NOX4 (NADPH Oxidase 4) and Poldip2 (Polymerase -Interacting Protein 2) Induce Filamentous Actin Oxidation and Promote Its Interaction With Vinculin During Integrin-Mediated Cell Adhesion

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NOX4 and Poldip2 induce F-actin oxidation and promote its interaction with vinculin during integrin-mediated cell adhesion

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Abstract

Objective—Actin cytoskeleton assembly and organization, as a result of focal adhesion formation during cell adhesion, are dependent upon reactive oxygen species (ROS) and the cellular redox environment. Polymerase delta interacting protein 2 (Poldip2), a novel regulator of NADPH oxidase 4 (NOX4), plays a significant role in ROS production and cytoskeletal remodeling. Thus, we hypothesized that endogenous ROS derived from Poldip2/NOX4 contribute to redox regulation of actin and cytoskeleton assembly during integrin-mediated cell adhesion.

Approach and Results—Using vascular smooth muscle cells, we verified that H2O2 levels increase during integrin-mediated cell attachment due to activation of NOX4. F-actin was oxidized by sulfenylation during cell attachment, with a peak at 3 hours (0.80±0.04 vs. 0.08±0.13 AU at time zero), which was enhanced by overexpression of Poldip2. Depletion of Poldip2 or NOX4 using siRNA, or scavenging of endogenous H2O2 with catalase, inhibited F-actin oxidation by 78±26, 99±1, and 98±1%, respectively. To determine the consequence of F-actin oxidation, we examined the binding of F-actin to vinculin, a protein involved in focal adhesion complexes that regulates focal adhesion maturation. Binding to vinculin during cell adhesion and migration capacity was inhibited after transfection with actin containing two oxidation-resistant point
mutations (C272A and C374A). Silencing of Poldip2 or NOX4 also impaired actin-vinculin interaction, which disturbed maturation of FAs and inhibited cell migration.

**Conclusions**—These results suggest that integrin engagement during cell attachment activates Poldip2/Nox4 to oxidize actin, which modulates focal adhesion assembly.

**Keywords**

Cell adhesion molecule; Oxidation; Cytoskeletal Dynamics; NADPH oxidase

**Introduction**

Actin is the principal component of the cytoskeleton with an indispensable role in maintaining form, internal organization and function of cells, including crucial processes such as adhesion, migration and contraction. Actin filaments (F-actin) are dynamic structures made up of G-actin monomers that assemble and disassemble in response to external stimuli into higher-order structures of the cytoskeleton. Their association with other cell structures such as the plasma membrane is controlled by interaction with numerous actin-binding proteins. An important subgroup of these molecules is focal adhesion (FA) proteins that serve as adaptors linking the actin cytoskeleton via integrins to extracellular matrix (ECM). FA assembly, maturation and disassembly have central roles in cellular adhesion and migration.

Cell adhesion is a complex stepwise process. Formation of local protrusions in association with rapid F-actin assembly leads to cell contact with the ECM. Subsequently, integrins bind to ECM proteins, thereby activating a signaling mechanism that recruits FA adaptor proteins to form nascent FAs. Nascent FAs consist of talin, paxillin and focal adhesion kinase. In the next step, recruitment of vinculin and other actin binding proteins leads to formation of a flexible bridge between the integrins and the actin cellular network. Further strengthening and maturation of FAs is characterized by the increased presence of α-actinin, VASP and zyxin. Mature FAs mechanically couple the actin cytoskeleton to the ECM, allowing transmission of force. Once a mechanical link between the actin cytoskeleton and the ECM is established, cell spreading occurs. In addition, further organization of actin filaments provides force for tissue integrity or cell migration.

Integrin signaling has a crucial role in coordinating complex changes during processes of cell adhesion and migration. These transmembrane receptors can act as bidirectional signal transducers. Interaction of their cytoplasmic tails with adaptor proteins such as talin and vinculin increases affinity of integrins for ECM ligands. Conversely, binding of ECM ligands triggers a signaling cascade leading to FA formation and maturation through integrin clustering, the recruitment of adaptor proteins such as zyxin, and cytoskeletal rearrangement.

There is growing evidence that reactive oxygen species (ROS) function as important second messengers in integrin-activated signaling pathways during cellular adhesion and migration, but the source of integrin-triggered ROS production is less clear. Some data suggest mitochondria or 5-lipoxygenase produce ROS during signal transduction initiated by...
integrin engagement, but NADPH oxidases (NOXs) appear to be a more likely source. However, since ROS react promiscuously with numerous biomolecules, they require subcellular compartmentalization for specificity. However, since ROS react promiscuously with numerous biomolecules, they require subcellular compartmentalization for specificity. 

