Angiotensin II-Dependent Chronic Hypertension and Cardiac Hypertrophy Are Unaffected by gp91phox-Containing NADPH Oxidase

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Abstract—The gp91phox-containing NADPH oxidase is the major source of reactive oxygen species (ROS) in the cardiovascular system and inactivation of gp91phox has been reported to blunt hypertension and cardiac hypertrophy seen in angiotensin (Ang) II-infused animals. In the current study, we sought to determine the role of gp91phox-derived ROS on cardiovascular outcomes of chronic exposure to Ang II. The gp91phox-deficient mice were crossed with transgenic mice expressing active human renin in the liver (TTRhRen). TTRhRen mice exhibit chronic Ang II–dependent hypertension and frank cardiac hypertrophy by age 10 to 12 weeks. Four genotypes of mice were generated: control, TTRhRen transgenics (TTRhRen), gp91phox-deficient (gp91/H11002), and TTRhRen transgenic gp91phox-deficient (TTRhRen/gp91/H11002). Eight to 10 mice/group were studied. ROS levels were significantly reduced (P<0.05) in the heart and aorta of TTRhRen/gp91/H11002 and gp91/H11002 mice compared with control counterparts, and this was associated with reduced cardiac, aortic, and renal NADPH oxidase activity (P<0.05). Systolic blood pressure (SBP), cardiac mass, and cardiac fibrosis were increased in TTRhRen versus controls. In contrast to its action on ROS generation, gp91phox inactivation had no effect on development of hypertension or cardiac hypertrophy in TTRhRen mice, although interstitial fibrosis was reduced. Cardiac and renal expression of gp91phox homologues, Nox1 and Nox4, was not different between groups. Thus, although eliminating gp91phox-associated ROS production may be important in cardiovascular consequences in acute insult models, it does not prevent the development of hypertension and cardiac hypertrophy in a model in which the endogenous renin-angiotensin system is chronically upregulated. (Hypertension. 2005;45:530-537.)

Key Words: angiotensin ■ blood pressure ■ fibrosis ■ mice

Activation of the renin-angiotensin-aldosterone system plays a major pathophysiological role in the genesis of hypertension and cardiac hypertrophy.1,2 Processes underlying these phenomena have been attributed, in part, to increased generation of reactive oxygen species (ROS), particularly superoxide (O2−) and hydrogen peroxide (H2O2),3,4 which function as important second messengers.5,6 The major source of Ang II-stimulated ROS generation in the cardiovascular system is NADPH oxidase. All vascular cell types as well as cardiomyocytes and cardiac fibroblasts express components of the prototypical NADPH oxidase, including its cell membrane-associated subunits, p22phox, gp91phox (Nox2), or Nox2 homologues (Nox1 and Nox4), and cytosolic subunits, p47phox, p67phox, and p40phox.7,8 The functional significance of each component has not yet been fully elucidated, but p47phox plays an important role in cytosolic subunit translocation and in initiation of NADPH oxidase assembly.9,10 The gp91phox is the catalytic subunit and the major membrane component to which the p47-p67-p40phox complex binds.9,10 Ang II-mediated -O2− generation requires functionally active gp91phox, p22phox, p47phox, and p67phox because downregulation or absence of these subunits results in attenuated or abrogated Ang II–induced cell growth, contraction, and inflammation,11,12 whereas up-regulation has been associated with enhanced effects.13 The importance of NADPH oxidase in processes associated with Ang II–mediated hypertension and cardiac hypertrophy has also been demonstrated in Ang II–infused animal models. Inhibition of NADPH oxidase activity with apocynin or gp91ds-tat, a chimeric peptide inhibitor that interferes with assembly of vascular NAD(P)H oxidase components, attenuates blood pressure increase, regresses cardiac and vascular remodeling, and improves endothelial function in Ang II–infused rodents.14,15 In p47phox-deficient mice, Ang II fails to induce cardiac hypertrophy16 and blood pressure elevation is blunted.17 In gp91phox-deficient mice, basal blood pressure is lower than wild-type counterparts,18 and subpressor doses of Ang II infused over 2 weeks fail to induce cardiac hypertension.
hypertrophy.\textsuperscript{19} However, these studies were performed in models in which high Ang II concentrations were infused over a short (2 weeks) period and during which time blood pressure increase and cardiovascular remodeling occurred relatively acutely. The importance of NADPH oxidase in models of chronic Ang II–dependent cardiovascular disease, in which Ang II levels are only modestly increased, and which represent a better clinical correlate than acute Ang II–mediated hypertension, remains unclear.

