Objective—We tested the hypothesis that p47phox associates with the actin cytoskeleton, enabling site-directed activation of NAD(P)H oxidase, and assessed whether these actions influence reactive oxygen species (ROS) generation and signaling by angiotensin II (Ang II) in vascular smooth muscle cells (VSMCs) from human resistance and coronary arteries.

Methods and Results—Electroporation of anti-p47phox antibody into VSMCs abrogated Ang II–mediated O2\textsuperscript{-} generation, establishing the requirement for p47phox in this response. Immunofluorescence confocal microscopy demonstrated a cytosolic distribution of p47phox in basal conditions. After Ang II stimulation, p47phox rearranged in a linear fashion, colocalizing with F-actin. Co-immunoprecipitation studies confirmed an association between p47phox and actin and demonstrated an interaction with the actin-binding protein cortactin. Cytoskeletal disruption with cytochalasin prevented p47phox:actin interaction and attenuated ROS formation and p38MAP kinase and Akt phosphorylation by Ang II. Intracellular ROS generation in response to LY83583 (O2\textsuperscript{-} generator) or exogenous H2O2 and Ang II–induced ERK1/2 activation were unaltered by cytochalasin.

Conclusions—The p47phox:actin interaction, through cortactin, plays an important role in Ang II–mediated site-directed assembly of functionally active NAD(P)H oxidase, ROS generation, and activation of redox-sensitive p38MAP kinase and Akt, but not ERK1/2. These findings demonstrate the importance of an intact actin–cytoskeleton in NAD(P)H oxidase regulation and redox signaling by Ang II in human VSMCs. (Arterioscler Thromb Vasc Biol. 2005; 25:512-518.)

Key Words: reactive oxygen species ■ cytochalasin B ■ cortactin ■ LY83583 ■ hydrogen peroxide.
and p22phox, resulting in assembly of the active oxidase, which generates O$_2^-$. In polymorphonuclear leukocytes, p47phox is essential in the assembly process. This is exemplified in cells of patients with chronic granulomatous disease, in which no translocation occurs because of p47phox deficiency. The p47phox also plays a major role in functionally active NAD(P)H oxidase activity in cardiovascular cells, as evidenced by studies using p47phox$^{-/-}$ mice. Lavigne et al demonstrated that aortic VSMCs derived from aorta of p47phox$^{-/-}$ mice failed to generate O$_2^-$ in response to phorbol esters, Ang II, or platelet-derived growth factor (PDGF). Li and Shah reported that Ang II regulates pre-assembled NAD(P)H oxidase in endothelial cells, which is inhibited in cells from p47phox$^{-/-}$ mice. We recently demonstrated in VSMCs from human resistance arteries that c-Src–mediated phosphorylation of p47phox is a prerequisite for assembly and translocation of the p40-p47-p67 phox complex. What remains unclear is how the p47phox-regulated cytosolic complex migrates to the cell membrane to facilitate site-directed complex formation with p22phox and gp91phox/nox and consequent activation of NAD(P)H oxidase.

Studies in polymorphonuclear leukocytes and endothelial cells suggest that the cytoskeleton may play a role. In activated neutrophils, p47phox, p67phox, and p40phox associate with filamentous (F)-actin. In endothelial cells, p47phox localizes with the cytoskeleton to induce tumor necrosis factor-α signaling. However, van Bruggen et al recently reported that p67phox and Rac2 translocation do not depend on a rearrangement of the actin cytoskeleton in phagocytic cells. To our knowledge, nothing is known about the relationship between the cytoskeleton and NAD(P)H oxidase, specifically p47phox, in Ang II–mediated ROS regulation and signal transduction in human VSMCs.

We tested the hypothesis that Ang II–activated p47phox associates with cytoskeletal proteins, which facilitates site-directed targeting of functionally active NAD(P)H oxidase in VSMCs.

**Methods**

**Cell Culture**

VSMCs derived from human small arteries from gluteal biopsy samples were cultured as previously described. Human coronary VSMCs (Clonetics San Diego, Calif) were also studied.

