Expression of a Functionally Active gp91phox-Containing Neutrophil-Type NAD(P)H Oxidase in Smooth Muscle Cells From Human Resistance Arteries

Regulation by Angiotensin II

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Abstract—A major source of vascular smooth muscle cell (VSMC) superoxide is NAD(P)H oxidase. However, the molecular characteristics and regulation of this enzyme are unclear. We investigated whether VSMCs from human resistance arteries (HVSMCs) possess a functionally active, angiotensin II (Ang II)–regulated NAD(P)H oxidase that contains neutrophil oxidase subunits, including p22phox, gp91phox, p40phox, p47phox, and p67phox. mRNA expression of gp91phox homologues, nox1 and nox4, was also assessed in HVSMCs, human aortic smooth muscle cells, and rat VSMCs. HVSMCs were obtained from resistance arteries from gluteal biopsies of healthy subjects. gp91phox and nox4, but not nox1, were detected in HVSMCs. Nox1 and nox4, but not gp91phox, were expressed in human aortic smooth muscle cells and rat VSMCs. All NAD(P)H oxidase subunits were present in HVSMCs as detected by reverse transcriptase–polymerase chain reaction and immunoblotting. Ang II increased NAD(P)H oxidase subunit abundance. These effects were inhibited by cycloheximide. Acute Ang II stimulation (10 to 15 minutes) increased p47phox serine phosphorylation and induced p47phox and p67phox translocation. This was associated with NAD(P)H oxidase activation. In cells transfected with gp91phox antisense oligonucleotides, Ang II–mediated actions were abrogated. NADPH-induced superoxide generation was reduced by gp91ds-tat and apocynin, inhibitors of p47phox-gp91phox interactions. Our results suggest that HVSMCs possess a functionally active gp91phox-containing neutrophil-like NAD(P)H oxidase. Ang II regulates the enzyme by inducing phosphorylation of p47phox, translocation of cytosolic subunits, and de novo protein synthesis. These novel findings provide insight into the molecular regulation of NAD(P)H oxidase by Ang II in HVSMCs. Furthermore, we identify differences in gp91phox homologue expression in VSMCs from rats and human small and large arteries. (Circ Res. 2002;90:1205-1213.)

Key Words: superoxide ■ renin-angiotensin system ■ cultured cells

Reactive oxygen species (ROS), including superoxide (\(\cdot O_2^-\)), hydrogen peroxide (\(H_2O_2\)), nitric oxide (NO\(-\)), and peroxynitrite (ONOO\(-\)), are important signaling molecules that regulate vascular tone and structure. Emerging evidence supports a role for ROS in pathological processes underlying cardiovascular diseases, such as hypertension, atherosclerosis, and restenosis.\(^1\)\(^-\)\(^3\) In the normal vascular wall, \(\cdot O_2^-\) is produced primarily in vascular smooth muscle cells (VSMCs) and fibroblasts.\(^3\) The major enzyme responsible for vascular \(\cdot O_2^-\) appears to be NAD(P)H oxidase,\(^4\) which catalyzes the production of \(\cdot O_2^-\) by the 1-electron reduction of oxygen with the use of NAD(P)H as the electron donor: \(2O_2^-+NAD(P)H\rightarrow2O_2^-+NAD(P)^+H^+\).

