Angiotensin II Induces \( p67^{\text{phox}} \) mRNA Expression and NADPH Oxidase Superoxide Generation in Rabbit Aortic Adventitial Fibroblasts

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Abstract—Superoxide radical (O\(_2^–\)) is ubiquitously critical to the bioactivity of endothelial nitric oxide. In angiotensin-dependent hypertension, vascular O\(_2^–\) levels rise and impede endothelium/nitric oxide–dependent vascular relaxation. We have reported that the major O\(_2^–\) source in the rabbit aorta is adventitial fibroblast phagocyte-like NADPH oxidase and shown that angiotensin (Ang II) treatment of adventitial fibroblasts causes a concentration-dependent increase in particulate NADPH-dependent O\(_2^–\). From cultured rabbit aortic adventitial fibroblasts treated or not treated with Ang II, we prepared particulate fractions and measured lucigenin-enhanced chemiluminescence. Because [Sar\(^1\),Thr\(^8\)]-Ang II, a generalized antagonist of Ang II and plausible inhibitor of the conversion of Ang II, reversed Ang II (10 nmol/L)–induced NADH- and NADPH-dependent O\(_2^–\) to basal levels, we tested the effect of the inhibitor of aminopeptidase N, amastatin (10 \( \mu \)mol/L), and found no effect on Ang II–stimulated O\(_2^–\). Ang(1-7), Ang III, and Ang IV also were not effective in stimulating O\(_2^–\) levels at concentrations similar to those of Ang II. Kinetic analysis showed a rise in NADPH oxidase O\(_2^–\) production in response to Ang II, which peaks at 3 hours and returns to basal levels by 16 hours. \( p67^{\text{phox}} \), a cytosolic factor, appears to be affected at both the level of transcription and protein synthesis because actinomycin and cycloheximide individually inhibited the observed effect. A partial sequence of \( p67^{\text{phox}} \) was recovered by reverse transcriptase from mRNA harvested from cultured rabbit aortic adventitial fibroblasts. Furthermore, the \( p67^{\text{phox}} \) mRNA transcript in aortic fibroblasts is induced by Ang II before the peak of NADPH oxidase by Northern analysis and ribonuclease protection assays. These data suggest that Ang II stimulates NAD(P)H oxidase O\(_2^–\) generation in fibroblasts of aortic adventitia via transcriptional activation of \( p67^{\text{phox}} \). These data also provide preliminary evidence for the regulation of factors of the NADPH oxidase and potentially provide a novel means by which to abrogate the development of O\(_2^–\)-dependent hypertension. (Hypertension. 1998;32:331-337.)

Key Words: rabbits ■ angiotensin II ■ superoxide ■ free radicals ■ reactive oxygen species ■ NADPH oxidoreductases

Several studies demonstrate O\(_2^–\) involvement in hypertension. Superoxide dismutase was shown to lower blood pressure in SHR.\(^1\) Contractile effects of acetylcholine in the mesenteric artery of SHR are blocked by an inhibitor of O\(_2^–\) production via the cyclooxygenase pathway.\(^2\) Likewise, in rats with acute Ang II–induced hypertension, superoxide dismutase and catalase inhibit vascular hyperpermeability and cellular damage related to the hypertension\(^3\) indicative of the involvement of O\(_2^–\) and hydrogen peroxide, respectively. In both SHR and Ang II–dependent hypertensive rats, endothelium-dependent relaxations are impaired\(^4,5\) and endothelium-dependent contractions are enhanced.\(^6\) In vivo treatment with angiotensin-converting enzyme inhibitors in SHR normalized aberrant ex vivo aortic relaxations and contractions.\(^4,5\) Implicating a role for O\(_2^–\) in these 2 forms of hypertension, recent reports show that Ang II can stimulate O\(_2^–\) levels from phagocytes and rat aortic smooth muscle cells via NADPH oxidases.\(^7,8\)