NADPH oxidases of the NOX family are important enzymatic sources of ROS. This family of enzymes includes 7 members, NOX1-NOX5, DUOX1 and DUOX2. NOX4 is a major source of intracellular hydrogen peroxide (H₂O₂) in vascular smooth muscle cells (VSMCs) and we have previously shown that NOX4 and its regulator Polymerase delta-interacting protein-2 (Poldip2) have an important role in regulating FA turnover and cell migration. In addition to altering the function of multiple redox sensitive signaling molecules involved in integrin-signaling, ROS can also directly oxidize the actin cytoskeleton or actin-binding proteins. A select group of cysteine (Cys) residues on proteins are typical targets of oxidative modification because of their low pKa thiols within certain protein microenvironments. Oxidation of critical cysteines in various signaling proteins is considered an important posttranslational regulatory signaling event. Among the six cysteine residues of human β-actin, Cys272 and Cys374 are of particular interest as targets for oxidation because of their location on the surface of the molecule. High, non-physiological concentrations of ROS can cause irreversible cysteine oxidation producing sulfenic and sulfonic acid derivatives, which inhibit actin polymerization and migration. However, cysteine oxidation to sulfenic acid, triggered by low physiological levels of cellular oxygen species like H₂O₂, is a reversible process suitable to function as a redox switch and may promote migration, and by inference, adhesion. In addition to their effect on actin polymerization, oxidation of cysteine residues may affect binding of regulatory proteins to actin. Interestingly, it was previously shown that low concentrations of H₂O₂ promote the formation of stress fibers and vinculin-containing FAs, pointing to an important role of ROS in cell adhesion to the substrate.

Vinculin is a crucial FA adaptor protein, which in concert with other bridging proteins, such as talin, paxillin and α-actinin, tethers actin filaments to integrins in FAs. The role of vinculin in FA formation is multifaceted. In addition to linking integrins and actin, vinculin serves as a scaffold with binding sites for 17 other proteins including talin, α-actinin, actin-related protein (ARP) 2/3, zyxin and paxillin. Vinculin in concert with talin activates integrins and promotes actin nucleation and crosslinking. In the present study, we report that activation of integrins during cellular adhesion triggers a Poldip2/NOX4 signaling pathway leading to oxidation of Cys residues in F-actin. Cysteines 272 and 374 are involved in this mechanism, which enhances binding of vinculin to actin and thereby promotes FA maturation.

Materials and methods

Detailed materials and methods can be found in Supplemental Material.

Primary rat aortic smooth muscle cells (RASMs) were prepared locally and human aortic smooth muscle cells (HASMs) were purchased from Clonetics.
Intracellular \( \text{H}_2\text{O}_2 \) was monitored using live confocal microscopy by ratiometric fluorescence of HyPer-3 probe.\textsuperscript{49} NADPH oxidase activity in membrane fractions was measured by detection of \( \text{H}_2\text{O}_2 \) using CPH as an ESR spin trap.\textsuperscript{50, 51} Oxidation of cysteine residues in actin was measured using DCP-Bio1 assays.\textsuperscript{52, 53}

Transmission electron microscopy and immunoperoxidase staining was used to detect Nox4 in vesicles using an antibody given by Dr. Lambeth.\textsuperscript{54} Some sections were processed using tannic acid to improve images of vesicles.\textsuperscript{55, 56}

Cells were transduced with adenoviruses prepared using the pAdEasy system\textsuperscript{57} or transfected with plasmids by electroporation. Cells were transfected with siRNA or inactive fluorescent siRNA using lipid reagents.

Assays for mRNA and protein expression were performed using RT-qPCR\textsuperscript{58} and Western blotting.

Protein subcellular localization was investigated by immunofluorescence staining and confocal microscopy. Focal adhesion assembly was assessed by measuring the zyxin/paxillin ratio.\textsuperscript{59, 60} Interaction of proteins was measured using proximity ligation assays and confocal microscopy.

Cell migration was investigated in vitro using confocal microscopy in a wound healing assay.

Statistical analysis using one or two-way ANOVA was performed using GraphPad Prism software.

