Increased Expression of NAD(P)H Oxidase Subunit p67phox in the Renal Medulla Contributes to Excess Oxidative Stress and Salt-Sensitive Hypertension

Di Feng,1 Chun Yang,1 Aron M. Geurts,2,3 Terry Kurth,1 Mingyu Liang,1 Jozef Lazar,2 David L. Mattson,1 Paul M. O’Connor,1 and Allen W. Cowley, Jr.1,3,*
1Physiology Department
2Human and Molecular Genetics Center
3Cardiovascular Center
Medical College of Wisconsin, Milwaukee, WI 53226, USA
*Correspondence: cowley@mcw.edu
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SUMMARY
NAD(P)H oxidase has been shown to be important in the development of salt-sensitive hypertension. Here, we show that the expression of a subunit of NAD(P)H oxidase, p67phox, was increased in response to a high-salt diet in the outer renal medulla of the Dahl salt-sensitive (SS) rat, an animal model for human salt-sensitive hypertension. The higher expression of p67phox, not the other subunits observed, was associated with higher NAD(P)H oxidase activity and salt sensitivity in SS rats compared with a salt-resistant strain. Genetic mutations of the SS allele of p67phox were found in the promoter region and contributed to higher promoter activity than that of the salt-resistant strain. To verify the importance of p67phox, we disrupted p67phox in SS rats using zinc-finger nucleases. These rats exhibited a significant reduction of salt-sensitive hypertension and renal medullary oxidative stress and injury. p67phox could represent a target for salt-sensitive hypertension therapy.

INTRODUCTION
Essential hypertension is a multifactorial disease affecting nearly 27% of the world population (Kearney et al., 2005) and is a major risk factor for stroke, heart failure, and end-stage renal disease. Excess dietary salt intake is an important environmental factor in the etiology of hypertension, and the susceptibility of individuals to salt intake (salt sensitivity) is, in part, genetically determined. Therefore, it is of great interest to identify genes contributing to salt sensitivity.

The Dahl salt-sensitive (SS) rat, which shares many phenotypic traits seen in African American hypertensive patients (Cowley and Roman, 1996; Cowley et al., 2001; Jones et al., 2002; Rostand et al., 1982), has been used extensively to dissect the genetic complexity and related mechanisms contributing to salt-sensitive hypertension. Our interest in p67phox, one of the cytosolic subunits of NAD(P)H oxidase, resulted from characterizing a panel of congenic strains derived from the SS rat and the salt-resistant Brown Norway (BN) rat (Moreno et al., 2007). One of the congenic strains, SS.13BN26, containing a 16.0 megabase (Mb) genomic region from the BN rat substituted into Chromosome 13 of SS rats, was found to have a significant reduction of salt-sensitive hypertension and renal injury compared with the SS rat (Lu et al., 2010). p67phox was found to be located in the BN introgressed region of this congenic strain.

For many decades, the only pathological condition in which NAD(P)H oxidase was recognized to be of importance was chronic granulomatous disease (Kuhns et al., 2010). It is now recognized that this enzyme also plays an important role in the development and progression of cardiovascular disease (Brandes et al., 2010), including hypertension (Landmesser et al., 2002; Laursen et al., 1997; Rajagopalan et al., 1996). NAD(P)H oxidase is a multisubunit enzyme comprised of the membrane subunits gp91phox and p22phox and the cytosolic subunits p47phox, p67phox, p40phox, and Rac 1 or 2 (Paravacini and Touyz, 2008). All of the cytosolic subunits assemble on the membrane upon activation, allowing the enzyme to generate superoxide (O2·−). Most functional studies concerning the role of this enzyme in hypertension have focused on the central nervous system (Infanger et al., 2006) and peripheral vasculature. However, there is evidence that NAD(P)H oxidase contributes to oxidative stress that occurs in the renal outer medulla (OM) with increased salt intake in SS rats (Taylor et al., 2006). Direct interstitial infusion of an NAD(P)H oxidase inhibitor apocynin into the OM of SS rats attenuated salt-sensitive hypertension by nearly 50%. Since p67phox is the only known NAD(P)H oxidase subunit located in the introgressed region of congenic SS.13BN26, we determined whether genetic variances in p67phox of the SS rat could increase expression of the gene and thereby upregulate the activity of the enzyme, contributing to increased salt sensitivity in SS rats. We then generated a p67phox-null mutant (p67phox−/−) rat and demonstrated that disruption of p67phox in SS rats reduced salt-sensitive hypertension and renal oxidative stress and injury.

