Reduced NAD(P)H Oxidase in Low Renin Hypertension
Link Among Angiotensin II, Atherogenesis, and Blood Pressure

Ming-Sheng Zhou, Ivonne Hernandez Schulman, Patrick J. Pagano, Edgar A. Jaimes, Leopoldo Raij

Abstract—Endothelial dysfunction (ED) complicates hypertension and is a precursor of atherosclerosis. Reduced NO bioactivity, because of increased reduced NAD(P)H oxidase–derived reactive oxygen species (ROS), plays a critical role in ED. gp91phox, predominantly expressed in the endothelium and adventitia, is a subunit of NAD(P)H oxidase important for its activation in response to angiotensin (Ang) II. Human atherosclerotic plaques are heavy laden with gp91phox. We have shown that in Dahl salt-sensitive (DS) rats, a paradigm of low renin salt-sensitive (SS) hypertension in humans, Ang II receptor blockade normalizes ROS production and endothelium-dependent relaxation (EDR) without significantly affecting systolic blood pressure (SBP). To additionally elucidate the mechanisms involved in the functional association of Ang II in SS hypertension, we administered a cell-permeable inhibitor of the assembly of p47phox with gp91phox in NAD(P)H oxidase, gp91ds-tat (10 mg/kg body weight, 3 weeks by minipump), to DS rats fed a 4% salt diet. Control rats received either vehicle or an inactive scramb-tat peptide. Vehicle-treated DS developed hypertension (SBP 168/110 mm Hg), left ventricular hypertrophy (LVH), proteinuria, impaired EDR, and increased aortic ROS production (superoxide 115% and peroxynitrite 157%) and expression of the proatherogenic molecules LOX-1 (130%) and MCP-1 (166%). gp91ds-tat, but not scramb-tat, normalized ROS and EDR, as well as LOX-1 and MCP-1, despite nonsignificant effects on SBP (159/110 mm Hg; P>0.05), left ventricular hypertrophy, and proteinuria. Our findings support the notion that in SS hypertension, activation of NAD(P)H oxidase promotes ED and atherogenesis via decreased nitric oxide bioactivity and increased LOX-1 and MCP-1, independent of blood pressure. (Hypertension. 2006;47:81-86.)

Key Words: endothelium ■ sodium ■ hypertension ■ free radicals ■ atherosclerosis

Accumulating evidence suggests that an imbalance between vasoactive factors, such as angiotensin (Ang) II and NO, promotes vascular inflammation and atherogenesis.1-2 Activation of vascular reduced NAD(P)H oxidase–derived reactive oxygen species (ROS) by Ang II may be a key factor in this process.3-5 Increased ROS production by Ang II contributes to the pathogenesis of vascular diseases by enhancing vascular smooth muscle cell growth, stimulating the synthesis of proinflammatory proteins, and inactivating NO and transforming it into the pro-oxidant species peroxynitrite (ONOO-).3-5

Hypertension is a well-established risk factor for the development and acceleration of atherosclerosis. Clinical studies have demonstrated that up to 50% of hypertensives can be reproducibly classified as salt sensitive (SS).6 SS hypertensive subjects display a cluster of cardiovascular risk factors associated with the development of atherosclerosis, including a higher incidence of endothelial dysfunction (ED), insulin resistance, and microalbuminuria.7 SS hypertension has traditionally been considered a “volume-dependent hypertension,” in which the role of Ang II was presumed to be inconsequential because of low-plasma renin level concentration. However, studies in Dahl SS (DS) rats, a paradigm of low-renin SS hypertension in humans, have shown that SS hypertension is accompanied by activation of the local tissue Ang system.8-10 Our recent studies, and those of others, support the notion that low-renin SS hypertension is a specific vascular diathesis characterized by decreased NO bioavailability, which is manifested by impaired NO-mediated vascular relaxation and upregulation of Ang II action at the local tissue level, as evidenced by ED and increased ROS synthesis that are normalized by Ang II type 1 receptor blockade.8,11

gp91phox (Nox2) is a subunit of NAD(P)H oxidase that is principally located in the endothelium and adventitia and is important for activation of this enzyme in response to Ang II.12-15 In the present study we used gp91ds-tat, a cell-permeable, specific inhibitor of the assembly of p47phox with...
gp91\textsuperscript{phox} in NAD(P)H oxidase,\textsuperscript{16,17} to investigate in DS rats in vivo the role of NAD(P)H oxidase–derived ROS in the modulation of blood pressure and the upregulation of the proinflammatory and proatherogenic molecules monocyte chemoattractant protein-1 (MCP-1)\textsuperscript{18} and lectin-like oxidized low-density lipoprotein (LDL) receptor-1 (LOX-1).\textsuperscript{19} We hypothesized that these studies would provide additional understanding of the mechanisms that link hypertension and atherogenesis.

