NADPH Oxidase Contributes to Vascular Inflammation, Insulin Resistance, and Remodeling in the Transgenic (mRen2) Rat

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Abstract—Reduced insulin sensitivity is characteristic of various pathological conditions such as type 2 diabetes mellitus and hypertension. Angiotensin II, acting through its angiotensin type 1 receptor, inhibits the actions of insulin in the vasculature which may lead to deleterious effects such as vascular inflammation, remodeling, endothelial dysfunction, and insulin resistance. In contrast, insulin normally exerts vasodilatory, antiinflammatory, and prosurvival actions. To explore the impact of angiotensin II on insulin signaling, NADPH oxidase–derived reactive oxygen species formation, vascular inflammation, apoptosis, and remodeling, we used transgenic TG(mRen2)27 (Ren2) rats, which harbor the mouse renin transgene and exhibits elevated tissue angiotensin II levels. Compared with Sprague-Dawley controls, Ren2 aortas exhibited greater NADPH oxidase activity, reactive oxygen species levels, C-reactive protein, tumor necrosis factor-α expression, apoptosis, and wall thickness, which were significantly attenuated by in vivo treatment with angiotensin type 1 receptor blockade (valsartan) or the superoxide dismutase/catalase mimetic (tempol). There was substantially diminished Akt and endothelial NO synthase activation in Ren2 aortas in response to in vivo insulin stimulation, and this was significantly improved by in vivo treatment with valsartan or tempol. In vivo treatment with valsartan, but not tempol, significantly reduced blood pressure in Ren2 rats. Further, there was reduced insulin induced Akt activation and increased tumor necrosis factor-α levels in vascular smooth muscle cells from Ren2 and Sprague-Dawley rats treated with angiotensin II, abnormalities that were abrogated by angiotensin type 1 receptor blockade with valsartan or antioxidant N-acetylcysteine. Collectively, these data suggest that increased angiotensin type 1 receptor/NADPH oxidase activation/reactive oxygen species contribute to vascular insulin resistance, endothelial dysfunction, apoptosis, and inflammation. (Hypertension. 2007;50:384-391.)

Key Words: Ren2 rat ■ NADPH oxidase ■ ROS ■ vascular inflammation ■ insulin resistance ■ apoptosis

There is accumulating evidence that inflammation and insulin resistance play fundamental roles in the development of vascular disease.1–3 Activation of the renin-angiotensin system (RAS) is crucial in the pathogenesis of hypertension, insulin resistance, and vascular disease.3–5 Angiotensin II (Ang II), the major component of RAS, has proinflammatory activity that induces the expression of proinflammatory cytokines, chemokines, growth factors, and adhesion molecules, and regulates vascular cell growth, apoptosis, migration, inflammation, and fibrosis via binding to type 1 receptor (AT1R) or interacting with G protein and cytokine receptors.4–8 The effects of Ang II depend on duration (acute or chronic), tissue levels, and the tissue/cell types on which it acts. Accumulating evidence suggests that reactive oxygen species (ROS) is an important mediator for Ang II–induced vascular effects.4–7 ROS can diminish the bioactivity of nitric oxide (NO) by conversion of locally released NO to peroxynitrite (ONOO−). These highly reactive molecules oxidize lipids, cause cellular injury, and enhance vascular contraction.5,6,8 ROS activates transcription factors (eg, nuclear factor-kappa B [NF-κB] and activator protein 1 [AP-1]) and upregulates expression of proinflammatory genes such as tumor necrosis factor (TNF)-α, monocyte chemoattractant protein (MCP)-1, interleukin (IL)-6, and C-reactive protein (CRP) leading to vascular inflammation. TNF-α impairs insulin action,9 suppresses the expression of endothelial NO synthase (eNOS),10 and causes vascular cell apoptosis,11,12 which further increases vascular injury and contractile reactivity.13

Insulin is a pivotal regulator of the metabolism on glucose, fat, and protein. In addition, insulin has important nonmetabolic, hemodynamic actions as well, eg, peripheral vasodila-
tation through NO release, and increasing local blood flow, and antioxidant and antiapoptotic actions through stimulating the Akt-signaling cascade, and antiinflammatory effects via inhibition of NF-κB. Indeed, insulin resistant states are characterized by an impaired vasodilator response to insulin. RAS activation and insulin resistance may perpetuate each other and coordinately contribute to endothelial dysfunction, vascular inflammation, injury, and remodeling, consequently predisposing to hypertension and vascular disease.