The prototypical NAD(P)H oxidase is found in neutrophils, which is composed of five subunits: p40phox (the term phox is derived from phagocyte oxidase), p47phox, p67phox, p22phox, and gp91phox. In unstimulated cells, p40phox, p47phox, and p67phox exist in the cytosol, whereas p22phox and gp91phox are located in the membranes, where they occur as a heterodimeric flavoprotein, cytochrome b558.\(^5\) On cell stimulation, p47phox becomes phosphorylated, and the cytoplasmic complex migrates to the membrane, where it associates with cytochrome b558 to assemble the active oxidase, which then transfers electrons from the substrate to \(O_2^-\), leading to the generation of \(\cdot O_2^-\).\(^5\)\(^-\)\(^6\) Activation also requires participation of two low-molecular-weight guanine nucleotide–binding proteins, Rac 2 (or Rac 1) and Rap1A.\(^5\)\(^-\)\(^5\) Although it is evident that cells of the vasculature contain functionally active NAD(P)H oxidase, which of the neutrophil NAD(P)H oxidase subunits is present in vascular...
cells is still unclear. In adventitial fibroblasts and endothelial cells, mRNAs for gp91phox, p22phox, p47phox, and p67phox have been demonstrated.\(^7\,\,12\) mRNA for gp91phox is barely detectable in rat aortic VSMCs,\(^13\) All phox subunits have been identified in rabbit aortic adventitia.\(^7\,\,14\) Rat aortic smooth muscle cells express p22phox, p47phox, and rac1, but not gp91phox.\(^10\,\,14\,\,16\) Whether a similar situation exists in human VSMCs is unclear. Gorlach et al\(^10\) demonstrated a gp91phox-containing NADPH oxidase in human umbilical endothelial cells but not in human aortic smooth muscle cells (ASMCs). Because p22phox and gp91phox are essential for NAD(P)H oxidase activity, the possibility arose that there are gp91phox isoforms that are functionally active in VSMCs. Homologues of gp91phox, nox1 (this term is derived from NADPH oxidase or nonphagocytic NADPH oxidase) and nox4, have been identified in rat aortic smooth muscle cells.\(^13\,\,17\) Most studies were performed in cells from large arteries of experimental animal models. The status of gp91phox as well as the other major neutrophil NAD(P)H oxidase subunits in human VSMCs, particularly from peripheral resistance arteries (vessels that are important in blood pressure regulation), has not been fully investigated.

The aims of the present study were to determine whether smooth muscle cells from human small arteries (HVSMSCs) express gp91phox, nox1, and/or nox4 and to investigate whether p22phox and the cytoplasmic subunits of neutrophil NAD(P)H oxidase are present. Because angiotensin II (Ang II) has been implicated as a major mediator of vascular oxidative stress, we investigated the regulatory role of Ang II on the expression of NAD(P)H oxidase subunits and assessed whether Ang II induces translocation of cytoplasmic subunits to the cell membrane. p47phox is the subunit that is chiefly responsible for transporting the cytosolic complex to the membrane during oxidase activation. However, before the cytosolic oxidase components can be translocated, p47phox must be phosphorylated. Accordingly, we also determined whether Ang II stimulation influences the phosphorylation status of p47phox in VSMCs from human resistance arteries.

Materials and Methods

Cell Culture

The present study was approved by the Ethics Committee of the Clinical Research Institute of Montreal (IRCM). Healthy volunteers (aged 30 to 65 years, n = 7 [4 males]) were recruited at the IRCM Hypertension Clinic. Gluteal biopsies of subcutaneous tissue were obtained under local anesthetic. Vessels were dissected as described.\(^18\) RVSMCs were isolated as described.\(^19\) ASMCs were from Clonetics. VSMCs from mesenteric arteries of Wistar-Kyoto rats (RVSMCs) were isolated as described.\(^19\) Human colon carcinoma cells (CaCo2), which express several neutrophil oxidase subunits, were from the American Type Culture Collection. Cells from mesenteric arteries of Wistar-Kyoto rats (RVSMCs) were isolated as described.\(^18\)

Confocal Immunofluorescence Microscopy

To characterize cells as VSMCs, immunofluorescence studies were performed. Cells were labeled with smooth muscle–specific monoclonal antibodies (anti-α-smooth muscle actin, anti-smooth muscle myosin, and anti-calpainin), fibroblast-specific antibody (monoclonal anti-human fibroblast surface protein, clone 1B10), and endothelial cell–specific antibody (anti–von Willebrand factor). Cells were identified as VSMCs if they were positively labeled with antibodies to α-smooth muscle actin, heavy chain myosin, and calpainin and negatively labeled with anti-fibroblast and anti–von Willebrand factor antibodies. Cells were also dual-labeled with phalloidin-TRITC (actin specific) and anti-gp91phox antibody. Confocal microscopy was performed with an LSM 510 system (Zeiss).