**Methods**

**Animals and Experimental Protocols**

We studied separate groups of 6-week-old DS rats (Brookhaven strain, Harlan) during the prehypertensive (5 days) and hypertensive (3 weeks) phases of a high-salt (HS, 4% NaCl) diet (Figure 1).\textsuperscript{11} All of the rats underwent implantation of a minipump containing gp91ds-tat [cell-permeable inhibitor of the assembly of p47\textsuperscript{phox} with gp91\textsuperscript{phox} in NAD(P)H oxidase],\textsuperscript{16} scramb-tat (a peptide control),\textsuperscript{16} or saline. The DS rats studied during the prehypertensive phase (5 days) were divided into 4 groups: NS, DS rats fed a normal sodium (NS, 0.5% NaCl) diet (n=6); HS, DS rats fed a HS diet (n=6); HS/gp91, DS rats fed a HS diet plus gp91ds-tat (10 mg/kg per day, SQ by minipump, n=5); and HS/scramb, DS rats fed a HS diet plus scramb-tat (10 mg/kg per day, SQ by minipump, n=5). Rats studied during the hypertensive phase were fed either a NS (n=6) or HS diet for 5 days; thereafter, HS rats were divided into 3 groups and given gp91ds-tat (HS/Gp91, n=5), scramb-tat (HS/scramb, n=5), or saline (HS, n=6) for 14 days. Systolic blood pressure (SBP) was measured by the tail-cuff method.\textsuperscript{11} On the twelfth day, the mice underwent implantation of a minipump containing Ang II (0.75 mg/kg per day) or saline for 12 days. SBP was measured in conscious mice by tail-cuff 1 day before, and on the fifth and twelfth day after implantation of the minipump. On the twelfth day, the mice were anesthetized with a mixture of ketamine (30 mg/kg per day), xylazine (6 mg/kg), and acepromazine (1 mg/kg) given IP. The right carotid artery was cannulated with polyethylene tubing (PE-10). The tubing was connected to a pressure transducer and mean arterial pressure (MAP) recorded by Powerlab. After a 30-minute equilibration period, MAP was continuously measured for 60 minutes.

**Determination of Plasma Renin Concentration**

Plasma (50 μL) was incubated with sheep angiotensinogen in buffer [0.1 mol/L sodium phosphate, 0.02 mol/L sodium EDTA, and 0.05% PMSF (pH 6.5)] at 37°C for 2 hours. The incubation was stopped by boiling in water for 10 minutes, the mixture centrifuged at 1680 g for 15 minutes, and the supernatants stored at −20°C until assayed. Generated Ang I was measured with a clinical RIA kit (DiaSorin), and the results were expressed as Ang I ng/mL plasma per hour.

**Detection of Aortic O$_2^-$ and ONOO$^-$ Generation**

The levels of O$_2^-$ and ONOO$^-$ in intact aortic rings were determined using lucigenin (5 μmol/L) and luminol (50 μmol/L) enhanced chemiluminescence, respectively, as described previously.\textsuperscript{11,20}

**Organ Chamber Experiments**

Endothelial function was examined in aortic rings in an organ chamber, as described previously.\textsuperscript{11,21,22} Endothelium-dependent relaxation (EDR) in response to acetylcholine (10$^{-9}$ to 10$^{-7}$ mol/L) was studied in aortic rings precontracted to 70% of maximal contraction to norepinephrine in the presence or absence of N-nitro-1-arginine methyl ester (l-NAME, 100 μmol/L), an NO synthase inhibitor.

**LOX-1 and MCP-1 mRNA Expression**

Total RNA (2 μg) was extracted from rat aorta and reverse transcribed using the SuperScript II RT First Strand Synthesis kit (Gibco, BRL), according to the manufacturer’s directions, as described previously.\textsuperscript{20} Using TagDNA polymerase (Gibco, BRL) and a primer pair specific to rat LOX-1 or MCP-1, 2.5 μL of product were amplified by PCR. The PCR product was visualized on a 1.5% agarose gel containing ethidium bromide and quantified using a GAPDH mRNA reference and Imagequant software.

