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Redox Control of Vascular Smooth Muscle Migration

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Abstract

Vascular smooth muscle cell migration is important during vascular development and contributes to lesion formation in the adult vasculature. The mechanisms regulating migration of this cell type are therefore of great interest. Recent work has shown that reactive oxygen species (ROS) derived from NADPH oxidases are important mediators of promigratory signaling pathways. ROS regulate the intracellular signals responsible for lamellipodia formation, actin cytoskeleton remodeling, focal adhesion turnover, and contraction of the cell body. In addition, they contribute to matrix remodeling, a critical step to initiate and support vascular smooth muscle cell motility. Despite these recent advances in our understanding of the redox mechanisms that contribute to migration, additional work is needed to evaluate fully the potential of ROS-sensitive molecular signals as therapeutic targets to prevent inappropriate smooth muscle cell migration. *Antioxid. Redox Signal.* 12, 625–640.

Introduction

Directed cell migration is an integrated, dynamic, and cyclic process that guides the morphogenesis of the embryo during development. In the adult, cell migration plays a key role in mounting immune responses and the repair of injured tissues. In vascular remodeling associated with diseases such as hypertension, atherosclerosis, hyperlipidemia, diabetes, and postangioplasty restenosis, one of the most relevant cellular events underlying this process is the dedifferentiation of vascular smooth muscle cells (VSMCs) into a synthetic phenotype. A major characteristic of this latter phenotype is that it recoups its capacity to migrate and proliferate in response to a variety of extracellular stimuli.

Reactive oxygen species (ROS) production has been implicated in nearly every cardiovascular pathology, from hypertension to atherosclerosis and restenosis after angioplasty (53). Indeed, ROS mediate neointimal hyperplasia during restenosis (95, 171), angiotensin II–induced hypertension (39, 146), impaired endothelium-dependent vasorelaxation (96), and heart failure (16). Moreover, the expression of NADPH oxidase subunits is upregulated in aortas of hypertensive animals (46) and during restenosis in experimental models (175), suggesting an association between these oxidases and ROS-mediated events.

The strong relation of oxidant stress with vascular remodeling establishes a connection between ROS production and VSMC proliferation, hypertrophy, and migration. Although the role of ROS in vascular growth has been investigated in detail, surprisingly, only limited information is available regarding the role of ROS in VSMC migration.

This review summarizes the current knowledge of the impact of ROS-mediated signaling on a variety of molecular targets that participate in VSMC migration. The repercussions for pathology and the potential directions for future research are discussed.

Reactive Oxygen Species

ROS is the common name given to a heterogeneous group of highly reactive small molecules. An important fraction of these compounds have unpaired valence shell electrons in the oxygen atom, explaining, in part, their increased reactivity. Another important ROS is hydrogen peroxide (H_2O_2), which is not a free radical. Although it is one of the most stable ROS, it has a strong oxidizing capacity based on its high reduction potential. The reaction of H_2O_2 with thiol-containing proteins is a key redox-signaling event (202). Very often, the term ROS also is used to refer to reactive nitrogen species (RNS) such as nitric oxide (NO) and peroxynitrite ($ONOO^-$), which are also highly reactive and have high oxidant capacity, but instead depend on the presence of a reactive nitrogen atom within the molecule.

ROS are produced from a sequential one- or two-electron reduction of molecular oxygen. Among the major sources of intracellular ROS are the mitochondria, where they are produced as a by-product of the electron-transport chain (principally in complexes I and III) during cell metabolism (124). In addition to the mitochondrial respiratory chain, VSMCs contain abundant sources of ROS, including xanthine oxidase (205), lipoxygenases (126), nitric oxide synthases (155), and NADPH oxidases (51). VSMCs also contain hemoxygenases (122), which produce the Fe^{2+} that reacts with H_2O_2 to create

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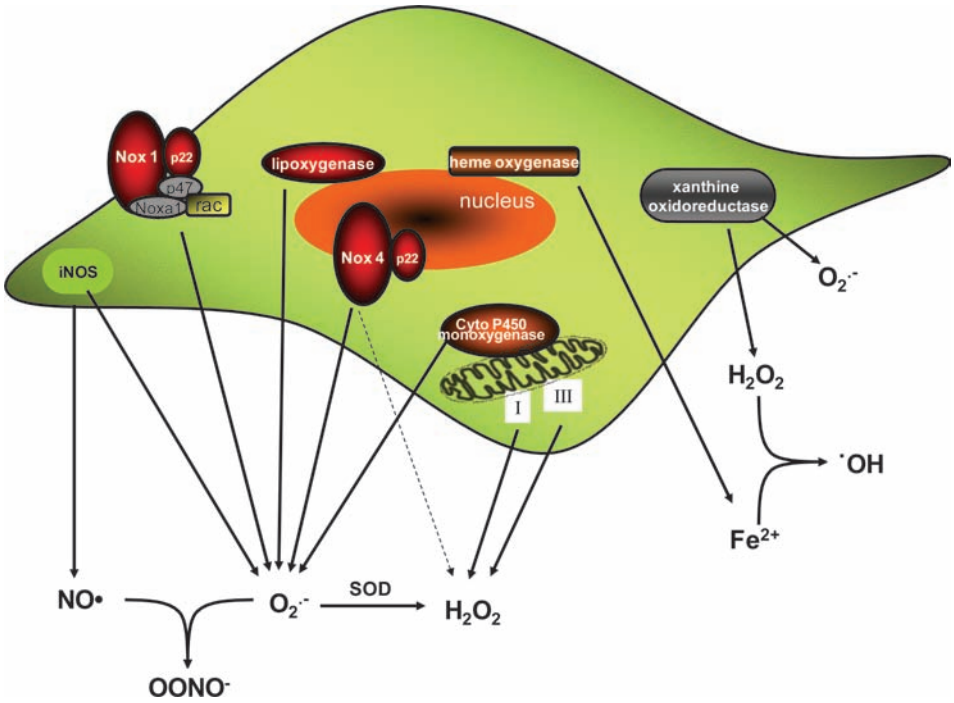

FIG. 1. VSMCs contain multiple sources of ROS. ROS-producing enzymes include NADPH oxidases, lipoxygenases, xanthine oxidase, iNOS, mitochondrial electron-transport chain, and cytochrome p450 monoxygenase. VSMCs also contain hemoxygenase, which produces Fe that can react with H_2O_2 and generate hydroxyl radical ($^{\cdot}OH$) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

the highly reactive hydroxyl radical through the Fenton reaction (103). Sources of ROS and the possible interactions among them are summarized in Fig. 1.