## Results

**Intracellular \( \text{H}_2\text{O}_2 \) levels increase during cell adhesion**

It was previously shown that production of ROS is an important signaling mechanism in the process of cellular adhesion,\textsuperscript{21} profoundly affecting FA formation and turnover.\textsuperscript{26} In order to assess the time course of ROS generation during cellular adhesion, we utilized the cyto-Hyper3 probe, a genetically-encoded, permuted yellow fluorescent protein fused to a modified, \( \text{H}_2\text{O}_2 \)-sensitive, bacterial transcription factor OxyR. Cyto-Hyper3 functions as a ratiometric fluorescent sensor which allows dynamic, reversible measurement of low physiological concentrations of intracellular \( \text{H}_2\text{O}_2 \) with high selectivity and sensitivity.\textsuperscript{49, 61}

The pseudo-colored overlay images in Figure 1 represent the ratio of cyto-HyPer3 fluorescent signals, with blue color corresponding to low, green to medium, and red to high level of \( \text{H}_2\text{O}_2 \) concentrations. We found that during a 6 hour period after seeding, cells expressing cyto-Hyper3 show an initial increase in the fluorescence ratio (Figure 1A, C) peaking at 150 minutes. This change reflects a 55±14% increase in intracellular \( \text{H}_2\text{O}_2 \) levels compared to time zero. After 150 minutes, the HyPer signal decays, correlating with a decrease in the levels of \( \text{H}_2\text{O}_2 \), which return to baseline at 6 hours post seeding.

Previously, it was reported that ROS production during cellular adhesion is controlled by outside-in signaling pathways triggered by integrin engagement of ECM.\textsuperscript{23} To further
explore that possibility, we used the tetrapeptide Arg-Gly-Asp-Ser (RGDS) to activate the integrin-mediated signaling pathways, as this sequence is a part of the integrin binding site of fibronectin. The inactive analog Arg-Gly-Glu-Ser (RGES) was used as a control. VSMCs transfected with cyto-Hyper3 treated with 50 μM RGDS for 30 minutes present a change in fluorescence ratio, which correlates with a 44±5% increase in intracellular H$_2$O$_2$ levels (Figure 1B, D). Control cells treated with 50 μM RGES had no significant change in intracellular H$_2$O$_2$ levels over the same time period (101±2% control).

**NOX4 and Poldip2 promote H$_2$O$_2$ generation during integrin activation and NOX4 localizes in vesicles along stress fibers**

NOX4 is a major source of intracellular H$_2$O$_2$ in VSMCs. In order to investigate the role of NOX4 in the production of H$_2$O$_2$ during integrin engagement, we measured NADPH-dependent H$_2$O$_2$ production using electron spin resonance (ESR) and a spin probe/HRP detection system as described in Methods. VSMCs transfected with control siRNA and treated with RGDS showed significantly increased NADPH-dependent H$_2$O$_2$ production compared to cells treated with RGES (1.36±0.09 vs. 0.77±0.08 AU). This effect was attenuated in cells transfected with siNOX4 (0.65±0.05 vs. 0.43±0.03 AU) (Figure 1E). We have previously shown that Poldip2 can act as a regulator of NOX4, stimulating ROS production. Thus, we hypothesized that Poldip2 may also participate in regulation of NADPH-dependent H$_2$O$_2$ production upon integrin activation. As shown in Figure 1F, RGDS-induced NADPH-dependent H$_2$O$_2$ production was suppressed after transfection of siPoldip2 (0.91±0.09 vs. 0.57±0.05 AU), similar to the effect of siNOX4. Neither RGES nor RGDS had any effect on the expression of Poldip2 and NOX4 (Figure 1G, H).

Interestingly, transmission electron microscopy (TEM) analysis of VSMCs demonstrated abundant vesicles alongside the stress fibers at the bottom of the cell (evident from the electron-dense material which represents extracellular matrix after staining with tannic acid) (Figure 1I left). Some vesicles of this type are positive for NOX4 (Figure 1I, right), suggesting that ROS from NOX4 can target the cytoskeleton. Taken together, these results suggest that integrin activation-induced H$_2$O$_2$ production is dependent on Poldip2 and NOX4 and occurs adjacent to cytoskeletal elements. The mechanism is, however, not based on increased expression of these molecules, but rather results from activation of the oxidase complex.

