Spontaneous One-Kidney Rats are More Susceptible to Develop Hypertension by DOCA-NaCl and Subsequent Kidney Injury Compared to Uninephrectomized Rats

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ABSTRACT

There is little clinical data of how hypertension may influence individuals with nephron deficiency in the context of being born with a single kidney. We recently developed a new rat model (the HSRA rat) that is born with a single kidney and exhibits progressive kidney injury and decline in kidney function with age. We hypothesized that deoxycorticosterone acetate (DOCA)-salt would induce a greater increase in blood pressure and therefore accelerate progression of kidney injury in rats born with a solitary kidney compared to rats that have undergone unilateral nephrectomy. Time course evaluation of blood pressure, kidney injury, and renal hemodynamics was performed in 6 groups of animals from week 13 -18: (1-2) DOCA+S or Placebo+S (solitary kidney); (3-4) DOCA+C or Placebo+C (two-kidney control); and (5-6) DOCA+UNX8 or Placebo+UNX8 (two-kidney control that have undergone uninephrectomy). DOCA+S treated rats demonstrated a significant rise (p<0.05) in blood pressure (192±4 mmHg), proteinuria (205±31 mg/24hr) and decline in GFR (600±42 µl/min/gKW) relative to DOCA+UNX8 (173±3 mmHg, 76±26 mg/24hr, 963±36 µl/min/gKW) and DOCA+C (154±2 mmHg, 7±1 mg/24hr, 1484±121 µl/min/gKW). Placebo groups showed no significant change among the three groups. An assessment of renal injury markers via real-time PCR/western analysis and histological analysis was concordant with the measured physiological parameters. In summary, congenital solitary kidney rats are highly susceptible to the induction of hypertension compared to uninephrectomized rats, suggesting that low nephron endowment is an important driver of elevated blood pressure, hastening nephron injury through transmission of elevated systemic blood pressure, and thereby accelerating decline in kidney function.
INTRODUCTION

Unilateral renal agenesis, also known as congenital solitary kidney is a developmental defect that occurs in 1:500 to 1:1000 births and is frequently associated with other urogenital defects. (5, 37, 41) While there appears to be no overt clinical symptoms in the majority of children, there have been multiple studies that found that some patients are at an increased risk to develop hypertension, proteinuria, and renal failure as an adult. (16, 24) This may not only be related to the loss of one kidney (reduced nephrons), but also influenced by other factors such as hypertension and diabetes (e.g. “second hit”). While an inverse relationship has been observed between nephron number and kidney injury/blood pressure (lower nephron numbers are associated with glomerulosclerosis and increased blood pressure), the 10-fold variation of nephron numbers observed in normal human kidneys confounds a direct connection between the two. (7, 10, 15, 23) Thus, a better understanding of the impact of hypertension in the context of a congenital single kidney could provide important insight into the role of reduced nephron numbers in the onset and progression of kidney and cardiovascular disease.

The pathophysiological consequences of hypertension on the development of renal disease has been well-investigated in nephron deficiency models generated by surgical intervention. For example, studies utilizing rat models of deoxycorticosterone acetate (DOCA)-salt, in combination with unilateral nephrectomy, have demonstrated that hypertension promotes significantly more kidney injury in uninephrectomized compared to two-kidney control rats. (3, 4) However, a major limitation of these studies is the use of an invasive procedure after birth (i.e., animals develop with two functional kidneys and then undergo nephrectomy) which may not be representative of patients born with a single kidney. We recently developed and characterized a new rat model, the heterogeneous stock derived model of unilateral renal
agenesis (HSRA) rat, which exhibits congenital solitary kidney (50-75% in offspring) and susceptibility to develop significant kidney injury and decline in renal function with age. (35) A major characteristic of these single kidney rats is that they have fewer nephron numbers from birth than would be expected by loss of one-kidney (i.e., single kidney rats exhibit ~20% less nephrons versus comparable kidney from two-kidney control rats). While the mechanism of injury between the HSRA and nephrectomized models is similar (i.e., hyperfiltration), the reduced nephron endowment in the HSRA model appears to lead to greater susceptibility to injury compared to nephrectomized animals where loss of nephrons occur after birth. (35) In this context, the HSRA model provides a unique opportunity to investigate the impact of confounding factors on renal function in three different groups from the same model: nephron deficient (solitary kidney), two-kidney (control), and/or nephrectomized animals.

In the present study, we examined the hypothesis that treatment with DOCA combined with sodium loading would elicit a greater rise in blood pressure and accelerate onset and progression of kidney injury in spontaneous single kidney rats. The major conclusion is that nephron deficiency that occurs during the developmental period of the HSRA model leads to greater susceptibility to develop hypertension, kidney injury and decline in renal function compared to both uninephrectomy and two-kidney controls.