Although they were originally known for their detrimental role in oxidation of biomolecules, such as proteins, lipids, and DNA, it is now widely accepted that ROS function as important intracellular and intercellular second messengers to modulate many downstream signaling molecules. ROS influence signaling molecules by altering the intracellular redox state and by oxidative modification of proteins, such as protein tyrosine phosphatases (30, 31), protein tyrosine kinases (52), transcription factors (155), mitogen-activated protein kinases (189), and ion channels (104, 151, 176). It is now well established that ROS such as superoxide ($O_2^{\cdot-}$) and H_2O_2 play important roles regulating physiologic and pathophysiologic processes in vascular biology (52). Of importance for our review, they have been shown to have profound effects on VSMC growth and migration (65, 99, 119, 157, 173, 199, 208).

We and others have established that the ROS responsible for PDGF-induced migratory signaling is H_2O_2 (22, 173, 199). This makes sense because of its longer half-life and lower reactivity than other ROS. As noted earlier, despite its higher stability, H_2O_2 can induce protein oxidation, such as thiol modifications, that alter the activation state of proteins (207). Certain proteins contain cysteine thiols with a low pK_a that are easily oxidized by H_2O_2 to form sulfenic (SOH), sulfinic (SO_2H), and sulfonic (SO_3H) acids or protein disulfides (PrSSPr).

Among the nonmitochondrial oxidases, NADPH oxidases are a major source of $O_2^{\cdot-}$ and H_2O_2 within the vessel wall (117, 145, 185, 208). NADPH oxidases produce ROS both extracellularly and intracellularly within endosomal compartments (113, 133). As a result, NADPH oxidase–derived ROS have been shown to modulate intracellular signaling pathways in a paracrine, autocrine, or even intracrine manner (145, 187, 193, 194). NADPH oxidases are multisubunit enzymes of which the catalytic subunit consists of one of the Nox proteins. The best-studied NADPH oxidase mediates the respiratory burst of neutrophils. The catalytic moiety of this enzyme is $gp91^{phox}$ (Nox2), which contains one FAD and two hemes, and catalyzes NADPH-dependent reduction of O_2 to form $O_2^{\cdot-}$. It is dormant in resting neutrophils and becomes activated on assembly with the cytosolic regulatory proteins $p47^{phox}$, $p67^{phox}$, and the small GTPase Rac (9).

In VSMCs, NADPH oxidase activity is centered around novel $gp91^{phox}$ homologues as the catalytic subunits (93). VSMCs from large arteries express Nox1 and Nox4, whereas resistance and coronary arteries express Nox2 (56, 185), and human VSMCs also express Nox5 (14). In all vascular cells, these oxidases are low-output enzymes whose capacity is about one third that of the neutrophil (54), making them good candidates for participation in signaling. The kinetics of activation with cellular stimulation are also unique: $O_2^{\cdot-}$ is produced in minutes to hours, rather than in seconds to minutes as in the neutrophil (51, 134, 137). Like Nox2, Nox1 and Nox4 also interact with $p22^{phox}$ (4, 62, 190), and agonist-stimulated Nox1 activity requires Rac1 activation (92, 163, 197). Although the exact identity of the vascular Nox1 complex has not been proven in a single study, Lavigne *et al.* (97) showed that genetic deletion of $p47^{phox}$ attenuates angiotensin II– and PDGF-induced radical production in aortic VSMCs (which has been shown to be Nox1 dependent), whereas Ambasta *et al.* (4) found that $p67^{phox}$ mRNA is barely detectable in VSMCs and is instead functionally replaced by the $p67^{phox}$ homologue Nox-activator 1 (Noxa1). Taken together, these studies suggest that the VSMC Nox1 complex consists of Nox1, $p22^{phox}$, $p47^{phox}$, Noxa1, and Rac1. In contrast, Nox4 does not require any of the known cytosolic subunits for activity (17) but instead uses Polip2 as an activator (105). Structures of NADPH oxidases expressed in VSMCs are shown in Fig. 2.

The need for more than one NADPH oxidase complex within the same cell is somewhat paradoxic. One likely explanation may be that the location of $O_2^{\cdot-}$ production is

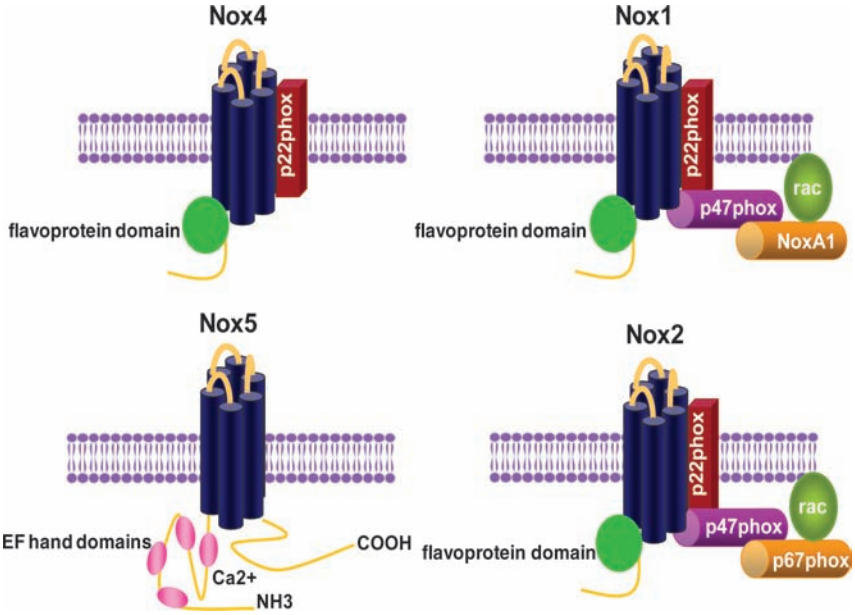

FIG. 2. Structures of NADPH oxidases found in VSMCs. NADPH oxidases are a family of multisubunit enzymes whose catalytic subunit consists of one of the Nox proteins. VSMCs from large arteries express Nox1 and Nox4, as well as Nox5 in humans. VSMCs from resistance arteries express Nox2 and Nox4. The Nox1 NADPH oxidase associates with two cytosolic factors, $p47^{phox}$ and Nox activator 1 (Noxa1), as well as the small-molecular-weight G protein Rac. Nox2 is regulated by $p47^{phox}$ and $p67^{phox}$, whereas Nox4 is not. In the case of Nox1 and 2, activity requires assembly with the cytosolic subunits (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

important, as expected when dealing with a signaling molecule with an extremely short half-life and diffusion distance. We have found that VSMC Noxes have different subcellular localizations (68) and differential regulation by agonists (92), suggesting different functions in VSMC biology as well. Similar observations have been made for Nox2 and Nox4 in endothelial cells (5). This is potentially extremely important for the regulation of migration, because, as noted later, subcellular location is exquisitely important in migratory signaling.

