Angiotensin II Stimulation of NAD(P)H Oxidase Activity
Upstream Mediators

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Abstract—Angiotensin II (Ang II)–stimulated hypertrophy of vascular smooth muscle cells is mediated by reactive oxygen species (ROS) derived from NAD(P)H oxidases. The upstream signaling mechanisms by which Ang II activates these oxidases are unclear but may include protein kinase C, tyrosine kinases, phosphatidylinositol-3-kinase, and Rac, a small molecular weight G protein. We found that Ang II–stimulated ROS production is biphasic. The first phase occurs rapidly (peak at 30 seconds) and is dependent on protein kinase C activation. The larger second phase of ROS generation (peak at 30 minutes) requires Rac activation, because inhibition of Rac by either Clostridium difficile toxin A or dominant-negative Rac significantly inhibits Ang II–induced ROS production. Phosphatidylinositol-3-kinase inhibitors (wortmannin or LY294002) and the epidermal growth factor (EGF) receptor kinase blocker AG1478 attenuate both Rac activation and ROS generation. The upstream activator of EGF receptor transactivation, c-Src, is also required for ROS generation, because PP1, an Src kinase inhibitor, abrogates the Ang II stimulation of both responses. These results suggest that c-Src, EGF receptor transactivation, phosphatidylinositol-3-kinase, and Rac play important roles in the sustained Ang II–mediated activation of vascular smooth muscle cell NAD(P)H oxidases and provide insight into the integrated signaling mechanisms whereby Ang II stimulation leads to activation of the growth-related NAD(P)H oxidases. (Circ Res. 2002;91:406-413.)

Key Words: angiotensin II ■ reactive oxygen species ■ vascular smooth muscle ■ NAD(P)H oxidase ■ Rac

Angiotensin II (Ang II) is an important mediator of vascular smooth muscle cell (VSMC) biology. Many of the effects of Ang II are mediated by the generation of reactive oxygen species (ROS). In particular, it has been shown that Ang II–induced hypertrophy is inhibited by overexpression of catalase, which hydrolyzes hydrogen peroxide (H₂O₂), or by inhibition of vascular NAD(P)H oxidase (NOX)-dependent responses in VSMCs, strongly implicating NAD(P)H oxidase as an activator of NOX-1 and NOX-4. Although several growth-related downstream targets of ROS have been identified, the signaling pathways that mediate the production of ROS remain incompletely understood.

Vascular NAD(P)H oxidases are similar in structure to the phagocytic NAD(P)H oxidase but differ in output and activation kinetics, suggesting differences in the regulation of activation. In neutrophils, the respiratory burst NADPH oxidase consists of a membrane-associated cytochrome b₅₅₈ (gp91phox and p22phox), the cytosolic components p47phox, p67phox, p40phox, and the small G protein Rac.7 Agonist exposure leads to assembly and activation, which are dependent on the production of phosphatidic acid by phospholipase D, protein kinase C (PKC)-dependent phosphorylation of p47phox (leading to conformational rearrangement and translocation of the cytosolic subunits to the membrane), and guanine nucleotide exchange of Rac-GDP to form Rac-GTP.8 The VSMC NAD(P)H oxidase consists of p22phox, p47phox, and novel homologues of gp91phox, Nox-1 and Nox-4.2,9,10

The role of the other cytosolic subunits is unclear, because p67phox and p40phox have not been identified in these cells.11 Although Rac, a small molecular weight G protein, has been shown to be important in the activation of the oxidase in other nonphagocytic cells,12–14 its role in oxidase activation in VSMCs remains to be conclusively proven. Because its binding partner p67phox is absent in VSMCs, it is unclear whether Rac would be expected to modulate VSMC oxidase. Wassmann et al15 showed that atorvastatin, which inhibits geranylgeranylation of small G proteins, inhibited Ang II–induced ROS generation, suggesting the involvement of a small G protein. Additionally, Herkert et al16 showed that dominant-negative Rac inhibited NAD(P)H oxidase–dependent responses in VSMCs, strongly implicating Rac in oxidase activation in these cells. However, Touyz and Schiffrin17 showed that Ang II–induced ROS generation in VSMCs is partially inhibited by blockers of phospholipase D and PKC, suggesting that other pathways of activation are involved as well.