MATERIAL AND METHODS

Animals

All experimental procedures were approved by the University of Mississippi Medical Center (UMMC) Institutional Animal Care and Use Committee (IACUC). All experiments utilized the HSRA strain (heterogeneous stock derived model of unilateral renal agenesis), which is
maintained at UMMC. The HSRA model was developed from a single breeding pairs of heterogeneous stock (HS) rats whose offspring demonstrated a high degree (>60%) of unilateral renal agenesis (32). Brother-sister mating was initiated to establish an inbred strain that demonstrates consistent unilateral renal agenesis in 50-75% of offspring (35). At 6 weeks of age, kidney status was determined by palpitation. This method has been previously validated by comparison with ultrasound measurements and all animals were confirmed to have a single kidney via visual inspection after euthanasia. Offspring that exhibited a single kidney were denoted as S and those born with two kidneys as C. As previously reported, renal agenesis occurs on both the left and right side, with a higher incidence on the right side (2/3 offspring right side and 1/3 on the left). Thus, for consistency and to minimize any impact of the sidedness of kidney loss on the study, only male animals that exhibited right side agenesis (i.e. left side present) were studied.

Uninephrectomy (-UNX) was performed using two-kidney rats (HSRA-C) and sham operations were performed on littermate HSRA-S animals as done previously (35). Briefly, animals were anesthetized with 2-3% isoflurane/O2 and under aseptic conditions, an incision on the right flank was made. The right kidney was gently lifted and a single ligature was tied tightly around the renal vessels and ureter. The distal portions of the renal vessels and ureter were then cut, and the kidney was removed. The incision was closed by continuous subcutaneous stitch and additional closure of the skin was done using independent sutures. For consistency with HSRA-S animals, nephrectomy was performed on the right kidney. Animals used for this studied were caged under controlled temperature, humidity, and 12h light/12h dark conditions.

Measurement of blood pressure, renal injury and renal hemodynamic parameters. To investigate the impact of deoxycorticosterone acetate (DOCA) induced hypertension on renal
hemodynamics, renal injury, and renal function in the HSRA model, six groups of animals (n=6 per group) were studied (Fig. 1A): (1) DOCA+S (spontaneous single kidney); (2) DOCA+C (two-kidney control); (3) DOCA+UNX8 (two-kidney control uninephrectomized at 8 weeks of age, fully developed kidney and sexually mature); (4) Placebo+S; (5) Placebo+C; and (6) Placebo+UNX8.

Blood pressure (BP), measured by telemetry and urinary protein excretion (proteinuria) were assessed weekly from week 13-18. Renal hemodynamic and histological analysis were performed at the end of the study (Fig. 1B). Telemetry transmitters (model PAC40, Data Sciences International) were implanted as previously described. Briefly, at week 12, surgeries were performed under 2-3% isoflurane/O₂, and the catheter of the device was inserted into the left femoral artery and guided upstream to the aorta. The body of the telemetry unit was placed in the lateral cavity of the left leg and sutured to the musculature. The skin was closed by independent sutures. Animals were provided Baytril (10mg/kg) to prevent infection and long acting analgesic Rimadyl (5mg/kg) to control for any operative pain. After one week of recovery, baseline (week 13) blood pressure was measured. BP was measured for 4 hours (from 11AM-2PM) one day each week for weeks 13-18.

DOCA [200mg, 21 days release (Innovative Research of America] and placebo pellets were implanted at the lateral side of the neck under the skin at week 13. (40) Another pellet was added after 21 days (week 16). All animals were provided 1% NaCl in their drinking water during the 5 week study period. Each week, animals underwent a 24-hour urine collection for the determination of proteinuria. (35, 36) At week 18, renal blood flow (RBF) and glomerular filtration rate (GFR) were measured as previously described. (36, 39) The concentration of FITC-inulin in the plasma and urine was determined using a Fluorescent Bio-Tek plate reader.
and GFR calculated as done previously. (36, 39) RBF was measured using an ultrasound flow probe (Transonic System, Ithaca, NY) on the renal artery. At the end of each experiment, the kidney(s) and heart (ventricle) were removed and weighed. Tissues were processed for isolation of RNA and histological examination.

**Real Time PCR, Western Blot Analysis, and ELISA**

Gene expression differences were evaluated using SYBR-green dye chemistry on Bio-Rad CFX96 (n=6 per group) on RNA isolated from a piece of kidney that contained both cortex and medulla (week 18). RNA quality was assessed by Experion™ Automated Electrophoresis System (RQI>9). RNA was reverse-transcribed to cDNA using the iScript™ cDNA Synthesis Kit and real-time PCR (Bio-Rad CFX96) was performed using SsoFast™ EvaGreen® Supermix (Bio-Rad). Western blot analysis was performed using antibodies for tubular injury (NGAL, α-Actin) and pro-inflammatory factors (TGF-ß1) purchased from Santa Cruz Biotech as previously described. (35, 36) Urinary excretion rates of nephrin and podocalyxin were determined by ELISA (Exocell, Philadelphia, PA) per manufacturer’s instructions.