ROS and VSMC Migration: An Overview

The first clue that ROS might be important in VSMC migration came from a seminal study of Sundaresan *et al*. (173), who showed that H_2O_2 was required for PDGF-induced migration in VSMCs (173). We and others have expanded these observations, showing that PDGF-induced ROS production is dependent on Nox1 activation (22, 173, 181, 199). Subsequently, it was shown that migration in response to other agonists, such as phenylephrine and VEGF, is also ROS sensitive, as it is prevented by catalase treatment and antioxidants [*N*-acetylcysteine (NAC) and pyrrolidine dithiocarbamate] (127, 195). Moreover, thrombin-stimulated migration is blocked by the flavin-containing oxidase inhibitor diphenylene iodonium (DPI) and the NADPH oxidase inhibitor apocynin, implicating NADPH oxidase–derived ROS in this response (196). Recently, it was proven that the Nox1-based NADPH oxidase is required for VSMC migration induced by basic fibroblast growth factor (161) and PDGF (99, 157). Although Nox1 seems to be unequivocally involved in agonist-induced migration in VSMCs, Nox4 may play a role as well. It was recently published that Nox4 mediates insulin-like growth factor-I–induced migration (111) and angiotensin II–induced myofibroblast migration (65).

Peroxiredoxins are a family of multifunctional antioxidant thioredoxin-dependent peroxidases that eliminate H_2O_2. One of the members of this family, peroxiredoxin II (Prx II), has been shown to be a negative regulator of PDGF signaling. Prx II overexpression in VSMCs inhibits migration *in vitro* (32).

In parallel with the *in vitro* studies, data obtained in animal models support the role of ROS in VSMC migration. Superoxide and lipid peroxidation are elevated immediately after vascular injury, during the migratory phase of neointimal formation (8, 132, 171). In addition, an increase in nitrotyrosine, which is formed by reaction of nitric oxide and $O_2^{\bullet -}$, has been detected with immunohistochemistry after vascular injury (184). Functionally, a number of investigators have shown that administration of antioxidants (75, 76, 89, 99, 132, 180), treatment with the NADPH oxidase inhibitor gp91ds-tat (74), genetic deletion of NADPH oxidase homologues (99), or treatment with the xanthine oxidase inhibitor allopurinol (205) significantly reduces neointimal hyperplasia formation during repair of vascular injury, a response that is heavily dependent on VSMC migration and proliferation. Likewise, it was recently reported that gene transfer of redox factor 1 inhibits neointimal formation *in vivo* because it blocks ROS-mediated protein tyrosine kinase activity in VSMCs (98). Similar results were obtained by adenovirus-mediated overexpression of peroxisome proliferator–activated receptor-γ coactivator (PGC)-1α, a protein that regulates mitochondrial antioxidant capacity and biogenesis. PGC-1α overexpression greatly reduced neointima formation in balloon-injured rat carotid artery (143). Conversely, wire-injured carotid arteries from Prx $II^{-/-}$ animals develop a thicker layer of neointima when compared with wild-type animals (32).

The Cycle of Migration

Our knowledge of the molecular mechanisms regulating VSMC migration is still somewhat limited, but much can be inferred from studies in fibroblasts. Fibroblast migration is a dynamic process that requires specialized signaling domains at the front and rear of the cell (11, 25, 144). First, a cell must sense a gradient and establish polarity (94). Plasma membrane is then extended in the direction of eventual movement in the form of lamellipodia (168). New focal complexes are established in the front of the cell under the protrusion by restructuring of the actin cytoskeleton. Then, a mechanical

contraction force is induced by phosphorylation of myosin II, and the body of the cell contracts, moving it forward. Subsequently, focal adhesions in the rear of the cell are detached, and the trailing edge retracts. Finally, adhesion receptors are recycled by endocytosis and vesicular transport (164). These individual events are directed by activation of specific signals in the relevant subcellular compartment. Therefore, specialized signaling domains exist that serve to distinguish the front and rear of the cell (11, 25, 144). Successful migration is thus dependent on many molecules, the activation and actions of which are carefully timed in the pertinent subcellular compartments. In the remainder of this review, we consider how ROS regulate each of these steps in migration, starting with their effects on the actin cytoskeleton and microtubules, which are involved in all aspects of migration.

Cytoskeleton Dynamics and ROS

Actin-filament dynamics and reorganization are essential for cell-shape change, polarity formation, and all phases of cell migration (59). Organized and directed movement of the cell is based on an exquisite local and temporal regulation of the actin cytoskeleton. The Rho family of low-molecular-weight G proteins (especially, cdc42, Rho, and Rac) are intimately involved in most aspects of actin-filament turnover and assembly. Depending on the identity of the GTPase, different changes in the actin cytoskeleton will be induced. Cdc42 activation induces the formation of actin-rich surface protrusions called filopodia (90, 131). Rho activation leads to the assembly of contractile actin–myosin filaments (stress fibers) and of associated focal adhesion complexes (148). Finally, Rac induces the assembly of a meshwork of actin filaments at the cell periphery to produce lamellipodia and membrane ruffles (129–131, 148). As discussed in more detail later, ROS can influence actin dynamics both directly and indirectly through the alteration of intracellular signaling pathways during specific phases of migration.

ROS as Direct Regulators of Actin Polymerization

As noted earlier, H_2O_2 can oxidize reactive thiols in proteins. Oxidized thiols can also react with glutathione (GSH) to form glutathiolated disulfides (PrSSG). *S*-glutathiolation is reversible by enzymatic reduction through glutaredoxins, thioredoxin, or peroxiredoxins (207). Obviously, a number of proteins involved in cytoskeletal reorganization are potential targets for oxidation or glutathiolation, but oxidation of only a few has been verified, including Src (49), C-terminal Src kinase (Csk) (114), actin (35), and a number of phosphatases (PTP-PEST, LMW-PTP, and SHP-2) (30, 177). Of importance, β-actin itself can be directly oxidized, and this posttranslational modification has been shown to affect polymerization. *In vitro* treatment of β-actin with high (millimolar) concentrations of H_2O_2 or *tert*-butyl hydroperoxide decreases the maximal rate of polymerization, increases both the delay time and the time required for half-maximal assembly, decreases the elongation rate, increases the critical monomer concentration for polymerization, and inhibits binding of the actin capping protein filamin (35, 36, 115). Extensive mutational and mass-spectrometry analysis showed that the C-terminal cysteine (Cys374) of α- or β-actin can be oxidized in either G-actin monomers or after polymerization of F-actin (35, 36). Of importance, this C-terminal region of the molecule is the binding site for several actin-binding proteins (152). Cys374 has also been shown to be glutathiolated (81), which also leads to a reduced rate of polymerization, a relative instability of F-actin filaments, and a corresponding enhancement of steady-state ATPase activity *in vitro* (40, 172).