**Determination of Blood Pressure in Wild-Type and gp91\textsuperscript{phox(-/-)} Mice**

Male gp91\textsuperscript{phox(-/-)} and C57BL/6J control mice, 8 weeks of age, were obtained from Jackson Laboratories (Bar-Harbor, ME). All of the mice underwent implantation of a minipump containing Ang II (0.75 mg/kg per day) or saline for 12 days. SBP was measured in conscious mice by tail-cuff 1 day before, and on the fifth and twelfth day after implantation of the minipump. On the twelfth day, the mice were anesthetized with a mixture of ketamine (30 mg/kg per day), xylazine (6 mg/kg), and acepromazine (1 mg/kg) given IP. The right carotid artery was cannulated with polyethylene tubing (PE-10). The tubing was connected to a pressure transducer and mean arterial pressure (MAP) recorded by Powerlab. After a 30-minute equilibration period, MAP was continuously measured for 60 minutes.

**Data Analysis**

Relaxation of aortic rings was expressed as a percentage inhibition of norepinephrine-induced constriction. The maximal response to acetylcholine and the concentration of acetylcholine required for a half-maximal response were determined from the concentration–response curve, using the best fit to a logistic sigmoid function. Statistical analyses were performed by ANOVA (Stat-View). Bonferroni and Scheffe’s tests for multiple comparisons were used.

**Experimental Protocol**

<table>
<thead>
<tr>
<th>Groups</th>
<th>NS</th>
<th>HS</th>
<th>HS/gp91</th>
<th>HS/scramb</th>
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<tbody>
<tr>
<td>Prehypertensive DS Rats</td>
<td>NS for 5 days</td>
<td>HS for 5 days</td>
<td>HS plus gp91 ds-tat for 5 days</td>
<td>HS plus scramb-tat for 5 days</td>
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<tr>
<td>Hypertensive DS Rats</td>
<td>NS for 3 weeks</td>
<td>HS for 3 weeks</td>
<td>HS for 5 days then switched to HS + gp91ds-tat for 3 weeks</td>
<td>HS for 5 days then switched to HS + scramb-tat for 3 weeks</td>
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NS: Normal salt (0.5% NaCl) control diet, HS: High salt (4% NaCl) diet
Scramb-tat is a control peptide without inhibitory effect on NAD(P)H oxidase
pllected inhibited EDR to acetylcholine in both the HS and NS groups of rats (Figure 3B), indicating that impaired EDR to acetylcholine in aortic rings of hypertensive DS rats is because of impaired NO bioavailability.

mRNA Expression of LOX-1 and MCP-1 in Aortic Rings

Both LOX-1 and MCP-1 mRNA levels, determined by RT-PCR, were significantly higher (by 130% and 166%, respectively) in DS rats in the hypertensive phase, compared with NS control. Treatment with gp91ds-tat, but not scramb-tat, normalized both LOX-1 and MCP-1 mRNA levels (Figure 4), despite not modifying SBP.

Blood Pressure Response to Ang II in Wild-Type and gp91phox(−/−) Mice

To additionally elucidate the role of ROS in the development of hypertension, we investigated the blood pressure

<table>
<thead>
<tr>
<th>Characteristics of Experimental Animals in DS Rats</th>
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<tr>
<td>Characteristic</td>
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<tr>
<td>------------------------------------------</td>
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<tr>
<td>Prehypertensive stage (5 days)</td>
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<tr>
<td>SBP (mm Hg)</td>
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<td>LV weight (mg/100 g BW)</td>
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<td>Hypertensive stage (3 weeks)</td>
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<td>SBP (mm Hg)</td>
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<tr>
<td>LV weight (mg/100 g BW)</td>
</tr>
<tr>
<td>Proteinuria (mg/24 h)</td>
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<tr>
<td>EDR to acetylcholine</td>
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<tr>
<td>Emax (%</td>
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Emax indicates maximal response to acetylcholine; ED50, the concentration causing half maximal response to acetylcholine. n = 5 to 6.

*P<0.05 vs HS.
†P<0.05 vs HS/scramb.

where appropriate for post-hoc analyses. Results were considered significant when P<0.05.

Results

Plasma Renin Concentration in DS Rats

Plasma renin concentration was 1.42±0.22 ng/mL per hour in hypertensive DS rats and 2.0±0.16 ng/mL per hour in normotensive DS rats (P<0.05), confirming that the DS rat is a model of low-plasma renin hypertension.

SBP, Left Ventricular Hypertrophy, and Urinary Protein Excretion

High-salt intake for 5 days (prehypertensive phase) did not affect SBP or left ventricular weight-to-body weight ratio (LVW/BW), as compared with normal salt control (Table). On the other hand, in a separate group of animals, high-salt intake for 3 weeks (hypertensive phase) significantly increased SBP, LVW/BW ratio, and proteinuria. Neither treatment with gp91ds-tat nor with scramb-tat resulted in a significant reduction in SBP, LVW/BW ratio, or proteinuria in either phase (Table).