The phagocyte NADPH oxidase or respiratory burst oxidase is a well-characterized reactive oxygen species–generating system that catalyzes the 1-electron reduction of oxygen to O\(_2^–\). It is a multicomponent enzyme complex that includes the 2 membrane-spanning polypeptide subunits p22\(^{\text{phox}}\) and gp91\(^{\text{phox}}\), which comprise flavocytochrome b\(_{558}\), and 3 cytoplasmic polypeptide subunits, p40\(^{\text{phox}}\), p47\(^{\text{phox}}\), and p67\(^{\text{phox}}\).\(^9,10\) Moreover, the cytosolic guanine nucleotide–binding protein Rac2, a member of the Ras family of peptides, is required for oxidase activation.\(^11\) Exposure of the cell to a variety of agonists induces the association of the cytosolic with the membrane-associated components and causes activation of the normally dormant oxidase.\(^9,10\)

Previous reports have shown that NAD(P)H oxidase(s) exists in nonphagocytic cells, including carotid body cells,\(^12\) mesangial cells,\(^13\) vascular smooth muscle cells,\(^8,14\) endothelial cells,\(^15,16\) and fibroblasts.\(^17\) In vascular smooth muscle cells of the rat aorta, an NADH oxidase has been described.
that interferes with vascular relaxation, and these cells express the mRNA for 1 of the cytochrome b55 subunits found in phagocyte membranes, p22phox. In cultured rat aortic smooth muscle cells, NAD(P)H oxidase O₂⁻ activity is stimulated by Ang II. It appears to be involved in the hypertrophic response, and was subsequently suggested to be involved in the development of hypertension. Likewise, endothelial cells of the bovine pulmonary artery, as well as the human umbilical vein, appear to contain an O₂⁻-generating NADH oxidase.

We have reported that the adventitia is the major site of O₂⁻ production in the rabbit aorta and contains phagocyte-like NADPH oxidase and all 4 of its major subunits. Moreover, we showed that adventitial fibroblasts contain an NADPH oxidase that is enhanced by Ang II and also contain the unique p67phox, which is essential for activity. Because of the potential importance of an apoptotic NADPH oxidase in the regulation of NO function and vascular physiology, we have focused our studies on the regulation of the aortic adventitial NADPH oxidase. The purpose of this study was 2-fold: (1) to determine whether metabolic conversion of Ang II to metabolites could account for induction of NADPH oxidase activity and (2) to examine a transcriptional effect of Ang II on NADPH oxidase in adventitial fibroblasts.

**Methods**

**Materials**

Lucigenin was solubilized in physiological buffer; leupeptin and aprotinin were solubilized in 0.9% saline; PMSF was solubilized in DMSO; Trizma base, EDTA, diethyldithiocarbamate, and Tiron were solubilized in H₂O. The above compounds, as well as isopropanol, chloroform, phenol, formaldehyde, formamide, and sodium pentobarbital, were purchased from Sigma Chemical Co. DMEM, fetal bovine serum, trypsin-EDTA, collagenase, elastin, and penicillin/streptomycin were purchased from GIBCO-BRL. PBS was purchased from Mediatech. β-NADPH was purchased from Boehringer Mannheim and was solubilized in buffer. Diphenylene iodonium was purchased from Biomol and solubilized in DMSO. Heparin was purchased from Elkins-Sinn. Losartan was obtained from Merck, Inc. PD 123,319 was obtained from Research Biochemicals International. Ang II, Ang(1–7), Ang III, and Ang IV were obtained from Sigma; all were diluted in 0.9% saline with 0.001N acetic acid.

**Tissue Culture of Aortic Adventitial Fibroblasts**

Thoracic aortas were removed sterilily from 7 male New Zealand White rabbits and placed in DMEM/Ham’s F-12 containing 0.17 mmol/L penicillin and 0.07 mmol/L streptomycin. Vessels were then cultured on gelatin-coated plates. After one passage of growth, adventitial fibroblasts were isolated and cultured to the third passage as previously described. Forty-eight-hour quiescent cells were made quiescent by stepwise decrease in fetal bovine serum to 0.67% confluent P₁ cells were harvested and fractions prepared. In experiments examining activation by angiotensins, angiotensin or vehicle was added to quiescent cells for designated time periods and then harvested for particulate fraction or RNA preparation.