Among the important growth-related signaling pathways activated by Ang II are those initiated by transactivation of the epidermal growth factor (EGF) receptor (EGFR).19 EGFR transactivation is Src dependent19,20 and, in turn, activates phosphatidylinositol-3 kinase (PI3-K).21 In other nonphago-
cytic cells. NAD(P)H oxidase activation is accomplished by PI3-K–dependent mechanisms. Bae et al\textsuperscript{13} showed that PI3-K is required for oxidase activation by platelet-derived growth factor (PDGF) in HepG2 cells, and phosphatidylinositol 3,4,5-trisphosphate (PIP\textsubscript{3}), the product of PI3-K, has been suggested to bind directly to p47phox\textsuperscript{22} and to activate Rac-mediated guanine nucleotide exchange.\textsuperscript{23,24} On the basis of these observations, we hypothesized that Ang II generation of ROS requires Src-dependent EGFR transactivation, followed by PI3-K–mediated activation of Rac. We found that these pathways are in fact involved in ROS production, and our data suggest an integrated signaling mechanism whereby stimulation of VSMCs by Ang II leads to activation of the growth-related NAD(P)H oxidase.

Materials and Methods

Materials
PP1, a Src kinase inhibitor, was obtained from Biomol Corp. Wortmannin, AG1478, AG43, LY294002, diphenylene iodonium (DPI), and Clostridium difficile toxin A were obtained from Calbiochem. Dichlorofluorescein diacetate (DCF-DA) was obtained from Molecular Probes. Anti-Rac antibody (clone 23A8) and the Rac activation assay were obtained from Upstate Biotechnology. All other chemicals and materials, including DMEM with 25 mmol/L HEPES were obtained from Sigma Chemical Co.

Cell Culture
VSMCs were isolated from male Sprague-Dawley rat thoracic aortas by enzymatic digestion as described previously.\textsuperscript{25} Cells were grown in DMEM supplemented with 10% calf serum, 2 mmol/L glutamine, 100 U/mL penicillin, and 100 \(\mu\)g/mL streptomycin and were passaged twice a week by harvesting with trypsin/EDTA and seeding into 75-cm\(^2\) flasks. For experiments, cells between passages 6 and 15 were used.

Flow Cytometry Measurement of ROS
DCF-DA–stained cells, as described previously by others.\textsuperscript{26–28} In brief, VSMCs were grown to 70% confluence in serum-enriched DMEM, made quiescent with 0.1% serum-containing media for 48 hours, and stimulated with Ang II (100 nmol/L) for the indicated times. Cells were then incubated in the dark with DCF-DA (7.5 \(\mu\)mol/L) for 10 minutes at 37°C, scraped, and resuspended in HBSS at a concentration of 1 \(\times\) 10\(^6\) cells/mL. For the short time course, VSMCs were preincubated with DPI (7.5 \(\mu\)g/mL) for 10 minutes at 37°C before stimulation with Ang II. Fluorescence was measured using a flow cytometer (Becton-Dickinson FACSort). The mean of DCF fluorescence intensity was obtained from 10,000 cells using 480-nm excitation and 540-nm emission settings. By use of the same settings, the fluorescence was collected from each experimental group. Fluorescence data were expressed as net fluorescence increase or as percent increase over unstained samples. To validate the assay, the fluorescence of DCF-DA to increasing doses of \(H_2O_2\) was measured. A linear curve was obtained for 0.1 to 20 \(\mu\)mol/L \(H_2O_2\) \((P=0.0004)\). This signal was abolished by 24-hour preincubation with polyanethylen glycol–catalase (data not shown). The fluorescence signal obtained from VSMCs treated with Ang II was within the linear portion of the curve.

Transfection
VSMCs were infected overnight with recombinant dominant-negative Rac adenovirus\textsuperscript{29} (AdRacN17) at a multiplicity of infection of 1 to 5 in culture medium with 0.1% calf serum and incubated for another 48 hours in the same medium without virus before stimulation with Ang II.