Vascular O2− and ONOO− Generation

High-salt intake for 5 days significantly increased O2− and ONOO− levels in aortic rings without significantly elevating SBP (Figure 2A). The levels of O2− and ONOO− increased to a greater extent during the hypertensive phase of DS rats fed high-dietary salt (Figure 2B). Treatment with gp91ds-tat prevented the increase in aortic O2− and ONOO− during both phases (Figure 2B), whereas treatment with scramb-tat did not affect the production of O2− or ONOO− in either phase.

EDR to Acetylcholine in Aortic Rings

We confirmed our previous findings that during the prehypertensive phase, the EDR response to acetylcholine remains unaffected, whereas during the hypertensive phase, the EDR response to acetylcholine is significantly impaired.11 Treatment with gp91ds-tat, but not scramb-tat, normalized the impaired EDR to acetylcholine in hypertensive DS rats (Figure 3A and Table). Preincubation with L-NAME completely inhibited EDR to acetylcholine in both the HS and NS groups of rats (Figure 3B), indicating that impaired EDR to acetylcholine in aortic rings of hypertensive DS rats is because of impaired NO bioavailability.

Blood Pressure Response to Ang II in Wild-Type and gp91phox(−/−) Mice

To additionally elucidate the role of ROS in the development of hypertension, we investigated the blood pressure

Figure 2. Superoxide (O2−) generation determined by chemiluminescence of lucigenin and peroxynitrite (ONOO−) generation determined by luminol-enhanced chemiluminescence in aortic rings of prehypertensive (5 days, A) and hypertensive DS rats (3 week, B). NS: normal salt diet; HS: high-salt diet; HS/gp91: high-salt diet plus gp91ds-tat treatment for 5 days or high-salt diet for 5 days, then adding gp91ds-tat treatment for 2 weeks; HS/scramb: high-salt diet plus scramb-tat treatment for 5 days or high-salt diet for 5 days, then adding scramb-tat treatment for 2 weeks. Data were expressed as mean±SE. *P<0.05 vs NS and HS/gp91 groups (n = 5 to 6).
response to Ang II in gp91phox(−/−) and wild-type (WT) mice. It has been demonstrated that Ang II increases vascular O$_2^-$ production in WT but not in gp91phox(−/−) mice. Baseline SBP measured by the tail-cuff method showed no significant difference between gp91phox(−/−) and C57BL/6J WT mice (116±5 mm Hg versus 118±7 mm Hg; P>0.05). Ang II increased SBP from 115±7 to 149±5 mm Hg on the fifth day and 150±2 mm Hg on the twelfth day in gp91phox(−/−) mice and from 117±4 mm Hg to 146 mm Hg on the fifth day and 152±7 mm Hg on the twelfth day in WT mice (Figure 5A). The results were additionally confirmed by intraarterial measurement of MAP. Ang II infusion for 12 days increased MAP by 30% in gp91phox(−/−) mice and by 35% in WT mice (Figure 5B).

**Discussion**

The major new findings in this study are that, in a model of low-renin SS hypertension, activation of vascular NAD(P)H oxidase contributes to the genesis of ED and upregulation of proatherogenic molecules, namely, MCP-1 and LOX-1. Of note, these proatherogenic vascular effects mediated by NAD(P)H oxidase-derived O$_2^-$ are largely independent of the hemodynamic stress of high blood pressure.

ED is an early risk factor for the development of atherosclerosis. Ang II plays a critical role in the generation of oxidative stress leading to ED. Ang II–induced production of ROS is accomplished through activation of vascular NAD(P)H oxidase, which is the main source of O$_2^-$ in endothelial cells. We have demonstrated previously that the ED in DS rats was because of NAD(P)H oxidase activation. Vascular ROS generation and endothelial function were normalized by Ang II type 1 receptor blockade independent of blood pressure (Figure 6), suggesting an upregulation of Ang II action at the local tissue level. gp91phox (Nox2) is a subunit of NAD(P)H oxidase that plays a critical role in the activation of this enzyme by Ang II in the endothelium and adventitia but not in the aortic or resistance artery vascular smooth muscle of the rat. It has been reported that vascular smooth muscle cells derived from human resistance arteries do express gp91phox, whereas human aortic vascular smooth muscle cells do not. Human atherosclerotic plaques are heavy laden with gp91phox but not with Nox4, a subunit of NAD(P)H oxidase that is active primarily in vascular smooth muscle.

