Evidence of the Importance of Nox4 in Production of Hypertension in Dahl Salt-Sensitive Rats

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Abstract—This study reports the consequences of knocking out NADPH (nicotinamide adenine dinucleotide phosphate) oxidase 4 (Nox4) on the development of hypertension and kidney injury in the Dahl salt-sensitive (SS) rat. Zinc finger nucleic acid injection of single-cell SS embryos was used to create an 8 base-pair frame-shift deletion of Nox4, resulting in a loss of the ≈68 kDa band in Western blot analysis of renal cortical tissue of the knock out of Nox4 in the SS rat (SS<sup>Nox4</sup>−/−) rats. SS<sup>Nox4</sup>−/− rats exhibited a significant reduction of salt-induced hypertension compared with SS rats after 21 days of 4.0% NaCl diet (134±5 versus 151±3 mm Hg in SS) and a significant reduction of albuminuria, tubular casts, and glomerular injury. Optical fluorescence 3-dimensional cryoimaging revealed significantly higher redox ratios (NADH/FAD [redated nicotinamide adenine dinucleotide/flavin adenine dinucleotide]) in the kidneys of SS<sup>Nox4</sup>−/− rats even when fed the 0.4% NaCl diet, indicating greater levels of mitochondrial electron transport chain metabolic activity and reduced oxidative stress compared with SS rats. Before the development of hypertension, RNA expression levels of Nox subunits Nox2, p67phox, and p22phox were found to be significantly lower (P<0.05) in SS<sup>Nox4</sup>−/− compared with SS rats in the renal cortex. Thus, the mutation of Nox4 seems to modify transcription of several genes in ways that contribute to the protective effects observed in the SS<sup>Nox4</sup>−/− rats. We conclude that the reduced renal injury and attenuated blood pressure response to high salt in the SS<sup>Nox4</sup>−/− rat could be the result of multiple pathways, including gene transcription, mitochondrial energetics, oxidative stress, and protein matrix production impacted by the knock out of Nox4. (Hypertension. 2016;67:440-450. DOI: 10.1161/HYPERTENSIONAHA.115.06280.) • Online Data Supplement

Key Words: Dahl salt-sensitive rat ■ hypertension ■ Nox4 ■ oxidative stress ■ renal injury

Kidney function, which plays a key role in hypertension, can be significantly compromised by pathways of oxidative stress, particularly if an imbalance between production of nitric oxide and reactive oxygen species (ROS) develops in the kidney.1-7 Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (Noxs) are a major source of ROS within all regions of the kidney,7-19 with the renal outer medulla exhibiting the greatest levels of Nox enzyme activity based on the rate of O<sub>2</sub>·<sup>−</sup> production per gram tissue.12 Elevations of O<sub>2</sub>·<sup>−</sup> levels, specifically in the renal medulla of normal Sprague-Dawley rats, have been shown to result in sodium retention and reductions of medullary blood flow, leading to hypertension.13,20,21 Conversely, local reduction of Nox by chronic medullary interstitial infusions of apocynin resulted in a reduced hypertension in Dahl salt-sensitive (SS) rats which express elevated levels of medullary Nox2 and tissue ROS.7 The renal medulla of SS rats naturally exhibits excess medullary ROS production with tissue O<sub>2</sub>·<sup>−</sup> and H<sub>2</sub>O<sub>2</sub> concentrations nearly twice that of salt-resistant consomic SS,13<sup>80</sup> control rats,6,7 even in the prehypertensive, low salt–fed state.5 Yet, the relative abundance, localization, regulation, and functional roles within the kidney of the various Nox isoforms have only begun to be elucidated. Nox1 and Nox5 (not expressed in rodents) are expressed at low levels within the kidney, and no specific functions have been yet ascribed to them,19,22 despite clear implications that they play an important role in vascular pathology of a variety of disease states.8,23 Nox2, the prototypical Nox isoform, has been the most widely studied and is found in the vasculature, heart, brain, and kidneys. Nox4 has been found to be the most abundant Nox isoform in the kidney,18,19 although its distribution and functional relevance to kidney function and in hypertension has remained poorly understood. The present study examined the contribution of the Nox4 isoform of Nox in salt-induced hypertension and renal injury of the SS rat, a model that recapitulates many aspects of hypertension in African Americans.24 To define the role of Nox4 in SS rats, we have produced a ubiquitous knock out (KO) of the Nox4 in the SS rat (SS<sup>Nox4</sup>−/−) using zinc finger nucleic acid technology.25,26 Additionally, given recent evidence that chemiluminescence signals in tissue/cell homogenates in

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the presence of enhancers, such as lucigenin, may not accurately reflect important sources of cellular ROS production, the redox state of the kidneys in the present study was assessed by optical fluorescence 3-dimensional (3D) cryoimaging, a novel technique we have recently described. Together, the results of our studies demonstrate that Nox4 contributes importantly to the development of salt-induced hypertension in the SS rat via alterations of mitochondrial electron transport chain (ETC) and redox state and through transcription effects on several Nox subunits and the intrarenal production of collagen.

Methods

Experimental Animals

Male rats were obtained at weaning from colonies developed and maintained at the Medical College of Wisconsin under controlled environmental conditions with parents and offspring fed a purified AIN-76A rodent food (Dyets, Bethlehem, PA) containing 0.4% NaCl with water provided ad libitum until the experimental period of 4.0% NaCl diet (high salt; Dyets, Bethlehem, PA). All experimental protocols were approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee.

Development of SS Rat With Nox4 (SSNox4−/−) Knocked Out

An SSNox4−/− was developed using zinc finger nucleases designed by Sigma to target the Nox4 exon 7 sequence GGTTACAGCTTCTACatatg AATAAGGTAAGGGTC, where zinc finger nucleases bind to each capitalized sequence on complementary strands. A similar approach was used successfully to study the function of p67phox, renin, and Rag1 in SS rats, and some details of the breeding strategy are included in the online-only Data Supplement. To confirm the mutation in homozygous animals, Nox4 cDNA of SS and SSNox4−/− products were amplified from kidney cortex samples and sequenced. The Nox4 gene in the KO rat was found to be missing 8 nt (CCTATGCA) at site +535 in exon 7. The frame shift caused by this deletion introduces 4 amino acids (aa) and an early stop codon. The SS Nox4 protein has 578 aa, whereas the deleted form in the KO rat contains N-terminal 178 aa containing the first 4 transmembrane domains but missing most of the C-terminal, including D, E loop, and FAD, NADPH-binding domains (see online-only Data Supplement for details).