Although much has been learned about the vascular effects of Ang II from acute and chronic infusion of this peptide in rodent models, there are limited data in genetic models of tissue Ang II overexpression as it relates to inflammation, apoptosis, and remodeling, particularly in the insulin resistant state. The transgenic Ren2 rat harbors the mouse renin gene and exhibits increased Ang II tissue levels with subsequent severe hypertension, cardiovascular remodeling, insulin resistance, and albuminuria. Thus, the Ren2 rat provides a unique model to investigate the impact of chronic endogenous elevations of Ang II on inflammation, insulin metabolic signaling, apoptosis, and vascular remodeling. We hypothesized that overexpression of tissue Ang II will result in these vascular abnormalities via AT1R-mediated stimulation of NADPH oxidase. We further hypothesized the effects of Ang II could be attenuated by in vivo treatment with AT1B or tempol treatment (data not shown).

Figure 1A). There were no effects on SBP of SD rats with AT1B or tempol treatment (data not shown).

**Systolic Blood Pressure**
Systolic blood pressure (SBP) was measured in triplicate using the tail-cuff method (Harvard Systems, Student Oscillometric Recorder) before and after treatment.

**Cell Culture**
Aortic vascular smooth muscle cells (VSMCs) were obtained from SD and Ren2 rats. Cells were cultured in DMEM and Ham’s F12 (1:1) supplemented with 15% fetal bovine serum (FBS). Cells of the second to fourth passage were used for experiments.

**Materials**
Details of the materials used in this study are available in a data supplement available online at http://hyper.ahajournals.org.

**In Situ Detection of Vascular Superoxide Anion**
Superoxide (O$_2^-$) anion generation in aorta was evaluated by using the fluorescent dye DHE. Details of the vascular superoxide detection are available in the data supplement.

**Measurement of Oxidative Stress Marker**
Anti-4-HNE antibody was used to detect lipid peroxidation as a marker of ROS generation. Details of the measurement of oxidative stress markers are available in the data supplement.

**NADPH Oxidase Activity Assay**
Details of the determination of NADPH oxidase activity are available in the data supplement.

**Western Blot Analysis**
Details of the western blot analysis of p67phox, Rac1, TNF-α, caspase-3, Akt, Ser1177 phospho-Akt, eNOS, Ser1177 phosphor-eNOS are available in the data supplement.

**Immunofluorescence**
Aorta sections were incubated with anti-Nox 2 (formerly gp91phox), p22phox, Rac1 antibodies (1:100) for overnight at room temperature. After wash, second antibody conjugated with Alexa Fluor 647 was added for 4 hours at room temperature. Images were acquired and gray scale intensity was measured as described above.

**Endothelial NOS Activity Assay**
Details of the eNOS activity assay are available in the data supplement.

**Evaluation of Apoptotic Cell Death by Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End-Labeling**
Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay was performed using In Situ Cell Death Detection Kit per manufacturer instruction. TUNEL-positive and -negative cells were counted at 5 random fields. Results are expressed as number of TUNEL-positive cell/total cells×100%.

**TNF-α ELISA**
Primary VSMCs were cultured as described above. Supernatants were collected and analyzed by ELISA as the manufacturer’s instruction. VSMCs were isolated from 3 to 4 rats for each group and were cultured in triplicate.

**Statistical Analysis**
All data are reported as the means±SEM. Dunnett test or Student t test were used to determine the significance among groups. A value of P<0.05 was considered to be statistically significant.

**Results**

**Systolic Blood Pressures**
The initial SBP of Ren2 rats at age 6 weeks was higher than SD rats (153.8±5.9 mm Hg and 112.5±4.1 mm Hg, respectively, P<0.001). In addition, SBP was dramatically increased in Ren2 rats at age 9 to 10 weeks compared with age 6 weeks (P<0.001). SBP was significantly lower in Ren2 rats treated with ATB for 3 weeks compared with untreated Ren2 rats (150.8±9.4 and 208.4±9.3 mm Hg, respectively, P<0.001). SBP in Ren2 rats treated with tempol for 3 weeks was not significantly different from untreated Ren2 rats (182.4±9.6 and 208.4±9.3 mm Hg, respectively, P=0.06, Figure 1A). There were no effects on SBP of SD rats with ATB or tempol treatment (data not shown).

