NAD(P)H Oxidase Mediates Angiotensin II–Induced Vascular Macrophage Infiltration and Medial Hypertrophy

Jianhua Liu, Fang Yang, Xiao-Ping Yang, Michelle Jankowski, Patrick J. Pagano

Objective—Our preliminary data suggested that angiotensin II (Ang II)–induced reactive oxygen species are involved in intercellular adhesion molecule-1 (ICAM-1) expression and leukocyte infiltration in the rat thoracic aorta. Other reports demonstrating reactive oxygen species–induced cell growth suggested a potential role of NAD(P)H oxidase in vascular hypertrophy. In the present study, we postulate that NAD(P)H oxidase is functionally involved in Ang II–induced ICAM-1 expression, macrophage infiltration, and vascular growth, and that oxidase inhibition attenuates these processes independently of a reduction in blood pressure.

Methods and Results—Rats were infused subcutaneously with vehicle or Ang II (750 μg/kg per day) for 1 week in the presence or absence of gp91 docking sequence (gp91ds)-tat peptide (1 mg/kg per day), a cell-permeant inhibitor of NAD(P)H oxidase. Immunohistochemical staining for ICAM-1 and ED1, a marker of monocytes and macrophages, showed that both were markedly increased with Ang II compared with vehicle and were reduced in rats receiving Ang II plus gp91ds-tat. This effect was accompanied by an Ang II–induced increase in medial hypertrophy that was attenuated by coinfusion of gp91ds-tat; however, gp91ds-tat had no effect on blood pressure.

Conclusions—Ang II–enhanced NAD(P)H oxidase plays a role in the induction of ICAM-1 expression, leukocyte infiltration, and vascular hypertrophy, acting independently of changes in blood pressure. (Arterioscler Thromb Vasc Biol. 2003;23:776-782.)

Key Words: NADPH oxidoreductase ■ NAD(P)H oxidase ■ hypertrophy ■ inflammation ■ angiotensin II

Angiotensin II (Ang II) has been shown to increase vascular adhesion molecule expression by stimulating the production of reactive oxygen species (ROS). Mediators of increased adhesion molecule expression include activation of nuclear factor-κB (NF-κB) and activator protein-1 transcription factors. Ang II has been implicated in vascular dysfunction, acting via a variety of mechanisms, including (1) stimulation of superoxide anion (O₂⁻) production by large and small blood vessels through activation of vascular NAD(P)H oxidases, leading to impairment of endothelial function; (2) induction of adhesion molecules, such as vascular cell adhesion molecule-1, via activation of NF-κB–dependent gene expression; and (3) hypertrophy and remodeling. A number of studies have shown that a phagocyte-like NAD(P)H oxidase is a major source of O₂⁻ in vascular tissue. Ang II stimulates NAD(P)H oxidase O₂⁻ production by neutrophils as well as the vascular endothelium, medial smooth muscle cells, and adventitial fibroblasts, and increases vascular mRNA levels of p22phox and p67phox. We reported upregulation of “anchoring” oxidase component gp91phox in aortas from Ang II–infused mice, and Lassègue et al recently demonstrated that Ang II can induce transcription of its homologue nox1 in vascular smooth muscle cells. It is well known that activation of NAD(P)H oxidase in neutrophils is triggered via protein kinase C–mediated phosphorylation of cytosolic p47phox, which then binds to membrane-associated gp91phox. Regardless of whether the anchoring component is gp91phox or another nox isoform, a similar process has been inferred in the vasculature.
human neutrophils, small peptide sequences of gp91phox, which are involved in the binding of gp91phox to p47phox, inhibit O$_2^-$ formation in cell-free assays. We selected the sequence found to be most potent in cell-free human neutrophil assays and then determined the corresponding sequence from the gp91phox rat and mouse clone, calling it gp91 docking sequence (gp91ds). Because we intended to deliver this peptide to either the whole animal or intact vessels, we linked it to a specific 9-amino-acid peptide of HIV viral coats (HIV-tat), which is known to be internalized by all cells and has been shown to deliver conjugated proteins after intravenous injection (gp91ds-tat). We have previously shown that gp91ds-tat is effective at attenuating Ang II–induced vascular O$_2^-$ and blood pressure elevation in the mouse.