Rac Activation Assay
VSMCs were grown to 75–80% confluence and made quiescent in 0.1% calf serum for 48 hours before stimulation with Ang II (100 nmol/L). Cells were lysed with ice-cold lysis buffer (pH 7.5) containing 25 mmol/L HEPES, 150 mmol/L NaCl, 1% Igepal CA-630, 0.25% sodium deoxycholate, 10 mmol/L MgCl\textsubscript{2}, 10% glycerol, 25 mmol/L NaF, 1 mmol/L EDTA, 1 mmol/L sodium orthovanadate, 10 \(\mu\)g/mL leupeptin, 10 \(\mu\)g/mL aprotinin, and 1 mmol/L phenylmethylsulfonyl fluoride. Activated (GTP-bound) Rac was affinity-purified with p21-activated kinase-1 (PAK-1) protein binding domain peptide,\textsuperscript{30} which binds only to Rac-GTP and not Rac-GDP. PAK-1 protein binding domain agarose (PAK-1 PBD, 7.5 \(\mu\)g/mL cell lysate) was added, and the reaction mixture was gently rocked at 4°C for 60 minutes. The agarose beads were collected by pulsing for 5 seconds in a microcentrifuge at 14 000g, and the beads were washed three times with 0.5 mL lysis buffer. The agarose beads were resuspended in 40 \(\mu\)L of 1\(\times\) SDS sample buffer and boiled for 5 minutes. The supernatant was separated by SDS-PAGE on a 12% gel, and the proteins were transferred to nitrocellulose. After blocking for 1 hour in PBS containing 5% nonfat dry milk and 0.1% Tween 20, the membrane was incubated with anti-Rac antibody (1:1000 dilution) overnight. After incubation with the secondary antibody, Rac was detected by enhanced chemiluminescence.

Statistical Analysis
The data were analyzed by ANOVA, followed by the Bonferroni post hoc test with the use of Prism 3.0 (Graphpad Software).

Results

Ang II Stimulates NAD(P)H Oxidase Activity in VSMCs
We have previously shown that Ang II stimulates NADPH oxidase activity in VSMCs, leading to superoxide (\(O_2^-\)) and \(H_2O_2\) generation.\textsuperscript{1,3} In the present study, we used DCF fluorescence as a reflection of oxidase activity (Figure 1A). Ang II (100 nmol/L) increased DCF fluorescence in a time-dependent manner (Figure 1B). After a delay of 10 seconds, ROS generation increased significantly at 30 seconds (60±12% over control, \(P\leq0.05\)), declined again by 1 minute, and then further increased until a plateau was reached at 30 minutes (164±8%). The DCF-DA signal induced by Ang II was inhibited 84±14% \((P=0.04)\) by preincubation with DPI (10 \(\mu\)mol/L, 30 minutes), an inhibitor of flavin-containing oxidases such as the NAD(P)H oxidase (Figure 1C). DPI also inhibited ROS production at 30 seconds (88±16% inhibition).

Differential Role of PKC in Biphasic Activation of the Oxidase
PKC has been shown to be involved in the generation of ROS in neutrophils.\textsuperscript{? To define the role of PKC in Ang II–induced oxidase activation, we studied the effect of PKC inhibition on the biphasic production of \(H_2O_2\) (peaks at 30 seconds and 30 minutes) induced by Ang II with the use of GF10920X (10 \(\mu\)mol/L), a PKC inhibitor that we have previously shown to abolish Ang II–stimulated PKC activity.\textsuperscript{31} Blockade of PKC profoundly inhibited Ang II–induced ROS production at 30 seconds by 80±11% \((P=0.001)\), whereas at 30 minutes, only partial inhibition was observed (51±12%, \(P=0.01\); Figure 2). This suggests that PKC mediates the early rapid production of \(H_2O_2\) and is only partially involved in the later phase.
Involvement of Rac in Ang II–Dependent Oxidase Activation

To begin to elucidate the signaling mechanisms regulating the sustained phase of ROS production, we first wanted to unequivocally establish the role of Rac in ROS generation in Ang II–stimulated VSMCs. Incubation of VSMCs with Ang II increases Rac-GTP by 2.1±0.2-fold over unstimulated cells at 1 minute, and activated Rac remains elevated at 30 minutes (2.4±0.8-fold, Figure 3A). This time course is consistent with the temporal production of ROS by Ang II (Figure 1B).