**Aortic Hypertrophy**
Aortic wall thickness was substantially greater in Ren2 compared with SD controls. There was significant medial hypertrophy (49%) and increased thickness of adventitia...
Increased ROS Formation in Ren2 Aorta

ROS have been shown to act as important signaling molecules in the cardiovascular system and activate many redox signaling pathways such as NF-κB.\textsuperscript{25,26} To evaluate ROS formation in Ren2 aortas, we used DHE fluorescence and 4-HNE immunostaining. Intracellular superoxide converts DHE to ethidium which binds to double-stranded DNA resulting in nuclear red fluorescence. Compared with SD rats, Ren2 aorta exhibited increased DHE staining intensity ($P<0.01$), which was significantly reduced in the aortas of rats treated with AT\textsubscript{1}B or tempol ($P<0.05$, Figure 1B and 1C), suggesting a contribution of the AT\textsubscript{1}R in ROS formation. Immunostaining for 4-HNE, a marker of lipid peroxidation, also showed a significant increase of staining intensity in Ren2 aortas compared with SD and was significantly decreased in Ren2 aortas treated with AT\textsubscript{1}B or tempol (Figure 2C). This was particularly notable in the vessel media.

Aortic NADPH Oxidase Activity

To determine whether increased ROS accumulation in Ren2 aorta was attributable to NADPH oxidase activation, the enzyme activity was measured by detecting the rate of NADPH consumption as described in the Methods section. Ren2 aortas exhibited greater NADPH oxidase activity compared with SD ($P<0.05$), and that was completely inhibited by AT\textsubscript{1}B or tempol treatment for 3 weeks ($P<0.01$, Figure 3A) compared with untreated Ren2. As there are several enzymes or molecules that can produce ROS,\textsuperscript{27} enzyme inhibitors were added to the in vitro assay to differentiate the source; eg, DPI (nonspecific flavoprotein and NADPH oxidase inhibitor), or apocynin (specific inhibitor for NADPH oxidase), or rotenone (mitochondrial inhibitor), or L-NAME (NOS inhibitor). DPI and apocynin dramatically blocked NADPH consumption, but not rotenone or L-NAME (Table). These data suggest that elevated ROS generation in Ren2 aorta tissue was primarily mediated by increased NADPH oxidase activation.

NADPH Oxidase Subunit Expression

NADPH oxidase is a multi-subunit enzyme complex including the membrane-bound components Nox2, and p22\textsubscript{phox}, cytosolic subunits p47\textsubscript{phox}, p67\textsubscript{phox}, p40\textsubscript{phox}, and the small GTP-binding protein Rac1/Rac2. Cytosolic subunits assembly with the membrane components appears to be essential for activating the NADPH oxidase complex.\textsuperscript{28,29} To deter-

Figure 1. A, SBP. *$P<0.01$ vs SD; **$P<0.01$ vs RC. B, H&E staining of aortic wall cross-sections. C, Bars represent media and adventitia thickness. *$P<0.05$ vs SD; **$P<0.05$ vs RC.

Figure 2. DHE and 4-HNE staining were used to estimate ROS generation. A, Representative DHE fluorescent photomicrographs of aortic sections. B, Graph show a quantitative densitometry of DHE fluorescence intensity as average gray scale. C, Representative 4-HNE immunofluorescent photomicrographs, an indicator of lipid peroxidation of ROS. *$P<0.01$ vs SDC; **$P<0.05$ versus RC.
mine whether increased NADPH oxidase activity is related to the protein levels of the enzyme, we evaluated the expression of NADPH oxidase subunits by immunoblot and immunofluorescent staining. Rac1 and p67phox were higher (by 3.08- and 4.06-fold, respectively) in Ren2 aorta compared with SD by Western blots (P<0.05; Figure 3D and 3E, respectively). Increased intensity of immunofluorescence for Rac1, Nox2, and p22phox was observed in Ren2 aortas (Figure 3B and 3C). Treatment with AT1B or tempol dramatically reduced these proteins levels (P<0.05, Fig 3B and 3C). There was minimal immunostaining for macrophages (CD68) and T-lymphocytes (CD3) in aortic sections (data not shown).

Reduction of Phosphorylation of eNOS and eNOS Activity in Ren2 Aorta

To determine whether increased ROS in Ren2 aorta affects eNOS activation, NOS activity and Ser1177 phosphorylation of eNOS (activation) were measured by 3H-labeled arginine, immunofluorescence, and immunoblot, respectively. NOS activity (75%, P<0.01) and eNOS Ser1177 phosphorylation (50%, P<0.01) were significantly decreased in Ren2 aorta compared with SD (Figure 4A and 4C). Treatment with AT1B or tempol reversed NOS activity and eNOS Ser1177 phosphorylation in Ren2 aortas compared with untreated Ren2 (P<0.05, Figure 4A and 4C). Immunofluorescent staining showed that Ser 1177 phosphorylation of eNOS was predominantly expressed by endothelial cells and the staining was diminished in Ren2 aorta (Figure 4B).