This effect of oxidants to cause cytoskeletal disorganization or impairment of actin–myosin functionality has also been demonstrated in cells and tissues treated with strong oxidants. Incubation of cardiomyocytes with 2,2-dithiodipyridine reduces contractile-force generation in parallel with oxidation of actin (67), whereas treatment of permeabilized rabbit psoas muscle fibers with 50 m*M* H_2O_2 decreases fiber contractility and impairs actomyosin enzyme activity (142). However, disruption of the actin cytoskeleton by oxidants is not a universal finding. Treatment of macrophage-like P388D1 cells with 1–5 m*M* H_2O_2 increases stress-fiber formation while decreasing actin nucleation activity (136). Slow oxidation of G-actin produces intermolecular disulfide-bonded actin dimers that can be incorporated into F-actin during polymerization, generating cross-links between actin filaments and thus enhancing the elasticity of the F-actin network (178). Moreover, additional work has shown that when oxidizing conditions favor sulfhydryl oxidation, a greater rate and extent of actin polymerization is observed (69).

It should be noted that all of these studies were performed with very high concentrations of oxidants, which do not necessarily mimic the physiologic state. When intact cells are exposed to millimolar concentrations of H_2O_2, cells undergo apoptosis or cell cycle arrest but not migration (38, 101). Conversely, generation of lower, physiologically relevant concentrations of H_2O_2 seems to promote actin polymerization and formation of stress fibers. For example, endothelial cells actively migrating into a wound produce elevated levels of ROS, and reduction of these molecules with DPI or the SOD-mimetic MnTMPyP abolishes actin monomer incorporation at the barbed end of growing actin filaments (118). Because these studies were performed in intact cells, it was not possible to determine whether ROS exert their effects by directly oxidizing actin or by affecting the oxidation state, phosphorylation, or binding of actin-binding proteins. However, the effects of oxidants on the actin cytoskeleton may be cell-type specific. Huot *et al*. (71) showed that the same concentration of H_2O_2 that induces fragmentation of F-actin in fibroblasts induces a reorganization of F-actin in endothelial cells, leading to the accumulation of stress fibers, the recruitment of vinculin to focal adhesions, and the loss of membrane ruffles. Fiaschi *et al*. (42) found that administration of an inhibitor of ROS generation during cell adhesion and spreading on fibronectin prevents the necessary remodeling of the actin cytoskeleton. They found that engagement of integrin receptors results in a transient glutathiolation of actin that is required for cytoskeletal reorganization. Similarly, our work showed that depletion of Nox4 by using siRNA results in dissolution of smooth muscle α-actin–based stress fibers (33), but the mechanism remains unclear. Clearly, more work is needed to determine the potential role of actin oxidation in VSMCs.

The relation between the actin cytoskeleton and ROS seems to work both ways. Thus, cortactin, an actin-binding protein that has traditionally been found to regulate polymerization of the actin cortex, has also been shown to mediate $p47^{phox}$ translocation to the membrane during angiotensin II– and

hyperoxia-induced of NADPH oxidase activation (186, 188). Moreover, actin activates Nox2 in neutrophils in a cell-free system, implying a direct effect on NADPH oxidase enzyme activity, and destabilization of the actin cytoskeleton robustly enhances the neutrophil respiratory burst activity (19, 121). A more complete understanding of this bidirectional relation between NADPH oxidases and the actin cytoskeleton may shed further light on how ROS mediate migration.

Microtubules, ROS, and Migration

Active remodeling of microtubules also is required during multiple phases of migration. Microtubules are the strongest of the cytoskeletal polymers and are made up of α/β-tubulin heterodimers. Microtubules are essential, not only because they reorganize the microtubule cytoskeleton during cell-cycle progression and cell motility, but also because they participate in the modulation of signal transduction within the cell and regulate remodeling of the actin cytoskeleton.

To migrate directionally, cells must be polarized. The microtubule-organizing center (MTOC) and other microtubule-containing apparatuses orient toward the direction of migration. Treatment with the microtubule-stabilizing agent taxol has been shown to inhibit VSMC migration *in vivo* and *in vitro* (7, 169). The pathways involved in microtubule dynamics in VSMCs have not been well studied, and no direct evidence suggests that ROS participate in those dynamics. Most of the available mechanistic information has been inferred from the mechanism of action of microtubule inhibitors. As in the relation between actin and NADPH oxidases, microtubules seem both to influence ROS production and to be regulated by it. It has been reported that in melanoma cells, taxol induces downregulation of uncoupling protein 2, thus increasing mitochondrial ROS production, in a mechanism that involves activation of the JNK and p38 pathways, and is blocked by *N*-acetylcysteine (NAC) (162). In addition, it has been shown that taxol promotes ROS generation by enhancing the activity of NADPH oxidase in neurons (77) and in cancer cells through translocation of Rac1 (3). Moreover, the flavoenzyme inhibitor diphenylene iodonium (DPI), which blocks NADPH oxidases and mitochondrial ROS production, has been shown to inhibit mitotic cell division by impairment of centrosome maturation (159). Finally, depolymerization of microtubules activates NF-κB (150), a transcription factor widely implicated in the regulation of oxidative stress–related proteins. Because of the critical role of microtubules in migration in other cell types, clearly the relation of ROS with microtubules during VSMC migration deserves further study.

ROS and the Initiation of Migration

VSMC migration is influenced by many factors, but *in vivo*, PDGF is the major promigratory stimulus (55, 73, 78), largely as a consequence of PDGF-β receptor activation (24). PDGF-α and PDGF-β are expressed at very low or undetectable levels in normal vessels (66). Likewise, PDGF-α and PDGF-β receptor mRNAs are present in SMCs in the vessel wall (108), but their proteins are barely detectable (43, 153). In atherosclerosis, during the initial response to injury or even during the phenotypic transformation of VSMCs in culture, PDGF-β receptor synthesis is induced (15, 108). This may in part be mediated by ROS as a result of a positive feedback of the increased ROS production in these conditions (63, 138). Agents that inhibit the PDGF-induced ROS formation in VSMCs (139) are also capable of blocking autocrine PDGF-β synthesis in mesangial cells (154). Furthermore, preincubation of VSMCs with NADPH oxidase inhibitors DPI and apocynin partially blocks PDGF-induced PDGF-β–receptor phosphorylation (98), suggesting that ROS may be involved as early as the initial activation of the receptor.

Several migratory stimuli can induce a positive redox feedback in the expression of other migratory signals. For instance, after the engagement of PDGF with its receptor, Nox1-mediated ROS are produced over minutes to hours (92, 99). Not only do these ROS mediate cytoskeletal-associated signal transduction (see later), but they also participate in the induction of other promigratory growth factors (109), such as FGF-2 (21, 140). Likewise, angiotensin II, which is a weak migratory factor that also activates Nox1, can enhance EGF-receptor expression levels through an ROS-mediated mechanism in a nontumorigenic human keratinocyte cell line (125). Thus, in the course of their action, and most likely through ROS-dependent pathways, growth factors and cytokines can stimulate the synthesis of other promigratory stimuli, amplifying their cellular responses.

