Distinct Subcellular Localizations of Nox1 and Nox4 in Vascular Smooth Muscle Cells

Lula L. Hilenski, Roza E. Clempus, Mark T. Quinn, J. David Lambeth, Kathy K. Griendling

Objective—Reactive oxygen species (ROS) that act as signaling molecules in vascular smooth muscle cells (VSMC) and contribute to growth, hypertrophy, and migration in atherogenesis are produced by multi-subunit NAD(P)H oxidases. Nox1 and Nox4, two homologues to the phagocytic NAD(P)H subunit gp91phox, both generate ROS in VSMC but differ in their response to growth factors. We hypothesize that the opposing functions of Nox1 and Nox4 are reflected in their differential subcellular locations.

Methods and Results—We used immunofluorescence to visualize the NAD(P)H subunits Nox1, Nox4, and p22phox in cultured rat and human VSMC. Optical sectioning using confocal microscopy showed that Nox1 is co-localized with caveolin in punctate patches on the surface and along the cellular margins, whereas Nox4 is co-localized with vinculin in focal adhesions. These immunocytochemical distributions are supported by membrane fractionation experiments. Interestingly, p22phox, a membrane subunit that interacts with the Nox proteins, is found in surface labeling and in focal adhesions in patterns similar to Nox1 and Nox4, respectively.

Conclusions—The differential roles of Nox1 and Nox4 in VSMC may be correlated with their differential compartmentalization in specific signaling domains in the membrane and focal adhesions.

Key Words: Nox1, Nox4, NAD(P)H oxidase, caveolae, focal adhesions

Reactive oxygen species (ROS), including superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$), are important determinants of vascular function, acting not only as signaling intermediates to promote growth and differentiation but also as modulators in pathological processes such as hypertension, atherosclerosis, and diabetic microvascular disease. Major sources of ROS in the vascular wall are NAD(P)H oxidases, multi-subunit enzymes that differ structurally and biochemically from the prototypical phagocyte NAD(P)H oxidase active in host defense. One structural difference is that vascular smooth muscle cells (VSMC) express two proteins, Nox1 and Nox4, which are homologues to the gp91phox catalytic subunit in phagocytes. Biochemical differences include kinetics of activation, output, and regulation of NAD(P)H-dependent ROS production. In contrast to the activated phagocyte oxidase, which produces large quantities of ROS into an extracellular (phagosomal) compartment in inducible bursts, Nox1 and Nox4 generate low-level, predominantly intracellular ROS constitutively and in response to agonists. In VSMC, Nox1 is inducible and upregulated by growth factors and hormones, whereas Nox4 is downregulated by these agonists. Furthermore, in an animal model of restenosis in which O$_2^-$ production is increased, Nox1 and Nox4 are induced at different times after carotid injury, suggesting different functions of the two Nox proteins in redox-sensitive arterial remodeling.

The differential expression and growth factor-related responses of multiple Nox proteins in VSMC imply distinct mechanisms of Nox1 and Nox4 regulation. This might involve specific signaling pathways such as the biphasic production of angiotensin II-mediated ROS consisting of a rapid PKC-dependent phase, followed by a sustained Rac/Src/PI3 kinase-dependent phase, and may implicate more than one Nox enzyme in ROS generation in VSMC. Another potential mechanism for the differential regulation and function of the Nox proteins is the targeting of the isoforms to specific subcellular signaling domains. Two candidate sites for NAD(P)H oxidase localization are caveolae’ flask-shape plasma membrane invaginations, which are enriched in cholesterol and an array of signaling molecules, and focal adhesions, which are major sites of tyrosine kinase signaling that link the extracellular matrix to cytoskeletal proteins through transmembrane integrins. Caveolae and...
focal adhesions are proposed sites for growth factor

We tested the hypothesis that the opposing growth and senescence functions for Nox1 and Nox4 are caused by their differential subcellular locations. We found that Nox1 and Nox4 are positioned in caveolae and focal adhesions, respectively, each with its own repertoire of signaling components. This targeting of NAD(P)H oxidase subunits to specific multi-molecular signaling domains provides a possible explanation for their apparent differential roles in normal growth, differentiation, and disease.

Methods

Cell Culture

Rat VSMC were isolated from male Sprague-Dawley rat thoracic aortas by enzymatic digestion and cultured as previously described. For experiments, cells between passages 7 to 9 were seeded onto round glass coverslips and serum-starved in 0.1% serum or serum-free media for 18 to 20 hours before treatments.

Human VSMC were cultured in SmGM2 media (Clonetics) supplemented with 5% fetal bovine serum and growth factors according to the supplier’s instructions. Cells between passages 3 to 7 were used and were plated for experiments as described.