**Integrin activation stimulates F-actin oxidization**

Cysteine residues on proteins are typical targets of oxidative modification because of their low pKa thiol in particular protein microenvironments. Oxidation of protein cysteine residues by physiological levels of cellular oxygen species such as H$_2$O$_2$ yields sulfenic acid, which is reversible. To determine if actin is a target of H$_2$O$_2$ induced by integrin activation, we used the dimedone-based chemical probe DCP-Bio1 to evaluate actin sulfenylation. This reagent covalently traps sulfenic acids but not disulfide bonds (which can be generated subsequent to sulfenic acid formation in proteins, or through thiol-disulfide exchange processes). Thus, it is ideal for detecting sulfenic acids generated during active oxidation processes (prior to subsequent reactions and generation of alternative products) and those which are stabilized within their protein microenvironment. VSMCs
were harvested at 0, 1, 3, and 6-hour time points after seeding, then lysed in the presence of DCP-Bio1, and F-actin and G-actin were separated by centrifugation. Western blot analysis of streptavidin-agarose pull-down samples revealed an increase in the F-actin oxidation with a peak at 3 hours. In contrast, G-actin was not oxidized during cell attachment (Figure 2A). We next examined F-actin oxidation following integrin engagement. Western blot analysis of VSMCs treated with RGDS for 30 minutes showed an increase in F-actin oxidation compared to RGES treated cells. Of importance, integrin-mediated oxidation of F-actin was suppressed by PEG-catalase, which depletes intracellular H$_2$O$_2$ (Figure 2B). Similar to the cell adhesion experiment, G-actin was not oxidized by integrin activation (Figure 2B).

**Integrin mediated F-actin oxidation requires NOX4/Poldip2**

To explore the role of NOX4 in F-actin oxidation during cell adhesion, we used siRNA. Cells transfected with siControl showed a typical pattern of F-actin oxidation with a peak at 3 hours post seeding, as previously observed. However, F-actin oxidation in VSMCs transfected with siNOX4 was completely blocked (Figure 3A). Similarly, RGDS-induced F-actin oxidation was suppressed by NOX4 knockdown (Figure 3B), indicating a role for NOX4 in integrin-mediated actin oxidation.

We have previously shown that Poldip2 can act as a regulator of NOX4, stimulating ROS production. Based on our previous results, we hypothesized that Poldip2 may participate in regulation of NOX4 mediated F-actin oxidation. As expected, Poldip2 knockdown in VSMCs inhibited both F-actin oxidation during cell adhesion (Figure 4A) and RGDS induced F-actin oxidation (Figure 4B). The reverse was also true. Adenoviral-mediated overexpression of Poldip2 (AdPoldip2) in VSMCs increased F-actin oxidation compared to cells transfected with control adenovirus (AdGFP) (Figure 4C). These results suggest that the Poldip2/NOX4 signaling pathway may be an effector of F-actin oxidation following integrin activation during cellular adhesion.

**Actin oxidation on Cysteine 272 and/or 374 is involved in F-actin-vinculin complex assembly**

To further analyze the role of F-actin redox regulation during cellular adhesion, we designed two plasmids to express either myc-tagged wild type (WT) human β-actin or an oxidation-resistant mutant of β-actin, with both cysteine 272 and 374 substituted with alanine (C272A and C374A). These residues were selected because of their location on the surface of the molecule. We hypothesized that cysteine sulfonylation may affect interactions between F-actin and its binding proteins. As vinculin is a critical actin binding protein involved in FA formation, we examined the role of cysteine 272 and 374 oxidation on the interaction between β-actin and vinculin using the proximity ligation assay. VSMCs transfected with either the WT or oxidation-resistant mutant of β-actin were re-plated for 1, 3 and 6 hours. We observed that the F-actin-vinculin complex formation (green) increases between 1 and 3 hours and then begins to return to baseline at 6 hours in cells expressing WT actin. In contrast, in cells expressing the oxidation-resistant C272A, C374A β-actin mutant, complex formation is impaired (Figure 5A-C). This result suggests that actin oxidation on Cys272 and/or Cys374 is important for F-actin-vinculin complex assembly. Note that both recombinant WT and mutant β-actin (green) were incorporated to the same degree into

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cellular F-actin labeled by phalloidin (red) (Figure 5D), indicating that the C-terminal myc tag and the cysteine mutations do not prevent actin polymerization.

**Mutant cysteine 272A and/or 374A on β-actin impairs VSMCs migration**

To investigate the effect of Cysteine 272A and 374A mutation of β-actin on VSMC migration, we performed a wound healing assay. VSMCs transfected with either the WT or the oxidation-resistant mutant of β-actin (C272A/374A) were grown for 72 hours to confluence. Subsequently, a wound was introduced with a sterile pipette. Upon stimulation with 10 ng/ml PDGF for 6 hours, VSMCs transfected with the oxidation-resistant mutant of β-actin migrated less into the wound area than did WT β-actin transfected cells (Figure 5E).