RESULTS
Genetic Variances in the Promoter Region of p67phox Affect Promoter Activity and Gene Expression
We performed real-time quantitative RT-PCR (qRT-PCR) to analyze messenger RNA (mRNA) expression of p67phox. Rats...
that the genetic variances in the promoter region may

cessence was 1.7-fold higher in the SS construct than in the

tions (SNPs) compared to SS.13BN26 rats (Figure S1 available
rats had a 204 bp deletion and four single-nucleotide polymor-

tively. The analysis of the promoter sequence revealed that SS

introduced rat medullary thick ascending limb cells (raTAL).

construct. The promoters were transfected into

containing all the genetic variances (Figure 1B) were cloned

were fed on a 0.4% NaCl diet (low salt, LS) since weaning. At

6 weeks of age, a subgroup of these rats was switched to an

8% NaCl diet (high salt, HS). Outer medullary tissue was

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days after their diets were switched. The average mRNA levels of

p67phox were increased in both the SS rat and congenic SS.13BN26

rats on the LS diet and greater salt sensitivity in SS rats compared

with greater levels of renal medullary NAD(P)H oxidase activity

on the HS diet and SS rats (Figure 2C).

p67phox-Null Mutant SS Rat Is Generated

with Zinc-Finger Nuclease Technology

To determine the functional relevance of p67phox in the develop-

ment of salt-sensitive hypertension in SS rats, we generated a p67phox−/−

model in the genomic background of SS rats using zinc-finger nuclease (ZFNs) (Geurts et al., 2009; Geurts et al.,

2010) (for details, see the Experimental Procedures). Genomic

DNA of p67phox-null mutant (p67phox−/−) rats was sequenced. Seque-

cing results suggested there was a 5 bp deletion (GAGAA) in the genomic sequence of p67phox−/− rats (Figure 3A). The

p67phox−/− rats were also validated by western blot experiments (Figure S3). In order to further demonstrate that there

was no functional p67phox in p67phox−/− rats, respiratory burst experiments were performed. p67phox is known to be critically

involved in the respiratory burst of macrophages (Maehara et al., 2009; Noack et al., 1999). We obtained macrophages from

the peritoneal space of 6- to 7-week-old p67phox−/− rats and wild-type (WT) littermates fed the HS diet for 14 days (De

Miguel et al., 2010). The respiratory burst response to phorbol 12-myristate 13-acetate (PMA) stimulus in the p67phox−/− rats

was completely abolished compared to the WT littermates (Figure 3B). We concluded that we had successfully generated the

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Cell Metabolism

*p67phox* in Salt-Sensitive Hypertension

**Figure 2. Greater *p67phox* Expression Is Associated with Greater NAD(P)H Oxidase Activity and Salt Sensitivity in the SS Rat**

(A) Western blot densitometric analyses of *p67phox* (β-actin normalized) of rats on both 0.4% LS and day 7 of HS diet with the associated gel shown (n = 5 rats/strain/time point). *p < 0.05 significant difference from SS.

(B) NAD(P)H oxidase activity of rats on both LS and D7-HS (n = 11–12 rats/strain/time point). *p < 0.01 significant difference from LS within strain; *p < 0.01 significant difference from SS.

(C) Mean arterial pressure (MAP) of 5–6-week-old rats on both 0.4% and 8% salt diet (n = 8–10 rats/strain). |p| < 0.001 significant difference between strains at that time point. Data are presented as mean values ± SE. See also Figure S2.

*p67phox*−/− Rats Show Significantly Reduced Salt Sensitivity and Renal Oxidative Stress

We first examined the effect of disrupting *p67phox* on blood pressure. Baseline MAP was not different between the WT and *p67phox*−/− rats at 6 weeks of age when fed the LS diet (Figure 4A). MAP between WT and *p67phox*−/− rats was statistically different on days 10 and 14 of the HS diet (p < 0.001). By the end of day 14, the MAP of *p67phox*−/− rats was 30 mmHg lower than their WT littermates. These results suggest that *p67phox* had a profound effect on the development of salt-sensitive hypertension in SS rats.