Here, we sought to determine whether gp91phox-containing NADPH oxidase plays a role in the development of hypertension and cardiac hypertrophy in a model in which the endogenous renin-angiotensin system is chronically up-regulated. We previously generated transgenic mice that produce human active renin in the liver under the control of the transthyretin promoter.\textsuperscript{20} These mice, termed TTRhRen, have slightly elevated plasma Ang II levels (1- to 2-times normal), are chronically hypertensive and have frank cardiac hypertrophy by 10 to 12 weeks of age.\textsuperscript{20} TTRhRen transgenic mice were bred with gp91phox-deficient mice to generate gp91phox-deficient mice with a chronic Ang II–mediated cardiovascular insult.

**Materials and Methods**

**Mice**

The study was approved by the Animal Ethics Committee of the Clinical Research Institute of Montreal. The generation of the TTRhRen mice was described previously.\textsuperscript{20} Briefly, the human prorenin cDNA was expressed primarily in the liver of transgenic mice under control of a 3-kb region of the transthyretin gene promoter. To generate active human renin, a cleavage site for the ubiquitous protease furin was inserted at the juncture of the prosegment and the active renin molecule, resulting in prostate removal by endogenous proteases in the secretory pathway of expressing cells. TTRhRen mice are chronically hypertensive and exhibit frank cardiac hypertrophy by 10 to 12 weeks of age. TTRhRen-A3 line was originally derived in the FVB strain of mice, but was transferred by back-crossing into the C57BL/6J strain. The gp91phox-deficient mice were purchased from the Jackson Laboratory (Bar Harbor, Me) and were maintained from the F5 back-cross generation and are referred to as TTRhRen.

The gp91phox-deficient mice (and all other mice) were purchased from the Jackson Laboratory (Bar Harbor, Me) and were maintained in the C57BL/6J strain. The gp91phox gene is on the X chromosome. For that reason, males are not actually X\(^{+}\), they are hemizygous (X\(^{+}\)/Y).

The 10- to 12 week-old male mice that were either heterozygous for the TTRhRen transgene or hemizygous for the gp91phox deletion or both and control littermates were used for all of the described experiments. The genotype of cross-bred mice was verified by polymerase chain reaction analysis of tail DNA biopsy specimens. Four genotypes of mice were generated: control, TTRhRen transgenic (TTRhRen), gp91phox-deficient (gp91\(^{-}\)), and TTRhRen transgenic gp91phox-deficient (TTRhRen/gp91\(^{-}\)). Eight to 10 mice were studied/genotyped.

**Measurement of Blood Pressure and Cardiac Hypertrophy**

Systolic blood pressure was measured by tail-cuff plethysmography (model BP-2000; Visitech Systems, Apex, NC) as previously described.\textsuperscript{20} Briefly, mice were trained to the apparatus for 7 continuous days and measurements were recorded only for the last 3 days. Cardiac hypertrophy was assessed by measuring the ratio of combined ventricular wet weight to total body weight.

**Treatment with AT\(_1\)-R Blockers**

Control and TTRhRen mice (n=4/group) were treated for 5 consecutive days with candesartan (15 mg/kg per day in gum Arabic) by gavage. Blood pressure was measured daily by tail-cuff.

**Histochemistry**

Mice were anesthetized by intraperitoneal injection with 3 mg pentobarbital sodium (MTC Pharmaceuticals). Blood was chased from major vessels by whole-body perfusion of saline solution through the heart, followed by in situ organ fixation using 40 mL Bouin fixative solution (0.9% picric acid, 10% formaldehyde, and 5% glacial acetic acid). Organs were quickly removed and postfixed for 5 hours. Fixed tissue was stored in 70% ethanol at 4°C until analyzed.