**Poldip2 and NOX4 promote F-actin-vinculin complex assembly**

Additionally, we were interested in determining the role of Polidp2/NOX4 in F-actin-vinculin complex formation during cell attachment. We depleted Poldip2 or NOX4 in VSMCs by transfecting cells with the corresponding siRNA. The efficiency of knockdown was confirmed by qPCR and western blot (Figure 6A, B). Results showed that β-actin-vinculin interactions were only 47±7% and 76±4% of the control signal upon knockdown of NOX4 and Polidp2, respectively (Figure 6C,D). These results suggest that both NOX4 and Poldip2 depletion can impair F-actin-vinculin complex assembly during cellular adhesion. To further explore interdependence and causality of Poldip2 and NOX4, we simultaneously performed knockdown of NOX4 and overexpression of Poldip2. Overexpression of Poldip2 in cells transfected with control siRNA significantly enhanced β-actin-vinculin interactions. However, this effect was attenuated in Poldip2 overexpressing cells transfected with siNOX4 (Figure 6E-F). This result indicates that the effect of Poldip2 on the formation of β-actin-vinculin complexes depends on enhanced NOX4 activity.

**Poldip2 and NOX4 promote FA maturation**

We have previously reported that overexpression of Poldip2 inhibits migration by strengthening FAs and consequently impairing FAs turnover. However, knockdown of Poldip2 or NOX4 can also inhibit migration by inducing a loss of FAs. As noted above, nascent FAs consist of talin, paxillin and focal adhesion kinase, while mature FAs are characterized by increased presence of α-actinin, VASP and zyxin. Therefore, we examined maturation of FAs during cell attachment by measuring zyxin-paxillin co-localization 1, 3, and 6 hours after seeding. In comparison to siControl transfected cells, depletion of Poldip2 or NOX4 significantly inhibited recruitment of zyxin in FAs (Figure 6G-H), indicating disturbed maturation of FAs. Consistent with our previous results, knockdown of either Poldip2 or NOX4 impairs FA maturation.

**Discussion**

In this study, we examined the effect of integrin-mediated actin oxidation on actin-vinculin complex assembly during cell adhesion. Our principal findings are as follows. First, integrin engagement leads to a Poldip2/NOX4-mediated transient increase in H₂O₂ production during cell adhesion. Second, the increase in H₂O₂ production during cell adhesion causes oxidation of redox-sensitive cysteine residues on F-actin. Lastly, oxidation of Cys-272 and

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Cys-374 on F-actin promotes actin-vinculin complex assembly during cellular adhesion and cell migration. Our results suggest that actin oxidation via activation of Poldip2/NOX4 is an important step in cytoskeletal organization during cell adhesion. In addition to altering the function of multiple redox-sensitive signaling molecules involved in integrin-signaling, ROS can also directly oxidize actin-binding proteins or the actin cytoskeleton itself. Our study provides evidence that ROS can regulate processes of cellular adhesion and migration via direct oxidation of actin, leading to increased binding of vinculin.

One of the most interesting findings in our study is that both integrin- and adhesion-mediated oxidation of actin predominantly affects F-actin, but not G-actin. This specificity of freely diffusible ROS is intriguing. Interestingly, a recent study examining the role of actin oxidation and subsequent S-glutathionylation in integrin-mediated cell adhesion reported that protein disulfide isomerase (PDI) binds to glutathionylated G-actin. This is followed by reversal of glutathionylation and increased polymerization of actin, leading to an increase in the F-actin content. A similar effect of deglutathionylation is observed in cells stimulated by epidermal growth factor (EGF). Reversible protein S-glutathionylation is an important post-translational modification in which thiol groups of cysteine residues oxidized to sulfenic acid form a mixed disulfide with glutathione. In this way, glutathionylation of β-actin can occur selectively by a thiol-exchange mechanism. However, as glutathionylation of cysteine will prevent binding of the dimedone-based DCP-Bio1 probe we used in our experiment, the fact that we observed oxidation of F-actin, but not G-actin, may indicate that in our system, cysteine residues in F-actin are oxidized, but do not undergo glutathionylation (at least rapidly enough to out-compete DCP-Bio1 trapping). This is in accordance with findings that glutathionylation leads to instability and breakdown of F-actin. Alternatively, Cys272 and Cys374 may have different fates after exposure to H₂O₂. Our results also suggest additional regulatory effects of ROS during cellular adhesion, as we have shown that actin oxidation leads to an increase in vinculin binding.