We then measured NAD(P)H oxidase activity and total superoxide levels using outer medullary tissue homogenate. OM was harvested at the end of blood pressure measurement on day 14 of the HS diet. The *p67phox*−/− rats demonstrated significantly lower renal medullary NAD(P)H oxidase activity (p = 0.0049) (Figure 4B). We used the portion of O$_2^−$ that was inhibited by diphenylene iodonium (DPI) as an index of NAD(P)H oxidase activity as previously described (Taylor et al., 2006). Since DPI is recognized as a nonselective inhibitor of all FAD-containing enzymes, including xanthine oxidase, cytochrome p450 oxidoreductase, NADH dehydrogenase, and others (Drummond et al., 2011), the residual O$_2^−$ level in the *p67phox*−/− rats was likely derived from non-specific DPI inhibition of other O$_2^−$ generating enzymes. Total renal medullary O$_2^−$ levels was also significantly lower in the *p67phox*−/− rats (p = 0.01) (Figure 4C).

To further validate that oxidative stress was reduced in *p67phox*−/− rats, outer medullary hydrogen peroxide (H$_2$O$_2$) levels were measured in anesthetized rats. In a separate group of *p67phox*−/− rats and WT littermates, microdialysis techniques were used to collect interstitial fluid from the outer medullary region, and H$_2$O$_2$ levels were measured with the Amplex Red assay (Jin et al., 2009). As shown in Figure 4D, H$_2$O$_2$ concentrations in the medullary interstitial fluid of *p67phox*−/− rats were significantly lower than WT littermates (p = 0.019).

*p67phox*−/− Rats Show Significantly Reduced Renal Injury

To evaluate the degree of renal injury, proteinuria and microalbuminuria were measured. Urine was collected overnight (18 hr) on the LS diet and on days 7 and 14 of the HS diet. In rats fed the LS diet, no differences were observed between WT and *p67phox*−/− rats. Protein excretion of WT rats increased from 14.4 ± 8.7 mg/day on the LS diet to 122.8 ± 18.7 mg/day on day 7 and to 331.2 ± 50.5 mg/day on day 14 of the HS diet. In contrast, *p67phox*−/− rats exhibited only half of these levels in response to the HS diet (Figure 5A). Microalbumin excretion followed the same trend (Figure 5B).

Histological analyses of the kidneys collected on day 14 of the HS diet were also used to quantify the renal injuries between WT and *p67phox*−/− rats. Trichrome staining was used to quantify protein casts (stained in red), an index of tubular necrosis. Protein casts were mostly observed in the OM region at levels that were 7-fold higher in WT compared with *p67phox*−/− rats (p < 0.001) (Figure 5C). Interstitial fibrosis was quantified by α-SMA immunostaining (Figure 5D), and infiltrated macrophages were quantified by ED-1 immunostaining (Figure 5E). α-SMA and ED-1 were stained in brown. In the outer medullary area, the levels of interstitial fibrosis and infiltrated macrophages in *p67phox*−/− rats were half the levels found in WT rats (p = 0.008 and 0.008, respectively). Glomerular injury was quantified by scoring 50 superficial cortical
and 20–30 juxtamedullary glomeruli (0–4 scale) of p67phox−/− rats and their WT littermates (Mori and Cowley, 2004). As shown in Figure 5F, the glomerular injury was significantly reduced in the superficial cortical glomeruli of p67phox−/− rats (similar reduction in the juxtamedullary glomeruli; data not shown). Overall, p67phox−/− rats showed dramatic reductions of renal injury in both the OM and cortex. These results indicate that p67phox plays a causal role in the development of salt-sensitive hypertension and renal oxidative stress and injury.

**DISCUSSION**

p67phox is known to be a key activator of NOX2-containing NAD(P)H oxidase in phagocytes (Maehara et al., 2009; Noack et al., 1999). However, the direct role of p67phox in the development of salt-sensitive hypertension and renal injury has not been explored previously. The results of the present study using p67phox−/− rats provide strong evidence of an important role for this gene in salt-sensitive hypertension. Even in the face of very large increases of salt intake, suppression of this gene in salt-sensitive hypertension. Even in the face of very large increases of salt intake, suppression of this gene in salt-sensitive hypertension. Even in the face of very large increases of salt intake, suppression of this gene in salt-sensitive hypertension. Even in the face of very large increases of salt intake, suppression of this gene in salt-sensitive hypertension. Even in the face of very large increases of salt intake, suppression of this gene in salt-sensitive hypertension. Even in the face of very large increases of salt intake, suppression of this gene in salt-sensitive hypertension. Even in the face of very large increases of salt intake, suppression of this gene in salt-sensitive hypertension.