Tissues were dehydrated, embedded in paraffin blocks, cut into 5-μm sections, and mounted on 3-aminopropyltriethoxysilane–coated slices (Sigma Chemical). Sections were deparaffinized, rehydrated, and washed with H\(_2\)O.

**Cardiac Collagen Quantification**

Cardiac sections were stained with Sirius Red F3BA (0.5% in saturated aqueous picric acid; Aldrich Chemical Company) for assessment of interstitial and perivascular collagen content, as previously described.\textsuperscript{21} Quantification of fibrosis was performed using an image analysis system (Northern Eclipse 5.0; EM-PIX Imaging Inc). A single investigator unaware of the experimental groups performed the analysis.

**Detection of Vascular, Cardiac, and Renal NADPH Oxidase Activity by Lucigenin Chemiluminescence**

The lucigenin-derived enhanced chemiluminescence assay was used to determine NADPH oxidase activity in aortic, cardiac, and renal (cortical) tissue as previously detailed.\textsuperscript{22} NADPH (10\(^{-4}\) mol/L) was added to the suspension (400 μL, 30 to 50 μg protein) containing lucigenin (5 μmol/L). This concentration of lucigenin does not appear to be involved in redox cycling and specifically detects O\(_2\)\(^{-}\). Luminescence was measured for 3 minutes in a luminometer (AutoLumat LB 953; Berthold). A buffer blank was subtracted from each reading. Activity is expressed as counts/min per mg dry weight.

**Oxidative Fluorescent Microphotography to Assess Vascular Superoxide Generation**

Dihydroethidium (DHE), an oxidative fluorescent dye, which in the presence of O\(_2\) oxidized to ethidium bromide,\textsuperscript{23} was used to localize O\(_2\)\(^{-}\) in aortic segments in situ as previously described.\textsuperscript{14} Briefly, fresh unfixed vessel segments were snap-frozen. Transverse sections (30-μm-thick) were cut in a cryostat and placed on glass slides. Samples were then incubated at room temperature for 30 minutes with DHE (final concentration 2×10\(^{-4}\) mol/L, dissolved in 1 mL DMSO containing 16% (weight/volume) Pluronic I-127. Images were obtained using a Zeiss Axiosvert S100TV LSM 510 laser scanning confocal microscope.

**Protein Extraction and Immunoblotting**

Extracts of ventricular and renal (cortical) tissue were prepared in lysis buffer (1% nonidet p40, 0.5% sodium deoxycholate, 0.1% SDS, 1 μg/mL aprotinin, 1 μg/mL pepstatin, 1 mmol/L NaVO\(_4\)). Equal amounts of protein (25 μg/sample) were separated on 10% polyacrylamide gels and transferred to polyvinylidene difluoride membranes. Membranes were blocked with Tris-buffered saline and 0.1% Tween-20 containing 7.5% nonfat dry milk and incubated overnight at 4°C. The following antibodies were used: polyclonal anti-Nox1 (Mox1) antibody (1:1000; Santa Cruz Biotechnology, Inc, Santa Cruz, Calif), polyclonal anti-Nox4 antibody (1:1000; kindly provided by Dr J. D. Lambeth, Emory University, Atlanta, Ga), or anti-nitrotyrosine antibody (1:1000; Upstate, Lake Placid, NY). Membranes were washed 3 times with Tris-buffered saline–TWEEN and incubated with horseradish peroxidase-conjugated second anti-
body (1:2000; 1.5 hours, 24°C). Immunoreactive proteins were detected by chemiluminescence. Blots were analyzed densitometrically (Image-Quant software, Molecular Dynamics, Sunnyvale, Calif) with normalization for H9252-actin.

Statistical Analysis
Data are presented as means±SEM. Groups were compared using 1-way ANOVA or Student t test as appropriate. Tukey–Kramer correction was used to compensate for multiple testing procedures. P<0.05 was significant.