Cell Fractionation

Quiescent HVSMSCs were stimulated with Ang II for various times. Cells were washed in ice-cold PBS, scraped, and transferred to centrifuge tubes containing PBS. They were sonicated for 1 second and then ultra centrifuged (30 000g, 20 minutes at 4°C). The supernatant (cytosolic fraction) was removed, and the pellet was resuspended in lysis buffer (20 mmol/L monobasic potassium phosphate, 1 mmol/L EGTA, 10 μg/mL aprotinin, 0.5 μg/mL leupeptin, 0.75 μg/mL pepstatin, 0.5 mmol/L phenylmethylsulfonyl fluoride, and 1% Triton). The pellet suspension (membrane fraction) was dounced 20 to 30 times, and then incubated on ice for 30 minutes with intermittent vortexing.\(^21\) Protein content was measured in aliquots using Bio-Rad Protein Assay reagent (Bio-Rad Laboratories).

Preparation of Neutrophils

Because neutrophils possess all NAD(P)H oxidase subunits, these cells were used as positive controls.\(^22\)

Reverse Transcriptase–Polymerase Chain Reaction

Total cellular RNA was isolated from HVSMSCs, ASMCs, and RVSMCs by using Trizol Reagent (GIBCO-BRL), and reverse transcription was performed as described.\(^23\) After first-strand synthesis of DNA, 2 μL cDNA was amplified by using specific primers selected on the bases of published sequences and detailed in the online Table, which can be found in the online data supplement available at http://www.circresaha.org. Polymerase chain reaction (PCR) products were electrophoresed on a 1.5% agarose gel for 60 minutes at 9 V/cm gel. Bands corresponding to reverse transcriptase (RT)-PCR products were visualized by UV light.

Sequencing of PCR Products

Amplified PCR products were gel-purified and cloned with a Topo cloning kit according to manufacturer’s instructions (Clontech). Selected colonies were cultured and mini-prepped by using the QIA prep Spin kit (Quagen). DNA samples were sequenced automatically by a Dye Terminator Cycle Sequencer with the use of a CEO 2000 XL DNA analysis system (Beckman Coulter). Sequencing reactions were performed at least three times for each insert. Sequence comparisons were made with the NCBI web server (Blastn 2.1.2), available on the Internet.

Immunoprecipitation and Immunoblotting of NAD(P)H Oxidase Subunits

Immunoprecipitation and immunoblotting were performed as described.\(^19\)\,\,20 Membrane preparations were used to detect gp91phox and p22phox, whereas cytoplasmic fractions were used for p47phox, p47phox, and p67phox. To determine whether cytoplasmic subunits are translocated to the membrane after Ang II stimulation, expression of p40phox, p47phox, and p67phox was also assessed in membrane fractions. Previously characterized antibodies specifically recognizing p22phox (monoclonal, clone 44.1),\(^24\) gp91phox (monoclonal, clone 54.1),\(^24\) p47phox (polyclonal, R360), p67phox (polyclonal, R1497),\(^22\) and p40phox (monoclonal, clone 19) were used.

Immunoprecipitation and Serine Phosphorylation of p47phox

For immunoprecipitation of p47phox, the cytosolic fraction (100 μg protein) from unstimulated and Ang II–stimulated cells was transferred to microcentrifuge tubes, and anti-p47phox antibody (12 μg) was added and incubated for 60 minutes at 4°C. Twenty microliters of agaro conjugate (Protein G PLUS-Agarose, Santa Cruz Biotechnology) was then added and incubated for 60 minutes at 4°C. The sample was centrifuged at 12 000 rpm for 30 seconds, and the supernatant was subjected to immunoblotting as described above. Membranes were probed with rabbit polyclonal antibody to phos-
Measurement of NAD(P)H Oxidase Activity

Quiescent HVSMCs were stimulated with Ang II for 10 to 15 minutes. In some experiments, cells were preexposed for 30 minutes to gp91ds-tat, a novel competitive inhibitor of NADPH oxidase, before Ang II addition. Cells were fractionated as described above. The lucigenin-derived chemiluminescence assay was used to determine NAD(P)H oxidase activity in cell homogenates and membrane and cytosolic fractions. Activity is expressed as nmol $\cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$. To verify the specificity of the lucigenin assay for $\cdot \text{O}_2^-$ in our models, we examined the effects of superoxide dismutase (SOD, 120 U/mL), an enzymatic scavenger of $\cdot \text{O}_2^-$, and Tiron (10 mmol/L; Sigma Chemical), a nonenzymatic scavenger of $\cdot \text{O}_2^-$, on Ang II–stimulated NADPH oxidase activity.