In the present study, we examined the effects of endogenous NAD(P)H oxidase inhibition on Ang II–induced vascular inflammation and hypertrophy. We postulated that NAD(P)H oxidase inhibition attenuates these processes independently of a blood pressure–lowering effect.

Methods

Animals

Thirteen- to 16-week-old male Sprague-Dawley rats (Charles River Laboratories, Wilmington, Mass) were anesthetized with ketamine (80 mg/kg IP) and xylazine (7 mg/kg IP), and osmotic minipumps (Alza 2 ML1) were implanted for drug infusion. The present study was approved by the Henry Ford Hospital Institutional Animal Care and Use Committee.

Experimental Protocols

Infusion of Ang II and gp91ds-tat

Rats were divided into 3 groups for subcutaneous drug infusion with osmotic minipumps. Group 1 (n = 5) was infused with vehicle (saline with 0.01N glacial acetic acid) at a rate of 10 µL/h for 1 week. Group 2 (n = 7) was infused with Ang II (750 µg/kg SC per day, Bachem) dissolved in saline with 0.01N glacial acetic acid. Group 3 (n = 7) was infused with Ang II solution (750 µg/kg SC per day) containing gp91ds-tat (1 mg/kg SC per day) prepared as described previously.

SBP Measurement

Systolic blood pressure (SBP) was measured on day 0 (basal) and then every 2 days for 6 days using the standard tail-cuff method (ITC/Life Science Instruments).

Preparation of Tissue Samples

Tissue samples were prepared as described previously. Briefly, each animal was anesthetized with ketamine (80 mg/kg IP) and xylazine (7 mg/kg IP), given heparin (400 U IP per rat), and perfused with cold PBS (pH 7.4) via the left ventricle for 10 minutes. The aorta was removed and embedded in OCT compound, then immediately snap-frozen with liquid nitrogen and kept at −80°C until use.

Immunohistochemistry for ICAM-1, ED1, and HNE

Serial frozen sections (6 µm) of the thoracic aorta were fixed in aceton at −20°C for 30 minutes, followed by incubation in 0.5% H$_2$O$_2$ and 80% methanol for 30 minutes. The sections were incubated overnight at 4°C with primary antibody. Primary antibodies were monoclonal mouse anti-rat intercellular adhesion molecule (ICAM)-1 (1A29, Endogen), monoclonal mouse antibody against monocytes and macrophages (ED1, Accurate Chemical & Scientific Corp), and monoclonal mouse antibody against 4-hydroxy-2-nonenal (HNE) (OXIS). The secondary antibody was biotinylated horse anti-mouse IgG (Vector Laboratories). A Vectastain Elite ABC Kit (Vector Laboratories) was used according to the manufacturer’s protocol. A modified microwave method was used for antigen retrieval and background reduction. Cell nuclei were visualized by counterstaining with hematoxylin. The images were digitized and semiquantified using SigmaScan Pro 4.0 imaging software. For HNE quantification, 3 independent observers blinded to the study protocol scored the images.

Measurement of Vascular Hypertrophy

Sections were stained with hematoxylin. Cross-sectional area and radial thickness of the adventitia and media were measured using SigmaScan Pro 4.0. Cells were counted in 4 fields that encompassed the entire cross section of the aorta, and cell density (per 10 000 m$^2$) was determined (for detailed protocols, please see the online data supplement at http://atvb.ahajournals.org).

Statistical Analysis

All values are expressed as mean ± SEM, with n indicating the number of experiments in vivo. ANOVA was used to compare means among different factors. Where the factors were of a repeated nature, ANOVA with repeated measures was used. If an overall difference was found at the 0.05 level, appropriate multiple comparisons were used to determine whether pairwise differences existed. If unequal variance was observed, $t$ tests using Satterthwaite’s method were applied to calculate the probability value. In particular, Hochberg’s method of adjusting the $\alpha$ level was used to control the possibility of making an incorrect decision across the multiple tests.

