration can be inferred from the observation that the cortactin gene is upregulated after PDGF treatment in VSMCs [165]. Of importance, as we described in detail previously in this review, cortactin is a main target of direct oxidation. Not surprisingly, ROS have been shown to participate in cortactin regulation during integrin-mediated cell adhesion [90].

Focal Adhesion Kinase

FAK is a non-receptor tyrosine kinase that is activated by integrin receptors and phosphorylates paxillin and p130Cas, thereby regulating their translocation to FAs [154] and enhancing FA formation. At the same time, autophosphorylation of FAK on Y397 is essential for FAK-induced FA disassembly [155]. It is important to note that FA turnover is required for cell motility, so FAs must form and dissolve properly for normal migration. Indeed, depletion of FAK in fibroblasts results in enhanced FAs and impaired migration [166]. FAK has been shown to either activate RhoA by activating p190RhoGEF [167], or inhibit it by activating p190RhoGAP [168]. The precise role of FAK in regulating cell-substrate interactions is therefore context-specific, and may evolve temporally during migration. In this sense, local changes in ROS can indirectly regulate FAK activity after cell adhesion. Indeed, Chiarugi *et al*. have shown that in fibroblasts, Rac activity generates ROS after integrin engagement. At the same time they showed that ROS is necessary for cell adhesion since the incubation with DPI, a 5-lypoxigenase (LOX) inhibitor (nordihydroguaiaretic acid or NDGA) or an antioxidant N-acetylcysteine (NAC) dramatically delays the adhesion of cell to fibronectin [121]. Normally, LMW-PTP localizes to focal adhesions and dephosphorylates FAK; thus, ROS produced downstream of integrins after cell spreading induce the oxidation and inactivation of LMW-PTP, thereby increasing

the phosphorylation of FAK and other downstream effectors such as Src and MAPK [121, 169, 170]. Although LOX-mediated ROS seem to play a main role in the phosphorylation of FAK in this study [121], further work is necessary to estimate the contribution of NADPH oxidases in FAK activation during attachment.

In agreement with these results, H_2O_2 mediated increases in phospho-FAK levels correlate with an increase in stress fiber formation [171] and cell migration [172]. Moreover, in a model of oxidative stress-induced disruption of tight junctions in Caco-2 monolayers, FAK activation is mediated by Src and PI3K. Likewise, H_2O_2 induced migration is dependent on Src and PI3K activities [172]. A high migration rate after redox-induced FAK activation can be achieved after growth factor stimulation. Specifically, VEGF causes S-glutathionylation of LMW-PTP and subsequent FAK activation. Furthermore, the effect of VEGF by itself or in combination with low concentrations of peroxynitrite (0.1–0.2 mM) increase cell migration; however, VEGF in combination with higher concentrations of peroxynitrite (5mM) inhibit migration [173]. Interestingly, VEGF can induce ROS in leukemic cells by NADPH oxidases [174]. These results suggest that tight control of the redoxstate in the cell is necessary to control VEGF-driven migration.

Nox4 Regulation of FA Dynamics

In particular, Nox4 may play a critical role in FA dynamics as it has been shown to co-localize with vinculin and paxillin at FAs in VSMCs and monocytes respectively [175, 176]. Silencing either Nox4 or its activator, Poldip2, disorganizes FAs and stress fibers. Moreover, overexpression of Poldip2 results in an increase of both FA and stress fiber formation through a RhoA-dependent pathway and a decrease in cell migration in response to PDGF [34, 123]. By using a nocodazol-induced microtubule-dependent FA turnover assay, the authors demonstrate that Poldip2 overexpression impairs FA dissolution. At the same time, local levels of H_2O_2 in FAs remain high in Poldip2 overexpressing cells, which is consistent with the fact that an intracellular increase in H_2O_2 can activate FAK and subsequently stimulate cell adhesion. Indeed, the levels of FAK phosphorylated at Y397 decrease after the silencing of Poldip2 and Nox4 [123].