**Immunofluorescence Confocal Microscopy**

VSMCs were stimulated with Ang II (10^{-7} mol/L, 5 to 30 minutes). In some experiments, cells were pre-exposed to cytochalasin B (10^{-6} mol/L, 60 minutes), which arrests new F-actin assembly. Washed cells were fixed and incubated with primary antibody (anti-p47phox, anti-cortactin) and proteins detected with secondary antibody. Imaging was acquired with a Zeiss Axiovert S100TV LSM 510 laser scanning system.

**Immunoprecipitation and Immunoblotting**

**p47phox Immunoprecipitation**

Cells were lysed and p47phox was immunoprecipitated as previously detailed (online data). Western Immunoblotting

After SDS-PAGE separation of proteins, samples were transferred to polyvinylidene fluoride membranes. Membranes were incubated with the following antibodies: anti-smooth muscle cell α-actin, anti-cortactin, anti-p47phox, anti-phospho-ERK1/2, anti-phospho-p38MAP kinase, and anti-phospho-Akt. Washed membranes were incubated with horseradish peroxidase-conjugated second antibody. Immunoreactive proteins were detected by chemiluminescence.

**Electroporation**

VSMCs were electroporated with anti-p47phox antibody, anti-IgG, or in the absence of any antibodies as we previously described (online data).

**Viability Test**

Trypan blue dye exclusion, which was used to evaluate viability of electroporated VSMCs before plating them in DMEM.

**Detection of Electroporated Antibody by Immunofluorescence**

To determine efficiency of antibody incorporation, cells were also electroporated in the presence of secondary antibodies (Alexa fluor 488; Molecular Probes, Eugene, Ore). Fluorescence was assessed by epifluorescence microscopy just before experimentation. Cells that floresced were considered successfully electroporated.

**Measurement of ROS in Intact Cells**

Intracellular O$_2^-$ and H$_2$O$_2$ levels were measured with the fluoroprobes tempo-9-AC and CMH2-DCFDA, respectively, in unstimulated cells and in cells exposed to Ang II (10^{-6} to 10^{-4} mol/L) in the absence and presence of 10^{-6} mol/L cytochalasin B (10^{-6} mol/L, 60-minute pre-incubation). In some experiments cells were stimulated with LY83583 (O$_2$ generator, 10^{-5} to 10^{-4}, 10 minutes) or exogenous H$_2$O$_2$ (10^{-5} to 10^{-3} mol/L, 10 minutes), in the absence and presence of cytochalasin B (10^{-6} mol/L, 60-minute pre-incubation).

**Measurement of NAD(P)H Oxidase Activity**

Quiescent VSMCs were stimulated with Ang II for 5 to 15 minutes in the absence and presence of apocynin (3×10^{-3} mol/L, 20-minute pre-incubation), which blocks association of p47phox with membrane-associated subunits, p22phox and gp91phox, or with gp91ds-tat, a novel competitive inhibitor of NAD(P)H oxidase assembly (5×10^{-6} mol/L). The lucigenin-derived chemiluminescence assay was used to determine NAD(P)H oxidase activity in whole-cell homogenates as previously described. Data are presented as relative light units (RLU)/min per milligram of protein.

**Analysis**

Data obtained from digital imaging studies, in which multiple cells were examined in each experimental field, were calculated as the mean per experiment and then as the mean of multiple experiments. Each experiment was performed at least 3 times. Values are presented as means±SEM. Data were analyzed by ANOVA or Student’s t test. Tukey–Kramer correction was used to compensate for multiple testing procedures. P<0.05 was significant.

An expanded Methods section is available online. Please see http://atvb.ahajournals.org.

**Results**

Functionally Active p47phox Is Essential for Ang II–stimulated O$_2^-$ Production in VSMCs

To establish the functional importance of p47phox in the generation of intracellular ROS by Ang II, we disrupted p47phox function by electroporating cells with Alexa-labeled anti-p47phox antibody. When cells were exposed to a 1.3-ms pulse of 750 V/cm, >70% of cells survived and ~60% were brightly fluorescent after overnight incubation. In compari-
son, cells that were incubated in the presence of labeled
antibody without electroporation failed to fluoresce.