In our current study, we also observed that p67phox expression—not the expression of p22phox, gp91phox, p47phox or Rac1—in the outer medullary region is associated with higher NAD(P)H oxidase activity and greater salt sensitivity in SS rats. There is immunohistochemical evidence from kidneys of SHR rats suggesting that p67phox is expressed in the thick ascending limb, macula densa, distal convoluted tubule, cortical collecting duct, and perhaps the outer and inner medullary collecting ducts (Chabrashvili et al., 2002). We have confirmed these findings by immunostaining a single Sprague Dawley kidney. Additionally, we found no staining in the vasa recta of the outer medulla. Among different tubules in the outer medulla, the mTAL has been previously shown to produce greater amounts of O2− than the thin descending limb and outer medullary collecting duct (Li et al., 2002). Moreover, increased luminal flow rate and/or [Na+] can stimulate the generation of O2− in mTAL (Abe et al., 2006). Increased levels of O2− can enhance Na+ entry via Na+K+2Cl cotransport and therefore increase Na+ reabsorption (Juncos and Garvin, 2005).

It remains to be determined whether the mTAL of the SS rats exhibits higher levels of p67phox expression and NAD(P)H oxidase activity in response to a HS diet than the mTAL of congenic SS.13BN26.

Figure 3. The p67phox−/− Null Mutant Rat Model Is Validated

(A) Sequencing of genomic DNA of wild-type (WT) and p67phox−/− rats revealed a 5 bp deletion (GAGAA) in the genomic sequence of p67phox−/− rats (n = 3 rats/strain).

(B) Respiratory burst of macrophages isolated from p67phox−/− (n = 6) and WT (n = 4) rats in response to phorbol 12-myristate 13-acetate (PMA), a PKC activator, administered at time zero. Data are presented as mean values ± SE. See also Figure S3.
oxidative stress in the outer medulla remains to be determined.

Finally, despite the crucial role of renal \textit{p67-phox} in the development of salt-sensitive hypertension, the role of this gene should still be examined in vascular systems and the central nervous system. Although SNPs in human \textit{p67-phox} have not been found to be associated with hypertension in genome-wide association studies, the present study suggests that stratification of the populations based on salt-sensitivity may uncover an association. Our studies also suggest that inhibitors targeting \textit{p67-phox} for the treatment of salt-sensitive hypertension should be explored.

**EXPERIMENTAL PROCEDURES**

**Animals**

Male rats were obtained at weaning from colonies developed and maintained at the Medical College of Wisconsin under controlled environmental conditions. They were provided water ad libitum and maintained on a purified AIN-76A rodent food (Dyets, Bethlehem) containing 0.4% NaCl (LS) since weaning. Some groups were switched to an 8% NaCl diet (HS) at 6 to 7 weeks of age. All experimental protocols were approved by the MCW Institutional Animal Care and Use Committee. Inbred congenic SS.13BN26 was generated as previously described (Cowley et al., 2001; Moreno et al., 2007).

**Tissue Collection**

Rats were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg). Both kidneys were removed and hemisected. The outer medulla was quickly removed and snap-frozen in liquid N2 and stored at −80°C. Frozen tissues were used for either DNA or RNA extraction or for preparation of tissue homogenates to perform NAD(P)H oxidase activity assays or western blots.

**Western Blot**

Outer medullary tissue was homogenized and processed as previously described (Taylor et al., 2006). Protein concentrations were determined using a Coomassie blue protein assay (Biorad) with bovine serum albumin as a standard. Lysates were mixed with laemmli sample buffer (BIO-RAD) and were run on a 7.5%, 4%–20%, or 10%–20% Tris SDS-PAGE gel (BIO-RAD) and transferred to a PVDF membrane (Bio-Rad). Membranes were blocked for 1 hr in 1x TBS-T (TBS [BIO-RAD] + 0.1% Tween20 [Sigma]) containing 5% nonfat dry milk at room temperature and then incubated at 4°C overnight (16–18 hr). Membranes were incubated in a primary antibody at room temperature for 2 hr, and then washed 5 min each time for four times in 1x TBS-T. Membranes were incubated in a secondary antibody at room temperature for 1.5 hr, and washed 5 min each time for four times in 1x TBS-T. The films were developed with Immun-StarTM HRP peroxide reagent (BIO-RAD). The films were scanned and the intensities of the target bands were quantified using Imagequant program (Amersham Biosciences).