Western blot analysis using an antibody provided by Dr Ajay Shah and techniques previously described by his group was performed on renal cortex, and outer medulla tissue collected from SS and SSNox4−/− rats to confirm the absence of the Nox4 protein. Renal cortex and outer medulla tissue snap-frozen at time of collection were homogenized and protein quantified and the lysates prepared for loading of 30 μg of protein as we have previously reported. Proteins were then separated on a 4–20% SDS-PAGE gel (BioRad) and transferred to a polyvinylidene fluoride membrane (PVDF) prepared and probed as we have described in detail. Additional membranes were prepared from cortex and medullary tissues from rats fed 4.0% NaCl diet for 21 days and were probed with the antibody for the Nox2 (Becton Dickinson). A ChemiDox XRS+ imaging system (BioRad) was used for chemiluminescence detection of bands, and Image Laboratory software, version 5.1, was used for quantification by densitometry. The membrane was stained with Coomassie blue, and the total Coomassie blue intensity in each lane was used for normalization. As the sequencing of the homozygous Nox4 KO cDNA fragment indicated deletion of 8 nucleotides, there was a resulting shorter piece of Nox4 with N-terminal 178 aa plus 4 extra aa and molecular weight of ~25 kDa compared to the full length of 594 aa and molecular weight around 68 kDa. Western blot confirmed the loss of the specific Nox4 band (~68 kDa, as well as 28 kDa).

The short isoform of Nox4 (band at 25 kDa) was not observed because the antibody recognized only the C-terminal part of Nox4 protein. We also performed Western blots to determine the total abundance of S-nitrosylated products in the renal cortex and outer medulla of rats of the 2 strains. We used an NO2Tyr antibody (Abcam) to determine the nitrosylation of proteins in the samples and quantified as described earlier.

Chronic Measurement of Arterial Blood Pressure, Heart Rate, Collection and Analysis of Urine

Mean arterial pressure (MAP) and heart rate (HR) were measured by radiotelemetry in adult male rats. At 6 weeks of age on the day before surgery, rats were placed in metabolic cages for an overnight urine collection (18 hours) for the measurement of urine albumin. The following day, as we have described, rats were surgically prepared and then given a 5- to 7-day recovery period before recording of blood pressure (see online-only Data Supplement for more details). Baseline MAP was recorded 24 hours/d for 3 days while animals were maintained on the 0.4% salt diet. The diet was then switched from 0.4% to 4.0% NaCl diet. MAP and HR were recorded for 24 hours/d for 21 days. After the last recording of MAP and HR on day 21, the rats were again placed in a metabolic cage for a second overnight urine collection. Albuminuria was quantified using an Albumin Blue 580 (Molecular Probes) fluorescence assay.

Histology

Following the last day of 4.0% NaCl diet and measurement of blood pressure and collection of urine, rats were anesthetized; the left kidney was flushed with saline and fixed with a 10% buffered formalin solution. Kidneys were paraffin-embedded and 3 μm sections mounted and stained with Gomori’s trichrome and immunostained for α-smooth muscle actin (Dako Cytomation) for quantification of renal injury and fibrosis as we have been described in detail. One section stained from the left kidney of each rat studied was scored by a trained observer blinded to the strain/group. Tubular necrosis was determined by quantifying the percentage of the outer medullary tissue containing blocked tubules filled with protein using MetaMorph Image Analysis software (version 7.6; Universal Imaging System). For the glomerular scoring, 60 superficial glomeruli and 30 juxtamedullary glomeruli were selected and scored for each kidney section, and the percent of glomeruli with a score of ≥2 was calculated. The scoring of each glomerulus was a modification of the injury score described by Raji et al. Each glomerulus was examined and a score of 0 to 4 given according to the severity of the lesion (mesangial expansion and glomerulosclerosis), with a score of 1 representing an involvement of 25% of the glomerulus up to a score of 4 indicating 100% of the glomerulus was involved.

Quantitative Polymerase Chain Reaction

Tissue was collected from a separate group of rats of both strains maintained on 0.4% NaCl diet from birth. The outer medulla was dissected from the cortex, and samples of both regions were snap-frozen in liquid nitrogen and stored at −80°C. Total RNA was extracted from both the renal cortex and the outer medulla tissue using Trizol reagent. The quality of each sample was assessed by spectrophotometry (Nanodrop). Two microgram of total RNA was reverse-transcribed by random hexamer primers into cDNA (Thermo Scientific RevertAid First Strand cDNA synthesis kit) and real time polymerase chain reaction analysis performed using 8 to 10 ng total RNA as we have described using SYBR green chemistry on an ABI Prism 7900HHT (Applied Biosystems). All primer sequences are given in the online-only Data Supplement. Similarly, tissue was collected from the rats used for blood pressure recording at the end of the study after 21 days of 4.0% NaCl diet and renal cortex and outer medulla tissue for determination of gene expression.
Optical Fluorescence 3D Cryoimaging
In separate groups of SS (n=6–8/salt diet) and SSNox4−/− (n=5–9/salt diet) rats, kidneys were collected for the 3D imaging protocol. We have recently reported this technique for the determination of the oxidative state of the kidney in SS rats.28 The frozen kidneys, stored at −80°C until the day of study, were mounted in the cryoimager, a custom-designed and fabricated optical fluorescence imaging system at −80°C until the day of study, were mounted in the cryoimager, a custom-designed and fabricated optical fluorescence imaging system from the Biophotonics Laboratory at the University of Wisconsin-Milwaukee previously described in detail.28,30–32
Separate images for reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD) autofluorescence were obtained to probe the oxidative state of the metabolism in the tissue. These images were obtained from each of the sequentially sliced kidneys, and these images from each group of kidneys were analyzed using MATLAB (The Mathworks Inc). Representations of the kidneys were assembled into 3D images using z-stacks of all the image slices for both NADH and FAD signals, and the NADH to FAD redox ratio of each kidney was calculated as we have recently described.28 An intensity histogram distribution of NADH/FAD ratio for each kidney was calculated, and the mean value determined for the histogram of each kidney.28 A more detailed description of the calculation of the mean values of the histogram is given in the online-only Data Supplement along with other additional methodological descriptions.