Measurement of ROS in Intact Cells

Generation of ROS was measured with the fluoroprobe carboxymethyl-H$_2$-dichlorofluorescein diacetate (CM-H$_2$DCFDA, Molecular Probes) in unstimulated cells and in cells exposed to Ang II in the absence and presence of $10^{-5}$ mol/L diphenylene iodonium (DPI), a flavoprotein inhibitor that inhibits NADPH oxidase. Cells were pretreated with DPI for 20 minutes.

Statistical Analysis

Experiments were repeated three to six times in duplicate or triplicate. Results are presented as mean±SEM and are compared by ANOVA or by Student $t$ test, where appropriate. The Tukey-Kramer correction was used to compensate for multiple testing. A value of $P<0.05$ was considered significant.

An expanded Materials and Methods section can be found in the online data supplement available at http://www.circesaha.org.

Results

Immunofluorescence Studies

Figure 1 shows cells that were labeled positively with anti–α-smooth muscle actin antibody (A), anti-smooth muscle myosin monoclonal antibody (B), anti-calponin monoclonal antibody (C), monoclonal anti–human fibroblast surface protein, clone 1B10 antibody (D), anti–von Willebrand factor antibody (E), and dual labeling was with phalloidin (red fluorescence) and a monoclonal anti-gp91phox antibody (fluorescein conjugate, green fluorescence) (F). Images were taken at the midplane level (images A through E, ×63 objective; image F, ×100 objective).

VSMCs From Human Small Arteries Contain gp91phox mRNA

gp91phox mRNA was detected by RT-PCR in HVSMCs but not in ASMCs or RVSMCs (Figure 2). Neutrophils, which characteristically express gp91phox, were used as positive controls. Sequence analysis of cloned cDNA demonstrated that the obtained sequence of our samples was 99% identical to human CYBB (human gp91phox gene) cDNA (583 to 899). (Sequence is available in the online data supplement available at http://www.circesaha.org).

We detected nox1 mRNA in ASMCs and RVSMCs but not in HVSMCs. CaCo2 cells were used as a positive control for human nox1. Nox4 was present in HVSMCs, ASMCs, and RVSMCs. mRNAs for p22phox, p47phox, and p67phox were also detected in HVSMCs (Figure 2). Negative controls performed with RNA without RT did not yield any PCR products, indicating an absence of genomic DNA contamination.

Expression of NAD(P)H Oxidase Subunits in HVSMCs

Cell lysates were immunoprecipitated with anti-gp91phox, -p22phox, -p47phox, and -p67phox antibodies and then subjected to immunoblotting. Immunoprecipitated proteins were identified as gp91phox, p22phox, p47phox, and p67phox after probing with specific antibodies (Figure 3). In addition, the expression of all four oxidase proteins was...
confirmed by immunoblotting HVSMCs isolated from a number of different subjects (Figure 3). The presence of gp91phox in HVSMCs was also characterized by confocal microscopy. Figure 1F shows HVSMCs dual-labeled with phalloidin and anti-gp91phox antibody.

As shown in Figure 4, gp91phox and p22phox proteins were present in membrane fractions prepared from HVSMCs. gp91phox protein was not expressed in ASMCs or RVSMCs (data not shown). p40phox, p47phox, and p67phox were detected in the cytosolic fractions of HVSMCs. Long-term Ang II exposure (4 to 24 hours) increased the expression of NAD(P)H oxidase subunits.

### Ang II Stimulates Serine Phosphorylation of p47phox and Induces Translocation of Cytoplasmic Subunits

Phosphorylation of p47phox is critical for cytoplasmic complex formation and NAD(P)H oxidase activation in neutrophils. To determine whether a similar situation exists in HVSMCs, we immunoprecipitated p47phox from cytoplasmic fractions and then probed with an anti-phosphoserine antibody. As demonstrated in Figure 5A, Ang II (15 minutes) significantly increased serine phosphorylation of p47phox. Cytoplasmic subunits were weakly expressed in membranes in basal conditions (Figure 5B). Ang II (10 to 15 minutes) decreased the abundance of cytosolic p47phox and p67phox, whereas membrane content of these subunits was increased after stimulation. These data suggest that Ang II induces translocation of cytoplasmic subunits.