Regarding the mechanism by which Nox4 regulates FAs, recent work from our lab implicate the hydrogen peroxide-inducible clone-5 (Hic-5) and the chaperone protein Hsp27 [177]. Hic-5, first identified as a H_2O_2 and TGF-β-inducible gene [178], is a member of the paxillin family of proteins and localizes to FAs, where it reportedly binds to GIT1 [179], FAK [180], PTP-PEST [180] and Pyk2 [181]. For example, loss of Hic-5 impairs, and overexpression of Hic-5 increases, lysophosphatidic acid-induced migration of endothelial cells [182]. Overexpression of Hic-5 in mammary gland epithelial cells also stimulates migration [183]. Furthermore, we have recently shown that in VSMCs exposed to TGF-β, Hic-5 mediates cell adhesives forces and migration by a mechanism that is dependent on the production of H_2O_2 by Nox4 [177] (Fig. 2).

The precise mechanisms by which Hic-5 regulates FAs are unclear. As previously mentioned, Hic-5 forms a complex with Pyk-2 [181] in a Src-dependent manner [184] and becomes phosphorylated. This phosphorylation has been reported to release the Hic-5-mediated inhibition of Rac [185]. Another GTPase regulated by Hic-5 is RhoA. Indeed,

Hic-5 downregulation by siRNA inhibits RhoA activation by TGF-β in epithelial cells, which is accompanied by inhibition of stress fiber and FA formation [186]. Likewise, overexpression of Hic-5 in these cells leads to increased formation of stress fibers in a Rho kinase-dependent manner.

As previously indicated, another redox sensitive pathway that is implicated in Nox4 regulation of FAs involves the microfilament capping and actin polymerization inhibiting protein Hsp27 [187]. We have reported that in TGF-β-treated VSMCs Hsp27 is upregulated by a Nox4-dependent mechanism. Furthermore, we demonstrated that Hsp27 is indispensable for the localization of Hic-5 (but not paxillin) to FAs. Additionally, Hsp27 phosphorylation has been linked to its increased association with RhoA [188, 189] and to enhanced actin polymerization [188, 189]. Importantly, this pathway is redox-sensitive, as evidenced by its activation by H_2O_2 in endothelial cells [190], and is required for endothelial cell migration [191], PDGF-induced migration of hepatic myofibroblasts [192] and migration of tracheal smooth muscle cells induced by PDGF, IL-1β and TGF-β [193].

CONCLUSION

Given the important roles of Nox proteins in signaling pathways that contribute to normal cell physiology [6], it is not surprising that they also have a predominant role in the regulation of cytoskeletal dynamics. In fact, some well-known inhibitors of NADPH oxidases impact cell physiology via alterations of the cytoskeleton (Table 2). Cytoskeletal and cytoskeleton-associated proteins have a remarkable susceptibility to oxidization [1], based on the presence of reactive cysteine residues within their sequence [1]. At the molecular level, Cys modification is likely to impact protein structure and function, as well as interactions with other macromolecules.

Of the numerous oxidative modifications described, only a fraction of them have been linked to specific signaling pathways (Fig. 2). Similarly, most of the functional consequences of these modifications have yet to be determined. Nonetheless, it is clear that these effects are likely dependent on the source and localization of ROS, as well as the concentration. In this manner, ROS have the ability to elicit finely tuned temporal and spatial cellular responses to stimuli. It is the coordinated activity of ROS producing enzymes, in conjunction with other signaling molecules, which bring about the precise cytoskeletal changes required for complex cellular responses such as migration. Advances in understanding the structural changes induced by many of these modifications will be transcendental to improve the mechanistic understanding of redox signaling within the cytoskeleton, and is likely to impact a variety of aspects of human health and disease.