**Real-Time Quantitative Reverse-Transcription PCR**

Total RNA was extracted from OM with TRIzol (Invitrogen). The qRT-PCR reaction mixture contained 1x SYBR Green PCR master mix (Invitrogen), 0.25 U/μl MultiScribe reverse transcriptase (Applied Biosystems), 0.4 U/μl RNase inhibitor (Applied Biosystems), 400 nM forward and reverse primers, and 8 ng total RNA. qRT-PCR reactions were carried out with the ABI Prism 7900HT system (Applied Biosystems) (Morrison et al., 2004). 18S rRNA was used as an internal control. Primers to amplify complementary DNA (cDNA) of \textit{p67-phox} were designed by Primer Express version 2 (Applied Biosystems) and are as follows:

- Forward (fw) primer: 5’-AGAGCAAGACCGATTGAGCATTGG-3’
- Reverse (rv) primer: 5’-TGCTTTCCATGGCCTTGTG-TGC-3’

**Chronic Measurement of Arterial Blood Pressure**

MAP was measured by radiotelemetry (De Miguel et al., 2011). Five- to six-week-old male rats were anesthetized and surgically prepared with an implanted gel-filled catheter in the right carotid artery. This catheter was attached to a transmitter (DSI) that was anchored subcutaneously between the scapulae. Baseline MAP was obtained daily from 9 am to 12 pm over a 3 to 4 day period.
period following a 5 to 7 day surgical recovery after catheter implantation. The diet was then switched from 0.4% LS to 8% HS. MAP was measured on days 1–3, 7, 10, and 14 of the HS diet.

**Sequencing the Coding Region of Rat p67phox**

Total RNA was extracted from outer medullary tissue and reverse transcribed to cDNA with an oligo-dT primer (Invitrogen). PCR primers were designed by DNASTAR program. PCR products were cloned into the pCR4-TOPO vector (Invitrogen) and sequenced. Primers used to amplify the coding region were as follows:

fw primer: 5’-caccatctgttcctgtgataagc-3’
rv primer: 5’-ctatactttctggagagctcctc-3’.

**Sequencing Promoter Region of rat p67phox**

Genomic DNA was extracted from outer medullary tissue. PCR primers amplifying the 3000 bp prior to the translation start site were design by DNASTAR. PCR products were sequenced directly. A 1700 bp sequence prior to the translation start site was assembled and analyzed. Primers are as follows:

fw primer: 5’-gtgttaggtatgatgataagc-3’
rv primer: 5’-gtgttaggtctactagagcagac-3’.

**Building p67phox Promoter Constructs**

PCR products from genomic sequencing were used as the template to build promoter constructs. Primers to amplify the promoter region containing all of the genetic variances are as follows:

fw1 primer (for SS.13BN26): 5’-aactcgagttgctcttaaattttttatttattctca-3’
fw2 primer (for SS): 5’-aactcgagttgctcttaaatgctcagttgcagc-3’
rv primer: 5’-aaactgagttgctcttaaatggccagagtggc-3’.

The underlined sequences indicate restriction enzyme target sites. PCR products were cloned into the Xhol-BgII site of pGL4.81 [hRlucCP/Neo] vector (Promega) (Liu et al., 2009). Renilla luciferase reporter constructs containing either the SS or SS.13BN26 p67phox promoter segment were verified by sequencing. Promoter vectors were prepared with an E.Z.N.A. Endo-Free Midi Plasmid Kit (Omega).