Experimental studies have demonstrated previously that gp91ds-tat, a cell-permeable inhibitor of the assembly of p47phox with gp91phox in NAD(P)H oxidase, inhibited Ang II–induced aortic O$_2^-$ production in vivo and NAD(P)H oxidase–derived O$_2^-$ production both in endothelial cells and adventitial cells. In the present study, DS rats were administered gp91ds-tat to study the contribution of ROS, predominantly originating from gp91phox-containing NAD(P)H oxidase, in the modulation of endothelial function, expression of the proinflammatory/proatherogenic molecules LOX-1 and MCP-1, and blood pressure. Here we demonstrated the following: (1) in vivo administration of gp91ds-tat, but not scramb-tat, inhibited the increase in aortic O$_2^-$ and ONOO$^-$ generation, as well as the development of ED; and (2) inhibition of NAD(P)H oxidase with gp91ds-tat prevented the transcriptional upregulation of LOX-1 and MCP-1.

Atherosclerosis is associated with vascular inflammation and increased ROS generation. LOX-1, initially identified as the major receptor for oxidized LDL in endothelial cells, has a pivotal role in the pathogenesis of atherosclerosis. The mechanisms by which this receptor contributes to the atherosclerotic process is through uptake of oxidized LDL into endothelial cells, smooth muscle cells, and macrophages, decrease in NO production, increase in inflammatory cell recruitment, and increase in smooth muscle cell proliferation. In vivo, the expression of LOX-1 is enhanced in proatherogenic settings, such as hypertension, hyperlipidemia, and oxidized LDL.
diabetes and, indeed, is accumulated in atherosclerotic lesions of human carotid arteries and glomerulosclerotic lesions.\(^\text{19,30}\)

MCP-1 is another proatherogenic molecule that promotes chronic vascular inflammation via recruitment and activation of monocytes, which play a pivotal role in the development of atherosclerosis.\(^\text{18,31}\) Endothelial cells, vascular smooth muscle cells, and leukocytes present at the sites of vascular injury produce MCP-1.\(^\text{18}\) Oxidative stress, oxidized inflammatory lipids, and Ang II have been reported to increase MCP-1 expression.\(^\text{18}\) Mice lacking MCP-1 display reduced initial atheroma formation.\(^\text{32}\) Additionally, a recent clinical study in a large cohort of patients with acute coronary syndromes indicated that an increased baseline level of MCP-1 is associated with both traditional risk factors for atherosclerosis, as well as an increased risk for death or myocardial infarction, independent of baseline variables.\(^\text{33}\)

In our study, we also found that gp91ds-tat administration was not associated with a significant reduction in blood pressure. We additionally confirmed the lack of effect of NAD(P)H oxidase inhibition with gp91ds-tat on blood pressure in parallel studies of gp91\(^{phox(-/-)}\) and WT mice infused with Ang II for 2 weeks. Indeed, direct intraarterial blood pressure measurements showed that gp91\(^{phox(-/-)}\) and WT mice developed a similar degree of hypertension in response to Ang II infusion, suggesting dissociation between NAD(P)H oxidase-derived oxidative stress and elevation of blood pressure. These findings in gp91\(^{phox(-/-)}\) mice concur with those reported from another laboratory.\(^\text{15}\) Therefore, our results suggest that, in SS hypertension, activated vascular NAD(P)H oxidase drives the upregulation of proinflammatory/proatherogenic molecules in association with, but independent of, the hemodynamic stress of high blood pressure.

We found that neither LVH nor proteinuria was mitigated by NAD(P)H oxidase inhibition with gp91dstat. These findings support the notion that, in low-renin SS hypertension, the participation of other sources of ROS and/or hemodynamically related factors are more operative in the development of renal injury and LVH.\(^\text{34}\)

**Perspectives**

Experimental and clinical studies using angiotensin-converting enzyme inhibitors and Ang II receptor blockers have provided indirect evidence supporting the role of oxidative stress in the pathogenesis of ED and atherogenesis.\(^\text{11,17,35}\) In the current studies, we present evidence, to our knowledge for the first time, that in low-renin SS hypertension, selective inhibition of NAD(P)H oxidase normalizes endothelial function, vascular oxidative stress, and vascular LOX-1 and MCP-1 expression, without significantly lowering blood pressure, LVH, or proteinuria. In conjunction with our previous studies, these findings support the notion, that in low-renin SS hypertension, the participation of other sources of ROS and/or hemodynamically related factors are more operative in the development of renal injury and LVH.\(^\text{34}\)
Acknowledgments

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