Angiotensin II Induces p67phox mRNA Expression and NADPH Oxidase Superoxide Generation in Rabbit Aortic Adventitial Fibroblasts

Patrick J. Pagano, Stephen J. Chanock, Deborah A. Siwik, Wilson S. Colucci, Justin K. Clark

Abstract—Superoxide radical (O\textsubscript{2}\textsuperscript{−}) is ubiquitously critical to the bioactivity of endothelial nitric oxide. In angiotensin-dependent hypertension, vascular O\textsubscript{2}\textsuperscript{−} levels rise and impede endothelium/nitric oxide–dependent vascular relaxation. We have reported that the major O\textsubscript{2}\textsuperscript{−} 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\textsubscript{2}\textsuperscript{−}. From cultured rabbit aortic adventitial fibroblasts treated or not treated with Ang II, we prepared particulate fractions and measured lucigenin-enhanced chemiluminescence. Because [Sar\textsuperscript{1},Thr\textsuperscript{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\textsubscript{2}\textsuperscript{−} to basal levels, we tested the effect of the inhibitor of aminopeptidase N, amastatin (10 μmol/L), and found no effect on Ang II–stimulated O\textsubscript{2}\textsuperscript{−}. Ang(1-7), Ang III, and Ang IV also were not effective in stimulating O\textsubscript{2}\textsuperscript{−} levels at concentrations similar to those of Ang II. Kinetic analysis showed a rise in NADPH oxidase O\textsubscript{2}\textsuperscript{−} production in response to Ang II, which peaks at 3 hours and returns to basal levels by 16 hours. p67phox, 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 p67phox was recovered by reverse transcriptase from mRNA harvested from cultured rabbit aortic adventitial fibroblasts. Furthermore, the p67phox 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\textsubscript{2}\textsuperscript{−} generation in fibroblasts of aortic adventitia via transcriptional activation of p67phox. 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\textsubscript{2}\textsuperscript{−}–dependent hypertension. (Hypertension. 1998;32:331-337.)

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

Several studies demonstrate O\textsubscript{2}\textsuperscript{−} involvement in hypertension. Superoxide dismutase was shown to lower blood pressure in SHR.\textsuperscript{1} Contractile effects of acetylcholine in the mesenteric artery of SHR are blocked by an inhibitor of O\textsubscript{2}\textsuperscript{−} via NADPH oxidases.\textsuperscript{7,8}

Oxygen free radicals and hydrogen peroxide, respectively. In both SHR and Ang II–dependent hypertensive rats, endothelium-dependent relaxations are impaired\textsuperscript{5,6} and endothelium-dependent contractions are enhanced.\textsuperscript{6} In vivo treatment with angiotensin-converting enzyme inhibitors in SHR normalized aberrant ex vivo aortic relaxations and contractions.\textsuperscript{4,5} Implicating a role for O\textsubscript{2}\textsuperscript{−} in these 2 forms of hypertension, recent reports show that Ang II can stimulate O\textsubscript{2}\textsuperscript{−} levels from phagocytes and rat aortic smooth muscle cells via NADPH oxidases.\textsuperscript{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\textsubscript{2}\textsuperscript{−}. It is a multicomponent enzyme complex that includes the 2 membrane-spanning polypeptide subunits p22phox and gp91phox, which comprise flavocytochrome b\textsubscript{558}, and 3 cytoplasmic polypeptide subunits, p40phox, p47phox, and p67phox.\textsuperscript{9,10} Moreover, the cytosolic guanine nucleotide–binding protein Rac2, a member of the Ras family of peptides, is required for oxidase activation.\textsuperscript{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.\textsuperscript{9,10}

Previous reports have shown that NAD(P)H oxidase(s) exists in nonphagocytic cells, including carotid body cells,\textsuperscript{12} mesangial cells,\textsuperscript{13} vascular smooth muscle cells,\textsuperscript{8,14} endothelial cells,\textsuperscript{15,16} and fibroblasts.\textsuperscript{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 b$_{55}$ subunits found in phagocyte membranes, p22phox. In cultured rat aortic smooth muscle cells, NAD(P)H oxidase O$_2^-$ 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 O$_2^-$-generating NADH oxidase.

We have reported that the adventitia is the major site of O$_2^-$ 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 aortic NADPH oxidase in the regulation of NO function and vascular physiology, we have focused our studies on the regulation of the 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 H,O. The above compounds, as well as isopropanol, chloroform, phenol, formaldehyde, formamide, and sodium pentobarbital, were purchased from Sigma Chemical Co. DMEM, Ham's F-12, fetal bovine serum, trypsin-EDTA, collagenase, elastase, 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 steriley 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 cleared of adventitial adipose tissue and 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 P$_1$ cells were harvested and fractions prepared. In experiments exam-
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'-TACTTCCAAGGAGGTGCTC-3', and reverse, 5'-GCTCTTTAATCTCTGTGTCC-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 O2− 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 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, 1 μg of plasmid containing partial-length cDNA 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. 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 quantitated and then incubated with total cellular RNA at 42°C for 18 hours. 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 individual bands was normalized to rabbit GAPDH 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 T1 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 quantitated 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 a 2-way ANOVA and a Student-Newman-Keuls method for point comparisons. Statistical evaluation of the data for angiotensin metabolites was performed using a 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 inhibit Ang II–stimulated O2−. Figure 1 shows that pretreatment of quiescent fibroblasts with amastatin was without effect on Ang II–stimulated O2− generation. Also, toward a similar end, we tested whether Ang(1-7), Ang III, or Ang IV could stimulate O2− levels at concentrations similar to those of Ang II. Figure 2 shows a lack of effectiveness of each of the metabolites to stimulate O2− after 3 hours of incubation.