The production of ROS extracellularly can also increase migratory signals. For instance, nitrotyrosine can stimulate VSMC migration through a mechanism blocked by antioxidants or the SOD mimetic MnTBAP (123). Similarly, oxidant stress can increase levels of homocysteine (Hcys), an amino acid associated with a high risk for atherosclerosis and restenosis after angioplasty (110). Hcys activates MAP kinases and induces migration in VSMCs by a mechanism blocked by pretreatment with the flavoenzyme inhibitor DPI and the free-radical scavenger NAC (102). Moreover, in cultured VSMCs, Hcys can upregulate monocyte chemoattractant protein-1 (MCP-1), a potent chemokine that stimulates VSMC migration (192).

Finally, biomechanical forces such as hemodynamic changes also can affect VSMC migration (58). Although the mechanisms remain to be elucidated, focal adhesion sites, integrins, and cellular junctions can act as sensors of these mechanical changes, which have been reported to activate ROS-sensitive signal-transduction pathways (88).

Lamellipodium Formation and ROS

After the cell senses a signal, lamellipodia formation, or localized protrusion of the cell membrane in the direction of the chemotactic stimulus, is driven by the extension of F-actin–rich fibers (1, 28, 191, 204). Protrusion of such actin-rich lamellipodia in moving cells requires cycles of actin polymerization and depolymerization (actin polymerization transients). ROS-dependent pathways leading to lamellipodium formation are summarized in Fig. 3.

In lamellipodia, chemoattractants bind to receptors to activate a specific guanine nucleotide exchange factor (GEF), leading to an increase in the GTP-bound active form of Rac (10, 94, 116). Rac stimulates actin polymerization by several mechanisms, including NADPH oxidase–mediated ROS production (118), nucleation of new actin filaments by activation of WAVE/Arp2/3 (106, 112), or barbed-end uncapping and extension of existing filaments (64).

Lamellipodium formation *in vivo* has been shown to be essentially dependent on the formation of free barbed ends on

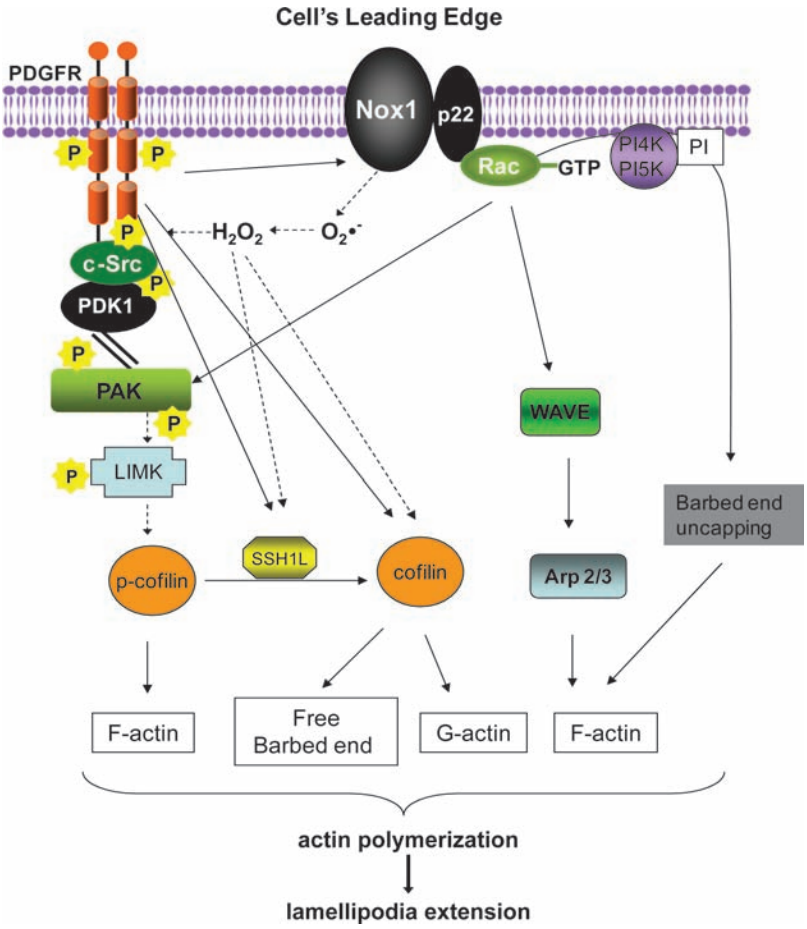

FIG. 3. ROS-dependent pathways leading to lamellipodium formation in VSMCs. After agonist stimulation, VSMCs initiate a cascade of events leading to lamellipodium formation. PDGF-induced ROS activate the Src/PAK/LIMK pathways to induce cofilin inactivation and therefore F-actin stabilization. PDGF-induced migration requires the activation of SSH1L, which activates cofilin and thus supplies G-actin and free barbed ends continuously to the leading edge. Branching and elongation of preexisting filaments are induced through Rac and WAVE/ARP2/3. *Interrupted arrows*, ROS-mediated pathways. SSH1L = slingshot phosphatase 1L; LIMK = LIM domain kinase 1; WAVE = WASP family verprolin-homologous protein; ARP2/3 = actin-related protein 2/3; PAK = p21-activated kinase. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

existing actin filaments (198). Cofilin, a key player in agonist-induced lamellipodium protrusion, is a protein capable of severing actin filaments at or near the pointed ends, and binds to both G- and F-actin to increase the rate of depolymerization of actin filaments (12, 13). This leads to the formation of free barbed ends, a continuous supply of actin monomers for polymerization, and rapid turnover of new actin filaments (27). Activation of cofilin has been shown to play an essential role in maintaining and protruding lamellipodia at the leading edge of migrating cells.

Cofilin activity is negatively regulated through phosphorylation at Ser-3 by the LIM kinase (LIMK) family of serine/threonine kinases (6, 182, 206). Suppression of cofilin activity by LIMK overexpression abolishes lamellipodium formation and polarized cell migration (37, 210). LIMKs are activated by phosphorylation in response to various extracellular stimuli, including lysophosphatidic acid (107), stromal cell-derived factor-1 (128), insulin (206), and PDGF (157). LIMK phosphorylation is mediated by Rho, Rac, Cdc42, and their downstream protein kinases, such as Rho kinase (ROCK) and p21-activated kinase (PAK) (128, 135, 206). It has been reported that ROCK is activated by ROS in VSMCs (80), and our work showed that PAK activation in PDGF-induced migration occurs through a ROS-sensitive Src activation (199).