Vinculin is one of the actin-binding proteins that can bind to the actin filament near Cys374. This complex protein has a unique structure consisting of a head linked by a proline rich sequence to the tail. In the inactive form of the molecule, interaction between the head and tail masks numerous binding sites for ligands. Upon recruitment to FAs, vinculin undergoes activation, characterized by a conformational change leading to the head separating from the tail and unmasking cryptic ligand binding sites. Interestingly, vinculin binding to F-actin is proposed to induce a conformational change, leading to its activation and dimerization. The binding of vinculin to actin is mediated by two regions located in the tail. In the inactive form of vinculin, one of the actin binding regions is available for interaction, while the access to second site is blocked by the head region. After activation of vinculin, the second site becomes available, and its binding strength to actin consequently increases, followed by dimerization of the vinculin tail. Our experimental data indicate that actin oxidation on Cys272 and/or Cys374 correlates with an increase in F-actin-vinculin complex assembly, most likely by inducing a conformational change that promotes vinculin binding and activation. It is likely that oxidation of additional cysteines or methionines is also involved, given that some interaction between F-actin and vinculin occurs even in Cys272/274 mutant expressing cells (Figure 5A). An interesting possibility arising from this observation is that oxidized actin could induce a vinculin conformational change that
activates its dimerization. Vinculin dimerization was shown previously to promote actin bundling. Consequently, vinculin dimerization would result in an increase in the vinculin-actin complex assembly as we observed with the proximity ligation assay, and would ultimately lead to FA strengthening and maturation. We have confirmed this effect by observing decreased recruitment of zyxin in FAs, as marker of FA maturation, in cells transfected with siPoldip2 or siNOX4. These results are in concordance with our previous findings that Poldip2/NOX4 regulate FA turnover during cell migration,26 as in our current study actin oxidation and vinculin binding are also controlled by a Poldip2/NOX4-mediated increase in H$_2$O$_2$ production. Furthermore, it appears that actin oxidation is an important component of Poldip2/NOX4 regulation of cellular migration as cells expressing the oxidation-resistant mutant of β-actin (Cys272A/374A) show impaired migration.

Of note, one study suggested that mitochondria and 5-lipoxygenase may be sources of ROS in integrin-mediated signaling.23 However, in our study we have shown that integrin mediated production of H$_2$O$_2$ during cellular adhesion depends on Poldip2 and NOX4. We have demonstrated that NOX4-containing vesicles are located along stress fibers, providing the compartmentalization necessary for controlled and specific action of H$_2$O$_2$ on actin. We have also shown that either Poldip2 or NOX4 knockdown can impair integrin-mediated production of H$_2$O$_2$ and consequent actin oxidation and vinculin binding. This effect of integrin activation is not dependent on increased expression of NOX4 or Poldip2. However, as Poldip2 knockdown prevents the integrin mediated increase in both NADPH oxidase-mediated H$_2$O$_2$ production and actin oxidation in a similar manner to NOX4 knockdown (Figure 1 E, F), it appears that Poldip2 primarily exerts its effect through regulation of NOX4 production of H$_2$O$_2$, although we cannot rule out the possibility that Poldip2 might also regulate trafficking of NOX4 to actin. Conversely, overexpression of Poldip2 leads to an increase in actin oxidation. However, if we simultaneously perform knockdown of NOX4 this effect is lost, indicating that the effects of Poldip2 depend fully on NOX4 activity. Thus, our previously published observation showing that a sustained increase in H$_2$O$_2$ levels due to overexpression of Poldip2 correlates with stabilization and increased strength of FAs may be in part due to an increase in the vinculin-actin complex assembly triggered by actin oxidation.

In summary, these results indicate that Poldip2/NOX4-mediated oxidation of Cys272 and/or Cys374 on F-actin may not just regulate F-actin-vinculin interaction during cellular adhesion leading to formation and strengthening of FAs, but also during cell migration. Because Poldip2/NOX4-mediated ROS signaling regulates both physiological cell migration in wound healing and angiogenesis72,73 as well as pathological migration in neointimal proliferation and atherosclerosis,74 further work is warranted to fully elucidate the contribution of actin oxidation to these processes.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.
Acknowledgements:

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Abbreviations

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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>RGDS</td>
<td>Arg-Gly-Asp-Ser</td>
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<td>RGES</td>
<td>Arg-Gly-Glu-Ser</td>
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<td>Cys</td>
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<td>ECM</td>
<td>Extracellular matrix</td>
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References:


Highlights:

1. ROS can regulate cellular adhesion via direct oxidation of F-actin.
2. NOX4-containing vesicles are located along stress fibers, providing the compartmentalization necessary for controlled and specific action of H_2O_2 on actin.
3. Actin oxidation on Cys272 and/or Cys374 correlates with an increase in F-actin-vinculin complex assembly during cell adhesion.
4. These studies uncover a vital role of Poldip2/NOX4 in F actin oxidation and the interaction of F-actin and vinculin that is indispensable in cytoskeleton organization and cell spreading during cell adhesion.
Figure 1. NOX4 produces H$_2$O$_2$ after integrin activation and localizes in vesicles along stress fibers.