### Regulation of NAD(P)H Oxidase Subunits by Ang II

The regulatory role of Ang II on NAD(P)H oxidase was further investigated by assessing whether Ang II influences the protein synthesis of the subunits. Exposure of the cells to cycloheximide, which inhibits protein synthesis by interfering with translation, decreased (P<0.01) the Ang II–induced effects on gp91phox, p22phox, p47phox, and p67phox (Figure 6). Actinomycin D, which inhibits translation, did not significantly alter expression.

### Activation of NAD(P)H Oxidase by Ang II in HVSMCs

Unstimulated cells exhibited some basal NAD(P)H oxidase activity (Figure 7A). Exposure of cells to Ang II increased NAD(P)H oxidase activation. Assessment of the different cell fractions demonstrated that activity was located primarily in the membrane fraction, suggesting that on stimulation, subunits become membrane-associated to activate the oxidase. To confirm that chemiluminescence measurements were attributable to •O_2^- generation, the effects of SOD and Tiron (•O_2^- scavengers) were determined. SOD and Tiron inhibited the lucigenin signal in Ang II–stimulated cells by 87% and 91%, respectively (Figure 7A).

### Ang II Stimulates Generation of ROS in HVSMCs

To evaluate the functional significance of Ang II–activated NAD(P)H oxidase, we measured the capacity of HVSMCs to generate ROS in the absence and presence of DPI, a flavoprotein inhibitor that reduces NAD(P)H oxidase activity. Ang II increased DCFDA fluorescence, indicating intracellular ROS generation (Figure 7B). DPI attenuated these effects, suggesting that effects are mediated via DPI-inhibitable enzymes, including NAD(P)H oxidase.

### gp91phox Is Essential for Ang II–Mediated NADPH Oxidase–Generated ROS

Transfection of HVSMCs with gp91phox antisense oligonucleotides reduced gp91phox expression, whereas in control conditions, gp91phox expression was unaltered (Figure 8A), demonstrating the efficiency of transfection. Equal loading of samples

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**Figure 2.** Representative RT-PCR products of RNA, extracted from HVSMCs, ASMCs, and RVSMCs. The human colon carcinoma cell line CaCo2 was used as a positive control for human nox1. Human and rat neutrophils (PMNs) were used as positive controls.
was confirmed by Coomassie blue staining of membranes. Figure 8B demonstrates the effects of Ang II on NAD(P)H oxidase activity in cells transfected with lipofectAMINE (Life Technologies, Invitrogen Canada Inc) alone (control cells) and sense and antisense oligonucleotides. Ang II–induced activation of NADPH oxidase was similar in control cells and sense-transfected cells. However, in antisense-transfected cells Ang II–stimulated responses were blunted.

**Association Between gp91phox and p47phox Is Important for NAD(P)H Oxidase Activation**

HVSMCs were exposed to different experimental conditions whereby gp91phox association to cytoplasmic subunits is blocked. First, cells were pretreated with a chimeric peptide that inhibits p47phox complex formation with gp91phox (gp91ds-tat). Second, cells were exposed to apocynin, a methoxy-substituted catechol, which inhibits NAD(P)H oxidase activation by preventing association between gp91phox and p47phox. Gp91ds-tat, but not the control peptide, scrambled-tat, reduced Ang II–induced activation of NAD(P)H oxidase (Figure 8C). Apocynin also abolished Ang II–mediated actions.

**Discussion**

The major findings of the present study demonstrate that VSMCs derived from human resistance arteries express gp91phox, which is essential for Ang II–regulated activation of NAD(P)H oxidase. The gp91phox homologue nox1 is expressed in ASMCs and RVSMCs but not in HVSMCs. Nox4, another gp91phox homologue, is present in HVSMC, ASMCs, and RVSMCs. We also demonstrate that the other major neutrophil NAD(P)H oxidase subunits (p22phox, p40phox, p47phox, and p67phox) are present in HVSMCs. Furthermore, we show that Ang II increases the expression of gp91phox, p22phox, p47phox, and p67phox by stimulating de novo protein synthesis at the posttranscriptional level. Acute Ang II stimulation resulted in the translocation of cytoplasmic subunits, the activation of NAD(P)H oxidase, and the production of ROS. In cells transfected with gp91phox antisense oligonucleotides, expression of gp91phox was reduced, and Ang II–stimulated NADPH-induced generation of ROS was attenuated. Taken together, these novel findings suggest that VSMCs from human small arteries possess a functionally active gp91phox-containing NAD(P)H oxidase that generates superoxide in response to Ang II.
II. In addition, we demonstrate differential mRNA expression of gp91phox, nox1, and nox4 in VSMCs from human small and large arteries. Whereas HVSMCs possess gp91phox, ASMCs and RVSMCs possess nox1. Nox4 appears to be expressed in VSMCs from both small and large human arteries as well as in RVSMCs.