Statistical Methods
Data are presented as mean values±standard error. A 2-way analysis of variance for repeated measures (repeated measurements in the same subjects) followed by a Holm–Sidak post hoc test were used to assess differences within and between the strains for the hemodynamic data and for the urinary excretion of albumin. For the comparison of the tissue redox ratio between the separate groups of collected kidneys from both strains on both salt diets, a 2-way analysis of variance followed by a Holm–Sidak post hoc test was used. Student’s t test was used to compare between the 2 strains for the histological analysis of glomerular injury and tubular necrosis and for the mRNA expression analysis and for the quantification of the nitrosylated proteins. A P<0.05 was considered significant with a power of >0.69 to 1.0 and a β<0.31.

Results
Validation of the Null Mutation of Nox4 in the SS Rat and Impact on Gene Expression
cDNA sequencing was performed on real-time polymerase chain reaction products amplified from the mRNA obtained from the renal cortex from SSNox4−/− rats and SS rats. Each of the SSNox4−/− rats (n=8) exhibited an 8 base-pair frame-shift deletion within exon 7, which resulted in a truncation of the 578 aa Nox4 protein with 178 aa remaining at the N-terminal (see online-only Data Supplement for details of sequence). Figure 1 (A) shows the Western blot membrane with no detectable Nox4 protein band at 68 kDa in the SSNox4−/− (n=5) renal cortical tissue collected after 21 days of 4.0% NaCl diet compared with tissue from the SS rat (n=5) on the same diet as quantified by densitometry (B). The molecular weight bands seen on our Western blot were previously observed by Anilkumar et al using this same antibody were absent in the SSNox4−/− rats, including those bands at 62, 28, 22, and 10 kDa. It has been proposed that the 28 kDa band represents a splice variant of Nox4 localized to the nucleus in vascular cells.15 Another group of bands in our blot was present in both strains and seems to be the result of nonspecific binding. In our preliminary Western blot, there was no signal for Nox4 detected in homogenized outer medullary tissue in the SS rat, and so the data are not shown.

Arterial Pressure Responses to the 4.0% NaCl Diet
As summarized in Figure 2, SSNox4−/− rats exhibited a marked reduction of salt-induced hypertension compared with SS rats. MAP 24 hours averages did not differ significantly between SS (n=8; 108±1 mm Hg) and SSNox4−/− rats (n=9; 105±1) on the last day of 0.4% salt diet. However, after 21 days of the high-salt (4.0% NaCl) diet, the average 24 hours MAP of SSNox4−/− rats was significantly lower (134±5 mm Hg; n=9) relative to SS rats (151±3 mm Hg; n=8; P<0.05). The continuous 24 hours pulsatile pressure data were analyzed to determine whether the circadian rhythm was modified in SSNox4−/− compared with SS rats. MAP was averaged in 12 hours bins to correspond to the day (6 AM to 6 PM) and night (6 PM to 6 AM) cycles. No significant differences in rhythmicity were observed between strains, although the amplitudes of cycles were greater in SS
rats fed the 4.0% NaCl diet (P<0.01; Figure S1 in the online-only Data Supplement). To control for the effect of pressure itself on the amplitude of the cycling, the amplitude data were normalized to the MAP. Analysis of these normalized data showed no significant differences between the strains during the control 0.4% NaCl diet or after the transition to the high-salt diet.

The 24 hours HR for SS rats maintained on the 0.4% NaCl diet averaged 446±5 and 455±3 bpm in SS Nox4−/− rats. After transition to high-salt diet, HR fell significantly in both strains by day 8 and remained reduced through day 21 of 4.0% NaCl diet (405±2 bpm in SS; 409±4 in SS Nox4−/−) and was not significantly different between strains (data not shown).

Indexes of Renal Injury

The daily excretion of albumin determined on the last day of 0.4% NaCl and after 21 days of 4.0% NaCl diet is summarized in Figure 3. Significantly lower levels of albumin excretion were observed in the SS Nox4−/− rats even when receiving the 0.4% NaCl diet when compared with the SS strain. Most evident, however, was the marked protection from severe albuminuria observed after 21 days of 4.0% NaCl diet at which time SS Nox4−/− rats excreted only about 1/7 as much albumin as SS rats.

Histological analyses of renal injury in the kidneys of both strains after 21 days of high salt (n=9–10/strain) included determination of tubular necrosis and glomerular injury. As summarized in Figure 4, SS Nox4−/− rats exhibited markedly less tubular necrosis as evidenced by a large reduction of tubular cast staining (Gomori’s trichrome; 2.4±0.3% positive strained region) compared with SS rats (12.6±1.3%). Glomerular injury was also significantly less (P<0.05) in SS Nox4−/− than in SS rats when comparing the percentage of glomeruli with an injury score of ≥2 (scale of 0 for no injury to 4 with marked glomerulosclerosis and mesangial expansion). Kidneys of SS Nox4−/− rats averaged 43±1.5% of the glomeruli compared with the SS kidneys with 65±3.9% of the glomeruli with an injury score of ≥2 in both the cortical and juxtamedullary areas. α-Smooth muscle actin immunostaining for renal fibrosis in the renal outer medulla was not significantly different between SS Nox4−/− (7.3±0.5%) and SS (7.9±1.0%) rats (data not shown).

NADH/FAD Redox Ratios and Determination of Oxidative Stress Levels

Figure 5A presents representative 3D images showing the NADH and FAD fluorescence signals and the tissue redox ratios (NADH/FAD) obtained from a SS and a SS Nox4−/− rat fed either the 0.4% NaCl diet or the 4.0% NaCl diet for 21 days. Higher NADH and lower FAD fluorescence signals (ie, higher redox ratios) are evident in the kidneys of SS Nox4−/− rats compared with SS rats fed either salt diet. Lower tissue redox ratios represent higher levels of oxidative stress, and the tissue redox ratio shows an inverse relationship with the oxidative stress level of the tissue. In both strains, the 4.0% NaCl diet resulted in lower redox ratios throughout the kidney, especially in the mitochondria-rich renal medulla as apparent by the pseudo-red color intensity. However, redox ratios remained comparatively greater in the SS Nox4−/− rats. Images of each of the kidneys analyzed for these studies are presented in Figure S2.