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Biography

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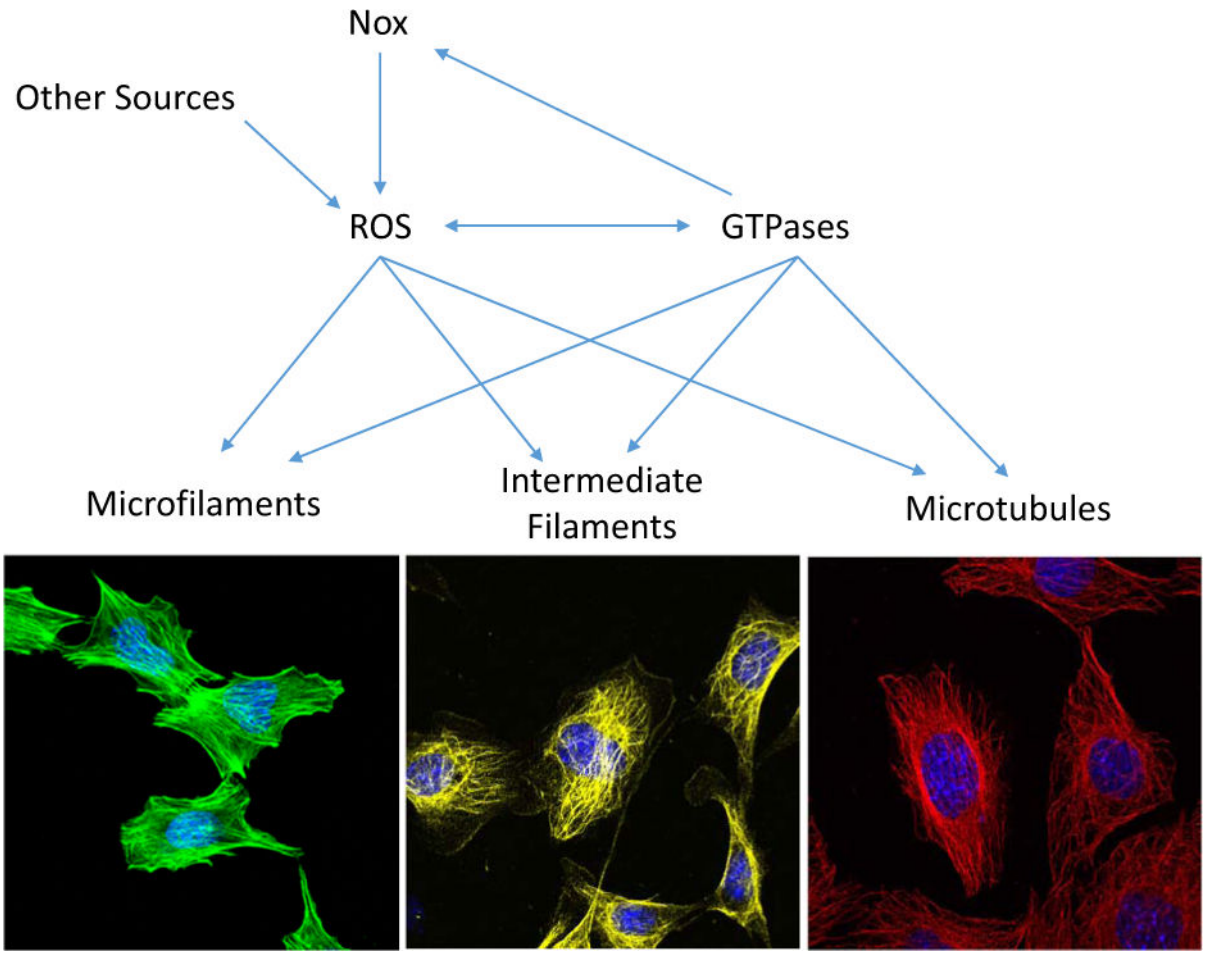

Fig. 1. Main cytoskeleton components that can be affected by ROS

ROS generated by Nox (*e.g.* Nox1, Nox2, Nox4) and from other sources, has a direct effect on cytoskeleton by modifying directly microfilaments, intermediate filaments and microtubules. Additionally, ROS regulates the activity of proteins that are associated with cytoskeleton dynamic such as Rho GTPases affecting cell adhesion, migration and invasion. Moreover, Rac GTPase can activate Nox and increase the levels of ROS. Images show mouse embryonic fibroblasts (MEFs) stained for actin microfilaments (green, Phallodin-Alexa488), Vimentin as an example of intermediate filaments (yellow, α-Vimentin/α-mouse-Alexa568), α/β-tubulin (red, α-tubulin/α-rabbit-Alexa 568), and DAPI as a marker of nucleus (blue).