Results

Blood Pressure
Consistent with our previous report, systolic blood pressure was significantly elevated in TTRhRen transgenic mice versus control littermates (P<0.001) (Figure 1a). Blood pressure elevation was Ang II–dependent, because treatment with the Ang II AT1 receptor blocker candesartan reduced blood pressure to control values (Figure 1b). TTRhRen/gp91 mice also exhibited significantly elevated blood pressure compared with control counterparts, with the magnitude of increase being identical to that seen in TTRhRen mice. Blood pressure was not different between gp91 and control mice.

Cardiac Hypertrophy
Heart weight-to-body weight ratios were identically and significantly increased in both groups of mice expressing the TTRhRen transgene (TTRhRen and TTRhRen/gp91), indicating that gp91phox deficiency does not protect against the cardiac hypertrophy observed in TTRhRen mice (Figure 2a). These findings were confirmed by gross cardiac histological studies, which demonstrated left ventricular hypertrophy in TTRhRen and TTRhRen/gp91 mice compared with the other groups (Figure 2b).

Cardiac and Perivascular Collagen Content
Sirius red staining for collagen demonstrated similar interstitial collagen content in control and TTRhRen groups, with slightly reduced content in gp91 mice (Figure 3a and 3b).

NADPH Oxidase Activity Is Attenuated in TTRhRen/gp91 Mice
To evaluate the functional impact of gp91phox deficiency, we measured NADPH oxidase activity in cardiac, aortic, and renal tissue. As shown in Figure 4, activation of NADPH oxidase was not increased in TTRhRen mice compared with controls but was significantly decreased in all tissues from gp91 mice. TTRhRen/gp91 mice also exhibited significantly reduced NADPH-driven generation of \( \cdot \text{O}_2 \) compared with TTRhRen mice.

In Situ ROS Production
To assess generation of ROS in situ and to confirm the results obtained with lucigenin chemiluminescence, we used DHE staining of frozen aortic sections. A low basal level of DHE...
fluorescence was detected in control mice, with higher fluorescence in aortic sections from TTRhRen mice (Figure 5). Both gp91phox-deficient groups exhibited weak DHE fluorescence.

Cardiac Nitrotyrosine Abundance
Superoxide can react with NO to form nitrotyrosine protein moieties, which may be used as a marker of oxidative stress. Nitrotyrosine content in cardiac tissue was similar in TTRhRen and control mice (Figure 6) and significantly reduced in both gp91phox-deficient groups.

Nox Expression
Because Nox2 homologues, Nox1 and Nox4, may compensate for gp91phox deficiency, expression of Nox2 homologues was assessed in cardiac and renal tissue. Expression of Nox1 and Nox4 was not significantly different between experimental groups. Nox2 (gp91phox) was undetectable in gp91− and TTRhRen/gp91− mice and levels were unaltered in TTRhRen mice compared with controls. Nox findings were similar in renal (Figure 7) and cardiac tissue (data not shown).

Discussion
Major findings from the present study demonstrate that gp91phox deficiency and associated reduction in NADPH oxidase-mediated production of ROS cannot prevent the development of hypertension and cardiac hypertrophy in a mouse model with chronic activation of the renin-angiotensin system. Moreover, the cardiac hypertrophy and hypertension that are seen in this model were not associated with increased ROS production in the tissues tested.
Many lines of experimental evidence have supported a role for NADPH oxidase-derived ROS in the pathogenesis of Ang II–mediated hypertension and cardiac remodeling. In Ang II–infused animal models and SHR (model of Ang II–dependent hypertension), NADPH oxidase activity is increased, ROS generation is enhanced, and redox-dependent signaling pathways are amplified.1,2,23 These processes, which are mediated through AT1R,24 are associated with overexpression of vascular and cardiac NADPH oxidase subunits, gp91phox, p22phox, p47phox, and p67phox, and increased systemic oxidative stress.25,26 Treatment with NADPH oxidase inhibitors, antioxidant vitamins, or superoxide dismutase mimetics attenuates development of hypertension and regresses cardiovascular remodeling in some animal models.14,15 These studies, together with supportive data from in vitro studies in cultured cells and isolated vessels, support a role for NADPH oxidase-derived ROS in the pathogenesis of Ang II–sensitive cardiovascular disease.