**Preparation of Particulate Fractions**

To determine effects of Ang II and its metabolites on NADPH oxidase activity and to examine the kinetics and mediators of such effects, we prepared particulate fractions enriched in NADPH oxidase activity of fibroblasts treated or not treated with angiotensins in the presence or absence of inhibitors. We have reported that Ang II causes a dose-dependent rise in fibroblast O₂⁻ production by NADPH oxidase and that this activity at the highest effective dose is inhibited by [Sar₁, Thr₈]-Ang II, yet not by losartan nor PD123,319. We now report that the combination of losartan and PD123,319 antagonists is also without effect (n = 2, not shown). Because [Sar₁, Thr₈]-Ang II could have potentially blocked conversion of Ang II to 1 of its metabolites, we attempted to characterize a potential angiotensin degradation metabolite that may have been responsible for the activity. Toward this end, we performed 2 experiments. In 1, we tested whether plausible metabolites of Ang II, i.e., Ang III, Ang IV, or Ang(1–7), were capable of eliciting a similar response as Ang II. Second, we reported that the major Ang II–metabolizing enzyme in fibroblasts is aminopeptidase N. We then tested whether amastatin, an inhibitor of aminopeptidase N, could inhibit Ang II–induced activity. In all of these experiments, treated or nontreated P₁ fibroblasts were harvested and the pellet was resuspended in 400 µL of ice-cold Tris-sucrose buffer; particulate fractions were prepared and used on the day of preparation as previously described.

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

Approximately 10 to 20 µg fibroblast particulate fraction was assayed for O₂⁻-dependent lucigenin chemiluminescence as described previously. To confirm that these measurements were not an artifact of the lucigenin assay, we also directly measured O₂⁻ using cytochrome c and obtained similar results.

**Isolation of Total Cellular RNA From Rabbit Aortic Fibroblasts**

Total RNA from P₁ cultured fibroblasts were chloroform extracted. After centrifugation, the upper layer was taken and subjected to isopropanol precipitation. Final total RNA concentration and purity was determined spectrophotometrically by measuring the absorbance at 260 and 280 nm.

**Reverse Transcriptase–Polymerase Chain Reaction**

RT-PCR was used to partially clone p67phox to (1) confirm its presence and determine its relationship by sequence homology to the human neutrophil counterpart and (2) use the partial clone as a probe for changes in mRNA levels of p67phox. cDNA was generated from 1 µg total RNA using Superscript II reverse transcriptase (GIBCO-BRL) and oligo (dT) primers (Promega). The RT reaction was carried out at 42°C for 30 minutes. Products of the RT reaction were subjected to PCR amplification using the forward primer 5'-TACCTCCAACGAGGGATGCTC-3' and the reverse primer 5'-AGCTTTCTCTCGGGGCTCT-3'. The conditions used for PCR were 94°C for 1 minute, 58°C for 1 minute, and 72°C for 2 minutes at 30 cycles. Products of PCR amplification were separated on a 1.5% (wt/vol) agarose gel containing ethidium bromide and visualized with ultraviolet transillumination.

**Sequencing of PCR Products**

Amplified PCR products were gel purified, ligated into a pCR 2.1 plasmid using the instructions provided by the Original TA Cloning kit (Invitrogen), and transformed into an INvA F° strain of Escherichia coli. Transformed plasmids resistant to ampicillin were screened for insertion of the vector using EcoRI restriction analysis. The plasmid containing the appropriate insert was selected and sequenced by the Brigham and Women's Automatic Sequencing and Genotyping Facility (Boston, Mass). The cDNA that was sequenced

**Selected Abbreviations and Acronyms**

Ang = angiotensin
Ang IV = Ang(31–78)
DMEM = Dulbecco’s modified Eagle’s medium
O₂⁻ = superoxide anion
RPA = ribonuclease protection assay
RT-PCR = reverse transcriptase–polymerase chain reaction
SHR = spontaneously hypertensive rat(s)