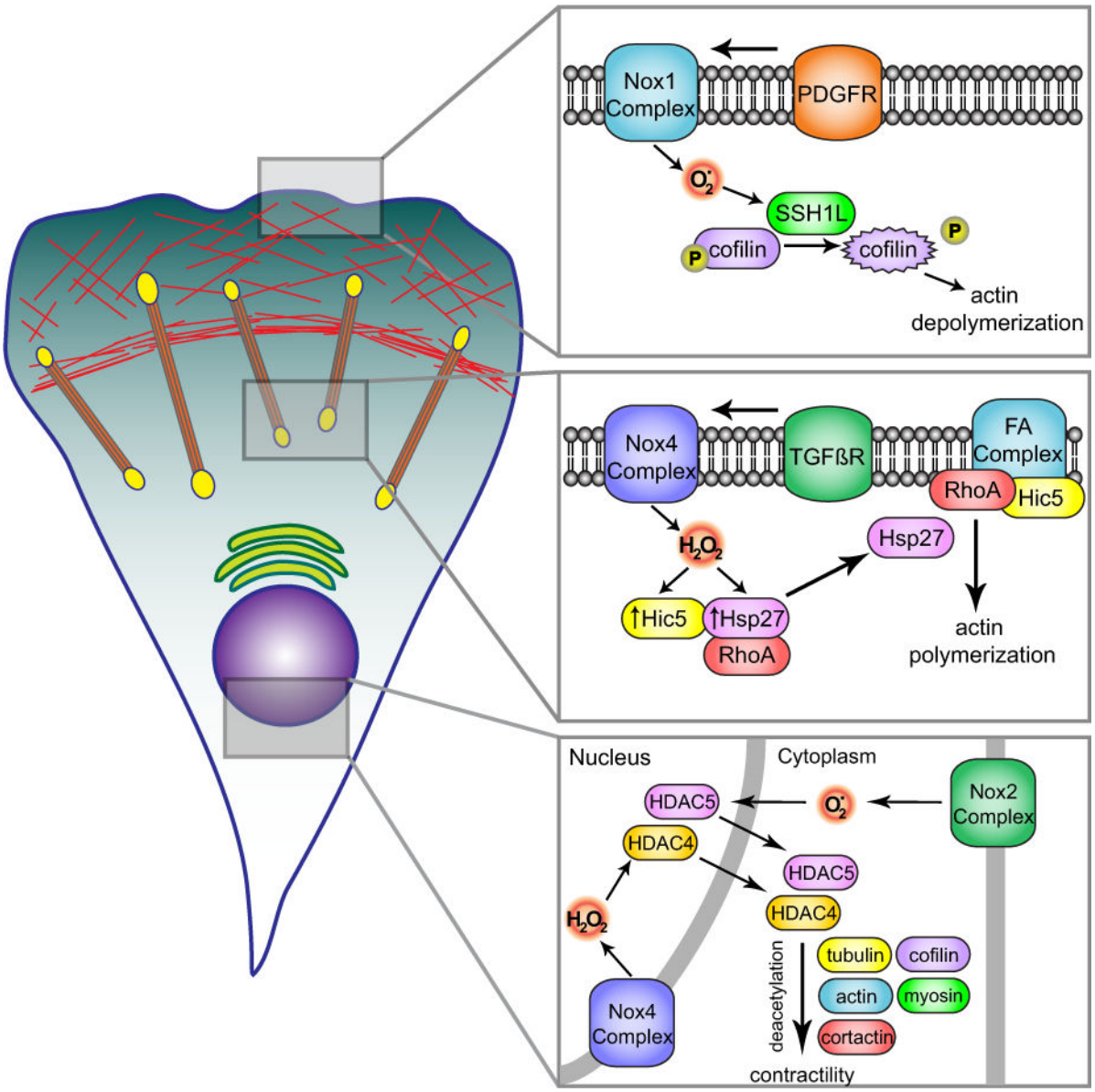

Fig. 2. Nox-mediated regulation of the cytoskeleton

Different cytoskeletal pathways are directly influenced by Nox-produced ROS. Top insert: PDGF-induced actin polymerization uses Nox1-derived superoxide that after being dismutased to H_2O_2, activates SSH1L phosphatase by oxidation of inhibitory 14-3-3 proteins. SSH1L phosphatase induces dephosphorylation and activation of the actin binding protein cofilin stimulating actin depolymerization. Middle insert: Participation of Nox4 in TGFβ-induced FA formation. After TGFßR activation, Nox4 produces H_2O_2, which mediates the expression of Hic-5 and the chaperone protein Hsp27. Hic-5 localizes to FA and mediates TGFβ induced adhesive forces while Hsp27 aids Hic-5 localization to FA and RhoA activation-induced actin polymerization. Bottom insert: Nox complexes participate in the redox-depending shuttling of HDACs between nucleus and cytosol. Nox2 and Nox4 oxidize HDACs leading to their translocation to the cytosol and the consequent

deacetylation of cytoskeleton-related proteins which leads to changes in cellular contractility.