Immunofluorescence

VSMC on glass coverslips were processed as previously described. Cells were incubated with anti-Nox1 (raised against Nox1 306 to 564 and 424 to 564, N-terminal 6X His fusion proteins, or Mox1; Santa Cruz), anti-Nox4, anti-p22phox R5554, anti-caveolin-1 (C13630; BD Biosciences), or anti-vinculin (V4505; Sigma) for 1 hour at room temperature and then incubated in either FITC-conjugated (Jackson ImmunoResearch, West Grove, PA) or Rhodamine Red X (RRX)-conjugated secondary antibodies for 1 hour at room temperature. Cells on coverslips were mounted in Vectashield (Vector Laboratories, Burlingame, CA) and examined using the 488-nm and 543-nm laser lines for the detection of FITC and RRX, respectively. The multi-tracking mode on a Zeiss LSM 510 using the 488-nm and 564-nm long pass filters, respectively, in the confocal imaging system (Bio-Rad MRC-1024). In double-labeling experiments, FITC and RRX images were scanned sequentially and merged using the Bio-Rad LaserSharp software or were scanned with the multi-tracking mode on a Zeiss LSM 510 using the 488-nm and 543-nm laser lines for the detection of FITC and RRX, respectively. Controls with no primary antibody showed no fluorescence labeling, and single-label controls were performed in double-labeling experiments.

Immunoblotting

VSMC were lysed in the presence of 1% Triton X-100 as described previously, and lysates were separated into Triton-soluble and insoluble fractions by centrifugation at 18 000g for 10 minutes. Fractions were separated using SDS-PAGE and transferred to nitrocellulose membranes, blocked, and incubated overnight at 4°C with anti-Nox1 (Santa Cruz) or anti-Nox4. After incubation with HRP-conjugated secondary antibody, proteins were detected by ECL chemiluminescence.

Purification of Caveolar Fractions

Caveolin-enriched membranes were isolated as described previously by Smart et al. Briefly, plasma membranes from lysed cells were isolated by Percoll gradient fractionation and disrupted by sonicaton. Caveolae membranes were separated from the plasma membrane by two OptiPrep density gradients, a continuous 23% to 10% OptiPrep gradient followed by centrifugation against a discontinuous OptiPrep gradient to concentrate the lightest material. Protein from each fraction was concentrated with acetone, re-suspended in 1% Triton X-100 lysis buffer, and separated on a 12% SDS-PAGE gel.

![Image](image.png)

Figure 1. Characterization of Nox4 and Nox1 antibodies by Western analyses. Immunoblot assays (a) of rat VSMC using anti-Nox4 show ~80-kDa and ~65-kDa bands that were blocked by preincubating the Nox4 antibody with excess antigenic peptide (blocking peptide = BP). Immunoblot assays (b) using anti-Nox1 on human VSMC and CaCo2 cells (positive control) show two bands (65-kDa and 50-kDa). Preincubation with excess antigenic peptide (BP) abolished the labeling in both cell types.

After transfer, membranes were blotted with anti-Nox1, anti-Nox4, and anti-caveolin-1 (C37120; BD Biosciences) as described.

Nuclear Fractionation

Isolation of nuclear fractions was performed as described previously by Neufeld and White. Briefly, rat VSMC were scraped into ice-cold PBS, rinsed, and re-suspended in 1-buffer (PBS containing 0.1% Triton X-100, 0.1% Nonidet P-40, pH 7.4) with protease inhibitors. Nuclei and non-lysed cells were pelleted at 1000g for 10 minutes at 4°C. The supernatant fraction was collected and classified as the non-nuclear fraction. The nuclear pellet was purified by passage through a 0.21-gauge needle 5 times and passage through a 0.85-sucrose cushion in PBS (20 800g, microfuge, 15 minutes at 4°C). Nuclei in the pellet were lysed by sonication, treated with DNase I (500 U total), and re-sonicated to prepare a nuclear lysate. Fractions were characterized by immunoblotting for β-tubulin (Sigma), a cytoskeletal marker, and histone H3 (Cell Signaling) nuclear proteins.

Results

To verify the quality of the antibodies used, Western analysis was performed in the presence and absence of the antigenic peptide. Western analysis of VSMC lysates using the Nox4 antibody showed an 80-kDa band that was blocked by excess peptide antigen (Figure 1a) and likely represents a tight association of the ~65-kDa Nox4 (lower band) with another protein (data not shown). The Nox1 antibody detected a 65-kDa band (the predicted size) and a band at 50 kDa, both of which were present in human colon carcinoma cells (CaCo2) and were blocked by preincubating the Nox1 antibody with excess antigen (Figure 1b).