A, C. Representative images (A) and quantified cytoplasmic H$_2$O$_2$ concentration (C) measured in HASMs at indicated times after plating using live fluorescence microscopy in single cells. Following transfection with cyto-HyPer3, the ratio of fluorescence signals is shown in false colors representing cytoplasmic H$_2$O$_2$ concentration. Summary data represent mean ± SEM of single cell measurements from 9 independent experiments. Scale bar: 20 μm, *p<0.05 vs. time zero. B, D. HASMs were exposed to 50 μM of RGDS peptide or its inactive control RGES starting 30 minutes after plating. HyPer3 fluorescence images were...
acquired at indicated subsequent times. B: representative images. D: mean ± SEM of data of single cell measurements from 16 independent experiments. Scale bar: 15 μm. * p<0.05, *** p<0.001 vs. RGES. E-F. HASMs were transfected with control siRNA (siControl), siNOX4 (E), or siPoldip2 (F). Cells were harvested after 72 hours and incubated in cell culture medium for 30 minutes with agitation before addition of 50 μM RGES or RGDS for 30 min. H₂O₂ was measured in membrane fractions by ESR using CPH as a spin probe. Bars represent mean ± SEM from 3 independent experiments. ### p<0.001 vs. siControl + RGES, ### p<0.01 vs. siControl + RGDS. G-H. HASMs were treated with 50 μM RGES or RGDS for 6 h. Poldip2 (G) and NOX4 (H) mRNA levels were measured by RT-qPCR and normalized to the housekeeping gene RPL mRNA. Summary data represent mean ± SEM of data from 3 independent experiments. I. Transmission electron micrographs showing vesicles located along stress fibers (SF) in sections parallel to the bottom edge of cultured RASMs. Scale bars: 0.5 μm. Left: The extracellular matrix and vesicles next to the substratum (arrowheads) are densely stained by tannic acid. In contrast, the plastic dish remains white at the top left and bottom right. Middle and Right: Immunoperoxidase staining. While vesicles stain lightly in the absence of primary antibody (Middle, arrowheads), they appear much denser to electrons after incubation with primary antibody against Nox4 (Right, arrowheads).
Figure 2. Integrin activation stimulates F-actin oxidation by H$_2$O$_2$.

A. RASMs were harvested 0, 1, 3 and 6 hours after plating. DCP-Bio-1 probe was used to label oxidized cysteine residues. F-actin (pellet) and G-actin (supernatant) were separated by centrifugation. Samples were analyzed by Western blot. Data represent mean ± SEM of 3 independent experiments. ** p<0.01, *** p<0.001 vs. 0 h.

B. RASMs were treated for 30 minutes with 50 μM of RGES, RGDS alone or RGDS after pre-treatment for 30 minutes with PEG-Catalase (200 U/ml). Data represent mean ± SEM from 3 independent experiments. *** p<0.001 vs. Control.

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Figure 3. Integrin mediated F-actin oxidation requires NOX4.
A. RASMs were transfected with control siRNA (siControl) or siNOX4. After 72 hours, cells were re-plated and harvested after 0, 1, 3 and 6 hours before measuring F-actin oxidation as in Figure 2. Data represent mean ± SEM from 3 independent experiments. ** p<0.01, *** p<0.001 vs. siControl at 0 h. B. RASMs were transfected with control siRNA (siControl) or siNOX4. After 72 hours, RGDS or RGES (50 μM) was added for 30 minutes before cell harvesting. F-actin was collected to measure cysteine oxidation, as above. Data represent mean ± SEM from 3 independent experiments. *** p<0.001 vs. RGES.
Figure 4. Integrin mediated F-actin oxidation requires Poldip2.
A. RASMs were transfected with control siRNA (siControl) or siPoldip2. After 72 hours, cells were re-plated and harvested after 1, 3 and 6 hours before measuring F-actin oxidation as in Figure 2. Data represent mean ± SEM from 3 independent experiments. ** p<0.01 vs. siControl at 0 h. B. RASMs were transfected with control siRNA (siControl) or siPoldip2. After 72 hours, RGDS or RGES (50 μM) was added for 30 minutes before harvesting. F-actin was collected to measure cysteine oxidation, as above. Data represent mean ± SEM of 4 independent experiments. * p<0.05 vs. siControl. C. Overexpression of Poldip2 induces F-
actin oxidation. RASMs were transfected with adenoviruses with no insert (AdGFP) or C-terminal myc-tagged Poldip2 (AdPoldip2) and harvested after 72 hours. Data represent mean ± SEM from 4 independent experiments. * p<0.05 vs. AdGFP.
Figure 5. Mutation of cysteines 272 and 374 inhibits F-actin-vinculin interaction and impairs cell migration.