Of the NADPH oxidase subunits, gp91phox seems to be particularly important in Ang II–dependent processes. In cultured vascular smooth muscle cells, vascular fibroblasts, endothelial cells, and cardiomyocytes, Ang II stimulation increases mRNA gp91phox expression and induces de novo protein synthesis.27 Ang II–dependent models of hypertension exhibit increased abundance of vascular gp91phox.28 Wang et al reported that in gp91phox-deficient mice, basal blood pressure is lower than wild-type counterparts and Ang II infusion fails to induce superoxide production and vascular hypertrophy.18 These findings are in contrast to our results in which basal blood pressure was similar in gp91phox-deficient mice and controls, even though NADPH oxidase activity and ROS generation were reduced. Reasons for these basal blood pressure discrepancies are unclear but may relate to different experimental conditions, specifically with respect to blood pressure measurement techniques and the fact that in the study of Wang et al, mice were treated with tetracycline, a matrix metalloproteinase inhibitor that may influence blood pressure.29 In gp91phox-deficient mice with renovascular hypertension, endothelial function is unaltered.30 Left ventricular hypertrophy induced by subpressor Ang II infusion is attenuated in gp91phox-deficient mice, indicating blood pressure-independent cardiac effects of gp91phox.19 This was further confirmed when gp91phox-deficient mice infused for 2 weeks with a pressor dose of Ang II had hypertension develop but no cardiac hypertrophy.31 However, all of the aforementioned studies were conducted in models of acute Ang II intervention, in which Ang II, at high doses, was infused over a relatively short time period. The importance of gp91phox in cardiovascular pathologies associated with slowly developing chronic Ang II–induced hypertension and cardiac hypertrophy remains unclear.

By 10 to 12 weeks of age, TTRhRen mice exhibit significant hypertension and gross cardiac hypertrophy.20 The AT1 receptor antagonist candesartan significantly decreased blood pressure, confirming that the TTRhRen mouse represents a model of Ang II–dependent hypertension. By breeding the TTRhRen transgene into the background of the mouse deficient for gp91phox, we were able for the first time to test for the cardioprotective effect of lifetime reduction of gp91phox.
derived ROS in the face of a lifetime activation of the renin-angiotensin system. As expected, NADPH oxidase activity was attenuated and production of vascular and nonvascular ROS was markedly reduced in all of the animals deficient for gp91phox regardless of the expression of the TTRhRen transgene. This was evidenced by decreased aortic DHE fluorescence indicating diminished vascular superoxide formation and by decreased lucigenin luminescence in cardiac, vascular, and renal tissue, demonstrating reduced NADPH oxidase activity. Further demonstration of attenuated superoxide generation was obtained by evaluating cardiac nitrotyrosine content. Formation of nitrotyrosine provides evidence of reaction of \( \cdot O_2^- \) with NO to form the short-lived, potent oxidant ONOO\(^-\) that can nitrosate tyrosine residue constituents of proteins. Cardiac nitrotyrosine content was significantly decreased in TTRhRen/gp91phox\(^-\) mice compared with TTRhRen littermates.

Despite gp91phox deficiency and downregulated ROS-producing NADPH oxidase, TTRhRen/gp91phox\(^-\) mice had hypertension and cardiac hypertrophy develop to a level that was equivalent to TTRhRen transgenic littermates with an intact gp91phox gene. Although Bendall et al reported a pivotal role of gp91phox-containing NADPH oxidase in cardiac hypertrophy induced by short-term (2 weeks) Ang II infusion,\(^ {19} \) others reported that gp91phox activity is not essential in overload-induced myocardial hypertrophy.\(^ {31,32} \) We considered the possibility that Nox 2 homologues, Nox 1 and Nox 4, may be upregulated in TTRhRen/gp91phox\(^-\) mice, which may compensate for gp91phox deficiency. However, Nox 2 homologue expression was unaltered in cardiac and renal tissue of TTRhRen/gp91phox\(^-\) mice, suggesting that systems other than gp91phox/Nox1/Nox4-containing NADPH oxidase-mediated ROS production contribute to cardiac hypertrophy and hypertension in this model.