**Histological Analysis**

Kidneys and hearts were fixed in 10% buffered formalin and embedded in paraffin, cut into 4-μm sections and stained with hematoxylin and eosin (H&E) and Masson’s trichrome. Glomerular injury and tubular injury were assessed using a semi-quantitative scoring system in 20 randomly selected images as done previously. (35, 36, 39) Glomerular injury was assessed for degree of mesangial matrix expansion and glomerulosclerosis on a scale from 0 (normal) to 4 (severe). Tubular injury was analyzed for degree of tubular atrophy, vacuolization, dilation, and protein casts on a scale from 0 (normal) to 4 (severe with > 75% tubules demonstrating injury) as previously described. (39) Tubulointerstitial injury was determined by evaluation of slides
stained with Masson’s Trichrome to quantify the percent fibrosis (blue staining) compared to background in 20 randomly selected images from cortex and 15 randomly selected images from medulla as previously done. (35, 36, 39)

Macrophage and T-cell infiltration was assessed by immunohistochemistry on unstained sections using primary antibodies directed at CD-68/ED-1 and CD-43 (Santa Cruz Biotechnology, etc.) and detected by DAB (Ultravision LPValue Detection System, Thermo Scientific). (36) Slides were counterstained with methyl green (n=4 section per group; 15-20 images). Images were captured using a Nikon 55i microscope with DS-Fi1 5-Meg Color C digital camera (Nikon, Melville, NY) and analyzed using Nis-Elements image analysis software (version 3.03, Nikon Instruments Inc., Melville, NY).

Statistical Analysis

The comparison of 6 groups of HSRA animals (S, C, and UNX8 with or without DOCA) experimental data (proteinuria, blood pressure, etc.) was evaluated by one-way or two-way ANOVA followed by either Dunnett’s or Bonferroni (GraphPad Prism 6, La Jolla, CA). A p<0.05 was considered to be statistically significant. All data are presented as mean ± standard error (SE).

RESULTS

Mean arterial pressure (MAP) steadily increased in all 3 groups of DOCA treated animals from week 13-18 (Fig. 2A). DOCA+S rats exhibited a more rapid increase in MAP compared to either the DOCA+UNX8 or DOCA+C groups, starting at week 15 (2 weeks post-treatment). By week 18, the DOCA+S group demonstrated the highest MAP (192±5 mmHg), followed by DOCA+UNX8 (173±3 mmHg) and DOCA+C (154±3 mmHg). Blood pressure for all 3 placebo
groups remained essentially unchanged and ranged between 100 – 110 mm Hg. Proteinuria increased before MAP for DOCA+S animals (week 14), but the increase in proteinuria was concurrent with MAP for DOCA+UNX8. The DOCA+C group didn’t exhibit any increase in proteinuria over baseline or the Placebo+C group over the course of the experiment (Fig. 2B). By the end of the study (week 18), the DOCA+S groups exhibited the most severe proteinuria (205±3 mg/24hr), which was 2.5 times more than that observed for the DOCA+UNX8 group (76±3 mg/24hr). All other groups demonstrated no significant change in proteinuria from week 13-18 as all stayed below ~10 mg/24hr.

The increased blood pressure observed in the DOCA treated groups was corroborated by increased cardiac hypertrophy compared to placebo groups (Fig. 3A). Despite the DOCA+S group demonstrating a greater rise in MAP compared to DOCA+UNX8, there was no significant difference in heart weight/body weight (HW/BW) ratio (3.8±0.1 and 3.8±0.1, respectively). However, both groups were significantly higher compared to DOCA+C (3.3±0.1) rats. There were no significant differences in HW/BW observed between the 3 placebo groups (average 2.8±0.1). In general, histological findings in the heart showed a low degree of fibrosis, but there were significant differences among groups (Fig. 3B-C). The DOCA+S group (2.4±0.2%) exhibited the greatest amount of cardiac fibrosis compared to all other groups, followed by DOCA+UNX8 (1.8±0.1%) and DOCA+C (1.4±0.2%), which were statistically higher than their respective placebo groups (average ~1%).

Renal hemodynamic measurements were performed at the end of the study (week 18) to investigate the impact of increased systemic hypertension on renal blood flow (RBF) and glomerular filtration rate (GFR). RBF (not corrected for kidney weight) was significantly
increased in Placebo+S and Placebo+UNX8 rats compared to Placebo+C, but when corrected for differences in kidney weight (KW) there were smaller differences between groups (Fig. 4A-B).

RBF (not corrected for kidney weight) was similar between DOCA+S (7.6±0.3 ul/min) compared to DOCA+C (7.8±0.3 ul/min) rats, whereas it was modestly increased in the DOCA+UNX8 (9.0±0.5 ul/min) group (Fig. 4A). However, when comparing each DOCA group with its placebo control, the DOCA+S group exhibited the largest decrease in renal blood flow (-40.7%), followed by DOCA+UNX8 (-18.3%), and DOCA+C (-13.4%). When RBF was analyzed to correct for KW, the DOCA+S group (2.9±0.32 ul/min/gKW) demonstrated a two-fold reduction compared to DOCA+C (6.1±0.45 ul/min/gKW) (Fig. 4B). The DOCA+UNX8 group (4.1±0.45 ul/min/gKW) was significantly lower than the DOCA+C, but not to the same degree as the DOCA+S group. The DOCA+S group exhibited the largest decrease in renal blood flow (-62.3%), followed by DOCA+UNX8 (-37.0%), and DOCA+C (-13.4%) compared to each placebo group, which was a larger decline than observed for uncorrected RBF values.