To define the role of Rac in NAD(P)H oxidase activation by Ang II, we used C difficile toxin A (C diff toxin A), an inhibitor of the small G proteins Rho, Rac, and cdc42.32 C diff toxin A (100 ng/mL) inhibits the Ang II–induced generation of ROS by 97±16% (P=0.05, Figure 3B). To further identify the small G protein, VSMCs were subjected to adenoviral transfection of a dominant-negative construct of Rac-1 (AdRacN17). AdRacN17 has previously been shown to inhibit ROS generated by PDGF stimulation of fibroblasts.12 In VSMCs, AdRacN17 inhibits Ang II–induced ROS generation by 82±22% (P=0.01), confirming that Rac is involved in the activation of the NADPH oxidase (Figure 3C). Adenoviral transfection with constitutively active Rac increases the basal production of ROS (14.0±0.3 arbitrary fluorescence units [AFU]) as well as the Ang II–stimulated production ROS (20.4±0.9 AFU) compared with uninfected cells (control, 10.8±0.2 AFU; Ang II, 16.5±2.6 AFU). As expected, DPI did not affect Ang II–induced Rac activation (data not shown), confirming that Rac is upstream from the oxidase.

Role of PI3-K in Rac Activation and ROS Production by Ang II

In HepG2 cells, it has been shown that PI3-K is required for PDGF-induced Rac-dependent H$_2$O$_2$ generation.13 We examined whether Rac activation by Ang II is also dependent on PI3-K in VSMCs. As shown in Figure 4A, the PI3-K inhibitor wortmannin, at a concentration of 100 nmol/L, inhibited Ang II–induced Rac activation by 75±18% (P=0.05). This concentration has been previously shown to block Ang II activation of Akt, the downstream target of PI3-K, by >95% in these cells.13 Similarly, LY294002 (10 μmol/L, a concentration that blocks Ang II–induced Akt activation) also inhibited Rac activation (data not shown).

PI3-K inhibitors were then tested for their effect on ROS generation by Ang II. Wortmannin (100 nmol/L) significantly inhibited Ang II–induced ROS generation by 84±19% (P=0.001). Similarly, the PI3-K inhibitor LY294002 (10

Figure 1. Flow cytometric analysis of ROS generation by Ang II in VSMCs. A, VSMCs were stimulated with 100 nmol/L Ang II for 30 minutes, stained with DCF-DA to detect ROS, and subjected to flow cytometry. ROS are expressed as a histogram of fluorescence generated by 10 000 cells. Solid histogram represents unstimulated VSMCs; open histogram, Ang II–stimulated cells. The rightward shift in the Ang II curve represents a 2-fold increase in ROS generation. B, VSMCs were prestained with DCF-DA and stimulated with 100 nmol/L Ang II for the indicated times. Values are mean±SE of 3 to 7 experiments. All values >10 seconds are significantly different from control (P<0.05). C, Flavin-containing oxidase inhibitor DPI (10 μmol/L) preincubated with VSMCs for 30 minutes inhibits Ang II (100 nmol/L, 30 minutes)–induced ROS generation measured by DCF–DA. Values are mean±SE of 4 experiments. *P<0.04 compared with Ang II alone.
μmol/L, a concentration that blocks Ang II–induced Akt activation; inhibited Ang II–induced ROS production by 89 ± 5% (P ≤ 0.01, Figure 3B). These inhibitor experiments suggest that PI3-K is upstream from Rac-dependent activation of NAD(P)H oxidase.

Figure 3. Ang II–induced ROS generation is mediated by Rac in VSMCs. A, VSMCs were stimulated with 100 nmol/L Ang II for the indicated times. Rac activity was measured by PAK-PBD affinity precipitation as described in Materials and Methods. Top, Representative immunoblot of GTP-bound Rac. Bottom, Densitometric analysis (mean ± SE) of immunoblots from 2 to 8 experiments expressed as fold increase over control. *P < 0.05 compared with control. B, VSMCs were preincubated with the small molecular weight G protein inhibitor C. diff toxin A (100 ng/mL) for 12 hours before stimulation with 100 nmol/L Ang II for 30 minutes. Cells were then stained with DCF-DA and analyzed for ROS production by flow cytometry. C. diff toxin A did not affect basal ROS production (11.6 ± 0.5 AFU compared to 10.7 ± 0.3 AFU in control cells). Results in the graph are expressed as mean ± SE (n = 3) of the percentage of maximum response to Ang II in untreated cells. *P ≤ 0.01.

