RNA Silencing In Vivo Reveals Role of p22phox in Rat Angiotensin Slow Pressor Response

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Abstract—The angiotensin II (Ang II) slow-pressor response entails an increase in mean arterial pressure and reactive oxygen species. We used double-stranded interfering RNAs (siRNAs) in Sprague Dawley rats in vivo to test the hypothesis that an increase in the p22phox component of NADPH oxidase is required for this response. Reactive oxygen species were assessed from excretion of 8-isoprostane prostaglandin F_2α and blood pressure by telemetry. Two siRNA sequences to p22phox (sip22phox) reduced mRNA >85% in cultured vascular smooth muscle cells. Rats received rapid (10 second) IV injections (50 to 100 μg) of 1 of 2 different sip22phox, control siRNA, or vehicle (TransIt in saline) during 14 day SC infusions of Ang II (200 ng · kg⁻¹ · min⁻¹) or sham infusions. In both groups, sip22phox, relative to control siRNA, led to significant (P<0.001; ≈50%) reductions in expression of p22phox mRNA and protein and of NADPH oxidase activity in the kidney cortex. In Ang II–infused rats, sip22phox decreased protein expression for Nox-1, -2, and -4 but increased p47phox. Three days after sip22phox, conscious rats infused with Ang II had a reduced excretion of 8-isoprostanate (10±1 versus 19±2 pg · 24 h⁻¹; P<0.01) and a reduced mean arterial pressure (142±5 versus 168±4 mm Hg; P<0.005). An increase in p22phox is required for increased renal NADPH oxidase activity, expression of Nox proteins and oxidative stress, and contributes ≤50% to hypertension during an Ang II slow-pressor response. (Hypertension. 2006;47:238-244.)

Key Words: hypertension, arterial arterioles oxidative stress kidney

Angiotensin II (Ang II) has been assigned a critical role in the generation and complications of human essential hypertension, yet plasma renin activity and plasma concentrations of Ang II are not remarkably elevated.1 Mice,2 rats,3,4 rabbits,5 or humans6 infused with Ang II at doses that are initially pressor develop a “slow-pressor response” in which the blood pressure (BP) increases progressively despite plasma Ang II concentrations that are increased only moderately.3 The kidney is implicated in the slow-pressor response, because the development of hypertension depends on salt intake.3 Moreover, rats or rabbits infused with Ang II have enhanced renal vasoconstriction to Ang II5,7 despite down-regulation of Ang II type 1 receptors. Reactive oxygen species (ROS) and superoxide anion (O_2⁻) have been implicated in the development of hypertension in the Ang II slow-pressor model, because hypertension is prevented by antioxidant molecules, such as a permeabilized form of superoxide dismutase or tempol, which is an superoxide dismutase mimic nitroxide.2,4,5,8 Infusions of Ang II increase the activity of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in blood vessels9 and the kidney cortex.4,10,11 This complex enzyme, which was first described in phagocytes and, later, in blood vessels and the kidney, is composed of membrane-associated components of the flavoprotein catalytic core, gp91phox (now named Nox-2) and p22phox.12 Activation requires phosphorylation of p47phox13 and its assembly with p67phox, Rac-1 at the membrane.12 Homologues of Nox-2 include Nox-1, which has been characterized in vascular smooth muscle cells (VSMCs),9 and Nox-4, which has been characterized in the kidney.15 VSMCs and kidneys have the phagocytic NADPH oxidase components.12 p22phox is expressed in the thick ascending limb, macula densa, distal convoluted tubule, collecting ducts, vasculature, and interstitial fibroblasts of the kidney.16 It is believed to dock the enzyme complex in the cell membrane and stabilize Nox proteins.12 There is colocalization of p22phox and O_2⁻ generation in atherosclerotic plaques from human blood vessels.17 The p22phox component is upregulated in the
kidneys\textsuperscript{4,10} of rats undergoing an Ang II slowpressor response. Antisense constructs targeted at p22\textsuperscript{phox} inhibit Ang II–induced hypertrophy of cultured VSMCs,\textsuperscript{18} and mice overexpressing p22\textsuperscript{phox} in their VSMCs have an exaggerated hypertrophic response to Ang II infusion.\textsuperscript{19} Whereas these observations demonstrate the importance of p22\textsuperscript{phox} in mediating the effects of Ang II on ROS and development of atherosclerosis, recent studies with mice overexpressing p22\textsuperscript{phox} in blood vessels show that, despite increased aortic expression of p22\textsuperscript{phox} and Nox-1 and increased \(O_2^-\) and \(H_2O_2\) generation, the basal BP is not increased\textsuperscript{20} and rises only slightly more rapidly during infusion of Ang II.\textsuperscript{19} Therefore, the role of p22\textsuperscript{phox} in the physiological response to Ang II is not yet clear. We tested the hypothesis that an increase in p22\textsuperscript{phox} is required for increased NADPH oxidase activity and development of oxidative stress and hypertension during an Ang II slowpressor response. Because specific pharmacological inhibitors or knockouts for p22\textsuperscript{phox} are not yet available, we used a RNA interference (RNAi) strategy with small (21-bp) double-stranded interfering RNAs (siRNAs)\textsuperscript{21,22} targeted to p22\textsuperscript{phox} (sip22\textsuperscript{phox}) to test its role in mediating responses to Ang II infusion in rats.