Impaired Insulin-Induced Akt Activation in Aorta and VSMCs From the Ren2 Rat

Insulin resistance is associated with decreases of vasodilatation, antioxidant, antiapoptotic, antiinflammatory effects, and upregulation of the local RAS activity. To determine whether increased ROS affects insulin signaling in Ren2 aortic tissues, in some rats, 2 units of insulin were administered by IV injection 5 minutes before sacrifice. Insulin-induced Akt Ser473 phosphorylation was significantly decreased in Ren2 aortas by (34%) compared with SD (P<0.05), and Akt activation was restored by valsartan or tempol treatment compared with untreated Ren2 aortas (P<0.05; Figure 4D). Insulin-mediated Akt Ser473 phosphorylation was dramatically reduced in the VSMCs isolated from Ren2 compared with SD rats (Figure 4E). Further, Ang II treatment impaired insulin-stimulated Akt activation in VSMCs from SD and valsartan or antioxidant NAC reversed Akt activation (Figure 4F).

Increased Expression of TNF-α in Aortas and VSMCs From the Ren2 Rat

ROS can activate NF-κB–mediated upregulation of inflammatory genes such as TNF-α. TNF-α impairs insulin action, suppresses the expression of eNOS, and activates NF-κB pathway. Expression of TNF-α was markedly up-
regulated in Ren2 aortas detected by immunofluorescence staining (Figure 5A) and Western blot analysis compared with SD (2-fold increase, \( P<0.01 \), Figure 5B). All layers of aorta expressed TNF-α, particularly in media and adventitia (Figure 5A). TNF-α expression was reduced treated Ren2 rats with AT1B or tempol compared with untreated Ren2 rats (\( P<0.01 \), Figure 5B). The primary VSMCs from Ren2 produced much more TNF-α compared with SD (\( P<0.05 \), Figure 5C). Further, Ang II induced significantly increase of TNF-α production in VSMCs isolated from SD aortas, and this was inhibited by valsartan or NAC cotreatment (Figure 5D).

### Increased Apoptotic Cell Death and Active Caspase-3 Expression in Ren2 Aortas

Apoptosis is a major contributor to the process of plaque rupture in atherosclerosis.\(^5\)\(^6\) TNF-α is known to cause apoptosis in VSMCs.\(^11\)\(^12\) There were increased TNF-α (Figure 5), ROS formation (Figure 2), impaired insulin signaling, and eNOS activation (Figure 4) in Ren2 aortas, all of which could contribute to increased apoptosis. Thus, vascular cell apoptosis was analyzed by TUNEL staining. There were more apoptotic cells (percentage TUNEL positive nuclei; both endothelial cells and smooth muscle cells) in Ren2 aortas compared with SD (\( P<0.05 \), Figure 6A) and that was significantly reduced in AT1B or tempol treated groups (\( P<0.05 \), Figure 6A). To confirm this finding, we also examined caspase-3 activation (an apoptotic cell death enzyme) by immunoblot. Activated caspase-3 levels were increased in Ren2 compared with SD (2-fold increase), and were dramatically reduced in Ren2 rats treated with AT1B or tempol (\( P<0.05 \), Figure 6B).

### Discussion

In the present investigation, we demonstrate that chronically elevated tissue Ang II levels promote NADPH oxidase–derived ROS formation leading to vascular inflammation, insulin resistance, reduced eNOS activity, apoptosis, and remodeling. These factors together contribute to the development of hypertension and vascular disease observed in Ren2 rat.

RAS and Ang II have pivotal roles in cardiovascular homeostasis. However, increased RAS activation and Ang II levels exert many detrimental effects primarily mediated by the AT1R. The AT1R is expressed in VSMCs, endothelial cells, fibroblasts, macrophages, and others. AT1R signaling induces cell growth, apoptosis, migration, inflammation, and fibrosis\(^34\) and impairs insulin signaling in many tissues including the vasculature.\(^35\) Those AT1R-mediated effects are...
mainly dependent on the duration, levels of Ang II, and tissues/organs involved. The intermediate signaling steps which mediate Ang II effects are complex and have not been fully elucidated. However, ROS formation appears to represent one critical mediator in the effects of Ang II on the vasculature. ROS formation induces insulin resistance and activates multiple redox signaling pathways including NF-kB. NF-kB provokes inflammation by upregulating inflammatory molecules: eg, TNF-α, IL-6, MCP-1, CRP. ROS production was significantly higher in Ren2 aorta, and was markedly inhibited by in vivo treatment with AT1B or the SOD/catalase mimetic tempol. However, AT1B treatment did not completely reverse ROS formation in Ren2 aorta suggesting some of ROS generation might have been independent of the Ang II stimulation of the AT,R. This suggests that other actions of Ang II are likely important mediators of ROS formation. For example, aldosterone increases ROS generation in many tissues/organs. The mouse renin transgenic heterozygous male Ren2 rat has elevated tissue RAS activity, which could increase aldosterone production. In fact, we have observed that Ren2 rats treated with the aldosterone antagonist spironolactone, have attenuated ROS production in heart and vessels (Wei and Sowers unpublished data, 2006).