Figure 5B summarizes the group data for the kidney tissue redox ratios of SS and SS Nox4−/− rats on the 2 salt diets. Kidneys of SS Nox4−/− rats exhibited significantly higher tissue redox ratios than kidneys of SS rats whether fed a 0.4% or the 4.0% NaCl diet, indicating reduced oxidative stress. The redox ratio of SS kidneys of rats fed the 4.0% NaCl diet (n=8) was significantly lower than those fed the 0.4% NaCl diet (n=6), 1.18±0.06 versus 1.34±0.03, respectively, as was the case in SS Nox4−/− rats (n=9), which averaged 1.38±0.05 when fed the 4.0% NaCl diet compared with SS Nox4−/− rats (n=5) which averaged 1.67±0.07 (P<0.001) when fed 0.4% NaCl diet. Based on a 2-way analysis of variance, there was a statistically significant difference between strains (P<0.001) and salt diet (P<0.001) and a significant interaction of the 2 (P=0.046). These data indicate that although oxidative stress, as assessed from the redox ratios, was increased with the high salt diet in both strains, it was buffered in the SS Nox4−/− rats.

These results were further supported by the determination of nitrosylated protein (NO2Tyr) by Western blots of the renal cortex and outer medulla from the SS and SS Nox4−/− rats.
on 0.4% and 21 days of 4.0% NaCl diet. In these studies, there was no significant difference between the abundance of NO2Tyr in the renal cortex or outer medulla of the SS (n=6) or SSNox4−/− (n=5) rats on 0.4% NaCl diet. However, the outer medulla of the SS rats fed 4.0% NaCl for 21 days had significantly higher abundance of NO2Tyr (1.3-fold higher; \( P < 0.005 \)) than the SSNox4−/− rats. The renal cortex of the 4.0% NaCl–fed SS rats was slightly but significantly less than the SSNox4−/− rats (\( P < 0.053 \)) (data not shown).

**Gene Expression Profiles of SSNox4−/− Rats**

Figure 6 summarizes the gene expression of the NADPH subunits and Nox4 in the renal cortex and renal outer medulla tissue collected in the prehypertensive state from rats maintained on the 0.4% NaCl diet. Expression of \( p67^{phox} \), \( p22^{phox} \), and Nox4 were significantly reduced (\( P < 0.05 \)) in the renal cortex and outer medulla of SSNox4−/− rats compared with SS rats. Nox2 was significantly less in the SSNox4−/− compared with the SS rats (\( P < 0.05 \)) but only in the renal cortex. Nox1 mRNA was detected in the renal tissue by real-time polymerase chain reaction but the expression was too low for quantitative real-time polymerase chain reaction to be run.

Shown in Figure S3 are the results of the Western blot analysis for Nox2 protein in homogenates prepared from the renal cortex and outer medulla tissue collected at the end of the study after 21 days of 4.0% NaCl diet. Nox2 protein expression was significantly reduced in the renal outer medulla of SSNox4−/− rats compared with SS rats. Consistent with the mRNA expression data, these results indicate that the reduction of Nox2 in SSNox4−/− rats was confined largely to the renal cortex after 21 days of 4.0% NaCl diet.

Because we did not detect a difference in \( \alpha \)-smooth muscle actin staining for renal fibrosis between strains, we tested expression levels of collagen. RNA expression levels of collagen 1a1, 1a2, and 4a2 were also significantly lower in SSNox4−/− compared with SS rats (\( P < 0.05 \)) in both the renal cortex and renal outer medulla in rats maintained on 0.4% NaCl diet and in rats after 21 days of 4.0% NaCl diet (see Figure S5 and S6).

Together these results indicate that KO of the Nox4 gene resulted in mRNA expression changes related to proteins critically important to the activity of Nox2 and matrix proteins of the cortex and medulla of the kidney. Remarkably, rather than observing compensatory increases of Nox2-related subunits indicated that Nox2 was reduced in the SSNox4−/− rats. As shown in Figure 7, analysis of mRNA expression of the NADPH subunits Nox2 and \( p47^{phox} \) from renal cortex found that SSNox4−/− rats (n=6) exhibited a nearly 50% reduction (\( P < 0.05 \)) of these isoforms in the renal cortical tissue, whereas no significant differences were observed in outer medullary tissue as compared with SS rats (n=6). In contrast to the rats fed 0.4% NaCl diet, no significant differences were observed in expression of \( p67^{phox} \) or \( p22 \) in the cortex in SS (n=6) versus SSNox4−/− (n=6) rats but there was a significant reduction of the \( p67^{phox} \) isoform in the outer medulla.

Figure S4 summarizes the results of the Western blots analysis for Nox2 protein in homogenates prepared from renal cortex and outer medulla tissue collected in those rats of both strains at the end of study after 21 days of 4.0% NaCl. Nox 2 protein expression significantly reduced in the renal cortex of SSNox4−/− rats (n=9) compared with SS rats (n=6). Consistent with the mRNA expression data, these results indicate that the reduction of Nox2 in SSNox4−/− rats was confined largely to the renal cortex after 21 days of 4.0% NaCl diet.
in the renal cortex from SSNox4−/− rats, the changes measured moved in parallel with the reduction of Nox4 expression in this mutated SS rat. Because these transcriptional changes were observed in the prehypertensive period when the blood pressure was the same in both rat strains before switching to the high-salt diet, it is evident that these changes were a consequence of the reduction of Nox4 rather than changes secondary to the high-salt diet and hypertension. The reduction of Nox2 expression and of the collagen matrix proteins would be expected to further enhance the protection of the kidneys from fibrotic injury and contribute to the blunting of salt-induced hypertension beyond that achieved by reduction of Nox4 alone.

**Discussion**

The role of Nox4 in hypertension and associated renal injury has remained largely unexplored. Nox4 is known to be abundantly expressed in the kidney as confirmed by the present study. We found that tissue levels of Nox4 mRNA in the kidney were expressed at levels nearly 7-fold higher in the renal cortex than medulla of prehypertensive SS rats fed a 0.4% NaCl diet. Western blotting further revealed that Nox4 is highly expressed in the cortex versus outer medulla in rats fed 4.0% NaCl diet. Most importantly, however, the KO of the Nox4 gene provided the SS rat significant protection from renal oxidative stress, renal injury, and hypertension. Moreover, the results revealed mechanistic insights whereby Nox4 exerted these protective effects, including alterations of cellular mitochondrial bioenergetics, alterations of the transcriptional regulation of Nox subunits, and transcriptional regulation of collagen synthesis within the kidney.