Although there is strong evidence that NAD(P)H oxidase is a major source of $\cdot \text{O}_2^\cdot$ in VSMCs, the enzyme has not been fully characterized in vascular cells, and there is much controversy regarding whether the classical neutrophil subunits, and particularly gp91phox, are present and functional in these cells.3,13,17,31 Recent studies have demonstrated that p22phox, p47phox, gp91phox, and rac are important in vascular NAD(P)H oxidase activity and in Ang II–mediated ROS generation.7–15,32 Because gp91phox and p22phox are essential for NAD(P)H oxidase activation,2 it is intriguing that VSMCs should not contain gp91phox. Results from the present study demonstrate that gp91phox is expressed at mRNA and protein levels. Sequencing analysis confirmed that the amplified RT-PCR products corresponded to gp91phox. With the use of monoclonal and polyclonal antibodies [against human neutrophil NAD(P)H oxidase] and carefully prepared membrane fractions, our data clearly show that gp91phox is expressed in HVSMCs. These findings were verified by confocal immunocytochemical microscopy, which demonstrated a reticular staining extending to the cell membrane. This pattern of labeling suggests that in the basal state, gp91phox is located in the cell membrane as well as intracellularly. gp91phox is primarily membrane-associated. The intracellular component that we detected may reflect gp91phox bound to intraorganelle membranes. In addition, it is possible that cytoplasmic labeling indicates newly synthesized gp91phox. Intracellular localization of gp91phox has also been demonstrated in endothelial cells.33

Although our gp91phox findings are in contrast to those of Gorlach et al,10 who did not detect gp91phox in human aortic cells, our nox1 findings in ASMCs confirm their results. Taken together, these data suggest that gp91phox, but not nox1, is present in HVSMCs, whereas nox1, but not gp91phox, is present in ASMCs. Reasons for the differential expression of gp91phox homologues between VSMCs from small and large arteries are unclear but may indicate heterogeneity of VSMCs derived from different vascular beds. Nox1 is a gp91phox homologue that has 56% identity with human gp91phox17 and, together with p22phox, is probably the functionally active component of cytochrome b558 in ASMCs and RVSMCs.13 Unlike gp91phox and nox1, which were differentially expressed, nox4 was ubiq-

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**Figure 5.** Activation and translocation of cytosolic subunits by Ang II. A, Effects of Ang II on serine phosphorylation of p47phox. A polyclonal p47phox antibody was used to immunoprecipitate (IP) p47phox from cytoplasmic fractions of vehicle-treated and Ang II–stimulated (15 minutes) HVSMCs. At the top is a representative immunoblot (IB), which was performed by using anti-phosphoserine antibody. At the bottom are corresponding bar graphs. Results are mean ± SEM of 3 preparations. *P < 0.05 vs control. B, Immunoblot analysis of p47phox and p67phox in cytosolic and membrane fractions after Ang II (10−7 mol/L) stimulation (10 to 15 minutes). Top graphs demonstrate decreased abundance of p47phox and p67phox in cytoplasmic fractions, and bottom panels demonstrate increased abundance of subunits after Ang II. Data are expressed as percent expression relative to control, taken as 100%. Results are mean ± SEM of 4 to 6 experiments. *P < 0.05 and **P < 0.01 vs control counterpart.
uitously expressed in all VSMCs studied. The exact function of this gp91phox homologue is unclear, but it may be antagonistic to nox1, as demonstrated in rat aortic smooth muscle cells, in which Ang II upregulates nox1 but downregulates nox4.13 Sorescu et al.34 recently reported that nox4 is intensely expressed in the media of human coronary arteries, particularly in VSMCs. On the basis of these findings, together with our data demonstrating high nox4 mRNA expression in HVSMCs, we cannot exclude the possibility that gp91phox antibody may react with nox4, especially because this homologue shares NADPH- and flavin-binding sites and 30% to 60% mRNA identity with gp91phox.35