GFR (not corrected for KW) was significantly higher in Placebo+S (+1.8-fold) and Placebo+UNX8 (+1.7-fold) compared to the Placebo+C group (Fig. 4C). DOCA+S and DOCA+UNX8 treated animals exhibited a significant decline in GFR following DOCA-NaCl treatment, whereas the DOCA+C group exhibited a modest increase in GFR compared to placebo control (-38.0%, -26.2%, and +25.7%, respectively). While there were clear differences between each DOCA group and its respective placebo control, no significant difference in GFR (uncorrected) between the DOCA treated groups was observed. In contrast, when GFR was corrected for differences in KW, there were no differences among the placebo groups, while the DOCA+S group exhibited a significant decline in renal function (600±42 µl/min/gKW) relative
DOCA+UNX8 (963±36 µl/min/gKW) and DOCA+C (1484±121 µl/min/gKW) (Fig. 4D). The DOCA+S group exhibited the largest decrease in GFR (-60.2%), followed by DOCA+UNX8 (-39.7%), and DOCA+C (+3.4%) compared to their respective placebo groups. The change in corrected GFR (placebo vs DOCA) was larger than the change observed for uncorrected GFR values.

Excretion rates of nephrin and podocalyxin, both urinary markers of glomerular injury, were evaluated at week 13, 15, and 18 (Fig. 5A-B). While excretion rates for nephrin were similar among the groups at week 13, by week 15, nephrin excretion in the DOCA+S group was significantly elevated compared to all other groups. At week 18, the DOCA+UNX8 group exhibited a significant increase (~15-fold) in nephrin compared to DOCA+C, while the DOCA+S was significantly higher than both groups (3.5-fold and 50-fold, respectively) (Fig. 5A). The detected differences between groups in podocalyxin were similar to nephrin (Fig. 5B). However, significant differences between the DOCA+S and DOCA+UNX8 group (compared to other groups) occurred earlier at week 13 and 15, respectively.

In addition to the examination of urinary biomarkers, renal injury was assessed by determination of renal hypertrophy and histological examination. There was a slight, but significant difference in the kidney weight/body weight (KW/BW) ratio between the Placebo+S (5.3±0.21) and Placebo+UNX8 (4.9±0.13) groups, and both were significantly greater (~1.5 fold) compared to the Placebo+C group (3.2±0.02) (Fig. 5C). Kidneys from DOCA+S rats exhibited significant renal hypertrophy (+1.3-fold) compared to the DOCA+UNX8 group (Fig. 5C). The
DOCA+S group exhibited the largest increase in KW/BW ratio (+92.6%), followed by DOCA+UNX8 (+66.5%), and DOCA+C (+51.6%) compared to their respective placebo control.

Morphologically, DOCA-NaCl treatment had a significant impact on glomerular and tubular injury between DOCA groups and placebo controls (Fig. 5D and 6A-B). Both the DOCA+S and DOCA+UNX8 groups demonstrated a significant increase (+3.2 and +2.6 fold, respectively) in glomerular injury (including hypertrophy, glomerulosclerosis, and mesangial expansion) compared to DOCA+C. There was a modest increase in glomerular injury (+1.2 fold) in the DOCA+S group compared to DOCA+UNX8. As expected, there was only a negligible degree of injury in all placebo groups (<0.3; scale 0-4).

Tubular injury (including atrophy, vacuolization, and presence of protein casts) was greatest in the DOCA+S (3.6±0.1), followed by DOCA+UNX8 (2.7±0.2) and DOCA+C (1.7±0.2) (Fig. 6-7), with very little injury or fibrosis in the placebo groups. For the DOCA groups, there was a significant increase in both cortical and medullary fibrosis (compared to placebo groups), consistent with the observed differences in tubular injury (Fig. 6A-B). The degree of tubular injury and fibrosis was confirmed by the measurement of kidney injury markers via real-time PCR (Kim-1 and Ngal) and western blot analysis (α-Actin and NGAL) using whole kidney (Fig. 6C-D). In the cortical region, kidneys from both DOCA+S (10.1±0.7%) and DOCA+UNX8 (9.2±1.3%) exhibited a similar level of fibrosis, but each were significantly higher than the DOCA+C group (5.5±1.2%). In contrast, in the medulla, the DOCA+S group (15.4±1.8%) exhibited 30% more fibrosis compared to DOCA+UNX8 (11.5±2.6%) rats (Fig. 7).
No difference in macrophage or T-cell infiltration was observed among groups in the absence of DOCA-NaCl treatment. Tubulointerstitial macrophage infiltration (CD68 positive cells) was significantly increased (+17-fold) in kidney of DOCA+S compared to placebo animals and +4.6 fold or +1.7 fold compared to DOCA+C and DOCA+UNX8, respectively (Fig. 8A). Kidneys from DOCA+S and DOCA+UNX8 also demonstrated an increase in T-cell infiltration (CD43 positive cells) compared to all the other groups (Fig. 8B). However, there was no difference in T-cell infiltration between DOCA+C and DOCA+UNX8 (representative images Fig. 9). The degree of inflammation in the kidney of each group was confirmed by the measurement of several inflammatory factors via real-time PCR (Ccr2, Il6, Tgfβ1 and Tgfβ2) and western blot analysis (CD-68 and TGFβ-1) (Fig. 8C-D). Kidneys from DOCA+S displayed the highest level of a number of inflammatory factors (e.g., Ccr2, Il6, Tgfβ1 and Tgfβ2) compared to all other groups, including the DOCA+UNX8 and DOCA+C.