**Nox4 Effects on Renal Mitochondrial Bioenergetics and Oxidative Stress**

The 3D cryofluorescence images show that the tissue redox ratio (NADH/FAD) was significantly higher in the kidneys of SSNox4−/− rats compared to SS rats whether fed the 0.4% or the 4.0% NaCl diet, similar to the differences we recently observed between the SS and SSp67phox−/− null mutant rats. Lower tissue redox ratios represent higher levels of oxidative stress, and the tissue redox ratio shows an inverse relationship with the oxidative stress level of the tissue. The higher NADH signal and lower FAD signal observed in the mitochondria-rich renal medulla of the SSNox4−/− rats indicates the greater presence of NAD in the reduced form (NADH) in the mitochondria and less FADH2 present in its oxidized form (FAD) compared to SSNox4−/− rats. Enhanced pairing of electrons with hydrogen and less leakage of electrons in the mitochondrial ETC to available oxygen would result in less production of ROS. Importantly, since NADH and FAD signals originate exclusively from the mitochondria, the present results indicate greater mitochondrial ETC metabolic activity and decreased oxidative stress in the mitochondria-rich renal medulla of the SSNox4−/− rats. It is recognized that in the presence of oxidative stress, the ETC does not function efficiently because of oxidative damage of ETC complexes. This leads to excess electron leak and ROS production, which leads to the accumulation of the mitochondrial coenzymes NADH and FADH2 in their oxidized forms NAD and FAD. Hence, a decrease in NADH fluorescence and an increase in FAD fluorescence results in a decreased redox ratio (NADH/FAD). Therefore, the inverse relationship between the redox ratio and oxidative stress shows that the lower redox ratios observed in the SS rats represent higher levels of renal oxidative stress. The presence of greater abundance of nitrosylated protein products (NO2Tyr) in the outer medulla of the SS rat fed 4.0% NaCl diet further supports the greater levels of oxidative stress in this strain than in the SSNox4−/− rats.

It is interesting that although we were able to detect greater Nox4 protein expression in cortical tissue homogenates than in medullary tissue of SS rats, alterations in the redox state by the mutation of the Nox4 were more apparent in the renal medulla. This seems to be explained by the insensitivity of the Nox4 antibody because preliminary functional studies in our laboratory have shown that Nox4 plays an important role in ROS production in the freshly isolated perfused medullary thick ascending limb of SS rat. Moreover, the results revealed mechanistic insights whereby Nox4 exerted these protective effects, including alterations of cellular mitochondrial bioenergetics, alterations of the transcriptional regulation of Nox subunits, and transcriptional regulation of collagen synthesis within the kidney.

**Figure 5. A** A composite of representative 3-dimensional rendered images of kidneys from each strain on both salt diets. The distribution of fluorescence signal for reduced nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD) and the tissue redox ratio (NADH/FAD) indicate those regions highest in red (see intensity scale) that are protected from the oxidative stress as demonstrated by the higher NADH/FAD ratio in the SSNox4−/− strain. **B** Summarizes the mean values of the tissue redox ratio of NADH/FAD for the separate groups of salt-sensitive (SS) and SSNox4−/− (Nox4−/−) rats on 0.4% (SS n=6; Nox4−/− n=5) and 4.0% NaCl diets (SS n=8; Nox4−/− n=9). *Significant difference within the strain between the 2 diets (P<0.05; **P<0.1). †Significant difference between strains on the same salt diet (P<0.05; **P<0.1). Two-way analysis of variance (ANOVA); Holm–Sidak post hoc.

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Fumarase is an enzyme in the TCA (tricarboxylic acid cycle), which facilitates a transition step in the production of energy in the form of NADH, and chronic infusion of a fumarate precursor into the medullary interstitium of a salt-resistant control strain (SS.13BN) restored blood pressure salt-sensitivity. It has recently been found that Nox4 can potently inhibit the enzyme fumarate hydratase, leading to accumulation of fumarate. The kidney tissue of diabetic mice and human subjects exhibited reduced expression of fumarate hydratase which could be restored in vitro by the administration of Nox1/Nox4 inhibitor GKT137831. Transgenic overexpression of Nox4 in mice resulted in overexpression of fumarate as found in diabetic mice, and this was associated with endoplasmic reticulum stress, matrix gene expression, expression of hypoxia-inducible factor-1a and transforming growth factor-β (TGF-β, events attenuated by NOX1/NOX4 inhibition in diabetic mice. These data suggest that the KO of Nox4 in SS rats may result in restoration of fumarase, which could account for the enhanced energy production and reduced mitochondrial ROS production observed in the SSNox4−/−.

Evidence of Nox4 Regulation on NADPH Oxidase Gene Transcription

It was found that SSNox4−/− rat kidneys expressed reduced levels of Nox2, p67phox, and p22phox mRNAs in the renal cortical tissue of rats fed the 0.4% NaCl diet and before the development of hypertension. As these subunits are critical to the activity of Nox2, the reduction of renal ROS in SSNox4−/− rat kidneys could be attributed to these observed changes, although Nox2 activity levels were not specifically quantified in the present study. The parallel reductions of the Nox2 subunits in the SSNox4−/− rat kidneys indicates that Nox4 is participating in the transcriptional regulation of these genes, whereby Nox4 and Nox2 may act in concert in determining renal function in the SS rat. It should be noted that Nox1 mRNA is expressed at nearly undetectable and unquantifiable levels in rodent kidneys, as we have also observed (data not shown).

These findings are of particular importance because, as the field moves forward and relies increasingly on the application of gene editing model systems, it is important to appreciate that the resulting phenotypes may not represent changes that can be directly attributable to the gene that was targeted. The present data are a good example of a response that was not a predictable compensatory response, which would offset the loss of function of the Nox4. Rather, the KO resulted in responses which would seem to amplify the effect of simply removing Nox4. It will not be easy to quantify the relative contribution of these interacting systems but important to recognize the complexity of these systems as we move forward.