Angiotensin II–metabolizing enzyme in fibroblasts is aminopeptidase N. We have reported that the adventitia is the major site of O₂⁻ production in the rabbit aorta and contains phagocyte-like NADPH oxidase and all 4 of its major subunits. Moreover, we showed that adventitial fibroblasts contain an NADPH oxidase that is enhanced by Ang II and also contain the unique p67phox, which is essential for activity. Because of the potential importance of an apoptotic NADPH oxidase in the regulation of NO function and vascular physiology, we have focused our studies on the regulation of the aortic adventitial NADPH oxidase. The purpose of this study was 2-fold: (1) to determine whether metabolic conversion of Ang II to metabolites could account for induction of NADPH oxidase activity and (2) to examine a transcriptional effect of Ang II on NADPH oxidase in adventitial fibroblasts.
was subamplified with 2 sets of primers and sequenced again to ensure sequence integrity. The sequence of the primers used for subamplification were forward, 5′-TACTTCCAACAGGGATGCTC-3′, and reverse, 5′-GCTCCTTAACCTCCTTCGTC-3′.

**Northern Blot Analysis**

Northern blot analysis was performed to determine whether molecular expression of p67phox is temporally related to Ang II–stimulated NADPH oxidase O$_2^·$ production. Total RNA (15 µg) was loaded in each lane on 1% denaturing formaldehyde agarose gels, and electrophoresis was performed. RNA was transferred to a GeneScreen-plus nylon membrane by capillary action and immobilized by ultraviolet cross-linking. Blots were prehybridized for at least 2 hours at 42°C in the following solution: 1 mol/L NaCl, 50 mmol/L Tris, 5× Denhardt’s solution, 50% formamide, 0.5% SDS, and 100 µg/mL sheared and denatured herring sperm DNA. Probe labeling was performed using the random-primed DNA-labeling kit from Boehringer Mannheim with [32P]dCTP. Unincorporated 32P was removed using Sephadex G-10 probe purification columns. The probe was incubated with the blot for 18 hours at 42°C. After autoradiography, the relative density of each band was determined using image densitometry. The density of each band was normalized to 18S ribosomal RNA.

**Ribonuclease Protection Assay**

In an attempt to avert degradation of mRNA and therefore detect a greater stimulation of p67phox mRNA levels with Ang II, an RPA was performed. [32P]dUTP-labeled antisense mRNA probe for p67phox was synthesized using the MAXiScript in vitro transcription kit (Ambion). Briefly, 1 µg of plasmid containing partial-length cDNA for p67phox was incubated with T7 RNA polymerase at 42°C for 1.5 hours. The reaction was terminated by heating for 5 minutes at 95°C, and the template DNA was digested using DNase. The radiolabeled probe was gel purified using a 5 mol/L urea/5% polyacrylamide gel. The method for the RPA was followed as instructed by the RPA II Kit (Ambion). Briefly, radiolabeled antisense probe was coprecipitated and then incubated with total cellular RNA at 42°C for 18 hours. RNase A and RNase T$_1$ were added and allowed to digest all single-stranded unhybridized RNA. After 1 hour at 42°C, the reaction was terminated. The reaction was concentrated via ethanol precipitation and run on a 5 mol/L urea/5% polyacrylamide gel. The protected fragments were visualized with autoradiography and quantified using image densitometry. The density of individual bands was normalized to rabbit GAPDH RNA.

**Statistical Analysis**

Data are expressed as mean±SEM; n represents the number of cellular experiments whose cells were derived from 1 or more animals. Cells were cultured from aortas to the second passage and frozen in separate aliquots, then grown for each experiment (n) as third-passage cells and used as such. Each n was performed on separate experimental days. Statistical evaluation of time-course relationships were performed using 2-way ANOVA and a Student-Newman-Keuls method for point comparisons. Statistical evaluation of the data for angiotensin metabolites was performed using 2-way ANOVA. All other differences were tested with Student’s t test for paired or unpaired comparisons. Statistical differences demonstrating P<0.05 were considered statistically significant.