The Ser-3–phosphorylated cofilin (p-cofilin) is dephosphorylated and thus activated by Slingshots (SSH), a relatively new family of protein phosphatases (83), or the novel HAD-type serine protein phosphatase chronophin (50). We recently showed that Slingshot1L (SSH1L) phosphatase activation is required for PDGF-induced cofilin activation (dephosphorylation) and VSMC migration (157). Intriguingly, we observed that cofilin activation by PDGF is dependent on Nox1 expression (99, 157) through ROS-mediated activation of SSH1L, in a mechanism that involves oxidation of its inhibitory partner protein 14-3-3 (85, 156). $\alpha_6\beta_4$ Integrin signaling through Rac-1 activation also participates in the regulation of SSH-mediated cofilin activation (87).

Focal Adhesion Assembly/Disassembly and ROS

After lamellipodia have formed, the next step in migration is the attachment of the leading edge of the cell to the substrate, which occurs through integrin engagement. Integrins are cell-surface receptors that serve to bridge the extracellular matrix (ECM) with the cell cytoskeleton (72). The specialized sites of cell attachment through integrins to the ECM are known as focal adhesions (FAs). It is important to note that FA turnover that is required for cell motility, so that FAs must form and dissolve properly for normal migration. FA-turnover pathways that use ROS as signaling molecules are summarized in Fig. 4.

Whether integrin activation occurs primarily through the engagement of ECM-bound promigratory stimuli (outside in), or as a consequence of an intracellular cascade initiated by a soluble factor (inside out), activated integrins cluster and consequently recruit actin filaments. This recruitment is

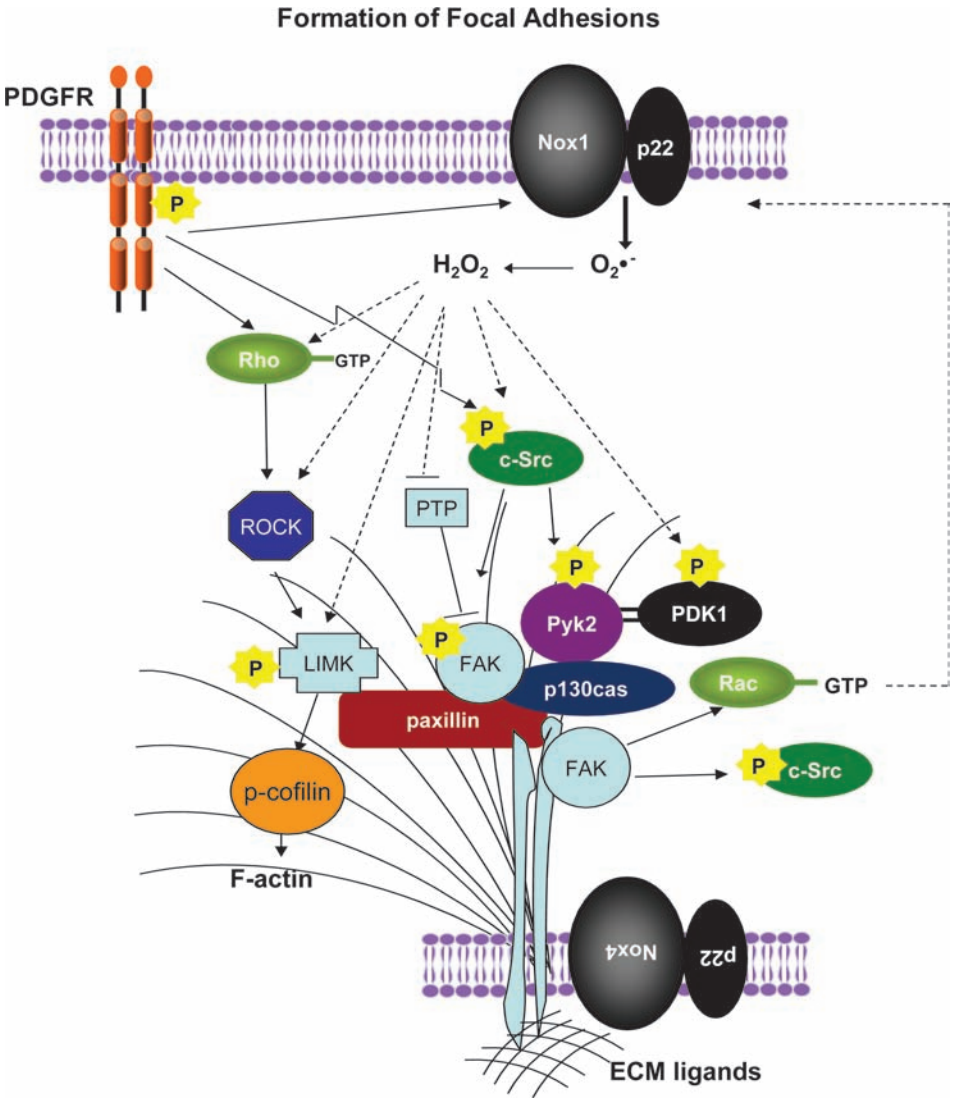

FIG. 4. ROS-dependent pathways leading to focal adhesion formation in VSMCs. Integrin stimulation through inside-out or outside-in stimuli induces clustering of FA proteins and strengthening of stress fibers. Simultaneous stimulation with PDGF activates a series of tyrosine kinases and inhibits phosphatases that contribute to FA formation. Both PDGF-mediated signaling and local production of ROS by Nox4 in FAs coordinate FA formation. *Interrupted arrows*, ROS-mediated pathways. FAs = focal adhesions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

achieved as the cytoplasmic domains of integrins associate with a group of effectors, which include talin, vinculin, α-actinin, filamin, and paxillin (34, 91, 209). One of the two homologues of LIMK, LIMK1, is also localized mainly to focal adhesions (2). Interestingly, in *Drosophila*, paxillin can negatively modulate LIMK function within focal adhesions by regulating the Rho pathway (26), which is activated by ROS in VSMCs (79, 80). These findings suggest a role for LIMK in FAs at the time that it participates in the formation of lamellipodia.

The FA protein complex organizes the actomyosin contractile apparatus and attracts signaling molecules such as focal adhesion kinase (FAK), Src family kinases, and integrin-linked kinase (91, 209). These kinases link integrins to the actin cytoskeleton and coordinate the formation and strengthening of FAs in the lamellipodium, as well as their recycling from the rear of the cell. FAK is of particular importance. Integrin-mediated activation of FAK leads to phosphorylation of paxillin and p130Cas, thereby regulating their translocation to FAs (203) and enhancing FA formation. At the same time, FAK autophosphorylates on tyrosine 397, which is essential for FAK-induced FA disassembly (60). Depletion of FAK in fibroblasts results in enhanced FAs and impaired migration (167).