Figure 6. mRNA expression was determined by quantitative polymerase chain reaction (qPCR) from RNA extracted from homogenates of renal cortical (A) and outer medullary (B) tissue collected from salt-sensitive (SS) rats (n=6) and SSNox4−/− (n=6) rats maintained on 0.4% NaCl diet. The RNA expression levels of the NADPH subunits gp91phox (Nox2), p47phox (p47), p67phox (p67), and p22phox (p22) and of Nox4. Tissues from both strains were run for any given gene on the same plate; thus, expression levels can be compared between the 2 renal regions. *Significant difference between strains (p<0.05; β<0.3); Students’ t test.
Although Nox4 is the most abundantly expressed Nox isoform in the kidney, we have shown that Nox2 also contributes importantly to renal dysfunction and hypertension in the SS rats. The null mutation of the p67phox gene in SS rats, a critical subunit of Nox2, also resulted in reductions of salt-induced hypertension and renal injury similar to those presently observed with the KO of Nox4. SSp67phox−/− null rats also exhibit a significant reduction of ROS production in the renal interstitium as determined by microdialysis and by optical fluorescence 3D cryoimaging. As p67 phox is important for the activation only of Nox2 (and not Nox4), it is evident that Nox2 contributes to hypertension in SS rats. Given the data from the present study showing that KO of Nox4 results in reductions of several of the key Nox2 subunits, it seems that the protective effects of Nox4 KO could be attributed in part to the parallel reduction of Nox2 activity.

Nox4 Effects on Transcriptional Regulation of Collagen Production

KO of Nox4 in SS rats on the 0.4% NaCl diet before hypertension in response to high salt resulted in reduced mRNA expression levels of collagens 1α1, 1α2, 4α2 in both the cortex and medulla. The reduced expression of these collagens indicate that Nox4 was indeed having a protective effect from fibrosis in these rats, even though the immunostaining and quantification of kidney α-smooth muscle actin did not demonstrate reduced levels of fibrosis in the SSNox4−/− rats after 4.0% NaCl diet for 21 days. We have previously reported that expression of collagen 1α1 was upregulated in SS rats as compared to the congenic salt-resistant SS.13th control strain. Collagen 1α2 and collagen 3α1 have been shown to be elevated in response to matrix metalloproteinase 7 and also in aging kidneys and in diabetes mellitus, where these proteins are thought to contribute to the development of fibrosis, leading to chronic kidney disease. TGFβ1, which is elevated in kidneys of SS rats and increases the progression of renal interstitial fibrosis independent of blood pressure, is associated with increases of fibronectin-1 and collagen 1α1. Based on these observations, the reduced expression of collagens 1α1, 1α2, and 4α2 would be expected to provide added protection from the salt-induced hypertension and renal injury in the SSNox4−/− rats.

Other Possible Contributors to Renal Protection in SSNox4−/− Rats

Given the global nature of the Nox4 KO, we also recognize that other sites of action of Nox4 (eg, vascular or central nervous system) could have contributed to the observed protection from hypertension and renal injury in SSNox4−/− rats. However, given the abundance of Nox4 in the kidney, it is likely that the reduced intrarenal actions of Nox4 contributed in large measure to the reduced oxidative stress and injury of the SSNox4−/− rats. Finally, it should be recognized that reduced renal injury in SSNox4−/− rats could also be a consequence of the blunting of hypertension because it has been shown using servo-control techniques that renal perfusion pressure contributes importantly to the renal injury in SS rats.
Nox4: A Unique Nox Isoform

The general biochemical features of the Nox family of enzymes have been well characterized and described by others. Nox4 is unique in that it does not require the binding of cytosolic p67phox, p47phox, or Rac1 or 2 proteins to produce ROS which is produced constitutively and mostly as H2O2. In addition to the cell membrane, Nox4 may also be localized to intracellular locations, such as the mitochondria, endoplasmic reticulum, the nucleus, and focal adhesions, but observations are variable and controversial given the lack of antibody selectivity, variations in staining approaches, splice variants, and possible transitioning between intracellular compartments.

The role of Nox4 in hypertension has not been previously studied in rats. Most studies related to the functional role of Nox4 have focused on the systemic and pulmonary vasculature, and largely in cultured cells, where this isoform has shown to be constitutively active, regulated at the gene level, and influenced by angiotensin II, shear stress/flow, hypoxia, and microRNAs. Nox4 was found to be a major source of oxidative stress in rat NRK-52E cells (a rat renal proximal tubular cell line) when stimulated with angiotensin II. In freshly isolated medullary thick ascending limb of Sprague–Dawley rats, Nox4 siRNA was found to reduce O2− responses to acute stimulation with either angiotensin II or increased luminal flow. In other studies, Nox4 has been importantly implicated in glomerular podocyte injury and apoptosis in a rat podocyte injury model induced by puromycin aminonucleoside treatment. Results of this study indicated that TGF-β1 induced mitochondrial Nox4 activation and ROS production through the TGF-β receptor-Smad2/3 signaling pathway. This conforms to the recent studies in Nox4 transgenic mice with specific expression in glomerular podocytes which resulted in accumulation of fumarate which is a key regulator of hypoxia-inducible factor-1α and TGF-β. In mice, KO of Nox4 has been found to provide protection from oxidative stress and injury of the systemic vasculature during ischemic or inflammatory stress induced by angiotensin II infusion. Deletion of Nox4 in streptozotocin-induced diabetic ApoE−/− mice also was found to confer renal protection from glomerular injury. Together, these observations are consistent with the present observations, indicating that the KO of Nox4 in SS rats results in protection from renal glomerular, tubular, and interstitial injury.

Perspectives

Nox4 is the most highly expressed Nox isoform in the kidney and seems to be involved in a various diseases, such as idiopathic pulmonary fibrosis, pulmonary arterial hypertension, diabetic nephropathy, complications of diabetic cardiomyopathy and neuropathy and retinopathy, and metastatic carcinoma. Little is known about the role of Nox4 in normal renal function and sodium homeostasis or in the pathophysiology of hypertension. The broad significance of the present study is as follows: First, an SS rat with a null mutation in Nox4 was producing enabling assessment of this Nox isoform in a hereditary model of SS hypertension. Second, data are presented showing that Nox4 contributes importantly to hypertension, renal oxidative stress, and renal injury observed in SS rats fed a high-salt diet. Third, mechanistic insights were obtained, indicating that Nox4 exerts its effects through multiple pathways, most notably by transcriptional regulation of Nox2 enzyme subunits, transcriptional regulation of collagen synthesis, and via its effects on mitochondrial energetics.

Acknowledgments

A. Cowley designed the study, participated in data analysis, and drafted the article. C. Yang, K. Sadovnikov, and V. Kumar performed the Western blot analysis and the RNA expression experiments. T. Kurrth performed the blood pressure studies. C. Yang, N. Zheleznova, F. Salehpour, and M. Ranji performed the tissue collection and 3D optical imaging studies. R. Ryan performed the histological analysis for the quantification of renal injury. L. Rein provided statistical consultation and analyses. A. Dayton and M. Hoffman performed the analysis of the circadian patterns. A. Staruschenko participated in the study design and data interpretation. M. Skelton performed data analysis and article preparation. A. Geurts produced the mutant rat strain and participated in the study design and data interpretation.