Optical sectioning of VSMC with laser scanning confocal microscopy revealed Nox1 labeling in a punctate surface.
distribution, often along cellular margins (Figure 2a), whereas Nox 4 was confined to the basal level where the cell contacts the underlying matrix († in a and c) in rat VSMC. Optical sectioning of the same cells using confocal microscopy (c and d) shows that in addition to the surface labeling (c), p22phox is found at the basal level of the cell (where the cell contacts the substratum) in elongated patches at the cell periphery († in d), a pattern similar to Nox4 labeling († in b). Nox4 labeling is also prominent in the nucleus (b). Incubation of Nox1 and Nox4 antibodies with their respective antigenic peptides abolished labeling (see Figure I, available online at http://atvb.ahajournals.org). † 20 μm.

Figure 2. Localization of Nox1, Nox4, and p22phox in VSMC. Indirect immunofluorescence labeling using antibodies to Nox1 (a) and p22phox (c) and FITC-conjugated secondary antibodies show a punctate surface distribution, often along the cellular margins († in a and c) in rat VSMC. Optical sectioning of the same cells using confocal microscopy (c and d) shows that in addition to the surface labeling (c), p22phox is found at the basal level of the cell (where the cell contacts the substratum) in elongated patches at the cell periphery († in d), a pattern similar to Nox4 labeling († in b). Nox4 labeling is also prominent in the nucleus (b). Incubation of Nox1 and Nox4 antibodies with their respective antigenic peptides abolished labeling (see Figure I, available online at http://atvb.ahajournals.org). † 20 μm.

In VSMC, Nox4 and p22phox co-localized with vinculin (Figure 3), a marker for focal adhesions.12 Nox4 was also strongly detected in the nuclei of these cells (Figure 2b). p22phox was found at the surface and at the basal level in patterns similar to Nox1 and Nox4, as revealed by optical sections taken at different planes within the cells (Figure 2c and 2d). These results indicate that Nox1 and Nox4 are preferentially localized in different subcellular compartments and that p22phox is found in both locations. Preincubation of Nox1 and Nox4 antibodies with their respective antigenic peptides abolished labeling (see Figure I, available online at http://atvb.ahajournals.org), in agreement with Western analyses (Figure 1).

In VSMC, Nox4 and p22phox co-localized with vinculin (Figure 3), a marker for focal adhesions.12 In some cells, Nox4 labeling appeared fibrillar, extending along actin fibers beyond the vinculin-positive focal adhesions († in Figure 3c).

To further verify the localization of Nox4 and p22phox in focal adhesions, we used cytochalasin D to induce alterations in the actin cytoskeleton with concomitant disruption of cytoskeletal-associated focal adhesions. Treatment of VSMC with 1 μmol/L cytochalasin D for 1 hour abolished Nox4 and p22phox labeling in focal adhesions, whereas the surface labeling of Nox1 and p22phox and the nuclear labeling of Nox4 were unaffected (Figure 4).

The labeling pattern of Nox1 in VSMC in punctate patterns on the cell surface and in patches concentrated at cell margins (Figure 2a) is reminiscent of that of caveolin, a scaffolding protein associated with caveolae.21,22 In VSMC, Nox1 is co-localized with caveolin (Figure 5a, 5b, and 5c). The co-localization (yellow) is evident in single confocal optical sections taken through the cell (Figure 5a, 5b, and 5c). Treatment of cells with 10 μmol/L methyl-β-cyclodextrin, which depletes cholesterol and disrupts caveolar structure, reduced the co-localization of caveolin with Nox1, as shown in optical sections taken at the level where the cells attach to the substrate (Figure 5d, 5e, and 5f). In these sections, Nox1, which often was found in ruffled membranes at the cell boundaries (Figure 5e and 5f), did not co-localize with caveolin.

To confirm localization shown by immunocytochemistry, caveolin-enriched fractions were isolated from VSMC by gradient centrifugation (Figure 6a). The 50- and 65-kDa forms of Nox1 were readily detectable in the cell lysate. The 65-kDa form was enriched in the caveolin fraction after separation of membranes, supporting the concept that this isoform and caveolin-1 co-localize.

Cell fractionation experiments provided support for a focal adhesion and a nuclear localization for Nox4. Although focal
adhesions are difficult to isolate biochemically, they fractionate with the cytoskeleton in the detergent-insoluble fraction after treatment of the cells with Triton X-100. Of interest, the 80-kDa band of Nox4 occurs in the Triton-soluble and Triton-insoluble fractions, whereas the 65-kDa form is found exclusively in the Triton-insoluble fraction (cytoskeletal fraction) (Figure 6b) and in the nuclear fraction (Figure 6c). These results suggest that it is the 65-kDa form of Nox4 that is associated with focal adhesions and the nucleus.