Electroporated cells were loaded with tempo-9-AC and
analyzed for O$_2$ production. Electroporation did not sig-
nificantly influence basal O$_2$ generation (Figure I, avail-
able online at http://atvb.ahajournals.org). In p47phox
antibody-electroporated cells, Ang II–stimulated O$_2$
formation was significantly reduced ($P<0.01$) compared
with cells electroporated with anti-IgG or no antibodies
(Figure I).

Interruption of p47phox–gp91phox/p22phox
Association Inhibits Ang II–Stimulated NAD(P)H
Oxidase Activation

To verify the importance of p47phox interaction with cell
membrane-associated subunits in the activation of NAD(P)H
oxidase in our cell model, effects of Ang II in the presence of
apocynin, a catechol-methyl derivative that blocks p47phox
interaction with gp91phox/p22phox, and gp91ds-tat, a com-
petitive inhibitor of p47phox-dependent NAD(P)H oxidase
assembly,$^3$ were assessed. Whereas Ang II significantly
increased NAD(P)H oxidase activation (3886±142 versus
basal 85±12 RLU/min per milligram ($\times 10^{4}$), $P<0.01$) in
control cells, Ang II–induced responses were significantly
attenuated ($P<0.01$) in cells pre-exposed to apocynin (65±14
RLU/min per milligram [$\times 10^{4}$] and gp91ds-tat (213±39 RLU/
min per milligram [$\times 10^{4}$]).

p47phox Is a Cytoskeletal-Associated Protein

Immunofluorescent image analysis demonstrated that in the
basal state, p47phox is localized in the cytoplasmic region
(Figure II, available online at http://atvb.ahajournals.org). After
Ang II stimulation, p47phox aligns in a reticular-type
pattern and migrates to the cell periphery. Costaining with
actin-microfilament–binding rhodamine phalloidin revealed
a somewhat linear colocalization of p47phox and actin
(Figure 1A to 1D), suggesting an association between
p47phox and F-actin in stimulated cells. Confocal immuno-
fluorescence images demonstrated that in addition to the actin-
binding protein cortactin in Ang II–stimulated cells (Fig-
ure 2A).

To further evaluate whether Ang II–stimulated p47phox
organization is related to actin association, cells were pre-
treated with cytochalasin B. As shown in Figure 1E to 1G,
p47phox remains mainly in the cytoplasmic area in cytocha-

Figure 1. Effect of chytochalasin B on p47phox and actin local-
ization in VSMCs. VSMCs were stimulated with Ang II (10$^{-7}$
mol/L, 5 minutes) and analyzed by confocal immunofluores-
cence microscopy using ×63 (A to C) and ×100 (D) oil immers-
sion objectives. Cells were labeled with monoclonal anti-
p47phox (blue fluorescence) and rhodamine-phalloidin for
F-actin detection (red fluorescence). A and B, Individual staining
patterns for actin and p47phox, respectively. C and D, Dual
staining pattern. VSMCs were pretreated with cytochalasin B
(10$^{-6}$ mol/L, 1 hour) and analyzed as described (E to G). E, 
Cytotochalasin B alone (p47phox, blue fluorescence; actin, red
fluorescence), ×63 oil immersion objective. F, Ang II plus
cytochalasin B, ×63 oil immersion objective. G, Ang II plus
cytochalasin B, ×100 oil immersion objective. H, No staining in
the negative control.

Figure 2. p47phox associates with the
actin-binding protein cortactin in Ang II–
stimulated cells. A, VSMCs were stimu-
lated with Ang II (10$^{-7}$ mol/L, 10 minutes),
followed by labeling with anti-cortactin
polyclonal antibody (Alexa-594, anti-rabbit
secondary antibody, red fluorescence) and
monoclonal anti-p47phox (Alexa-488 anti-
mouse secondary antibody, green fluores-
cence). Colocalization of cortactin and
p47phox are revealed as yellow–orange
fluorescence in the merged image. B to D,
Cortactin co-immunoprecipitates with
p47phox in Ang II–stimulated cells. Coron-
ary VSMCs were stimulated with Ang II,
immunoprecipitated (IP) with anti-p47phox
antibody, and immunoblotted (IB) with
anti-cortactin antibody (labeling at 80 to 85
kDa) (B). Membranes were reprobed with
anti-p47phox antibody and immunoblotted (IB) with
anti-cortactin antibody (labeling at 80 to 85
kDa) (B). Membranes were reprobed with
anti-p47phox antibody to demonstrate
equal p47phox protein content (C). Bar
graphs are means±SEM of 3 separate
experiments (D). Results are expressed as
percentage of control (H$_2$O-treated cells)
taken as 100%. *$P<0.05$ versus control.
IgG was used as a negative control.
Lasin B-treated cells and does not orientate in a linear alignment after Ang II stimulation, suggesting that cytochalasin B interferes with p47phox:actin interaction.