Kinetic analysis of the stimulation of O2− generation in response to Ang II demonstrates that the rise in O2− 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.
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 ± 16%; 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 ± 6% and 33 ± 18%, n = 3). The positive effect was lost by 6 hours (2 ± 16%; 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 p67phox mRNA was seen at 1 hour and was lost by 6 hours (see Figure 7). Moreover,
Ang II can act at the vascular level to increase \( \text{O}_2^- \) production. Previous results showed that the adventitia is the aortic site of greatest \( \text{O}_2^- \) generation in rabbit aortic adventitia. Our previous results also showed that Ang II increases \( \text{O}_2^- \) production 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 \( \text{p67}^{\text{phox}} \) 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 \( \text{O}_2^- \) 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 activation is modulated at the transcriptional and translational level.

We have reported that Ang II causes a dose-dependent rise in fibroblast \( \text{O}_2^- \) production by NADPH oxidase to the highest effective dose is inhibited by [Sar\(^1\),Thr\(^3\)]-Ang II, yet not by losartan or PD123,19,21 We now report that none of the plausible metabolites of Ang II, ie, Ang III, Ang IV, or Ang(1-7),22-24 was as efficacious in promoting \( \text{O}_2^- \)-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.20 We recently found that fibroblasts cultured from the aortic adventitia contain a phagocyte-like NADPH oxidase activity21 and showed the immunohistochemical presence of all 4 major components of the phagocyte NADPH oxidase, p22, gp91, \( \text{p67}^{\text{phox}} \), and \( \text{p47}^{\text{phox}} \), in the adventitia. We also showed by immunoblotting the presence of \( \text{p67}^{\text{phox}} \) in particulate fractions and by immunoprecipitation the essential role of this subunit in particulate \( \text{O}_2^- \)-generating activity.21 By RT-PCR, we have isolated a partial sequence of the rabbit \( \text{p67}^{\text{phox}} \), an essential cytosolic component for NADPH oxidase from aortic 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 \( \text{O}_2^- \)-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 \( \text{p67}^{\text{phox}} \) mRNA level and measured NADPH oxidase activity is striking. Importantly, an increase in NADPH oxidase activity does not require a measurable increase in protein.21,31 Several groups have suggested that in phagocytic cells, the amount of \( \text{p67}^{\text{phox}} \) may be the limiting factor for the assembly and activation of the NADPH oxidase.9,32 The projected \( K_m \) for \( \text{p67}^{\text{phox}} \) is estimated to be approximately 3 times lower than that of another major cytosolic subunit, \( \text{p47}^{\text{phox}} \), in normal cytosol.32 In support of this hypothesis, 1 group has reported a 2:1 ratio between \( \text{p47}^{\text{phox}} \) and \( \text{p67}^{\text{phox}} \) in isolated complex.33 Based on these observations, one could hypothesize that the percent increase observed in \( \text{p67}^{\text{phox}} \) mRNA would be significantly higher with respect to NADPH oxidase activity.

At the most fundamental level, we present evidence for the presence of \( \text{p67}^{\text{phox}} \) (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 \( \text{p22}^{\text{phox}} \) and that it modulates Ang II–dependent \( \text{O}_2^- \) generation in rat aortic smooth muscle.19 However, epitopes for \( \text{p22}^{\text{phox}} \) are ubiquitous, based on the general presence of cytochromes such as \( b_{598} \), of which it is part, and homology between the \( \text{p22}^{\text{phox}} \) and mitochondrial cytochromes.34 In contrast, there have been no reports of \( \text{p67}^{\text{phox}} \) 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 (\( \text{p67}^{\text{phox}} \)) is essential for activity.21 Hence, it is unlikely that we have demonstrated a coincidental rise in \( \text{p67}^{\text{phox}} \) 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 NADPH oxidase in rat aorta, which exhibits elevations in NADPH oxidase activity coincident with transcriptional changes in
p22<sub>phox</sub> expression after several days of Ang II infusion. Moreover, the peak of p22<sub>phox</sub> mRNA in the rat aorta followed the peak in NAD(P)H oxidase activity, which is suggestive of a positive induction of p22<sub>phox</sub> by O<sub>2</sub><sup>−</sup> 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 p67<sub>phox</sub> on a Western blot of particulate fraction. We propose 3 explanations for this: (1) that the p67<sub>phox</sub> 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, p22<sub>phox</sub>, gp91<sub>phox</sub>, and p47<sub>phox</sub>, 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 p67<sub>phox</sub> 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 O<sub>2</sub><sup>−</sup> generation at the gene level. Due to the apparent presence of p67<sub>phox</sub> exclusively in NADPH oxidase, inhibition of NADPH oxidase may be better served by specific knockout of the p67<sub>phox</sub> protein rather than p22<sub>phox</sub>.


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