It is well established that integrin signaling involves ROS, and, at the same time, that ROS can mediate integrin activation (29, 174). In different cell types, integrins have been shown to activate small Rho-GTPases (20, 61, 141). During fibronectin (FN)/integrin-mediated cell adhesion, ROS are dramatically increased by a Rac1-dependent activation of NADPH oxidase (31). Other sources of integrin-induced ROS include mitochondria (201) and lipoxygenase (177). As a result of this oxidative burst, the activity of low-molecular-weight protein tyrosine phosphatase (LMW-PTP) is transiently inhibited by thiol oxidation within the active site of the enzyme (30, 31). LMW-PTP also has been shown to associate with, dephosphorylate, and thus inactivate FAK (149). Therefore, LMW-PTP activity must be tightly regulated, probably through ROS-dependent mechanisms, to ensure proper focal complex/adhesion dynamics. Indeed, overexpression of LMW-PTP has been shown to inhibit VSMC migration (165). Similarly, the participation of ROS modulating the activity of PTPs has been studied in an *in vivo* model in which the participation of PDGF-induced ROS inhibition of PTP activity was reversed with the use of antioxidants (84).

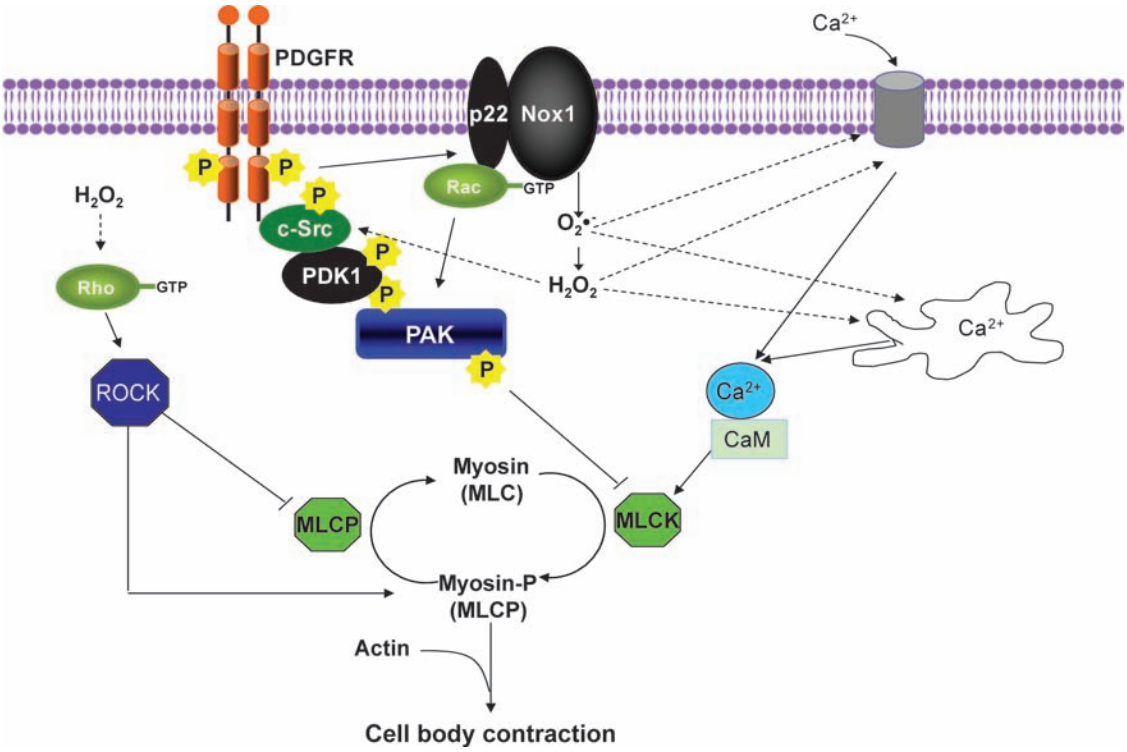

FIG. 5. ROS-dependent pathways leading to cell-body contraction in VSMCs. The final step in motility is the production of forward movement through regulation of myosin II phosphorylation and actin–myosin interaction. Several major regulators of myosin phosphorylation are ROS sensitive, including mobilization of calcium from intracellular or extracellular compartments, Src activation, and the Rho-ROCK pathway. *Interrupted arrows*, ROS-mediated pathways. Src = Rous sarcoma virus kinase homologue; ROCK = Rho-associated protein kinase. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article at www.liebertonline.com/ars).

FAs provide a support against which cell contraction can occur. They begin life as focal complexes, and the mechanisms regulating the conversion of focal complexes to FAs are unclear. Our work has shown that in angiotensin II–treated cells, ROS regulate the Src-dependent activation of PDK1, which is essential for this process (179). In addition, stress fiber formation and contraction, which are involved in FA strengthening, require activation of Rho (130, 200). Shinohara *et al.* (166) found that in Ras-transformed cells, Nox1-generated ROS mediate downregulation of Rho activity through oxidative inactivation of the LMW-PTP.

We recently found that Nox4 is also a key player in the regulation of stress fiber formation and focal adhesion turnover in VSMCs (33). Our group just reported the identification of Poldip2, a new regulator of Nox4 (105). Poldip2 is an activator of Nox4-mediated ROS production in VSMCs, and either upregulation or downregulation of Poldip2/Nox4 negatively affects FA turnover and inhibits VSMCs migration. These findings suggest a potentially novel mechanism, local ROS production, by which FA turnover is coordinated.

Contraction and ROS

The next phase in SMC motility is contraction of the cell body to create forward movement. As with cell contraction to regulate vascular tone, ATPase activity associated with myosin II is required for contraction to occur. After phosphorylation of the myosin regulatory light chain by a calcium-calmodulin (Ca^{2+}/CaM)-dependent myosin light-chain kinase (MLCK), actin activates myosin II ATPase activity, and contraction proceeds (47, 48, 170). In Fig. 5, ROS-mediated signaling pathways leading to VSMCs contraction are summarized.

In VSMCs, ROS appear to be both upstream and downstream of intracellular Ca^{2+} release and calcium influx. After growth-factor stimulation, VSMCs exhibit waves of cytosolic calcium release that are required for migration (160); blockers of calcium channels reduce both migration and ROS production. However, it also was shown that H_2O_2 and superoxide can increase the intracellular Ca^{2+} concentration in VSMCs and endothelial cells (104, 151, 176), apparently by regulating Ca^{2+} release from 1,4,5-trisphosphate–sensitive Ca^{2+} stores.