Co-immunoprecipitation of p47phox With Actin and Cortactin in Ang II–Stimulated VSMCs

To confirm these results and to evaluate whether the actin-binding protein cortactin plays a role in p47phox:actin association, p47phox was isolated by using affinity-purified anti-p47phox to immunoprecipitate p47phox from VSMC homogenate. The p47phox immunoprecipitates were analyzed by Western blotting for the presence of actin and cortactin using specific anti-actin antibody and anti-cortactin antibodies, respectively. As shown in Figures 2B to 2D and 3, cortactin and actin co-immunoprecipitated with p47phox, with enhanced effects in Ang II–stimulated cells compared with vehicle-treated cells. In the presence of cytochalasin B, Ang II–induced p47phox:actin co-immunoprecipitation was reduced compared with cells not exposed to cytochalasin B (Figure 3).

Cytochalasin B Attenuates Ang II–Stimulated ROS Production Without Influencing ROS Formation Induced by LY83583 and H2O2

To investigate the functional significance of p47phox:actin interaction, we investigated whether Ang II–mediated production of ROS is altered in cells in which actin assembly is arrested. Pretreatment of VSMCs with cytochalasin B significantly attenuated (P<0.01) Ang II–stimulated increase in DCFDA fluorescence (P<0.01) (Figure 4). However, responses were not completely abolished, indicating that cytochalasin B–independent events also contribute to Ang II–mediated O2·− generation.

Discussion

Ang II–mediated ROS production in the vasculature derives, in part, from VSMC NAD(P)H oxidase. Ang II activates p47phox to form complex with the NAD(P)H oxidase cytosolic subunits and promote translocation to cell membrane-associated flavocytochrome b555. Intracellular ROS are involved in redox-sensitive signaling pathways by Ang II and have been implicated as important second messengers in VSMCs. What remains unclear is how Ang II regulates p47phox migration and how it promotes site-direction of functionally active NAD(P)H oxidase. In the present study, we provide novel data indicating: (1) Ang II–activated p47phox associates with actin facilitating cytosolic complex translocation to the cell membrane to assemble active vascular NAD(P)H oxidase; (2) cortactin may be a putative adaptor protein...
linking p47phox and actin; (3) p47phox interaction with cytoskeletal proteins is important for NAD(P)H oxidase-mediated ROS generation in VSMCs; and (4) p38MAP kinase and Akt, but not ERK1/2, signaling by Ang II depends on an intact actin–cytoskeleton in VSMCs. The p47phox–cytoskeletal targeting may promote site-directed NAD(P)H-driven ROS production, which could contribute to efficient redox-dependent signaling by Ang II in VSMCs.

A relationship between NAD(P)H oxidase and the cytoskeleton has been demonstrated in neutrophils. Whether the cytoskeleton is also involved in NAD(P)H oxidase regulation and redox-sensitive signaling in non-phagocytic cells remains unclear. An association between p47phox and actin has been demonstrated in endothelial cells. In VSMCs, Nox1 and Nox4 have been shown to colocalize with caveolae and vinculin, respectively. Furthermore, Zuo et al recently showed that AT1R trafficking and ROS signaling involved in VSMC growth requires the integrity of microtubules. Here we demonstrate, using a multidisciplinary approach, the novel findings that p47phox is closely associated with F-actin in Ang II–treated human VSMCs. On Ang II stimulation, p47phox migrates from the cytosol along actin fibers to the cell periphery to assemble functionally active NAD(P)H oxidase. This is evidenced by immunofluorescence studies, p47phox:actin co-immunoprecipitation, and by the findings that cytochalasin B disrupts p47phox:actin interaction.