**Results**

To determine whether conversion of Ang II to one of its metabolites is involved in the activation of NADPH oxidase, we tested whether the inhibitor of aminopeptidase N amastatin (10 µmol/L) could affect Ang II–stimulated O$_2^·$ generation. Figure 1 shows that pretreatment of quiescent fibroblasts with amastatin was without effect on Ang II–stimulated O$_2^·$ generation. Also, toward a similar end, we tested whether Ang(1-7), Ang III, or Ang IV could stimulate O$_2^·$ levels at concentrations similar to those of Ang II. Figure 2 shows a lack of effectiveness of each of the metabolites to stimulate O$_2^·$ after 3 hours of incubation.

Kinetic analysis of the stimulation of O$_2^·$ generation in response to Ang II demonstrates that the rise in O$_2^·$ production to the maximally effective dose of Ang II peaks at approximately 3 hours and returns to basal levels by 16 hours (Figure 3). There is a 2-fold to 3-fold higher activity even after 10 minutes of Ang II concomitant with an observed rise in calcium. Protein synthesis and transcription appear to be involved in the stimulation by 3 hours, because cycloheximide (1.8 µmol/L) and actinomycin (0.4 µmol/L) were capable of inhibiting the rise (n=3, Figure 4). Cycloheximide and actinomycin were without effect on basal NADPH oxidase activity.

![Figure 2](http://hyper.ahajournals.org/) Lack of effect of amastatin on Ang II–induced particulate NADPH oxidase activity. Cells were either not treated or treated with Ang II (10 nmol/L). Ang II–treated cells were preincubated (30 minutes before the addition of Ang II) with 10 µmol/L amastatin or vehicle. Cells were then trypsinized, sonicated, and differentially centrifuged to obtain particulate fractions. Lucigenin chemiluminescence was measured using NADPH as cofactor, and units of chemiluminescence were converted to nanomoles O$_2^·$ generated per minute per milligram protein ±SEM (n=3 cell preparations). *Significance by a paired Student’s t test at P<0.01.