Results

SBP Measurements

SBP was significantly increased in rats receiving Ang II compared with vehicle. There were no significant differences in blood pressure measurements between Ang II and Ang II+gp91ds-tat groups at any time point (Table 1). Body weight appeared to decrease during the treatment period in the Ang II–treated groups, but the difference was not significant at any time point between vehicle-treated, Ang II--
treated, and Ang II+gp91ds-tat–treated rats or within groups between days 0 and 7 (Table 1).

**ICAM-1 Expression**
Intense ICAM-1 staining was seen in aortic sections from rats receiving Ang II alone, primarily in the endothelium (Figure 1B); coinfusion of gp91ds-tat caused a visible reduction in ICAM-1 (Figure 1C). There was also some staining in the adventitia, which appeared to be primarily associated with small vessels (Figure 1B). In contrast, faint ICAM-1 detection was observed in the adventitia, which appeared to be primarily associated with small vessels (Figure 1B). (For the negative control of ICAM-1 immunostaining, please see online Figure IB, available at http://atvb.ahajournals.org.) Digital quantification showed a significant increase in aortic ICAM-1 expression with Ang II compared with vehicle; gp91ds-tat decreased significant lowering of staining by 50% (Figure 1D). Intimal ICAM-1 staining reached 85.1±6.6% in the Ang II–treated group (Figure 1E). There was no significant increase in medial (Figure 1F) or adventitial ICAM-1 staining in Ang II–treated versus vehicle-treated rats.

**Detection of Macrophages**
ED1, a marker for monocytes and macrophages, was markedly increased in rats receiving Ang II alone, particularly in the adventitia (Figure 2B), and was dramatically lower in rats treated with vehicle or gp91ds-tat (Figure 2A and 2C). (For negative control of ED1 immunostaining, please see online Figure IB.) Digital quantification showed a significant increase in aortic infiltration in Ang II–treated versus vehicle–treated rats and marked attenuation in rats treated with gp91ds-tat (Figure 2D). Infiltration was greatest in the adventitia, reaching 5.8±1.1% in Ang II–treated rats, and was reduced to the same extent by gp91ds-tat (Figure 2E). Likewise, medial ED1 tended to increase with Ang II versus vehicle treatment, whereas it appeared to decrease with gp91ds-tat treatment, but no statistical difference was observed (Figure 2F). No increase in intimal staining was observed.

**In Situ ROS Detection**
Immunostaining showed a significant increase in HNE in rat aortas infused with Ang II compared with vehicle (Figure 3B versus 3A; Figure 3D) in all segments of the aortic wall. In rats coinfused with Ang II and gp91ds-tat, HNE staining decreased significantly in all vascular segments (Figure 3C).
and 3D). (For negative control of HNE immunostaining, please see online Figure IC).

Vascular Hypertrophy
Hematoxylin staining revealed Ang II–induced medial growth, which was reduced by gp91ds-tat, with a similar but nonsignificant effect on the adventitia (Figure 4). Medial cross-sectional area and radial thickness were significantly increased in aortas from Ang II–treated versus vehicle-treated rats and were significantly decreased with gp91ds-tat; adventitial thickness also appeared higher in Ang II–treated rats, but the difference was not significant (Table 2).

Discussion
The present study suggests that Ang II–induced ICAM-1 expression and leukocyte infiltration in the rat aorta are mediated by NAD(P)H oxidase–derived ROS. Moreover, our findings offer evidence of NAD(P)H oxidase involvement in Ang II–induced vascular hypertrophy by demonstrating that oxidase inhibition reduces medial cross-sectional and thickness, primarily in the media. The immunohistochemical data demonstrated that Ang II–enhanced ROS levels were significantly reduced by gp91ds-tat infusion, supporting the functional role of NAD(P)H oxidase. Thus, these data suggest an important role for NAD(P)H oxidase in Ang II–induced vascular inflammation and hypertrophy and suggest for the first time that these changes may be independent of changes in blood pressure.