Subcellular mechanisms whereby p47phox interacts with actin are unclear; however, p47phox contains numerous functional binding sites, including Src homology 3 (SH3) domains, a motif typically found in proteins known to associate with actin-binding proteins and Phox homology (PX) domains, which possess a PXXP motif, allowing binding to proteins containing SH3 domains. In fact, p47phox has been shown previously to associate with actin-binding proteins coronin, cofilin, and moesin. The interaction of p47phox with moesin, an ezrin-radixin-moesin family of F-actin–binding proteins, occurs through the PX domain. In our study, cortactin, a Src-regulated actin-binding protein, colocalized and co-immunoprecipitated with p47phox, particularly in Ang II–stimulated VSMCs. This was significantly attenuated by cytochalasin B. These results suggest cortactin as a putative scaffolding protein linking actin to cytosolic phox proteins. Of significance, we previously showed that Ang II regulates p47phox activation through c-Src–dependent actions. Accordingly, we suggest that c-Src may regulate NAD(P)H oxidase through multiple mechanisms, including phosphorylation of p47phox, as well as through translocation of the cytosolic phox complex by facilitating p47-phox-cortactin association. Cortactin is one of many actin-binding proteins and it is possible that other proteins are also involved. Exact molecular processes and identification of additional binding partners in this phenomenon await clarification.

Figure 5. Cytochalasin B attenuates Ang II–induced phosphorylation of p38MAP kinase and Akt, but not ERK1/2. VSMCs were stimulated with Ang II (10−7 mol/L) for 5 (Ang5) or 10 minutes (Ang10) in the absence and presence of cytochalasin B (10−6 mol/L, 1 hour). Phosphorylation of ERK1/2, p38MAP kinase, and Akt was assessed as described in the Methods. Immunoblots are representative of 3 different experiments. Data are percentage change relative to control taken as 100%. Results are means±SEM of 3 experiments. *P<0.05, **P<0.01 versus control (Cont) and cytochalasin B (cyt) groups. +P<0.05, ++P<0.01 versus Ang II counterpart.
p38MAP kinase and Akt was significantly downregulated by cytochalasin B. These data have at least 3 implications. First, not all Ang II–mediated cellular actions are cytoskeleton-dependent. Second, Ang II signaling is intact and functional in cytochalasin B-treated cells. Finally, p38MAP kinase and Akt, which are redox-sensitive kinases, depend on an intact actin cytoskeleton for activation by Ang II.

In addition to the cytoskeleton acting as a scaffold for p47phox, it is possible that phox proteins and ROS are involved in cytoskeletal reorganization accompanying \( O_2^- \) formation. In phorbol-activated polymorphonuclear leukocytes, cytosolic phox proteins associate with and regulate assembly of coronin, an actin-associated protein that accumulates at cortical sites of moving cells and contributes to the dynamics of the actin system.\(^7\) In cells from patients lacking p47phox or p67phox, rearrangement of F-actin and coronin in activated cells is absent or markedly diminished.\(^8\) Clements et al recently reported that peroxynitrite inhibits actin dynamics in phagocytic cells, with significant effect on actin-dependent cellular processes.\(^48\) In our study, we cannot exclude the possibility that p47phox and ROS may also influence VSMC function by altering cytoskeletal arrangement.

In conclusion, we provide several lines of evidence that p47phox associates with actin in VSMCs, possibly through scaffolding processes involving the actin-binding, c-Src-regulated protein cortactin. Interactions between cytosolic phox proteins and the cytoskeleton play an important role in NAD(P)H-driven \( O_2^- \) generation, which may contribute to site-directed activation of NAD(P)H oxidase and efficient redox-dependent Ang II signaling, such as Akt, in human VSMCs. These are not generalized phenomena because NAD(P)H oxidase-independent ROS formation and Ang II–mediated ERK1/2 signaling in VSMCs do not depend on an intact actin cytoskeleton.

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p47phox Associates With the Cytoskeleton Through Cortactin in Human Vascular Smooth Muscle Cells: Role in NAD(P)H Oxidase Regulation by Angiotensin II

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