Figure 4. PI3-K mediates Ang II–induced ROS generation in VSMCs. A, VSMCs were preincubated with 100 nmol/L wortmannin (Wort, a PI3-K inhibitor) or vehicle for 30 minutes and stimulated with 100 nmol/L Ang II for 1 minute, and Rac activity was measured by PAK-PBD affinity precipitation as described in Materials and Methods. Top, Representative immunoblot of GTP-bound Rac. Bottom, Densitometric analysis (mean ± SE) of immunoblots from 3 experiments expressed as fold increase over control. *P < 0.05 compared with Ang II alone. B, VSMCs were preincubated with wortmannin (100 nmol/L) or LY294002 (10 μmol/L) for 30 minutes, stimulated with Ang II (100 nmol/L), and analyzed for ROS production by flow cytometry with DCF-DA. Bars represent mean ± SE (n = 3 to 6). *P ≤ 0.05 and **P ≤ 0.01 vs Ang II alone.

EGFR Transactivation Is Upstream From Rac Activation and ROS Production

Although Ang II has been shown to activate PI3-K, the mechanisms by which this activation occurs are unknown. However, EGFR transactivation occurs rapidly after the addition of Ang II to VSMCs and is required for the activation of many downstream signaling pathways, including PI3-K–dependent activation of Akt (data not shown). Therefore, we hypothesized that EGFR transactivation might be upstream from Ang II stimulation of Rac and ROS generation.

AG1478 (250 nmol/L, 30 minutes), a specific inhibitor of EGFR kinase that completely blocks Ang II activation of the EGFR,20 was used to test the role of EGFR transactivation in Rac activation and ROS generation by Ang II. AG1478 inhibits Ang II–induced Rac activation by...
91±19% (P<0.01, Figure 5A). Consistent with the above results, AG1478 decreases ROS generation in response to Ang II by 78±23% (P<0.04, Figure 5B). An inactive tyrphostin, AG43, used as a negative control, had no effect on Ang II–induced oxidase activation. These results, taken together, suggest that EGFR transactivation is upstream from Rac and is required for oxidase activation.

If PI3-K is downstream from EGFR kinase activation, wortmannin should also inhibit EGF-induced ROS generation. Indeed, EGF (100 ng/mL) added to VSMCs for 30 minutes increased the ROS generation (23.1±4 AFU) compared with that in unstimulated cells (10.9±0.3 AFU). Preincubation of VSMCs with wortmannin (100 nmol/L) for 30 minutes abolished the EGF-induced ROS generation (8.8±0.8 AFU, P<0.01).

Figure 5. EGFR transactivation is required for Ang II–induced ROS production in VSMCs. A, VSMCs were preincubated with 250 nmol/L AG1478 (EGFR kinase inhibitor) or vehicle for 30 minutes and stimulated with 100 nmol/L Ang II for 1 minute, and Rac activity was measured by PAK-PBD affinity precipitation as described in Materials and Methods. Top, Representative immunoblot of GTP-bound Rac. Bottom, Densitometric analysis (mean±SE) of immunoblots from 3 experiments expressed as fold increase over control. *P<0.01 compared with Ang II alone. B, VSMCs were preincubated with AG1478 (250 nmol/L) or AG43 (250 nmol/L, inactive control) for 30 minutes, stimulated with Ang II (100 nmol/L, 30 minutes), and analyzed for ROS production by flow cytometry with DCF-DA. Bars represent mean±SE (n=4). *P<0.05 vs Ang II alone.

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410 Circulation Research September 6, 2002

Src Is Involved in Rac Activation and in ROS Generation

We have previously shown that c-Src mediates Ang II–induced EGFR transactivation. To determine whether c-Src is important in Rac and oxidase activation by Ang II, we used the Src kinase inhibitor PP1 (20 μmol/L). PP1 inhibits Rac activation by Ang II by 89±17% (P<0.001, Figure 6A). As expected, PP1 also inhibits Ang II–induced stimulation of ROS generation by 87±25% (P<0.03, Figure 6B). These results suggest that Rac activation and ROS generation occur in a Src-dependent manner.