**Discussion**

In the present study, we investigated the intracellular localizations of Nox1 and Nox4, two catalytic subunits of NAD(P)H oxidases that are major sources of \( \text{O}_2^- \) in VSMC. Our data indicate that Nox1 is co-localized with caveolin in caveolae-enriched fractions on the cell surface, Nox4 is co-localized with vinculin in focal adhesions and nuclei, and p22phox is found in patterns similar to Nox1 and Nox4. The localization of these two gp91phox homologues, Nox1 and Nox4, in two separate signaling domains may provide insights into their opposing functions and differential regulation by growth factors and hormones.

In phagocytes and VSMC, enzyme subunits are precisely assembled and targeted to specific sites within the cellular architecture for differential functions. In the classical phagocyte paradigm for NAD(P)H oxidase activation, the plasma membrane is the site for assembly of gp91phox, p22phox, and regulatory cytoplasmic phox proteins. The assembly of active enzyme on stimulation results in the release of deleterious ROS into the extracellular environment of the membrane-delimited phagosome. Recent evidence suggests that phagocytic NAD(P)H oxidase assembly and oxidant production occur not only on the plasma membrane for extracellular oxidant release but also on intracellular granules/vesicles for intracellular oxidant release, which is speculated to function in signal transduction. Therefore, the production of potentially damaging and/or regulatory ROS in phagocytes can be precisely controlled temporally and spatially by targeting NADPH oxidase assembly to the phagosome or to intracellular granules/vesicles. Our observations that Nox1 and Nox4, homologues to the gp91phox subunit of phagocites, are differentially localized in caveolae and focal adhesions in VSMC are consistent with these recent data implicating multiple sites of NADPH oxidase activation.
In VSMC, ROS generation is predominantly intracellular and is postulated to be compartmentalized within domains that would spatially confine their activities. Our observation that subunits of the NAD(P)H oxidase (Nox4, p22phox) are co-localized with vinculin suggests that focal adhesions may be one such domain in VSMC. A role for focal adhesions in NAD(P)H oxidase function is supported by data from phagocytes linking NAD(P)H oxidase regulation to multiple lipid products of PI3 kinase, a component of focal adhesions, and by recent data from VSMC linking PI3 kinase and Src, another tyrosine kinase found in focal adhesions, to angiotensin II-induced ROS production. Of particular interest is the biphasic nature of this ROS production in which a rapid PKC-dependent phase is followed by a Rac-mediated sustained phase leading to epidermal growth factor receptor (EGFR) transactivation and the growth response. These observations, together with data showing phosphorylated EGFR localization in focal adhesions, support the proposal that focal adhesions are sites where NAD(P)H oxidase subunits interact with signaling molecules. Thus multiple pathways and, by implication, multiple Noxes may be involved in generating intracellular ROS in VSMC.

The association of Nox4 with the cytoskeleton may have functional consequences in addition to providing compartmentalization of ROS production. Previous work showed an association of oxidase subunits and O$_2^·$/H$_2$O$_2$-producing activity with the submembranous actin network and with actin-associated proteins, including coronin and cofilin. Interactions between the actin-binding protein cortactin and subunits of NAD(P)H oxidase have been reported in hyperoxic human lung endothelial cells, suggesting that the actin cytoskeleton has a role in NAD(P)H oxidase activation and ROS generation. In vascular endothelial cells, all main subunits of the NAD(P)H oxidase are pre-assembled into an intracellular active enzyme complex associated with the cytoskeleton, which may provide a stable scaffold for constitutive production of low levels of ROS. In the present study, the abolition of Nox4 and p22phox-labeling in focal adhesions at the termini of actin filaments by cytochalasin D suggests that these NAD(P)H oxidase subunits in VSMC are also associated with or regulated by the actin cytoskeleton.

Another potential functional consequence of Nox4 association with the cytoskeleton is the regulation of focal adhesion proteins by ROS derived from this oxidase. Focal adhesions contain complexes of clustered integrins, tyrosine phosphorylated proteins, and Src kinases. Inhibition of NADPH oxidase or degradation of H$_2$O$_2$ by catalase inhibits the adhesion-dependent activities of Src kinases, whereas H$_2$O$_2$ produced by NADPH oxidase activates Src kinases. Thus, there are functional and physical links among the actin cytoskeleton, actin-associated proteins, focal adhesion proteins, including tyrosine kinases, and the NADPH oxidase. The actin cytoskeleton may play a role in translocating subunits to the membrane, in restricting the localization of oxidase activity, or in forming a scaffold for assembly of redox-sensitive signaling complexes, whereas the oxidase may influence the function of focal adhesion-associated signaling proteins.