![Figure 3](http://hyper.ahajournals.org/) Lack of effect of Ang II metabolites on particulate NADPH oxidase activity. Cells were either not treated or treated with Ang II metabolites (0, 1, and 10 nmol/L) for 3 hours. Cells were then trypsinized, sonicated, and differentially centrifuged to obtain particulate fractions. Lucigenin chemiluminescence was measured using NADPH as cofactor, and units of chemiluminescence were converted to nanomoles O$_2^·$ generated per minute per milligram protein ±SEM (n=2 to 6 cell preparations). A 2-way ANOVA was used to analyze the data and no significant differences were found.
An RT-PCR product of the 714-bp size expected for p67 phox (an essential cytosolic component for NADPH oxidase) was obtained by using primers derived from the human phagocytic p67phox sequence; RNA harvested from aortic fibroblasts was used as a template. The cDNA sequence shares a 94.6% homology with the human phagocytic p67phox. Interestingly, at the protein level, the homology is 89.1% and includes the first SH3 of p67 phox. Figure 5 shows the amino acid homology between the rabbit and human sequence. When used as a probe, the labeled RT-PCR product detected an mRNA species of comparable size on Northern blot, as shown in Figure 6. The p67phox message showed an increase in steady state levels compared with normalized control levels at 1 and 3 hours of Ang II treatment (34 ± 6% and 33 ± 18%, n = 3). The positive effect was lost by 6 hours (2 ± 6%, n = 3) and the 3-hour effect apparently blocked by 10 μmol/L [Sar1, Thr8]-Ang II (2 ± 12%, n = 1). The effect of Ang II on p67 phox mRNA is confirmed by the results of the RPA analyses, which were performed 5 times. This procedure showed a comparable effect, an increase in p67phox message before the time-dependent increase in activity. In the RPA analysis, significant stimulation in p67 phox mRNA was seen at 1 hour and was lost by 6 hours (see Figure 7). Moreover, at the protein level, the homology is 89.1% and includes the first SH3 of p67phox. Figure 5 shows the amino acid homology between the rabbit and human sequence. When used as a probe, the labeled RT-PCR product detected an mRNA species of comparable size on Northern blot, as shown in Figure 6. The p67phox message showed an increase in steady state levels compared with normalized control levels at 1 and 3 hours of Ang II treatment (34 ± 16% and 33 ± 18%, n = 3). The positive effect was lost by 6 hours (2 ± 10%, n = 3) and the 3-hour effect apparently blocked by 10 μmol/L [Sar1, Thr8]-Ang II (2 ± 12%, n = 1). The effect of Ang II on p67phox mRNA is confirmed by the results of the RPA analyses, which were performed 5 times. This procedure showed a comparable effect, an increase in p67phox message before the time-dependent increase in activity. In the RPA analysis, significant stimulation in p67phox mRNA was seen at 1 hour and was lost by 6 hours (see Figure 7). Moreover,
We have characterized a mechanism by which Ang II can increase O$_2^-$ in rabbit aortic adventitia. Our previous results showed that the adventitia is the aortic site of greatest O$_2^-$ via NADPH oxidase.\textsuperscript{21,28} The results reported here demonstrate that Ang II increases O$_2^-$ by increasing activity of NADPH oxidase in adventitial fibroblasts and suggest induction of the transcription of at least 1 phagocyte-like NADPH oxidase component. This result was established by generating a partial sequence of the rabbit p67phox that was used in 2 independent RNA analyses to demonstrate a correlative increase. The present results corroborate previous reports that Ang II can act at the vascular level to increase O$_2^-$ yet support evidence that this activation is adventitial in origin and derived from fibroblast NADPH oxidase. These results also suggest that this stimulation is caused through interaction with a receptor other than the classic type 1 and 2 and that this activity is modulated at the transcriptional and translational level.

We have reported that Ang II causes a dose-dependent rise in fibroblast O$_2^-$ production by NAD(P)H oxidase and that this activity at the highest effective dose is inhibited by [Sar$^1$,Thr$^4$]-Ang II, yet not by losartan or PD123,319.\textsuperscript{21} We now report that none of the plausible metabolites of Ang II, i.e., Ang III, Ang IV, or Ang(1–7),\textsuperscript{22–24} was as efficacious in promoting O$_2^-$ -generating activity, indicating that these metabolites are not responsible for the effect of applied Ang II. Moreover, amastatin, an inhibitor of aminopeptidase N (a major Ang II–metabolizing enzyme in fibroblasts), could not inhibit Ang II–induced activity. Thus, we conclude that Ang II was not being converted in any substantial amounts to an active metabolite but rather that Ang II was acting through an alternate receptor. In support of this contention is a report showing that the human cardiac hyperplastic response to Ang II is not mediated by an AT$_1$ or AT$_2$ receptor but rather by another receptor of differing binding characteristics.\textsuperscript{29} We recently found that fibroblasts cultured from the aortic adventitia contain a phagocyte-like NADPH oxidase activity\textsuperscript{21} and showed the immunohistochemical presence of all 4 major components of the phagocyte NADPH oxidase, p22, gp91, p67phox, and p47phox, in the adventitia. We also showed by immunoblotting the presence of p67phox in particulate fractions and by immunoprecipitation the essential role of this subunit in particulate O$_2^-$ -generating activity.\textsuperscript{21} By RT-PCR, we have isolated a partial sequence of the rabbit p67phox, an essential cytosolic component for NADPH oxidase from fibroblast RNA. Efforts are ongoing to clone and characterize the full-length cDNA. Interestingly, in our partial sequence of 714 bases, the degree of homology is high, 89.1\% and 94.6\%, at the levels of amino acid and nucleotide, respectively. The RT-PCR product was subcloned and used as a probe for Northern blot analysis; higher steady state levels compared with normalized control levels were detected 1 hour after treatment with Ang II, and this result precedes the peak of NADPH oxidase O$_2^-$ -generating activity. The net effect at the level of transcription confirmed by RNA protection assay was lost by 6 hours. Furthermore, the temporal association between the observed increase in p67phox mRNA level and measured NADPH oxidase activity is striking. Importantly, an increase in NADPH oxidase activity does not require a measurable increase in protein.\textsuperscript{30,31} Several groups have suggested that in phagocytic cells, the amount of p67phox may be the limiting factor for the assembly and activation of the NADPH oxidase.\textsuperscript{32} The projected $K_m$ for p67phox is estimated to be approximately 3 times lower than that of another major cytosolic subunit, p47phox, in normal cytosol.\textsuperscript{32} In support of this hypothesis, 1 group has reported a 2:1 ratio between p47phox and p67phox in isolated complex.\textsuperscript{33} Based on these observations, one could hypothesize that the percent increase observed in p67phox mRNA would be significantly higher with respect to NADPH oxidase activity.