A-C. HASMs were transfected with myc-tagged wild type (WT) or mutant (C272A and C374A) β-actin. After 72 hours, cells were re-plated and fixed during attachment at 1, 3 or 6 hours. Interaction between vinculin and myc-tagged β-actin was measured using a proximity ligation assay and confocal microscopy. A. Maximal intensity projection micrographs of consecutive Z-sections showing myc-tagged β-actin-vinculin interaction in green and nuclear staining with DAPI in blue. Scale bar: 20 μm. B. Negative controls for vinculin and myc-tag antibodies alone. Scale bars: 20 μm. C. Summary of image quantification representing mean ± SEM of data from 3–4 independent experiments. ** p<0.01 vs. WT at 3 h. D. Transfected β-actin was successfully incorporated into stress fibers in HASMs, as shown by confocal microscopy. Myc-tagged β-actin (green) and total actin, labeled by phalloidin (red), showed significant colocalization (yellow) in merged images. Scale bar: 20 μm. E. HASMs were transfected with myc-tagged wild type (WT) or mutant (C272A and C374A) β-actin. After 72 hours, a straight scratch wound was made and 10 ng/ml PDGF was added to stimulate cell migration. Cells were fixed after 6 hours and analyzed by confocal microscopy. The graph presents mean ± SEM of image quantification from 3 independent experiments showing numbers of myc-tagged cells in wound area. * p<0.05 vs. WT.
Figure 6. Knockdown of Poldip2 or NOX4 impairs F-actin-vinculin interaction and focal adhesion assembly.

A-D. HASMs were transfected with control siRNA (siControl), siPoldip2 or siNOX4 and co-transfected with inactive fluorescent siGLO. After 72 hours, cells were re-plated and fixed during cell attachment after 1, 3 or 6 hours. A. The knockdown of NOX4 mRNA by siNOX4 was confirmed by RT-qPCR and presented as mean±SEM of data from 4 independent experiments. * p<0.05. B. The knockdown of Poldip2 protein by siPoldip2 (siP), compared to siControl (siC) was confirmed by western blotting. These blots are representative of data from 4 experiments. C. Representative micrographs of proximity Vukelic et al. Page 23
ligation assays performed as in Figure 5 showing interaction (green) between vinculin and β-actin, siGLO (red) and nuclear staining with DAPI (blue). Scale bars: 20 μm. D. Summary of image quantification representing mean ± SEM of data from 3–4 independent experiments. Only transfected cells (positive for siGLO) were included. * p<0.05, ** p<0.01, *** p<0.001 vs. siControl. E- F. HASMs were transfected with control siRNA (siControl) or siNOX4 and co-transfected with inactive fluorescent siGLO on day 1. On day 2, cells were infected with adenoviruses expressing GFP and either no insert (AdGFP) or C-terminal myc-tagged Poldip2 (AdPoldip2). After 48 hours, cells were re-plated and fixed during cell attachment at 3 hours. E. Representative micrographs of proximity ligation assays showing interaction (white) between vinculin and β-actin, siGLO (red), GFP (green) and nuclear staining with DAPI (blue). Scale bar: 20 μm. F. Summary of image quantification representing mean ± SEM of data from 3 independent experiments. Only transfected cells (positive for siGLO and GFP) were included. ** p<0.01, *** p<0.001, ### p<0.001 vs. siControl+AdGFP. G-H. HASMs were transfected with control siRNA (siControl), siPoldip2 or siNOX4 and co-transfected with inactive fluorescent siGLO. After 72 hours, cells were re-plated and fixed during cell attachment after 1, 3 or 6 hours. G. Representative micrographs showing zyxin(green), paxillin (red), siGLO (purple) and nuclear staining with DAPI (blue). Scale bar: 20 μm. H. Summary of image quantification showing zyxin-paxillin colocalization in FA during cell attachment, including mean ± SEM of data from 3 independent experiments. * p<0.05, *** p<0.001 vs. siControl.