**DISCUSSION**

It is well established that hypertension can promote kidney injury and impaired renal function (8, 27), but there is limited experimental data of how hypertension directly impacts single kidney individuals. (28) While there have been several human studies that have demonstrated that individuals born with only one kidney are at an increased risk to develop hypertension and impaired renal function (16, 24), there are no studies that have examined the direct impact of hypertension on solitary kidney individuals. Previously, we developed and characterized the age-related changes (over 18 months) in cardiovascular and renal physiology in
the HSRA model. (35) We observed that congenital single kidney animals are born with significantly fewer nephrons (versus comparable individual kidney in two-kidney control), and while these rats develop significant hypertrophy at birth, there are no obvious histological changes compared to two-kidney control. In fact, there is essentially no change in blood pressure, renal injury (proteinuria), or renal function between these groups until 5 months of age. (35) After month 5, congenital single kidney animals exhibit a significant degree of kidney injury and gradual decline in renal function compared to both control and uninephrectomized animals group that becomes significantly impaired by month 18. (35)

The HSRA rat model provides a unique opportunity to address hypotheses that have not been previously possible as the model allows for a direct comparison between nephron deficient animals (solitary kidney), two-kidney control, and uninephrectomy in the same strain during a period of time when there are no obvious baseline differences in cardiovascular and renal function (<month 5) between the groups. For this study, we used the DOCA-salt induced hypertension experimental model as it is a well-established approach to generate hypertension and elicits more severe renal injury as compared to other experimental approaches such as ANGII and L-NAME. (13, 29) The lower susceptibility to injury in ANG II and L-NAME models is due to the strong vasoconstriction of the renal artery which reduces the transmission of high blood pressure into the kidney. (25, 26) This robust vasoconstrictive effect is not observed in the DOCA-salt model, making it an optimal model to investigate the direct impact of transmission of elevated blood pressure on kidney injury in the HSRA.

There were several major findings of this study, including: (1) DOCA+S animals demonstrated the most prominent increase in blood pressure over and above the DOCA+UNX8
group (corroborated by increased cardiac hypertrophy); (2) DOCA+S animals exhibited a greater degree of kidney injury (glomerular injury and proteinuria) and renal function decline compared to HSRA+UNX8 (and all other groups); (3) kidney injury in DOCA animals was more pronounced in the medullary region (vs cortical) and characterized by greater fibrosis, protein casts, and activation of immune genes, with this difference being greatest in the DOCA+S group; and (4) despite a significant rise in blood pressure in DOCA+C animals, they were strikingly resistant to develop renal injury as assessed by proteinuria, glomerular biomarkers, and histological analysis.

There were no significant differences in blood pressure between groups before week 15 (2-weeks post treatment). However, renal injury (demonstrated by proteinuria and increased nephrin and podocalxyn) was significantly greater in DOCA+S compared to all other groups during the same period. The most obvious explanation for this findings relates to solitary kidney animals having fewer nephrons and the inability of these kidneys to deal with increased water and salt retention compared DOCA+UNX8 (Fig. 10). (8) This is consistent with our previous study that found single nephron GFR in solitary kidney rats was significantly elevated over two-kidney control as well as our current finding of hyperfiltration (elevated total GFR) in either Placebo+S and Placebo+UNX8 compared with the Placebo+C. In support of this conclusion, studies have revealed that humans with less mean nephron numbers have increased risk of hypertension (6), and it is well established that increased glomerular capillary pressure (through transmission of systemic hypertension) can promote glomerular injury, proteinuria and tubular injury through alterations in vascular cells, proliferation of endothelial and mesangial cells, and cytokine generation/inflammation. (12, 30) This certainly appears to be the mechanism observed under baseline measurements of the HSRA model. In the current study, volume expansion likely
increased the degree of hyperfiltration during the initial phase of DOCA-NaCl treatment. Subsequently, this resulted in accelerated glomerular injury, reduced RBF, and GFR at only 18 weeks of age (5 weeks post treatment), whereas a similar degree of injury was previously observed at much a later time (>month 12) in the absence of hypertension (Fig. 10). Thus, the rapid decline in renal function exhibited by the HSRA-S leads to a cycle of increased volume expansion, hyperfiltration and injury, and elevated blood pressure compared to other DOCA groups (Fig. 10).