At the most fundamental level, we present evidence for the presence of p67phox (previously reported to be phagocyte specific) at the protein level and now the transcriptional level in aortic fibroblasts. Previous studies have shown the presence of p22phox and that it modulates Ang II–dependent O$_2^-$ generation in rat aortic smooth muscle.\textsuperscript{19} However, epitopes for p22phox are ubiquitous, based on the general presence of cytochromes such as b$_{558}$, of which it is part, and homology between the p22phox and mitochondrial cytochromes.\textsuperscript{34} In contrast, there have been no reports of p67phox presence in cells that lack other components of the oxidase. Moreover, unlike studies of the rat aortic NADPH oxidase, our previous study shows by immunoprecipitation that the protein subunit (p67$^{phox}$) is essential for activity.\textsuperscript{21} Hence, it is unlikely that we have demonstrated a coincidental rise in p67phox unrelated to NADPH oxidase activity.

The time course of the Ang II effect is indicative of an acute early response in stimulation of NADPH oxidase. This rapid activation of the NADPH oxidase system at the level of the gene distinguishes it from what was shown for NAD(P)H oxidase in rat aorta, which exhibits elevations in NADPH oxidase activity coincident with transcriptional changes in...
p22phox expression after several days of Ang II infusion. Moreover, the peak of p22phox mRNA in the rat aorta followed the peak in NAD(P)H oxidase activity, which is suggestive of a positive induction of p22phox by O2·− rather than a dependence on it.

We have also shown that cycloheximide can inhibit the rise in NADPH oxidase activity in the particulate fractions of fibroblasts, suggesting that translational activity is required. However, we were unable to observe a rise in protein for the p67phox on a Western blot of particulate fraction. We propose 3 explanations for this: (1) that the p67phox is loosely associated with the particulate and is lost during the preparation of the Western blot; (2) that a small but significant rise in protein cannot be resolved by the blotting and densitometric techniques; and/or (3) that cycloheximide is acting by inhibiting another essential component of the NADPH oxidase and that small increases in each synergistically enhance the response. Indeed, it is plausible that the other major components of the NADPH oxidase, p22phox, gp91phox, and p47phox, are transcriptionally and translationally induced, because the ratio of major subunit concentration in the active enzyme is generally regarded as equal.

These data demonstrate that a defining subunit for NADPH oxidase is present and that its expression is induced by Ang II in these cells. Because we have previously shown that p67phox is essential for fibroblast NADPH oxidase activity and here provide evidence that the expression of this significant subunit is induced by Ang II, the information should direct study to a better means by which to control basal and hormonally induced vascular O2·− generation at the gene level. Due to the apparent presence of p67phox exclusively in NADPH oxidase, inhibition of NADPH oxidase may be better served by specific knockout of the p67phox protein rather than p22phox.

A few studies demonstrate the physiological significance of phagocyte-like NADPH oxidase in the vasculature and in hypertension. Our group has recently found that nitric oxide–mediated relaxation of vascular smooth muscle cells.

Further identification of the molecular components of this system and investigation into their regulation are necessary.

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