Although Ca^{2+}-dependent activation of MLCK is the major mechanism initiating cell contraction, additional pathways have been shown to regulate actin–myosin function. One such pathway in VSMCs is PAK. After activation by either Rac or Cdc42, PAK1 phosphorylates MLCK, resulting in decreased MLCK activity, thereby inhibiting myosin light chain (MLC) phosphorylation and cell contractility (158). This mechanism may be ROS mediated, because PAK1 activation in VSMCs is dependent on Nox1-derived ROS (99). Another important mechanism that regulates the contractile apparatus is the Rho/ROCK pathway, which has shown to be activated by ROS in both aorta and VSMCs (79, 80). Importantly, this pathway promotes MLC phosphorylation by phosphorylat-

ing and thus inhibiting the regulatory subunit of myosin light chain phosphatase (79, 80, 86), and by direct ROCK-mediated myosin II phosphorylation in fibroblasts (183).

Actual movement of the cell occurs through engagement of actin–myosin interactions. 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 dissociated, allowing their components to recycle to the leading edge of the cell for the next wave of migration (11). Virtually nothing is known about the role of ROS in FA dissociation.

Extracellular Matrix and ROS

As mentioned earlier, cell adhesion and migration are dependent on integrin binding to the extracellular matrix (ECM). Cell migration is, in its essence, an invasive process that requires degradation of the ECM. This is achieved by activation of matrix metalloproteinases (MMPs) and simultaneous inhibition of tissue inhibitors of metalloproteinases (TIMPs). Accordingly, MMP inhibitors have been shown to attenuate migration and delay neointimal formation (18). Moreover, genetic deletion of either MMP-2 or MMP-9 reduces VSMC migration (82). MMP activity is regulated by transcriptional and posttranscriptional mechanisms, both of which are mediated by ROS. Although ROS has been reported to downregulate MMP2 and 14 activities (41), most of the compelling data indicate that ROS can directly or indirectly activate MMPs. In VSMCs, ROS activate MMP-9 (120) and MMP-2 (70). The stimulation of MMP-9 activity by direct incubation with H_2O_2 (147) proves unequivocally that the redox state of MMPs is at least part of their mechanism of regulation. MMP-2 activity also is increased by H_2O_2, as well as by $ONOO^-$ (147). Conversely, MnSOD and NO inhibit IL-1β–stimulated MMP-9 activity (57). It should be noted that MMP-7, an MMP with high degradative ability, is activated (45) or inactivated (44) by hypochloric acid ($HOCl^-$) depending on the system.

Like activity, expression of MMPs has shown to be sensitive to ROS. MMP-1, which is important in collagen degradation, is increased by angiotensin II stimulation through the redox-sensitive transcription factors NF-κB and activating protein-1 (AP-1) (23). TNF-α stimulation has similar effects (23). Similarly, 4-hydroxynonenal (HNE), a by-product of oxidative damage that frequently accumulates in atherosclerotic lesions, increases mitochondrial ROS production, and consequently enhances MMP-2 activity in VSMCs by a mechanism that involves the Akt/NF-κB signaling pathway (100). Thus, ROS not only regulate the mechanics of cell migration, but also regulate the expression and activity of the enzymes necessary to create a path for the migrating cell.

Conclusions and Future Directions

The findings discussed herein undoubtedly support a key role of ROS as signaling molecules that regulate VSMC migration. Because migration requires carefully coordinated, tightly regulated signaling within particular subcellular locations, ROS are potentially excellent candidates for such regulation. They have short half-lives and are degraded shortly after being produced, most likely only a few atomic ratios away from the site of production.

As is evident throughout this review, much work remains to be done to understand better the participation of ROS at different levels of cell migration. Particularly interesting will be to understand how the redox state of actin and actin-associated proteins affects their protein function/polymerization properties. It will also be important to understand the spatial and temporal relations of ROS production from specific sources of ROS and their specific subcellular targets. At this point, one of the major challenges is to be able to visualize these localized events. New live-cell imaging techniques and new probes that allow us to study this process in real time are essential.

We also need to understand the contribution of variations in cell type as well as particular extracellular environments that can differentially affect cellular movement. Our present paradigm of VSMC migration is based on a model developed in other cell types, principally, but not exclusively, fibroblasts. It is very likely that VSMCs, and even potentially VSMCs from different vascular beds, have unique regulatory mechanisms for their migratory behavior. Ultimately, knowledge gained in the *in vitro* systems will have to be translated to animal models to allow us to understand how ROS-mediated signaling contributes to phenotypic modulation and wound healing.

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Abbreviations Used

AP-1 = activating protein-1
Arp 2/3 = actin-related protein 2/3
Cdc42 = cell-division cycle 42
Csk = C-terminal Src kinase
DPI = diphenylene iodonium
ECM = extracellular matrix
FA = focal adhesion
FAD = flavin adenine dinucleotide
FAK = focal adhesion kinase
FN = fibronectin
GEF = guanine nucleotide exchange factor
GSH = glutathione
Hcys = homocysteine
H_2O_2 = hydrogen peroxide
$HOCl^-$ = hypochloric acid
JNK = JUN NH_2-terminal kinase
LIMK = LIM-domain kinase
LMW-PTP = low-molecular-weight protein tyrosine phosphatase
MCP-1 = monocyte chemoattractant protein-1
MLC = myosin light chain
MLCK = myosin light-chain kinase
MMP = metalloproteinase
MnTBAP = Mn(III)tetrakis(4-benzoic acid) porphyrin chloride
MTOC = microtubule organizing center
NAC = *N*-acetyl cysteine
NADPH = nicotinamide adenine dinucleotide phosphate
NF-κB = nuclear factor κB
NO = nitric oxide
Noxa1 = NADPH oxidase-activator 1
$O_2^{\bullet -}$ = superoxide radical
$ONOO^-$ = peroxynitrite
p38 = p38 mitogen-activated protein kinase
PAK = p21-activated kinase
PDGF = platelet-derived growth factor
PDK1 = 3′-phosphoinositide-dependent kinase-1
PGC-1α = peroxisome proliferator–activated receptor-γ coactivator
Poldip2 = polymerase (DNA-directed), delta-interacting protein 2
PrSSPr = protein disulfide
Prx II = peroxiredoxin II
PTP-PEST = PEST sequence containing protein tyrosine phosphatase
Rho = Ras homologue
RNS = reactive nitrogen species
ROCK = Rho-associated kinase
ROS = reactive oxygen species
SHP-2 = Src homology 2–containing protein tyrosine phosphatase
SOD = superoxide dismutase
SOH = sulfenic acid
SO2H = sulfinic acid
SO3H = sulfonic acid
SSH1L = slingshot 1L
TIMP = tissue inhibitor of metalloproteinase
TNF-α = tumor necrosis factor α
VEGF = vascular endothelial growth factor
VSMCs = vascular smooth muscle cell
Wave = Wiskott-Aldrich syndrome protein