The renal hemodynamic data (RBF and GFR) was presented both uncorrected and corrected for differences in KW. The uncorrected calculation provides a measure of renal hemodynamics without considering differences in kidney hypertrophy, whereas the corrected calculation accounts for differences in kidney size. While it is useful to view the hemodynamic measurements without considering kidney size, a significant correlation between GFR with BP or proteinuria is observed only when using GFR corrected for KW (r=-0.88 and -0.95, respectively; p<0.05), which suggests that these values may be the most relevant to consider for comparison among the groups. The most interesting observation from the renal hemodynamic measurements is not the decrease in GFR in either the DOCA+S or DOCA+UNX8, but that congenital solitary kidney rats exhibited a greater reduction in RBF and GFR with increased systemic blood pressure than would be expected based on incremental loss of nephrons (~>20%) over the nephrectomized group. In other words, the decrease in RBF and GFR for the nephrectomized group is consistent with the loss of half their nephron endowment (i.e., loss of one kidney after birth), whereas the DOCA+S exhibit a more substantial decrease in renal function considering a relatively modest difference in nephron numbers.
Histological analysis demonstrated a pronounced injury in the kidney medulla characterized by increased interstitial fibrosis and infiltration of immune cells (predominately macrophages), which was more severe than the injury observed in the cortex. This finding is consistent with the well-known consequences of arterial hypertension on renal vasculature. As a result of arterial hypertension, hyaline accumulates in the wall of small arteries and arterioles, thickening their walls and narrowing their lumens resulting in less blood flow. (33) Consequently, ischemia can facilitate tubular atrophy, interstitial fibrosis, glomerular alterations and fibrosis. (9) In the long term, this can result in impaired renal function and ultimately renal failure. (18) There have also been several studies that have demonstrated that the renal medulla has lower oxygen tension compared with the renal cortex, which renders the medulla more prone to hypoxic injury. (22)

There are a number of mouse models that exhibit various degrees of renal agenesis (14, 17, 19, 20), including those induced by genetic manipulation (e.g. knockout of Wt1, Gdnf, Ret, etc.) (reviewed in(31)). These types of models exhibit numerous abnormalities of the urogenital system and/or skeletal system, but the incidence of uni- or bilateral agenesis is variable, and typically below the incidence (50-75%) observed in the HSRA model. To our knowledge, none of the studies that have utilized these models have directly compared solitary rats with two-kidney control and nephrectomized rats in the same study. There are two reported models of uni- or bilateral agenesis in the rat, including the UUA (Unilateral Urogenital Anomalies) rat (<50%) (1, 2) and ACI (August × Copenhagen Irish) rat (5-15%). (11, 21) Again, neither of these models have been extensively characterized for long-term impact on renal or cardiovascular parameters for rats born with one-kidney. Typically, the ACI has been utilized as a control strain (normotensive and not susceptible for renal injury) for genetic studies, along
with the Fawn hooded hypertensive (FHH) to identify loci controlling hypertension and renal injury.\(^{(34)}\) The ACI is one of the eight progenitor strains used to derive the NIH heterogeneous stock (HS) rat from which the HSRA model was developed. It is likely that the HSRA and ACI rat do share a common genetic cause for renal agenesis, but based on the large disparity in the incidence of the single kidney phenotype observed between the two strains (75\% vs. 15\%, respectively), there are likely additional genetic factors in the HSRA (modifier genes) that contribute to the phenotype.

In summary, this study established a direct relationship between nephron deficiency and increased susceptibility to develop hypertension and subsequent renal injury by comparing physiological parameters between animals with one-kidney (congenital solitary rats and uninephrectomy in the same model) that differ in the number of nephrons. Future genetic, embryological, and physiological studies with the HSRA model will likely be useful to better understand the genetic basis of nephrogenesis/kidney development as well as investigate the impact of how other “second hits” such as diabetes/hyperglycemia, acute kidney injury, or other experimental models of hypertension (ANG II or L-NAME) could impact and/or modulate susceptibility toward kidney injury in single kidney individual.
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DISCLOSURES

None.


**FIGURE LEGENDS**

**Figure 1:** Overview of study design to evaluate the impact of deoxycorticosterone acetate (DOCA) hypertension on cardio-renal function in the HSRA model. **(A)** Groups of animals utilized for experiments and translation to human patients. Littermate animals for spontaneous one-kidney rats (S) and two-kidney littermates (C) were used for experiments. Two-kidney control animals (littermates from S) were used to generate uninephrectomized animals (C uninephrectomized at week 8; fully developed kidney and sexually mature). Spontaneous one-kidney rats exhibit ~20% less nephrons compared to individual kidney from two-kidney control. 

**(B)** Timeline of experimental protocol with weekly telemetry measured blood pressure and proteinuria from week 13-18 and terminal renal hemodynamics and histological analysis. n=6 in each group.