**Methods**

**Animal Preparation and Studies of Isoprostane Excretion, mRNA, and Protein Expression**

Studies were approved by the Georgetown University Animal Care and Use Committee. Experiments were performed on male Sprague-Dawley rats weighing 280 to 350 g maintained on a synthetic casein–based diet (Teckland Inc, Madison, WI) as described.\textsuperscript{23} Rats were maintained in individual cages under conditions of constant temperature and humidity and exposed to 12-hour cycles of light and dark with unrestricted water intake.

Under brief (10 minutes) anesthesia with 1% to 2% isoflurane, an osmotic minipump (model 2002, Alzet Corporation) was inserted subcutaneously in the nape of the neck and filled with vehicle (sham infused) or human Ang II (Peninsula Laboratories, San Carlos, CA) to deliver 200 \(\text{ng} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}\) (Ang II–infused). This protocol provides a gradual increase in mean arterial pressure (MAP) with Ang II over 2 weeks\textsuperscript{4} with increases in excretion of 8-isoprostane (8-Iso) prostaglandin F (PGF\textsubscript{2\alpha}) and malondialdehyde and increased oxidative stress and hypertension during an Ang II slowpressor response. Therefore, the role of p22\textsuperscript{phox} in the physiological response to Ang II is not yet clear. We tested the hypothesis that an increase in p22\textsuperscript{phox} is required for increased NADPH oxidase activity and development of oxidative stress and hypertension during an Ang II slowpressor response. Because specific pharmacological inhibitors or knockouts for p22\textsuperscript{phox} are not yet available, we used a RNA interference (RNAi) strategy with small (21-bp) double-stranded interfering RNAs (siRNAs)\textsuperscript{21,22} targeted to p22\textsuperscript{phox} (sip22\textsuperscript{phox}) to test its role in mediating responses to Ang II infusion in rats.

The aim of the first series was to assess the effects of an infusion of Ang II on renal expression of mRNA and protein for p22\textsuperscript{phox}, renal NADPH oxidase activity, excretion of 8-isoprostane (8-Iso) prostaglandin F (PGF\textsubscript{2\alpha}), and MAP. Eleven days after insertion of minipumps containing vehicle or Ang II, rats were placed in metabolic cages, and on day 12, a 24-hour urine was collected for excretion of 8-Iso. The next day, rats were euthanized for harvesting kidneys. Separate groups of rats were equipped with telemetric BP recorders (see below) and, after 2 weeks for recovery, received sham or Ang II infusions. MAP was recorded on day 12.

The aim of the second series was to assess the effects of sip22\textsuperscript{phox} on the renal cortical expression of mRNA and protein for p22\textsuperscript{phox} and NADPH oxidase activity. Rats received 2 injections, one of 100 \(\mu\text{g}\) of siRNA (or equivalent vehicle) on day 5 of Ang II or sham infusions and another of 50 \(\mu\text{g}\) on day 8. The siRNA was complexed with a polymer from TransIT in vivo gene delivery system and delivered according to the manufacturer’s recommendations (Mirus Inc). The injections of 6 mL were given via a catheter in a jugular vein over 10 seconds. Rats were euthanized 72 hours after the second injection of siRNA. Under thiobarbital anesthesia (Inactin; 100 mg \cdot kg \textsuperscript{-1} IP; Research Biochemicals Inc), the kidneys were flushed free of blood with PBS, harvested, and the cortex dissected and frozen for subsequent analysis of mRNA, protein, and NADPH oxidase activity.