**Transfection and Luciferase Activity Assay**

Immortalized rat medullary thick ascending limb cells (raTAL) were cultured in REGM Renal Epithelial Cell Growth Medium (BulletKit, Lonza) and passaged (Eng et al., 2007). Once 90% confluency was reached, the cells were seeded onto 96-well plates. When raTAL cells reached 40%–50% confluency, reporter constructs were transfected using Lipofectamine 2000 (Invitrogen). During transfection, REGM media was replaced by Opti-MEM I Reduced Serum Media (Promega) (Liu et al., 2009). A control plasmid pGL2 (Promega) was also cotransfected to adjust for transfection efficiency. Four hours after transfection, Opti-MEM I Reduced Serum Media was switched back to culture media REGM. Cells were incubated overnight, and the promoter luciferase activity was measured by a dual luciferase-reporter assay system (Promega). Luminescence was measured with a high-throughput Analyst HT 96.384 microplate reader (Molecular Devices) (Liu et al., 2009).

**Production of the p67phox-Null Mutant Colony**

Zinc Finger Nucleases (ZFNs) targeting rat p67phox exon 2 was designed by Sigma. The target sequence contains CTCTACTACAGCATGTAAGTGGTGTCGGAGTGTT, where each ZFN binds to each underlined sequence on complementary strands. mRNA encoding the p67phox ZFNs were injected into embryos from SS rats and transferred to pseudopregnant SS females. The SURVEYOR Nuclease Assay was used to detect ZFN-mediated genome editing (Geurts et al., 2009; Geurts et al., 2010). The SURVEYOR Nuclease Assay was used to detect ZFN-mediated genome editing (Geurts et al., 2009; Geurts et al., 2010). The PCR primers used to amplify the target site were as follows:

fw primer: 5’-tgttaaacagcggccacttgctcagttgctgaactc-3’
rv primer: 5’-aactgcgcagacagacaacag-3’.

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**Figure 5. Renal Injury Is Significantly Reduced in p67phox−/− Rats Compared to WT Littermates**

(A and B) Proteinuria (A) and microalbuminuria (B) of p67phox−/− and WT rats on LS, D7-HS, and D14-HS diet (n = 6 rats/strain). *p < 0.01 significant difference from WT at the same time point.

(C–E) Histological staining and quantitative analyses of renal injury markers in p67phox−/− and WT littermates on D14-HS diet. Trichrome staining for the quantification of protein casts (n = 7 rats/strain) (C), α-SMA immunostaining for fibrosis (n = 6 rats/strain) (D), and ED-1 immunostaining (n = 5 rats/strain) (E) are shown. *p < 0.05 significant difference from WT.

(F) Cortical glomerular injury of superficial glomeruli (n = 7 rats/strain), *p < 0.05 significant difference from WT.

Data are presented as mean values ± SE.
Sequencing results suggested one of the pups contained one allele with a 5 bp deletion and the other allele with an 11 bp deletion. This rat was backcrossed with an SS female. The offspring of the backcross were either heterozygous (5 bp deletion/+), or heterozygous (11 bp deletion/+). Female and male heterozygous rats (5 bp deletion/+ or 11 bp deletion/+ or 5 bp deletion/11 bp deletion) were chosen for intercross. Fluorescent genotyping was used to distinguish homozygous p67 phox-null mutant (p67 phox/−/−) rats from the WT littermates. PCR products amplifying the ZFNs target sites were mixed with LIZ-600 size standard (Applied Biosystems) and were loaded on an ABI3730 sequencer. The results were analyzed by GeneMapper 4.0 software (Applied Biosystems). WT animals amplified at 325 bp while homozygous mutant animals amplified at 320 bp.

**Respiratory Burst Assay**

Six- to seven-week-old WT and p67 phox−/−/− rats fed the HS diet for 14 days were used. Rats were anesthetized with an intraperitoneal injection of pentobarbital (0.1 ml/100 g body weight). Eighty milliliters of saline were injected into the abdominal cavity followed by a small midline incision. The excess fluid was drained in a beaker. The collected fluids were centrifuged at 400 g for 10 min. Histopaque-1083 (Sigma) was used to isolate mononuclear cells (De Miguel et al., 2010). Ninety percent of collected cells were viable confirmed by trypan blue (Bl) staining. Using flow cytometry, 70%–75% of the collected cells were identified as CD11b+ macrophages, the major cells that generate the respiratory burst. 10% of the collected cells were CD3+ T cells, and 10% were CD45Ra+ B cells. Collected cell pellets were resuspended in 1 ml Dulbecco’s Modified Eagle Medium (Invitrogen) and were aliquoted into three wells on a clear-bottom 96-well plate (Bioexpress). The plate was incubated at 37°C for 2 hr. Each well contained around 1 x 10⁶ cells. Media were then removed and replaced with 0.3 ml of 1 mM luminol derivative L-012 (Wako Pharmaceuticals) dissolved in Hank’s balanced salt solution (Invitrogen). Luminescence of L-012 was used as an index of superoxide production. Luminescence was measured at 37°C on FLUOstar Omega machine (BMG Labtech) for baseline recording. Phorbol 12-myristate 13-acetate (PMA), a PKC activator, was added to each well to yield a final concentration of 135 µM. Luminescence was then measured every 5 min for 30 min.