Table 1

Oxidation of Intermediate Filaments.

	Oxidation Induced by:	Cell/Tissue	Relevance	Refs.
Keratin 10	Ultraviolet A irradiation, hypochlorite, and benzoyl peroxide	Human stratum corneum	Skin damage	[194]
Keratin 1 and 10	Oxidative damage	Human skin	Potential biomarkers of oxidative skin damage	[195]
Cytokeratin 18	Nox1	Immortalized human epithelial	Prevents cytokeratin 18 degradation, Neoplasia	[196]
Vimentin	Hydrogen peroxide	Fibroblast-like (Type B) synoviocytes	n.d	[197]
	Sodium arsenite	Human umbilical vein endothelial cells (HUVECs)	n.d	[198]
Lamin A	Aging, hydrogen peroxide	Primary human dermal fibroblasts	Cellular senescence	[199]
Desmin	Punctual mutations	Muscle	Myofibrillar myopathies	[200, 201]
	Hydrogen peroxide	Rat heart	Myocardial pathologies	[202]
GFAP	Iron overload-induced ROS	Astrocytes	Aceruloplasminemia	[203]
	Neurodegenerative disorder	Striatum	Neurodegenerative Tau-related pathology	[204]
	Illness related ROS	Spinal cord	Axonal pathologies such as autoimmune encephalomyelitis and multiple sclerosis	[205]
	$ApoE^{-/-}$ -induced ROS	Mouse Brain	Alzheimer's disease	[206]
Neurofilaments: NF-H, NF-M, NF-L	Illness related ROS	Spinal cord	Axonal pathologies such as autoimmune encephalomyelitis and multiple sclerosis	[205]
	Serotonin/tryptamine-4,5-dione, Cytochrome C and Hydrogen peroxide	Neuroblastoma (SH-SY5Y)	Neurodegeneration processes	[207, 208]
	Hydrogen peroxide, Hdh140Q/140Q knock-in mice	Mouse striatal synaptosomes	Huntington's disease	[209]
	Salsolinol (Dopamine metabolism), Hydrogen peroxide, Cu,Zn-SOD/ H2O2	*In vitro*	Neurodegenerative disorders	[210] [211]
α-Internexin	Kainic acid-induced status epilepticus	Rat hippocampus	Neurodegenerative disorders	[212]

The table summarizes different intermediate filament proteins that can be oxidized in response to different stimuli. It is also shows the cell/tissue in which it was described and the possible relevance during physiological or pathological conditions. n.d: not determined.

Table 2

Nox inhibitors that target cytoskeletal-dependent structures and functions.

Inhibitor	Mechanism of action	Target	Refs.
ML171	Inhibits Nox1 (IC_{50} of 0.25 μM) and have a lower effect over other NADPH oxidases (IC_{50} >3 μM). Mechanism of action is not established.	-Impairs the formation of functional invadopodia in Colon cancer.	[213]
GKT136901	Inhibits Nox1 and Nox4 by an unknown mechanism of action. Structural similarity with NADPH suggests that it may act as a competitive inhibitor enzymatic inhibitor.	-Inhibits endothelial cell migration and tumor angiogenesis.	[126]
PR-39	Binds to SH3 domains of the p47phox subunit and prevents binding to p22phox.	-Inhibits endothelial cell migration/angiogenesis. -Alters invasive activity and actin structure of human hepatocellular carcinoma cells.	[214] [215]
Nebivolol	Induces the dissociation of p67phox and Rac1.	-Controls vascular tone through the Inhibition of RhoA activity.	[216]
Gliotoxin	Inhibits the phosphorylation of p47phox.	-Modulates of immune response by affecting actin cytoskeleton organization-mediated phagocytosis in human neutrophil. -Inhibits neointimal formation after vascular injury in rats.	[217] [218] [219] [220]
VAS2870	Inhibits NOX2 and NOX4 containing oxidases by an undefined mechanism of action.	-Reduces retrograde F-actin flow and neurite outgrowth. -Attenuates PDGF-dependent smooth muscle cell chemotaxis. -Reverses the over contraction of diabetic rat aorta.	[221] [137] [222]