**Figure 2:** Time course measurement of mean arterial pressure and proteinuria in HSRA model treated with/ without deoxycorticosterone acetate (DOCA) from week 13-18. **(A)** Measurement of blood pressure via telemetry in all 6 groups. Blood pressure remained unchanged for all placebo groups. After 5 weeks of DOCA+1% NaCl, the DOCA+S group exhibited the greatest increase in blood pressure followed by DOCA+UNX8 and DOCA+C. **(B)** Measurement of proteinuria. Proteinuria increased with time for DOCA+1% NaCl treated S and UNX8 groups. These groups were significantly elevated compared to the other 4 groups which demonstrated no change during the experiment. *, p<0.05, DOCA+S and DOCA+UNX8 from all other groups; †, p<0.05, each DOCA group from each other and placebo groups; ‡, p<0.05, DOCA+S and +UNX8 from each other and all other groups.

**Figure 3:** Measurement of cardiac hypertrophy and fibrosis at week 18. **(A)** Heart weight /body weight (HW/BW) ratio for all 6 groups. DOCA treated groups exhibited significantly increased
heart weight compared to placebo groups. DOCA+S and DOCA+UNX8 were significantly heavier compared to DOCA+C, corroborating the observed blood pressure differences. (B) Quantification of cardiac fibrosis in each group at week 18. DOCA+S exhibited the greatest degree of fibrosis compared to all other groups. (C) Representative images of cardiac tissue stained with Masson’s trichrome at 10X. *, p<0.05 DOCA treated groups compared to placebo groups; †, p<0.05, DOCA+S or DOCA+UNX8 compared to DOCA+C.

**Figure 4:** Impact of deoxycorticosterone acetate (DOCA) hypertension on renal blood flow and glomerular filtration rate in the HSRA model at week 18. (A) Measurement of renal blood flow (RBF). DOCA+1% NaCl treated groups demonstrated significantly lower RBF compared to placebo group. RBF for Placebo+S and Placebo+UNX8 were significantly elevated compared to Placebo+C. (B) RBF normalized to kidney weight. The DOCA+S group exhibited the largest reduction in RBF followed by DOCA+UNX8 and DOCA+C. (C) Measurement of glomerular filtration rate (GFR). GFR for DOCA treated –S and -UNX8 was significantly decreased compared to Placebo +S and Placebo+UNX8. (D) GFR normalized to kidney weight. The DOCA+S group exhibited the largest reduction in GFR compared to DOCA+UNX8, with both groups significantly lower DOCA+C. *, p<0.05 DOCA treated group compared to respective placebo group; †, p<0.05, placebo+S and placebo+UNX8 compared to +C; ‡, p<0.05, DOCA+S and +UNX8 from each other and DOCA+C

**Figure 5:** Impact of deoxycorticosterone acetate (DOCA) hypertension on kidney hypertrophy and glomerular injury parameters in the HSRA model. (A) Time course measurement of nephrin at week 13, 15, and 18. (B) Time course measurement of podocalyxin at week 13, 15, and 18. (C) Kidney weight/body weight (KW/BW) ratio at week 18. The DOCA+S group demonstrated the greatest kidney hypertrophy compared to +UNX and +C group. (D) Semi-quantitative
measurement of glomerular injury at week 18. A minimum of 15 randomly selected glomeruli were scored from each kidney section. DOCA treated groups exhibited more injury than placebo groups. *, p<0.05 DOCA treated groups compared to placebo groups; †, p<0.05, DOCA+S and +UNX8 from each other and DOCA+C; ‡, p<0.05, Placebo+S and Placebo+UNX8 compared to +C; ‡,

**Figure 6:** Semi-quantitative measurement of tubular injury, renal fibrosis and kidney injury markers at week 18. (A) Semi-quantitative measurement of tubular injury. Tubules were evaluated for degree of tubular atrophy, vacuolization, dilation, and protein casts on a scale from 0 (normal) to 4 (severe with > 75% tubules demonstrating injury). DOCA treated groups exhibited significantly more injury than placebo groups, with DOCA+S exhibiting the most severe injury. (B) Tubulointerstitial fibrosis in kidney cortex and medulla. DOCA+S demonstrated the most fibrosis (cortex +medulla) compared to other DOCA groups. Medulla fibrosis was more prominent than in the cortex. (C) Real-time PCR of kidney injury makers, Kim-1 and Ngal. (D) Western blot analysis of α-Actin and NGAL. A total of three gels were evaluated (for a total n=6 sample per group). The gel most representative of the average of the calculated protein/GAPDH ratio is show. Both the real-time PCR and western blot analysis confirm the histological findings of increased tubular injury and fibrosis. *, p<0.05 DOCA treated groups compared to placebo groups; †, p<0.05, DOCA+S compared to DOCA+C and UNX8.

**Figure 7:** Representative images of renal cortex and medulla stained with Masson’s trichrome at 10X.

**Figure 8:** Measurement of inflammatory cell infiltration and inflammatory markers at week 18. (A) Quantification of macrophage infiltration (CD-68). DOCA+S exhibited the largest number
of infiltrating macrophages. (B) Quantification of T-cell infiltration (CD-43). DOCA+S and DOCA+ UNX8 demonstrated significantly more T-cell infiltration compared DOCA+C and placebo groups. (C) Heat map of real-time PCR data of important inflammatory factors. For DOCA treated groups, almost all inflammatory related genes were significantly up-regulated (red) compared to placebo groups (green). (D) Representative western blot analysis of CD-68 and TGFβ-1. A total of three gels were evaluated (for a total n=6 per group). The gel most representative gel of the average calculated protein/GAPDH ratio is shown. Both the real-time PCR and western blot analysis is consistent with the immunohistological findings. *, p<0.05 DOCA treated groups compared to placebo groups; †, p<0.05, DOCA+ –S compared to DOCA+ –C and –UNX8.