**Detection of Total O2− and NAD(P)H Oxidase Activity by 2-Hydroxyethidium Fluorescence**

Outer medullary homogenate protein (20 µg) was incubated with dihydroethidium (20 µmol/liter), with salmon testes DNA (0.5 mg/ml), and with or without 100 µmol/liter diphenylene iodonium (DPI; Sigma), an inhibitor of NAD(P)H oxidase. Oxidethidium fluorescence was measured at an excitation of 485 nm and an emission of 570 nm on a SpectraFluor microplate reader (TECAN) every 5 min for 35 min (37°C) (Taylor et al., 2006). The maximal increase of fluorescence within 35 min was used as an index of total O2− level. The portion of oxidethidium fluorescence after DPI inhibition was used as an index of NAD(P)H oxidase activity.

**Acute Microdialysis**

WT and p67 phox−/−/− rats were anesthetized with ketamine (20 mg/kg, IM) and inactin (50 mg/kg, IP) and placed on a temperature-controlled surgical table maintained at 37°C. The femoral artery was cannulated to record arterial pressure. The femoral vein was cannulated to infuse saline containing 2% BSA in saline (0.1 ml/hr/100 g) to maintain constant blood volume. The left kidney was isolated and prepared for in vivo microdialysis (Jin et al., 2009). After a 1 hr equilibration period, dialysate effluent was collected over two 30 min intervals. Renal medullary interstitial H2O2 concentrations were determined by fluorescence spectrometry with the Amplex Red Hydrogen Peroxide Assay Kit (Molecular Probes).

**Histological Staining Analysis**

Rats were anesthetized and the left kidney was perfused with 10 ml 0.9% saline to flush the blood out, and the kidney was then removed and placed in phosphate buffer containing 10% formaldehyde. Kidneys were paraffin embedded in an automatic tissue processor (Microm). Three-micron cut sections were mounted on siliconized/charged slides. A robotic DAKO autostainer (Dako Cytomation) was used for all staining procedures. All images were captured with a Nikon E600 (Fryer) microscope equipped with a Spot Insight color CCD camera (Diagnostic Instruments). GoMori’s trichrome, α-SMA (Dako Cytomation) and ED1 (Serotec) staining and quantification were performed as previously described (Mori et al., 2008). Glomerular injury was quantified as previously described (Mori and Cowley, 2004).

**Measurement of Albumin and Protein in Urine**

Rats were placed in metabolic cages for overnight urine collection (18 hr). Microalbuminuria was quantified with an Albumin Blue 580 (Molecular Probes) fluorescence assay. Proteinuria was quantified with Weichselbaum’s biuret reagent on an ACE autoanalyzer (Alfa Wassermann).

**Statistical Analysis**

Data are presented as mean values ± one standard error. To assess the differences between two groups, t tests were performed. Empirical p values were obtained through 100,000 permutation tests. Two-way analysis of variance (ANOVA) for repeated-measures was used to analyze blood pressure, microalbuminuria and proteinuria data. For comparison of the difference between the strains on a specific day, a t test was used followed by 100,000 permutation tests. Multiple comparisons were adjusted with a Bonferroni correction. For other multiple group comparisons, a two-way ANOVA was performed. Multiple comparisons were adjusted with a Bonferroni correction. All of these data analyses were implemented using the R statistical package.

**ACCESSION NUMBERS**

Gene sequences have been deposited in NCBI’s GenBank and are accessible under accession numbers JN864041, JN864042, JN864043, JN864044, JN864045, and JN864046.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes three figures and can be found with this article online at doi:10.1016/j.cmet.2012.01.003.