Immunoblotting revealed that p40phox, p47phox, and p67phox are expressed in cytoplasmic fractions of HVSMCs. In addition, these subunits were detectable in membrane fractions in unstimulated cells, suggesting some translocation of the subunits and activation of the oxidase in the basal state. This was confirmed by our chemiluminescence studies demonstrating that NADPH oxidase is partially functional in unstimulated VSMCs. These findings are in contrast to leukocytes, which do not exhibit basal NAD(P)H oxidase activity.5 Within a few minutes of Ang II stimulation, the abundance of p47phox and p67phox in the cytoplasmic fraction decreased, whereas the content of these cytoplasmic subunits increased in the membrane fraction, indicating rapid translocation of p47phox and p67phox by Ang II. The physiological role of each subunit remains unclear, but p47phox phosphorylation seems to be pivotal, and the presence of p67phox is obligatory for NAD(P)H oxidase activation.6,32 p40phox may be an inhibitory oxidase subunit.5

Possible mechanisms whereby Ang II regulates NAD(P)H oxidase could be via its effects on the abundance of the oxidase subunits and/or by modulating phosphorylation of the proteins. In the present study, long-term Ang II stimulation (hours) increased NAD(P)H oxidase content. Cycloheximide, but not by actinomycin D, inhibited these effects, suggesting that Ang II regulates the de novo synthesis of NAD(P)H oxidase subunits at the posttranscriptional level. Pagano and colleagues7,11 reported that in rabbit aortic adventitial fibroblasts, p67phox is regulated by Ang II both at the level of transcription and at the level of translation. Similar findings have been shown for p22phox in rat aortic cells.36 Thus, Ang II probably influences the synthesis of NAD(P)H oxidase subunits at multiple levels.

It is also possible that Ang II regulates NAD(P)H oxidase activity by stimulating subunit phosphorylation. Serine phosphorylation of p47phox is critical for cytoplasmic subunit complex formation and translocation. During oxidase activation, serine S359 and/or S370 must first be phosphorylated, and then S379 is phosphorylated, allowing the cytosolic complex to translocate to cytochrome b558 (gp91phox and p22 phox). Finally, S303 and/or S304 is phosphorylated, endowing the oxidase with full catalytic activity.37 We demonstrate for the first time that Ang II induces the phosphorylation of serine residues of p47phox. These effects were evident within 10 to 15 minutes of stimulation, suggesting that p47phox phosphorylation is a rapid event. Interestingly, phospholipase A2, extracellular signal–regulated kinase 1/2, p38 mitogen-activated protein kinase, and phosphatidic acid, all downstream signaling molecules of...
Ang II, have been implicated in the phosphorylation of p47phox and in the activation of NAD(P)H oxidase. To demonstrate the functional significance of NAD(P)H oxidase, we determined whether Ang II activates the enzyme and whether it generates ROS in HVSMCs. The importance of gp91phox in these processes was assessed in cells transfected with gp91phox antisense oligonucleotides. Furthermore, cells were exposed to gp91ds-tat and apocynin, which inhibit association of gp91phox with p47phox. Ang II increased the activity of NAD(P)H oxidase and dose-dependently stimulated ROS production. Ang II actions were abrogated in antisense-transfected cells and reduced by gp91ds-tat and apocynin. These phenomena indicate that gp91phox plays a critical role in the activation of Ang II–regulated NAD(P)H oxidase in HVSMCs.

In conclusion, data from the present study demonstrate that VSMCs from human resistance arteries express gp91phox and nox4, but not nox1. In contrast, nox1, but not gp91phox, is present in ASMCs and RVSMCs. VSMCs from human small arteries also express the other major neutrophil NAD(P)H oxidase subunits (p22phox, p40phox, p47phox, and p67phox) at both mRNA and protein levels. Ang II increases the expression of NAD(P)H oxidase subunits by stimulating de novo protein synthesis. Moreover, we demonstrate that Ang II induces the phosphorylation of p47phox and the translocation of cytoplas-
mic subunits, with subsequent activation of NAD(P)H oxidase and generation of ROS. These novel findings suggest that VSMCs from human resistance arteries possess a functionally active Ang II–regulated gp91phox-containing neutrophil-like NAD(P)H oxidase, which is a major source of vascular-derived superoxide.

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