The aim of the third series was to assess the effects of sip22\textsuperscript{phox} on either the excretion of 8-Is0 or the MAP during the hypertensive phase of an Ang II slowpressor response. One group was infused with Ang II (\(n=6\) to 8 per group) and was habituated to metabolic cages on day 7 of Ang II infusion. Twenty-four–hour urine collections were undertaken on day 8 (before intravenous injections) and daily until day 13 of Ang II infusion for excretion of 8-Is0, as described previously.\textsuperscript{10} On day 9, rats received 6 mL of fluid IV containing either a vehicle or 50 \(\mu\text{g}\) of a control siRNA (siCont) or sip22\textsuperscript{phox} given by rapid IV bolus over 10 s as in series 2.\textsuperscript{24} The complex of siRNA and TransIT was incubated together for 5 minutes before injection. A second group was briefly anesthetized for insertion of BP telemeters (see below) and, after 14 days for recovery, was infused with Ang II and given 6 mL IV injections of siRNA or vehicle on days 5 and 8 as in the second series. This protocol was adopted from studies that had used in vivo siRNA successfully to target genes in mice\textsuperscript{21,24} and on results of pilot studies (see beginning of Results section).

The aim of the fourth group was to investigate potential off-target effects of siRNA in Ang II–infused rats.\textsuperscript{25} Rats were given intravenous injections of sip22\textsuperscript{phox} No. 1 targeted to a different part of the molecule (see below). Its effects on mRNA and protein expression for p22\textsuperscript{phox} and NADPH oxidase activity were compared with sip22\textsuperscript{phox} No. 1. The kidney cortex was also analyzed for mRNA expression of toll-like receptor 3 (TLR-3) and signal transducers and activators transcription-1 (STAT-1) to assess activation of the interferon (INF) response element pathway that can confound siRNA studies.\textsuperscript{26} An extended Methods section is available online at http://hyper.ahajournals.org for the following subsections: telemetric BP recording and injection of siRNAs; siRNA construction and validation; mRNA isolation and RT-PCR; protein isolation, quantification, and immunoblotting; NADPH oxidase activity; chemical methods; and statistical analysis.

**Results**

Pilot studies of isoprostane excretion of rats infused with Ang II were undertaken to establish optimal intravenous doses of sip22\textsuperscript{phox} constructs. Two days after 5–\(\mu\text{g}\) sip22\textsuperscript{phox}, the excretion of 8-Iso was reduced by 20±10% (\(n=6\)), whereas 50 \(\mu\text{g}\) reduced excretion more consistently and significantly by 50% (see below). A dose of 200 \(\mu\text{g}\) produced more variable results. siCont had no effect. Maximal effects were apparent at 36 to 72 hours after injection. Therefore, a dose of 50 to 100 \(\mu\text{g}\) was used, and organs were harvested 72 hours after injection.

In series I, we found that 12 days of Ang II infusion increased the MAP, NADPH oxidase activity, 8-Is0 excretion, and p22\textsuperscript{phox} mRNA and protein in the renal cortex (Figure 1). In series 2, we found that sip22\textsuperscript{phox} injection reduced the renal p22\textsuperscript{phox} mRNA and protein by 50% and had a similar effect on NADPH oxidase activity both in sham-infused (Figure 2A) in Ang II–infused rats (Figure 2B).

Before injection of vehicle or siRNA, the 8-isoprostane PGF\textsubscript{2\alpha} excretion of rats infused with Ang II averaged 12.5±1.8 \text{pg} \cdot \text{24 h}^{-1}. It increased progressively over 4 days in Ang II–infused rats that received a vehicle or siCont but not in those receiving sip22\textsuperscript{phox} (Figure 3A). This resulted in a \(~50\%\) (\(P<0.01\)) reduction in excretion of 8-isoprostane.
PGF$_2\alpha$ in the group receiving sip22$^{phox}$, relative to siCont or vehicle, from 48 to 96 hours after injection.

Ang II caused progressive increases in MAP after the first day of infusion (Figure 3B) to a plateau by day 9 of $\approx$170 mm Hg in rats receiving injections of vehicle or siCont. In contrast, there was no significant increase in MAP in those receiving sip22$^{phox}$ from the time of the first injection (day 5; 132±3 mm Hg) to 72 hours after the second injection (day 11; 140±5 mm Hg). There were no differences in heart rate between the groups 3 days after injections (vehicle: 339±14; siCont: 378±28; sip22$^{phox}$ No. 1: 355±11; sip22$^{phox}$ No. 2: 358±9 min$^{-1}$; P value not significant). The MAP of Ang
II–induced rats given sip22\textsuperscript{phox} remained significantly (\(P<0.005\)) above levels of sham-infused rats of 107 ± 4 mm Hg. Therefore, an increase in p22\textsuperscript{phox} expression and activity in the kidney can account for up to about one third of the increase in BP during a 12-day infusion of Ang II.