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Disclosures

Medical College of Wisconsin may one day receive royalties on the sales of genetically modified rats. The authors report no conflicts.

References

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**Novelty and Significance**

**What Is New?**

- The SS<sup>−∕−</sup> first rat model with a deletion of the NADPH oxidase 4 (Nox4) gene.
- The study represents the first to evaluate the specific contribution of Nox4 in a naturally occurring form of hypertension in the rat, Dahl salt-sensitive rats.
- Mechanistic insights were obtained indicating that Nox4 exerts its effects through multiple pathways, most notably by transcriptional regulation of Nox2 enzyme subunits, transcriptional regulation of collagen synthesis, and via its effects on mitochondrial energetics.

**What Is Relevant?**

- Nox4 was found to play an important role in salt-sensitive hypertension which is relevant to human disease.

- Deletion of Nox4 in the salt-sensitive Dahl rat resulted in a significant reduction of salt-induced hypertension.
- Nox4 is the most highly expressed NADPH oxidase isoform in the kidney, and the results of this study indicate that this largely unexplored Nox isoform plays an important role in fluid-electrolyte homeostasis and in hypertension.

**Summary**

The knockout of the Nox4 isoform of NADPH oxidase in the Dahl salt-sensitive rat revealed that Nox4 plays an important role in the development of this form of hypertension and the associated renal injury. It will now be important to determine the sites and mechanisms whereby Nox4 exerts this potent effect on blood pressure regulation.
Evidence of the Importance of Nox4 in Production of Hypertension in Dahl Salt-Sensitive Rats

Allen W. Cowley, Jr, Chun Yang, Nadezhda N. Zheleznova, Alexander Staruschenko, Theresa Kurth, Lisa Rein, Vikash Kumar, Katherine Sadovnikov, Alex Dayton, Matthew Hoffman, Robert P. Ryan, Meredith M. Skelton, Fahimeh Salepour, Mahsa Ranji and Aron Geurts

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EVIDENCE OF THE IMPORTANCE OF NOX4 IN PRODUCTION OF HYPERTENSION IN DAHL SALT-SENSITIVE RATS

Allen W. Cowley Jr.¹, Chun Yang¹, Nadezhda N. Zheleznova¹, Alexander Staruschenko¹, Theresa Kurth¹, Lisa Rein², Vikash Kumar¹, Katherine Sadovnikov¹, Alex Dayton¹, Matthew Hoffman¹, Robert P. Ryan¹, Meredith M. Skelton¹, Fahimeh Salehpour³, Mahsa Ranji³, Aron Geurts¹

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**Expanded Methods:**

Development of SS rat with Nox4 (SS\textsuperscript{Nox4-/-}) knocked out. \textit{In vitro}-transcribed mRNA encoding the Nox4 ZFNs were injected into embryos obtained from SS/JrHsdMcwi rats (SS) and the injected embryos transferred to pseudopregnant Sprague Dawley females as we have previously described\textsuperscript{1,2}. The SURVEYOR Nuclease Assay was used to detect six ZFN-induced mutant offspring. Founders were back crossed to the parental SS strain and siblings carrying the 8 nucleotide deletion were then intercrossed to establish the SS-Nox4\textsuperscript{em2Mcwi-/-} (SS\textsuperscript{Nox4-/-}) strain.

**Chronic measurement of arterial blood pressure and heart rate**

For surgical implantation of the arterial catheter, the rats were anesthetized using 2.5% isoflurane (remainder oxygen). The gel-filled catheter was inserted in the right carotid artery and this catheter was attached to a transmitter (model TA11PA-C40, Data Systems International, Minneapolis, MN) that was placed subcutaneously between the scapulae. Buprenorphine was administered post-operatively to provide analgesia and the rat returned to the home cage for a 5-7 day recovery period.

**Optical fluorescence 3D cryoimaging**

Rats were deeply anesthetized with sodium pentobarbital (60 mg/kg) and an aortic catheter inserted followed by a rapid infusion of 6-10 ml cold isotonic saline to remove blood from the kidneys. The left kidney was quickly removed, hemisected and dropped into a container of isopentane cooled by liquid nitrogen to minimize cracking of the tissue and preserve the metabolic state of the tissue at freezing moment. After 2 minutes, the frozen kidney was moved to liquid nitrogen before being stored at -80°C until cryoimaging.

Once the frozen tissue was mounted within the cryoimager, a motorized microtome blade sequentially sliced the tissue and for the present study 30 \( \mu \text{m} \) slices were made in the z direction, which resulted in 400 z-slices per kidney. During the slicing, tissue was maintained in the frozen state at -40°C. A mercury arc lamp (200 W, Oriel) is used as a light source and desired wavelengths selected by filtering the broad band light to excite the selected fluorophores from the exposed surface of the frozen tissue block. An emission filter is used to eliminate reflected and unwanted light.

The intensity at each pixel in the NADH and FAD images indicates the concentration of the fluorophore at that pixel and the 3D volume average NADH/FAD redox ratio of each kidney calculated voxel by voxel according to equation (1).

\[
\text{tissue redox ratio} = \frac{\text{NADH intensity}}{\text{FAD intensity}} \quad (1)
\]

The corresponding histograms for each group of kidneys representing the distributions of tissue redox ratio intensities in 3D volumes were plotted. Each histogram is a scaled probability density function of the redox ratio in a kidney for quantitative analogy between the groups. The arithmetic mean values of these histograms were then calculated according to Eq. (2)

\[
\text{Mean} = \frac{1}{N_x \times N_y \times N_z} \sum_{i=1}^{N_x} \sum_{j=1}^{N_y} \sum_{k=1}^{N_z} \text{KidneyVolume}(i,j,k) \quad (2)
\]

where \( N_x, N_y \) and \( N_z \) are the number of pixels in the x, y and z directions; the pixel size in x and y directions is 10 \( \mu \text{m} \), but in z direction is 30 \( \mu \text{m} \).
Sample size calculation-*post hoc*: We have consulted with our MCW biostatisticians and consequently performed a “post hoc” sample size calculation based on the Nox4 and SS data. With an alpha set at 0.05 and a desired power of 80% or more, the sample size to detect a difference across the 21 days of the study in the two strains of rats was calculated. In this analysis, we used a linear regression fit using generalized estimating equations (GEE) which allows us to account for correlation of repeated measures within rats over time. With the GEE approach we can specify a correlation structure for the repeated measurements – I chose an autoregressive working correlation structure. By comparing the change in blood pressure across the 21 days of the study, the number of animals would be n=7 with 80% power and n=9 with 90% power. In the study we are reporting, the Nox4 mutant rat strain had n=9 and the SS rat strain had n=8. We have indicated the $\beta>0.8$ in the methods, the results, and the figure legends for the blood pressure data.

