Spontaneous one-kidney rats are more susceptible to develop hypertension by DOCA-NaCl and subsequent kidney injury compared with uninephrectomized rats

Wang X, Johnson AC, Sasser JM, Williams JM, Woods LC, MR. Spontaneous one-kidney rats are more susceptible to develop hypertension by DOCA-NaCl and subsequent kidney injury compared with uninephrectomized rats. Am J Physiol Renal Physiol 310: F1054–F1064, 2016. First published March 2, 2016; doi:10.1152/ajprenal.00555.2015.—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 heterogeneous stock-derived model of unilateral renal agenesis rat) that is born with a single kidney and exhibits progressive kidney injury and decline in kidney function with age. We hypothesized that DOCA-salt would induce a greater increase in blood pressure and therefore accelerate the progression of kidney injury in rats born with a solitary kidney compared with rats that have undergone unilateral nephrectomy. Time course evaluation of blood pressure, kidney injury, and renal hemodynamics was performed in the following six groups of animals from weeks 13 to 18: 1) DOCA-treated rats with a solitary kidney (DOCA+S group), 2) placebo-treated rats with a solitary kidney, 3) DOCA-treated control rats with two kidneys (DOCA+C group), 4) placebo-treated control rats with two kidneys, 5) DOCA-treated rats with two kidneys that underwent uninephrectomy (DOCA+UNX8 group), and 6) placebo-treated rats with two kidneys that underwent uninephrectomy. DOCA+S rats demonstrated a significant rise (P < 0.05) in blood pressure (192 ± 4 mmHg), proteinuria (205 ± 31 mg/24 h), and a decline in glomerular filtration rate (600 ± 42 μl·min⁻¹·g kidney weight⁻¹) relative to the DOCA+UNX8 (173 ± 3 mmHg, 76 ± 26 mg/24 h, and 963 ± 36 μl·min⁻¹·g kidney weight⁻¹) and DOCA+C (154 ± 2 mmHg, 7 ± 1 mg/24 h, and 148 ± 121 μl·min⁻¹·g kidney weight⁻¹) groups. Placebo-treated groups showed no significant change among the three groups. An assessment of renal injury markers via real-time PCR/Western blot 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 with uninephrectomized rats, suggesting that low nephron endowment is an important driver of elevated blood pressure, hastening nephron injury through the transmission of elevated systemic blood pressure and thereby accelerating decline in kidney function.

fibrosis; high blood pressure; unilateral renal agenesis; chronic kidney disease; deoxycorticosterone acetate

UNILATERAL RENAL AGENESIS, also known as congenital solitary kidney, is a developmental defect that occurs in 1:500 to 1:1,000 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 (BP; lower nephron numbers are associated with glomerulosclerosis and increased BP), 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 insights 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 using rat models of DOCA-salt, in combination with unilateral nephrectomy, have demonstrated that hypertension promotes significantly more kidney injury in uninephrectomized compared with 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 a 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% fewer nephrons vs. comparable kidneys 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 with nephrectomized animals where loss of nephrons occurs 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 Na\(^+\) loading would elicit a greater rise in BP and accelerate the 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 a decline in renal function compared with both uninephrectomy and two-kidney controls.

**MATERIALS AND METHODS**

**Animals.** All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Mississippi Medical Center. All experiments used the HSRA strain, which is maintained at University of Mississippi Medical Center. The HSRA model was developed from a single breeding pair of heterogeneous stock 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 wk 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. As previously reported, renal agenesis occurs on both sides in 1/3 of offspring on the left side and 1/2 of offspring on the right side (35). At 6 wk 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. As previously reported, renal agenesis occurs on both sides in 1/3 of offspring on the right side and 1/2 of offspring on the left side. Thus, for consistency and to minimize any impact of the sidedness of offspring on the right side and 1/3 of offspring on the left side.

**Human Comparison**

Fig. 1. Overview of the study design to evaluate the impact of DOCA-induced hypertension on cardiorenal function in the heterogeneous stock-derived model of unilateral renal agenesis (HSRA model). A: groups of animals used 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 the S group) were used to generate uninephrectomized (UNX) animals (C animals uninephrectomized at week 8, with fully developed kidneys and sexually mature). Spontaneous one-kidney rats exhibit ~20% fewer nephrons compared with individual kidneys from two-kidney control rats (35). B: timeline of experimental protocol with weekly telemetry-measured blood pressure (BP) and proteinuria from weeks 13–18 and terminal renal hemodynamic and histological analyses. n = 6 animals/group. RBF, renal blood flow; GFR, glomerular filtration rate.