The renal cortical expression of the other NADPH oxidase proteins in angiotensin-infused rats is depicted in Figure 4. There were no differences in expression between rats receiving vehicle and siCont, but sip22\textsuperscript{phox} significantly reduced the expression of Nox-1, -2, and -4 and increased the expression of p47\textsuperscript{phox}.

As shown in Figure 5, the sip22\textsuperscript{phox} No. 1 and No. 2 (targeted to different regions of the p22\textsuperscript{phox} cDNA) produced equivalent reductions in p22\textsuperscript{phox} mRNA and protein expression and MAP. There were also equivalent reductions in NADPH oxidase activity (siCont: 54±7; sip22\textsuperscript{phox} No. 1: 29±4; sip22\textsuperscript{phox} No. 2: 30±5, 10\textsuperscript{5} counts \cdot mg protein\textsuperscript{-1} \cdot 5 min\textsuperscript{-1}). Therefore, data for sip22\textsuperscript{phox} No. 1 and No. 2 are presented together.

siRNA administration can activate off-target effects via cellular signaling through the TLR-3 with synthesis of interferons that signal via STAT-1. We found no significant increases in the expression of these mRNAs in the kidney cortex after injections of siCont or sip22\textsuperscript{phox}, relative to vehicle, in Ang II–infused rats (Figure 6), indicating that such off-target effects of our sip22\textsuperscript{phox} were minimal or absent.

**Discussion**

We confirm that Ang II infusion causes a progressive increase in MAP,\textsuperscript{2,3} accompanied by increased expression of mRNA and protein for p22\textsuperscript{phox} in the kidneys,\textsuperscript{5,10} increased renal cortical NADPH oxidase activity,\textsuperscript{11} and increased excretion of the lipid peroxidation marker 8-isoprostane PGF\textsubscript{2\alpha}.\textsuperscript{5,10} This study demonstrates the feasibility of "gene knockdown" in vivo in rats with a simple RNAi strategy using an RNA complexing solution and a rapid, relatively large-volume injection. This prevented increased expression of a target mRNA and protein in the kidney and thereby provided a model to investigate its biochemical and physiological actions. The main new findings are that injection of sip22\textsuperscript{phox} prevents an increase in expression of p22\textsuperscript{phox} mRNA and protein in the kidney and prevents an increase in NADPH oxidase activity, oxidative stress, and progressive rise in MAP of conscious rats during the second week of an Ang II infusion. In contrast, injection of a control siRNA sequence did not perturb these parameters. The effect is apparent between 48 and 96 hours after injection and was seen with 2 siRNAs targeted to different regions of the p22\textsuperscript{phox} cDNA. The reduction in renal cortical expression of p22\textsuperscript{phox} was accompanied by reduced expression of Nox-1, -2, and -4 but increased expression of p47\textsuperscript{phox}.

RNAi originated with the finding that double-stranded RNAs injected into *Caenorhabditis elegans* silenced genes with complementary sequences.\textsuperscript{22} Double-stranded RNA can be processed in cells into siRNAs, \(\approx 20\) to 22 nucleotides in length, by the enzyme Dicer,\textsuperscript{23} and are incorporated into an RNA-induced silencing complex, which silences complementary RNAs.\textsuperscript{21,27} Hydrodynamic transfection of siRNAs in mice involves rapid injection of a sufficiently large volume to transiently increase venous pressure\textsuperscript{24} to silence genes in organs with high blood flow, including the kidney\textsuperscript{28} and blood vessel.\textsuperscript{28} A modified strategy of rapid IV injection over 10 s of siRNAs in a gene complexing solution of TransIt in saline was effective in reducing the expression of the target mRNA and protein in vivo in the kidney cortex. The sip22\textsuperscript{phox} injection led to a 50\% reduction in p22\textsuperscript{phox} expression in the kidneys, matched by an equivalent reduction in the target enzyme activity in both sham and Ang II–infused rats. Apparently, the increase in NADPH oxidase activity in the kidney with Ang II depends on increased p22\textsuperscript{phox} protein...
expression. Additional studies will be required to determine whether similar effects of RNAi can be achieved in rats with this method in other organs besides the kidney and whether improved delivery or more stable constructs can increase the efficiency and prolong the duration of the therapeutic effects.