Figure 6. Ang II–induced ROS production involves Src-family kinases. A, VSMCs were preincubated with 20 μmol/L PP1 (Src inhibitor) or vehicle for 30 minutes and stimulated with 100 nmol/L Ang II for 1 minute, and Rac activity was measured by PAK-PBD affinity precipitation as described in Materials and Methods. Top, Representative immunoblot of GTP-bound Rac. Bottom, Densitometric analysis (mean±SE) of immunoblots from 3 experiments expressed as fold increase over control. *P<0.01 compared with Ang II alone. B, VSMCs were preincubated with PP1 (20 μmol/L) for 30 minutes, stimulated with Ang II (100 nmol/L, 30 minutes), and analyzed for ROS production by flow cytometry with DCF-DA. Bars represent mean±SE (n=4). *P<0.05 vs Ang II alone. Treatment with PP1 alone did not affect baseline (10.7±0.7 AFU compared with 10.8±0.8 AFU in unstimulated control cells).

Discussion

Activation of NAD(P)H oxidases is an integral component of tonic responses of VSMCs to Ang II. In the present study, we found that O$_2^-$ production in Ang II–stimulated VSMCs is biphasic. The first phase occurs rapidly (peak at 30 seconds)
and is dependent on PKC activation. The second phase of ROS generation is of much greater magnitude than the first and, as we have previously shown, continues for at least 6 hours.\(^1\) Thus, this phase is likely to most influence the long-term signaling events related to the growth response,\(^1\) so the signaling mechanisms responsible for the second phase of oxidase activity are of great interest. Our data suggest a novel pathway of oxidase stimulation that involves the activation of c-Src and PI3-K and the transactivation of EGFRs and is mediated by Rac, a small G protein. To our knowledge, this is the first evidence of the involvement of tyrosine kinases in the stimulation of NAD(P)H oxidases by G-protein–coupled receptors.

A full time course of oxidase activation in agonist-stimulated VSMCs has been difficult to obtain because of limitations in the methods of ROS detection. The recent development of fluorescent probes has permitted the detection of rapid ROS generation. Our data suggest that Ang II–induced ROS production occurs in two phases: (1) a low-level rapid production that peaks at 30 seconds and (2) a greater, more sustained generation that lasts from 20 minutes (Figure 1) to 6 hours.\(^1\) Schieffer et al\(^{35}\) also found that NAD(P)H oxidase activation occurs in minutes and begins to decline by 10 to 20 minutes in VSMCs on Ang II stimulation. They did not examine longer times, nor did they investigate the mechanisms of activation. In contrast, Touyz and Schiffrin\(^{17}\) showed that in human resistance vessels, Ang II increases H\(_2\)O\(_2\) production at 1 to 4 hours. In these tissues, sphinganine, a nonspecific phospholipase D inhibitor, attenuated DCF fluorescence, an effect that was partially reversed by the addition of phosphatidic acid. Two inhibitors of PKC also partially blocked ROS generation. However, none of these inhibitors completely abolished the Ang II response, suggesting that other signaling pathways are involved as well. Together, these observations indicate that the oxidase activation by Ang II is biphasic and involves multiple pathways of activation.

Thus, although some information is available concerning the proximal signaling pathways by which Ang II activates the NAD(P)H oxidase,\(^{17,36}\) an integrated picture remains elusive. The present data implicate a multistep pathway in which Ang II activates PKC, resulting in initiation of oxidase activity. The H\(_2\)O\(_2\) produced by this event activates c-Src,\(^{20}\) which in turn transactivates the EGFR, stimulates PI3-K, and activates the small molecular weight G protein Rac, leading to direct stimulation of oxidase activity (Figure 7). We have previously shown that c-Src is upstream from EGFR transactivation,\(^{20}\) and we now provide evidence that EGFR kinase activity is required for Rac stimulation (Figure 5A). Rac is activated within minutes of addition of Ang II (Figure 3) and is dependent on PI3-K and Src (Figures 4A and 6A). Using two different types of inhibitors, we showed that Rac is absolutely required for Ang II–stimulated oxidase activity (Figure 3). C diff toxin A has been shown to block the activation of several small G proteins (Rho, Rac, and Cdc42),\(^{32}\) but dominant-negative Rac inhibits only Rac. Both inhibitors dramatically attenuate ROS generation, indicating that Rac is the small G protein involved in oxidase activation in VSMCs. A role for Rho family GTPases in ROS generation in VSMCs has also been suggested by Wassmann et al,\(^{15}\) who found that atorvastatin, which inhibits geranylgeranylation of small G proteins, inhibited Ang II–induced ROS generation.