Numerous reports implicate Ang II–induced NAD(P)H oxidase O$_2^-$ in hypertension.Previously, we found that an NAD(P)H oxidase inhibitor at higher concentrations reduced Ang II–induced blood pressure elevation in mice. In fact, in previous studies in which NAD(P)H oxidase was implicated in vascular hypertrophy, this effect was not dissociated from an effect on blood pressure; ie, deletion of gp91$^{phox}$ significantly lowered blood pressure. In the present study, we observed no difference in SBP between rats treated with Ang II versus Ang II plus a 10-fold lower dose of the oxidase inhibitor gp91ds-tat. We believe that higher concentrations of the inhibitor would effectively lower blood pressure in the rat. However, in the present study, we were only interested in concentrations that had no effect on blood pressure. In our opinion, this represents an important difference between the present and other studies and may suggest that oxidase-induced vascular inflammation and hypertrophy are, in part, independent of blood pressure changes.

Vascular Inflammation
Studies have suggested a proinflammatory role for ROS in the vasculature, including increased adhesion molecule expression and chemoattraction of leukocytes. Indeed, ROS-activated NF-kB and activator protein-1 have been shown to increase the expression of adhesion molecules, and in vitro studies have suggested the involvement of NAD(P)H oxidase in this process.

Our present data suggest that ROS derived from vascular NAD(P)H oxidase are involved in Ang II–induced adhesion
molecule expression and macrophage infiltration in vivo, inasmuch as the NAD(P)H oxidase inhibitor gp91ds-tat significantly reduced the expression of Ang II–induced ICAM-1 and ED1 (a marker for macrophages) as well as Ang II–induced HNE. Total ICAM-1 staining across the aorta was significantly enhanced by Ang II and reduced by gp91ds-tat. ICAM-1 expression in the vascular endothelium clearly increased in response to Ang II, and this was partially but nonsignificantly reduced by gp91ds-tat, supporting the concept that vascular ICAM-1 expression is primarily endothelial.35,44 These data appear to suggest that NAD(P)H oxidase(s) in the endothelium mediates ICAM-1 expression and thus macrophage infiltration. The finding that macrophage infiltration decreased more than ICAM-1 expression in response to gp91ds-tat (Figure 2 versus Figure 1) may suggest that other redox-sensitive mediators of infiltration are involved in this response, including vascular cell adhesion molecule-1 and monocyte chemotactic protein-1.45,46 Interestingly, although Ang II–induced ICAM-1 expression was most visible in the aortic endothelium, as reported previously macrophage infiltration was greatest in the adventitia.4 A possible explanation for this finding is that the macrophages had infiltrated from small adventitial vessels of the vasa vasorum directly into the adventitia.

The ability of gp91ds-tat to inhibit NAD(P)H oxidase and its specificity for this class of enzymes have been reported previously.35,46 Our present findings are consistent with gp91ds-tat decreasing NAD(P)H oxidase–derived O$_2^-$.47 Immunohistochemical analysis of HNE, a marker of ROS production,47 indicated a general increase in ROS across the vascular wall in response to Ang II, which was substantially reduced by gp91ds-tat. These data are consistent with widespread enhancement of the contribution of vascular NAD(P)H oxidase–derived ROS to inflammation and hypertrophy. In fact, O$_2^-$ and H$_2$O$_2$, the 2 major ROS derived from vascular NAD(P)H oxidases,6,48–51 are capable of promoting peroxidation of cellular lipids.52 Thus, regardless of the particular ROS produced by vascular NAD(P)H oxidase, our data indicate a general increase in Ang II–induced NAD(P)H oxidase–derived ROS in these studies. Moreover, H$_2$O$_2$ and O$_2^-$ are both described as ROS mediators of redox-sensitive signaling leading to vascular growth.53