Potential problems with in vivo RNAi have been re-viewed. First, at high concentration, some siRNAs may act via TLR-3 to increase the INF response factor that stimulates INF and initiates cell signaling via STAT-1. Therefore, we undertook pilot studies in Ang II–infused rats to define a minimally effective dose of siRNA. We confirmed that siRNA injections did not change the renal mRNA expression for TLR-3 or STAT-1 in angiotensin-infused rats. Thus, it seems unlikely that activation of this pathway could account for our results. Second, there are off-target effects of RNAi because of silencing of genes with as few as 11 contiguous nucleotides of identity to the siRNA. Therefore, we limited the quantity of siRNA injected to 200 to 400 µg/kg (below the quantities used previously in mice), tested a control, nonsilencing sequence, and found that 2 active siRNAs targeted to different coding regions of the p22phox gene were equally effective in reducing p22phox expression, NADPH oxidase activity, and BP in Ang II–infused rats. Thus, the responses observed likely relate to reductions in p22phox mRNA and protein expression.

ROS have been assigned a critical role in vasoconstriction, endothelial dysfunction, vascular remodeling, and atherosclerosis accompanying hypertension. In the kidney, ROS can enhance many processes linked to the development of hypertension, including tubuloglomerular feedback, af-
ferent arteriolar vasoconstriction.\textsuperscript{11} NaCl reabsorption,\textsuperscript{30} and oxygen usage.\textsuperscript{23} Ang II can upregulate many of the components of NADPH oxidase in addition to \(p22^{phox}\) in blood vessels or kidneys, including Nox-1,\textsuperscript{9,10} Nox-2,\textsuperscript{31} \(p67^{phox}\),\textsuperscript{31} and \(p47^{phox}\).\textsuperscript{13} Mice with targeted deletions of \(p47^{phox}\) have a diminished increase in BP with Ang II.\textsuperscript{13} A Nox-2–blocking peptide reduces Ang II–induced hypertension in rats.\textsuperscript{32} whereas a Nox-2 knockout mouse has a reduced basal BP but a normal increase in BP with a full dose of Ang II despite a failure to increase aortic production of \(O_2^-\).\textsuperscript{33} The relative importance of Nox-1, -2, and -4 in mediating effects of Ang II requires additional study. \(p22^{phox}\) is expressed widely in the normal rat kidney in cells of the thick ascending limb of the loop of Henle, macula densa, distal tubule, cortical outer medullary and inner medullary collecting ducts, and vasa-luteative and interstitial fibroblasts.\textsuperscript{16} The present studies demonstrate that upregulation of \(p22^{phox}\) can account for about one third of the increase in BP, all of the increase in NADPH oxidase activity in the renal cortex, and the progressive increase in isoprostane excretion during an Ang II infusion.

Limitations of the present study include a lack of mechanism to explain how a reduction in \(p22^{phox}\) expression leads to a reduction in BP during Ang II infusion. Additional studies are needed to document the cellular sites of changes in \(p22^{phox}\) in resistance arterioles in systemic vessels and kidneys, in renal tubular epithelial cells and, perhaps, inflammatory cells, and the effects of these on salt transport, renin secretion, and vascular reactivity. A second limitation is that studies of NADPH oxidase expression and activity were confined to the kidneys. It is not clear to what extent \(p22^{phox}\) was reduced at other sites and the relative contributions of renal and extra-renal changes in \(p22^{phox}\) to the results obtained.

**Perspectives**

The \(p22^{phox}\) component interacts directly with Nox-1, -2, and -4 proteins in VSMCs to form a functional \(O_2^-\) generating NADPH oxidase.\textsuperscript{33,34} The reductions in Nox-1, -2, and -4 expression in rats with the \(p22^{phox}\) gene silenced may explain the reduced NADPH oxidase activity. Nox-2 is the prominent Nox isofrom expressed in human resistance vessels.\textsuperscript{39} Nox-1 is prominent in conduit vessels,\textsuperscript{9} and Nox-4 is expressed strongly in the kidneys.\textsuperscript{15} Because the resistance vessels and kidneys are prominent sites for Ang II–induced hypertension, this may explain the moderation of hypertension by silencing of \(p22^{phox}\) during Ang II infusion. Presently, there is no way to selectively prevent activation of NADPH oxidase. Drugs, such as diphenyliodinium, inhibit a wide range of flavin-containing enzymes in addition to NADPH oxidase. Pyocynin inhibits the activation of Nox-2 by \(p47^{phox}\) but \(p47^{phox}\) is not required for activation of Nox-1 or Nox-4.\textsuperscript{36} Thus, \(p22^{phox}\) appears to be the only unique requirement for activity of all Nox enzymes.\textsuperscript{34} This strategy of \(p22^{phox}\) silencing may have use in experimental studies and clinical settings in which a specific knockdown of all Nox enzymes is desirable. This study has, indeed, demonstrated that \(p22^{phox}\) plays a critical role in enhancing the expression of catalytic Nox components of NADPH oxidase during Ang II and may, therefore, be a useful target for drug development to obviate oxidative stress and its consequences in the hypertensive kidney.

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