ROS can be generated by several sources of enzymes and molecules. Data from this investigation indicate that NADPH oxidase activation may be the primary mediator of increased ROS generation in the Ren2 aorta. Furthermore, by using inhibitors to block different enzymes in an in vitro assay, NADPH consumption was abrogated by DPI (nonspecific NADPH oxidase inhibitor) or apocynin (specific NADPH oxidase inhibitor), but not by L-NAME (NOS inhibitor) or rotenone (mitochondria inhibitor). Collectively, these data indicate that NADPH oxidase was the major source for ROS generation in Ren2 aortas. However, it is not clear how the ROS scavenger tempol decreased NADPH oxidase activity and protein expression of the enzyme subunits. Perhaps this occurs through a positive feedback mechanism. Similar positive actions have been observed which increased ROS enhance mRNA expression of NADPH subunits in 3T3-L1 adipocytes.

NO is critical to maintain endothelial function and vasodilator tone. eNOS activity is regulated by various kinases leading to phosphorylation of specific sites. The phosphatidylinositol 3-kinase (PI3K)/Akt pathway activate eNOS and NO production. eNOS activity and eNOS phosphorylation at Ser1177 was greatly reduced in Ren2 aortas, and significantly improved with AT,B or tempol. Similarly, impaired Akt activation was also observed in Ren2 aortas and treatment with AT,B or tempol restored Akt phosphorylation at Ser473. The reduction of eNOS activation could be, in part, due to impaired PI3K/Akt signaling.

Insulin, besides its metabolic effects on glucose, fat, and protein, has important nonmetabolic actions: eg, vasodilatation, antioxidant, antiapoptotic, as well as antiinflamm-
addition, primary VSMCs from Ren2 produced more TNF-α than from SD an effect that was enhanced with Ang II treatment and attenuated after treatment with valsartan or the antioxidant NAC. In addition to TNF-α, CRP, an inflammatory marker of CVD and insulin resistance, was also elevated in Ren2 aortas. Lastly, we observed increased media smooth muscle cell proliferation and perivascular fibrosis in small arteries from Ren2 (data not shown). Therefore, increased oxidative stress, inflammation, impaired insulin signaling, as well as diminished eNOS activation collectively result in vascular injury leading to vascular remodeling and apoptosis. This notion was further supported by increased caspase-3, active caspase-3, and apoptotic cell death in Ren2 aortas, that was markedly attenuated in Ren2 rats treated with AT,B or tempol.

In summary, the results of this investigation indicate that elevated local Ang II levels and RAS activity in aortic tissue promotes NADPH oxidase activation/ROS production via the AT1R, which subsequently causes vascular inflammation, insulin resistance, vasoconstriction, and cell death leading to vascular remodeling observed in the Ren2 rat.

**Perspectives**

This investigation provides evidence in addition to that provided by acute and chronic Ang II infusion studies, that Ang II promotes vascular insulin resistance, inflammation, apoptosis, and remodeling. This study, in concert with prior reports in this genetic model of tissue Ang II overexpression, show that Ang II–induced NADPH oxidation is one mechanism linking hypertension to vascular inflammation, insulin resistance, and remodeling. This investigation also demonstrates that disparate therapeutic interventions, ie, AT1B and administration of a SOD/catalase mimetic can correct these abnormalities. Finally, data from this investigation help to explain why hypertension and vascular disease commonly exist in conditions characterized by metabolic abnormalities such as impaired insulin sensitivity and type 2 diabetes mellitus.

**Sources of Funding**

This work was supported by National Institutes of Health RO1-HL-073101-02 (to J.R.S.) and P01-HL-51952 (to C.F.), VA Merit 0018 (to J.R.S.); and Investigator Initiated Grant for Novartis Pharmaceutical (J.R.S.).

**Disclosures**

None.

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Hypertension. 2007;50:384-391; originally published online May 28, 2007;
doi: 10.1161/HYPERTENSIONAHA.107.089284

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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