Our data also clearly implicate PI3-K in Ang II–induced ROS generation (Figure 4B). This is consistent with our previous observation that Akt activation is PI3-K dependent and ROS sensitive.\(^{31}\) The finding that PI3-K mediates NAD(P)H oxidase activity is not universal. Bae et al\(^{13}\) found that ROS generation in response to PDGF is dependent on PI3-K in HepG2 cells. However, in macrophage-stimulating protein–activated macrophages, ROS generation is not inhibited by wortmannin.\(^{37}\) In some cell types, only the sustained phase of O\(_2^-\) production is mediated by PI3-K,\(^{38}\) whereas in others, intracellular, but not extracellular, release of ROS is PI3-K dependent.\(^{39}\) These data raise the possibility that PI3-K regulates those NAD(P)H oxidases that generate ROS inside the cell, such as the VSMC oxidases.

The mechanism by which PI3-K regulates oxidase activity is unclear. Our data show that PI3-K is upstream from Rac, inasmuch as wortmannin inhibited Rac activation by Ang II (Figure 3A). Thus, it is likely that the product of PI3-K, PIP\(_3\), is regulating a Rac-guanine nucleotide exchange factor, as suggested by Bae et al.\(^{13}\) PIP\(_3\) binding has been shown to enhance the activation of several Rac-guanine nucleotide exchange factors, including Vav and Sos, and thus to convert

\[ \text{H}_2\text{O}_2 \rightarrow \text{O}_2^- \]

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Rac to its active GTP-bound form. Another possibility is that the lipid products of PI3-K provide a direct molecular link to the components of the NAD(P)H oxidase. PIP2 binds the PX domain of p47phox, the cytosolic regulator of both neutrophil and nonphagocytic oxidases, possibly controlling localization to distinct intracellular compartments. An involvement of Src in activation of NAD(P)H oxidases has been suggested in eosinophils and macrophages, but its role in regulating novel Nox isoforms, such as those expressed in VSMCs, is not known. In eosinophils stimulated with leukotriene-B4, PPI inhibited both the rapid and sustained phases of O2− release, whereas in macrophages, it blocked macrophage-stimulating protein–mediated ROS generation. We have previously shown that Src is upstream from EGFR transactivation. Because EGFR transactivation is required for the stimulation of Rac activity by Ang II, as shown in the present study, it is not surprising that Src is also upstream from oxidase activation. However, in VSMCs, both Src phosphorylation and EGFR transactivation are redox sensitive, suggesting that they are downstream from NAD(P)H oxidase. It is likely that a feed-forward mechanism exists in VSMCs in which immediate low-level release of H2O2 mediated by PKC activates Src, which in turn initiates a signaling cascade leading to Rac-dependent NAD(P)H oxidase activation, the generation of additional ROS, further activation of Src, and the amplification of oxidase activity. Evidence supporting this comes from a recent study by Li et al., who showed that exogenous addition of H2O2 activates NAD(P)H oxidase in VSMCs. The origin of the initial intracellular H2O2 release is unclear but may result from differential activation of a specific Nox protein. This initial response may be due to phospholipase D–mediated activation of PKC and the production of arachidonic acid, as suggested by Touyz and Schiffrin and Zafari et al. Thus, attenuation of the initial phase of ROS generation by PKC inhibitors would be expected to partially inhibit, but not abolish, sustained ROS generation, which is in fact what we observed (Figure 2).

VSMCs express at least two NAD(P)H oxidases: a Nox–1–based oxidase and a Nox–4–based oxidase. The Nox family of proteins consists of gp91phox homologues that have been shown to support O2− generation, although the entire molecular structure of the functional oxidase complex remains unclear. We have previously shown that Nox-1 antisense abolishes ROS generation in VSMCs stimulated with Ang II for 30 minutes, but the role of Nox-4 in agonist-stimulated O2− generation has not been defined, nor is it clear whether the two oxidases are activated by identical signaling cascades. Although our data do not directly address this question because of the lack of suitable molecular tools, they do raise the possibility that the different Nox proteins mediate different phases of the response to Ang II.

NAD(P)H oxidase–derived ROS have defined multiple signaling pathways by which Ang II stimulates the growth of VSMCs, the interaction of these growth-related signaling pathways with NAD(P)H oxidases has not previously been understood. The present data provide a framework linking tyrosine kinase signaling to ROS production and afford insight into the interactions between the complex mechanisms by which Ang II contributes to vascular pathophysiology.

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References


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