Primer sequences for mRNA studies (Figure 6, 7, S5 and S6) summarized below:

<table>
<thead>
<tr>
<th>gene symbol</th>
<th>Primer name</th>
<th>primer sequence</th>
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<tr>
<td>Cybb</td>
<td>Nox2 RK</td>
<td>CTCAAAGAATTCGAAGACAC</td>
</tr>
<tr>
<td>Cyba</td>
<td>p22 F1</td>
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<td>Cyba</td>
<td>p22 Rv1</td>
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<tr>
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</tr>
<tr>
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<td>p67 5 race fw</td>
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<tr>
<td>Col4a2</td>
<td>ratcol4a2-rt-a</td>
<td>GGTGGGAGCAGGTCAGTG</td>
</tr>
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</table>

Sequence with deletions: Nox4 gene was amplified from kidney cortex tissues mRNAs of 2 control (SS) and 8 Nox4 KO rats, by using primers of Nox4clone2s (GTTGGGCTTCTAGGTGGATGTGTTGAG) and Nox4clone2a (TTGGCTTGGAGGCAGTAGTGA). PCR products were purified and sequenced by Nox4clone2a primers. The Nox4 gene in the KO rat was found to be missing 8 nt (CCTATGCA) at site +535 in exon 7. The frame shift caused by this deletion introduces a 4 amino acid and an early stop codon. The SS Nox4 protein has 578 aa while the deleted form in the KO rat maintains Nox4 N-terminal 178 aa containing the first 4 transmembrane domains but missing most of the C-terminal including D, E loop and FAD, NADPH binding domains. Nox4 cd given below is the cDNA sequence from NCBI.
Nz309KO  CTA--------ATAAGAGTTTTCTAATTATGATATCTTCTGGTATACTCACAACCTTTCT 443
Nz310KO  CTA--------ATAAGAGTTTCTAATTATGATATCTTCTGGTATACTCACAACCTTTCT 399
Nz311KO  CTA--------ATAAGAGTTTTCTAATTATGATATCTTCTGGTATACTCACAACCTTTCT 395
Nz312WT  CTACCTATGCAATAAGAGTTTTCTAATTATGATATCTTCTGGTATACTCACAACCTTTCT 426
Nz349KO  CTA--------ATAAGAGTTTTCTAATTATGATATCTTCTGGTATACTCACAACCTTTCT 377
Nz350KO  CTA--------ATAAGAGTTTTCTAATTATGATATCTTCTGGTATACTCACAACCTTTCT 427
Nz351KO  NTA--------ATAAGAGTTTTCTAATTATGATATCTTCTGGTATACTCACAACCTTTCT 379
Nz355KO  CTA--------ATAAGAGTTTTNTAATTATGATATCTTNTNGGTATACTCACAACCTTTCT 384
33WT    CTACCTATGCAATAAGAGTTTTCTAATTATGATATCTTCTGGTATACTCACAACCTTTCT 446
37KO    NTA--------ATAAGAGTTTTNTAATTATGATATCTTNTNGGTATACTCACAACCTTTCT 309
nox4cd  CTACCCTATGCAATAAGAGTTTTCTAATTATGATATCTTCTGGTATACTCACAACCTTTCT 592
References


Mean arterial pressure (MAP) was continuously measured for three days on 0.4% salt and 21 days after the diet was switched to 4.0% NaCl diet in SS (closed circles; n=8) and SSNox4-/-(open circles; Nox4-/-; n=9). MAP was averaged in 12 hr bins to correspond to the day (6am-6pm) and night (6pm-6am) cycles (A). Delta values were generated by calculating the absolute difference between adjacent 12 hr bins (B) which were then normalized to the mean pressures of the preceding bin (C), to account for the effect of pressure itself on the amplitude of the cycles. The results of the two way ANOVA with repeated measures performed on the raw (D) and normalized (E) data are summarized.
Figure S2:

Images of the tissue redox ratio (NADH/FAD) of the complete group of kidneys from both strains and on both salt diets.
Western blots were analyzed for Nox2 protein in renal cortical and outer medullary homogenates prepared from SS (black bars; n=6) and SS$^{Nox4-/-}$ (Nox4-/-; white bars; n=9) from rats fed 0.4% NaCl diet. * indicates significant difference between strains ($p<0.05$).
Figure S4:

Western blots were analyzed for Nox2 protein in renal cortical and outer medullary homogenates prepared from SS (black bars; n=6) and SS^{Nox4-/-} (Nox4-/-; white bars; n=6) from rats fed 4.0% NaCl diet for 21 days. * indicates significant difference between strains (p<0.05).
mRNA expression was determined by qPCR from RNA extracted from homogenates of renal cortical (A.) and outer medullary (B.) tissue collected from SS rats (black bars; n=6) and SS\textsuperscript{Nox4-/-} (Nox4-/-; white bars; n=6) rats maintained on 0.4% NaCl. The RNA expression levels (mRNA copy number) of Collagen 1a1, 1a2, and 4a2 were determined. Tissues from both strains were run for any given gene on the same plate thus expression levels can be compared between the two renal regions. *indicates significant difference between strains (p<0.05). (Students' t-test).
mRNA expression was determined by qPCR from RNA extracted from homogenates of renal cortical (A.) and outer medullary (B.) tissue collected from SS rats (black bars; n=5) and SS^Nox4-/- (Nox4-/-; white bars; n=9) rats fed 4.0% NaCl diet for 21 days. The RNA expression levels (mRNA copy number) of Collagen 1a1, 1a2, and 4a2 were determined. Tissues from both strains were run for any given gene on the same plate thus expression levels can be compared between the two renal regions. *indicates significant difference between strains (p<0.05). (Students' t-test)