Figure 9: Representative immunohistochemistry images (week 18) of macrophage (CD-68) and T-cell (CD-43) infiltration at 20X.

Figure 10: Overview of the physiological mechanism of kidney injury and development of hypertension in the HSRA model. HSRA-S animals develop with a single kidney and exhibit reduced nephrons versus comparable kidney in littermates born with both kidneys (HSRA-C). HSRA-C animals appear normal and exhibit no susceptibility to develop renal injury or hypertension. Based on previous long-term study of the HSRA model, HSRA-S animals develop progressive injury via hyperfiltration and glomerular hypertrophy that leads to glomerular injury and increased proteinuria (blue arrows). The increase in proteinuria, combined with tubular injury, results in reduced blood flow, promotes tubular injury (e.g. epithelial mesenchymal transition), inflammation, tubulointerstitial fibrosis, culminating in reduced renal function and elevated blood pressure.(35) This finding is similarly observed in HSRA-C animals subjected to uninephrectomy, but changes in renal function and blood pressure are significantly attenuated. In
the present study, overlaying hypertension (i.e. second hit) on top of nephron deficiency (HSRA-S) hastens glomerular injury and downstream tubular injury, culminating in rapid decline in renal function, volume expansion, and greater susceptibility to hypertension compared to uninephrectomy (red arrows).
Figure 1

A.

![Diagram showing kidney groups and statistics](image)

- **HSRA-S**: Spontaneous one kidney
  - Mean nephron numbers: 20373 ± 1039

- **HSRA-C**: 2-kidney control
  - Mean nephron numbers: 49026 ± 2122

- **HSRA-UNX8**: Nephrectomy at 8 weeks
  - Mean nephron numbers: 25093 ± 638

- **Born with one kidney/predisposed to hypertension**

- **Human Comparison**
  - Healthy control/predisposed to hypertension
  - Adult UNX/donor - predisposed to hypertension

B.

![Timeline showing weeks and telemetry](image)

- Week: 0, 8, 12, 13, 14, 15, 16, 17, 18
- HSRA-S
- HSRA-C
- HSRA-UNX8 UNX

- Telemetry Implantation
- Proteinuria
- And MAP
- RBF
- GFR
- Serum Tissue
- Histology
Figure 2

A. Mean Arterial Pressure (mmHg)

B. Proteinuria (mg/24 hours)
Figure 3

A. Heart Weight Body Weight Ratio (mg/g)

B. Percent Fibrosis

C. Microscopic images of DOCA and Placebo groups.
Figure 4

A. Renal Blood Flow (ml/min/gKW)

B. Renal Blood Flow (ml/min)

C. Glomerular Filtration Rate (μl/min/gKW)

D. Glomerular Filtration Rate (μl/min)

Groups compared:
- HSRA-S
- HSRA-C
- HSRA-UNX8

DOCA vs Placebo

Significance levels:
- * p < 0.05
- ** p < 0.01
- *** p < 0.001
- † p < 0.001
Figure 5

A. Log10 Nephrin (ug/24hrs)

B. Log10 Podocalyxin (ng/24hrs)

C. Kidney Weight Body Weight Ratio (mg/g)

D. Glomerular Injury Score
Figure 6

A. Tubular Injury Score

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<td>HSRA-C</td>
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<td>HSRA-UNX8</td>
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B. Percent Fibrosis

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<td>HSRA-UNX8</td>
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C. Normalized Fold Expression

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D. Protein/GAPDH (Densitometry Unit)

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*† Significance level
Figure 7

The images depict the histological analysis of different conditions: DOCA, Placebo, DOCA, and Placebo. The conditions are assessed in the Cortex and Medulla regions. The images are labeled with HSRA-S, HSRA-C, and HSRA-UNX8.
Figure 8

A. DOCA vs Placebo

Number of Macrophages (Foci per 20X)

B. DOCA vs Placebo

Number of T Cells (Foci per 20X)

C. Heatmap analysis

D. Western Blot analysis

Protein/GAPDH (Densitometry Unit)
Figure 9

DOCA  Placebo  DOCA  Placebo

HSRA-S  20X  20X

HSRA-C  20X

HSRA-UNX8  20X

CD-68  CD-43
Figure 10

Natural Progression

HSRA-S
Reduced Nephrons
(First Hit)
Birth

Hypertension

Hyperfiltration

>Month 20

Reduced Renal Function
(~800 ml/min/KW)

>Month 5 to 15

Glomerular Hypertrophy
Damage
Permeability

Proteinuria
Tubular Injury
Inflammation
EMT/Fibrosis

Month 4-5

Second Hit
Hypertension

volume expansion

Month 3

>Birth to Month 5

Reduced Renal Function
(~600 ml/min/KW)