Cardiac hypertrophy is a complex process involving cardiomyocyte hypertrophy, cardiac fibroblast growth (proliferation and hypertrophy), cardiac cell apoptosis, and interstitial fibrosis.\(^ {33} \) Histological assessment clearly demonstrated that left ventricular thickness was increased in TTRhRen mice irrespective of the presence of the gp91phox gene. However, interstitial fibrosis was markedly reduced in gp91phox-deficient TTRhRen mice, suggesting that gp91phox may influence processes associated with collagen deposition and degradation. Although mechanisms for this are unclear from the present study, altered matrix metalloproteinase activity and reduced collagen synthesis, which are redox-sensitive processes,\(^ {6,34} \) may be important. However, this awaits further clarification.

An interesting, and unexpected, finding in our study relates to the lack of significant oxidative stress and NADPH oxidase activation in TTRhRen transgenic hypertensive mice, especially because most studies have reported upregulation of NADPH oxidase-mediated ROS generation in Ang II–dependent hypertension. Reasons for these discrepancies may relate to various factors. First, TTRhRen transgenic mice have lifelong and chronic activation of the renin-angiotensin system, unlike acute models in which Ang II is infused for a relatively short time period. Second, plasma Ang II levels are only modestly increased in our model (1- to 2-times normal),\(^ {20} \) whereas in Ang II–infused mice, plasma levels increase markedly with associated increase in ROS formation. Third, unlike SHR, in which the pathogenesis of hypertension is multifactorial, the TTRhRen transgenic mouse is a pure Ang II–dependent model of hypertension. Considering the
chronicity of our model, compensatory mechanisms, such as upregulation of antioxidant systems, might come into play, which may normalize the redox state without influencing development of hypertension and cardiac hypertrophy. However, this awaits clarification. It is also possible that because Ang II levels do not reach levels as high as those in Ang II–infused mice, NADPH oxidase is not markedly activated. Transgenic models also exist that achieve higher Ang II concentrations locally in the heart and that might have led to increases in free radical formation similar to those seen in acutely infused models. However, the circulating levels of Ang II achieved in the TTRhRnRen mice are clearly sufficient to render the mice hypertensive and to lead to cardiac hypertrophy; therefore, we believe that these mice represent a reasonable model for human hypertension.

In summary, findings from the present study indicate that pathophysiological outcomes in a mouse model of chronic Ang II–dependent hypertension is not affected by gp91phox-containing NADPH oxidase activity and can occur in the context of significantly reduced tissue oxidative stress. There are several possible explanations for these findings. The lack of apparent effectiveness of antioxidant therapy on cardiovascular outcomes in clinical trials carried out to date have led to the suggestion that antioxidant therapy might need to be initiated earlier, maintained longer, and used at higher doses to be effective. However, in the model of gp91phox deficiency studied here, animals showed no apparent protection to hypertension and cardiac hypertrophy, although exposed to a lifetime reduction in circulating and tissue content of ROS. Conversely, the beneficial effect of gp91phox deletion that has been reported in acute Ang II insult models raises the possibility that ROS intervene early in cardiovascular remodeling, but that other mechanisms, such as hypertension, are able to overcome the beneficial effects in the chronic state. Alternatively, the sudden onset of hypertension characteristic of Ang II–infused models may induce inflammatory signals that are particularly sensitive to ROS reduction. Such processes may play a less important role in the slow-onset and chronic forms of hypertension. These findings are in contrast to acute models of Ang II–dependent hypertension in which NADPH oxidase-mediated ROS formation plays a key pathophysiological role in blood pressure elevation and cardiac hypertrophy. Our findings highlight the complexities relating to interactions between the renin-angiotensin system and ROS-generating systems and suggest that redox-dependent blood pressure-elevating mechanisms differ in acute and chronic settings of Ang II upregulation.

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