In vascular endothelial cells, all of the major NAD(P)H oxidase subunits and 50% of the total O$_2^·$ production occurs in a “nucleus-rich” fraction. Redox-sensitive targets have also been identified in the nucleus. The prominent labeling of Nox4 in the nucleus may provide a source of ROS that can potentially activate these targets, which include transcription factors such as AP-1 proteins c-Fos and c-Jun, implicated in

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**Figure 6.** Immunoblot detection of Nox proteins in subcellular fractions of VSMC. Human VSMC (a) were fractionated as described in Methods. For lane 1 (cell lysate=CL), 5 µg protein were loaded. For lanes 2 and 3, 2.5 µg protein were loaded. Blots were probed for Nox1 (top) and caveolin-1 (bottom) in CL, plasma membrane (PM), and caveolae-enriched (Cav) fractions. Triton-soluble (TS) and Triton-insoluble (TI) (cytoskeletal) fractions (b) were prepared from rat VSMC as described in Methods. Nox4 is detected in both fractions, but the 65-kDa form is enriched in the Triton-insoluble fraction. The distribution of Nox4 in non-nuclear (N-nu) and nuclear (Nu) fractions (c) from rat VSMC was characterized using antibodies directed against compartment-specific proteins: β-tubulin for cytoplasm/cytoskeleton and histone H3 for nucleus.
growth and differentiation processes, and NF-κB, implicated in inflammatory reactions and apoptosis. Alternatively, ROS production in the nucleus by Nox4 could lead to modification of DNA or chromosomes, such as telomere shortening, which is a major cause of cellular senescence induced by oxidative stress.

In addition to the colocalization of p22^phox with Nox4 in focal adhesions, there is evidence that p22^phox also associates with Nox1 in the caveolar signaling domain in VSMC. This p22^phox/Nox1/caveolin association is based on a recent study showing co-immunoprecipitation and co-localization of Nox1 and p22^phox by immunocytochemistry, and on the present study, showing that Nox1 associates with caveolin using immunocytochemistry and cell fractionation. These data suggest that in VSMC, Nox1 and p22^phox interact in caveolin-containing structures, presumably the caveolae.

The functional consequences of localization of NAD(P)H oxidases in caveolae are unknown but may be related to regulation of oxidase activation, as by the prototype G-protein–coupled angiotensin type 1 receptor (AT1). Previous studies have shown agonist-induced movement of the AT1 receptor into caveolin-enriched fractions. Thus, located within caveolae are G-proteins (Rac), AT1 receptors, Src family kinases, receptor tyrosine kinases, protein kinase C, and NAD(P)H oxidase subunits (Nox1 and p22^phox). Because all of these molecules are thought to be involved in ROS generation by angiotensin II, these data implicate caveolae as sites for Nox1 activation in VSMC. Interestingly, earlier work showed ROS production in caveolae of myoepithelial cells, which share features with smooth muscle cells. The positioning of Nox proteins in caveolae and focal adhesions suggests a role for NAD(P)H oxidases in growth factor and integrin signaling in VSMC. Furthermore, these data suggest that Nox1 and Nox4, by being positioned in two different signaling domains, may be regulated by different signaling cascades and thus may be responsible for different phases of agonist-induced responses in VSMCs.

The simultaneous expression of two homologous Nox proteins, Nox1 and Nox4, and their interactions with p22^phox suggest highly complex mechanisms for redox-sensitive events in VSMC, in which ROS generation can be constitutive and inducible and is important in normal physiology and pathophysiology. The importance of understanding the expression, activity, and signaling roles of these nonphagocytic NAD(P)H oxidases in vascular function and remodeling is underscored by the accumulating evidence linking oxidative stress and vascular diseases. Understanding the interplay among these components necessitates not only knowing which subunits are present and assembled in the active nonphagocytic enzyme but also knowing where within the subcellular architecture these components interact. This understanding is an essential step to designing possible therapeutic interventions in ROS-mediated vascular pathology and injury.

Acknowledgments
This work was supported by National Institutes of Health grants HL38206, HL58000, and HL 58863.


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Arterioscler Thromb Vasc Biol. 2004;24:677-683; originally published online December 11, 2003;
doi: 10.1161/01.ATV.0000112024.13727.2c
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272
Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
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