**Medial Hypertrophy**

In cultured rat aortic smooth muscle cells, NAD(P)H oxidase ROS are stimulated by Ang II and are involved in the hypertrophic response7,54 as well as the development of hypertension.7,55 Recently, Wang et al56 showed that deletion of gp91$^\text{phox}$ leads to decreased medial hypertrophy in the mouse aorta. However, there has been no other investigation, to our knowledge, indicating that the same mechanisms are viable independent of blood pressure changes in vivo. The present study showed a significant increase in medial cross-sectional area with Ang II infusion and a significant decrease in area with gp91ds-tat treatment concomitant with reductions in ROS. Likewise, medial thickness increased significantly in Ang II–treated versus vehicle-treated rats, and nuclei also appeared larger in medial cells from Ang II–treated versus vehicle-treated rats (not shown), consistent with cellular hypertrophy; similarly, increases in thickness were reversed by gp91ds-tat cotreatment. Measurements of cell density indicated no change in response to Ang II or Ang II+gp91ds-tat, suggesting expansion and contraction of the vascular media in response to cellular hypertrophy. Inasmuch as very few macrophages were detected in the media, the observed increase in medial thickness is most likely related largely to smooth muscle cell hypertrophy. In fact, on histological examination the cells undergoing hypertrophy appeared to be primarily smooth muscle cells. However, at this time we cannot rule out the possibility of a small contribution by an inflammatory cell infiltrate besides macrophages and neutrophils.

Overall, our findings indicate for the first time that NAD(P)H oxidase–derived ROS partially mediate Ang II–induced inflammation and hypertrophy in the rat aorta and that these changes do not appear to depend on changes in blood pressure. A ROS-mediated paracrine effect between various vascular segments has been reported,16,53,56 suggesting that the endothelial and adventitial ROS from vascular cells and particularly macrophages may contribute to the process of medial hypertrophy. However, future studies examining the temporal relationship between oxidase upregulation, inflammation, and hypertrophy will be necessary to carefully examine these interactions.

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**References**


Liu et al. NAD(P)H Oxidase, Inflammation, and Hypertrophy 781


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Detailed Methods (Online)

Immunohistochemistry

Serial frozen sections (6 µm) of the thoracic aorta were placed on Superfrost Plus slides (VWR) and stored at –20°C. They were fixed in acetone at –20°C for 30 min and then incubated in 0.3% hydrogen peroxide in 80% methanol for 30 min. Following this, they were blocked with 5% normal horse serum for 30 min, then incubated overnight at 4 °C with primary antibody (1:200 dilution for ICAM-1, 1:500 for ED-1, 10µg/ml for HNE in PBS containing 1% normal serum). Sections were incubated with biotinylated secondary antibodies for 30 min, and then with ABC reagent for 30 min (Vector). The reaction was visualized using diaminobenzidine tetrahydrochloride (DAB) (Vector Laboratories). Sections were counterstained with hematoxylin (Sigma). A modified method utilizing microwave technique\textsuperscript{1} was performed. Briefly, after fixation in acetone, the slides were microwaved (1000 W) in 0.01 M citric acid buffer (pH 6.0) for 6 minutes and cooled at room temperature for 30 minutes before proceeding to the next step. This step is necessary to significantly reduce the high non-specific staining caused by cross-reaction of anti-mouse IgG on rat tissues. Cell nuclei were visualized with hematoxylin staining.

Quantification of immunostaining

For ICAM-1 and ED1, 4 fields from one random section from each rat aorta were analyzed. The positively stained area (or cells) was measured using SigmaScan Pro 4.0 imaging software (SPSS Science), allowing quantification of expression and infiltration, respectively. ICAM-1- and ED1-positive staining were calculated as a percentage of the total vessel cross-section, the intima, media and adventitia. The intima is defined as the area between the lumen and internal
elastic lamina; the media as the area between the internal and external elastic lamina; the adventitia as the cellular area between the external elastic lamina and the adipose tissue.

For HNE, 4 fields from one random section of rat aorta were observed by three experienced observers in a blind fashion. Area and intensity of staining was scored semi-quantitatively from 0 to 4 as follows: 0, no visible staining; 1, faint staining; 2, moderate staining; 3, strong staining; 4, intense diffuse staining.

REFERENCE

Negative controls (A-C) for immunostaining of ICAM-1, ED1, and HNE, respectively. Original magnification ×400; scale bar = 20 μm.