**Fig. 2.** Time course measurement of mean arterial pressure and proteinuria in the HSRA model treated with or without DOCA from weeks 13 to 18. The following six groups of animals were studied: 1) DOCA-treated animals with a spontaneous single kidney (DOCA+S group), 2) DOCA-treated control animals with two kidneys (DOCA+C group), 3) DOCA-treated control animals with two kidneys that were uninephrectomized at 8 wk of age (fully developed kidneys and sexually mature; DOCA+UNX8 group); 4) placebo-treated animals with a spontaneous single kidney (placebo+S group), 5) placebo-treated control animals with two kidneys (placebo+C group), and 6) placebo-treated control animals with two kidneys that were uninephrectomized at 8 wk of age (placebo+UNX8 group). A: measurement of BP via telemetry in all six groups. BP remained unchanged for all placebo-treated groups. After 5 wk of DOCA + 1% NaCl, the DOCA+S group exhibited the greatest increase in BP followed by the DOCA+UNX8 and DOCA+C groups. B: measurement of proteinuria. Proteinuria increased with time for DOCA + 1% NaCl-treated S and UNX8 groups. These groups were significantly elevated compared with the other four groups, which demonstrated no change during the experiment. *P < 0.05, DOCA+S and DOCA+UNX8 groups compared with all other groups; †P < 0.05, each DOCA-treated group compared with each other and placebo-treated groups; ‡P < 0.05, DOCA+S and DOCA+UNX8 groups compared with each other and all other groups.
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 animals with solitary kidneys, nephrectomy was performed on the right kidney. Animals used for this study were caged under controlled temperature, humidity, and 12:12-h light-dark conditions.

Measurement of BP, renal injury, and renal hemodynamic parameters. To investigate the impact of DOCA-induced hypertension on renal hemodynamics, renal injury, and renal function in the HSRA model, the following six groups of animals (n = 6 animals/group) were studied (Fig. 1A): 1) DOCA-treated animals with a spontaneous single kidney (DOCA + S group), 2) DOCA-treated control animals with two kidneys (DOCA + C group), 3) DOCA-treated control animals with two kidneys that were uninephrectomized at 8 wk of age (fully developed kidneys and sexually mature; DOCA + UNX8 group), 4) placebo-treated animals with a spontaneous single kidney (placebo + S group), 5) placebo-treated control animals with two kidneys (placebo + C group), and 6) placebo-treated control animals with two kidneys that were uninephrectomized at 8 wk of age (placebo + UNX8 group).

BP, measured by telemetry, and urinary protein excretion (proteinuria) were assessed weekly from weeks 13 to 18. Renal hemodynamic and histological analyses were performed at the end of the study (Fig. 1B). Telemetry transmitters (model PAC40, Data Sciences) were implanted as previously described (38). 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 (10 mg/kg) to prevent infection and the long-acting analgesic Rimadyl (5 mg/kg) to control for any operative pain. After 1 wk of recovery, baseline (week 13) BP was measured. BP was measured for 4 h (from 11 AM to 2 PM) 1 day each week for weeks 13–18.

DOCA (200 mg, 21-day 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-wk study period. Each week, animals underwent a 24-h 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 previously described (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
Glomerular injury and tubular injury were assessed using a semiquantitative scoring system in 20 randomly selected images, as previously described (35, 36, 39). Glomerular injury was assessed for the degree of mesangial matrix expansion and glomerulosclerosis on a scale from 0 (normal) to 4 (severe). Tubular injury was analyzed for the 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 with the background in 20 randomly selected images from the cortex and 15 randomly selected images from the medulla, as previously described (35, 36, 39).

Macrophage and T cell infiltration were assessed by immunohistochemistry on unstained sections using primary antibodies directed at CD68/ED-1 and CD43 (Santa Cruz Biotechnology) and detected by diaminobenzidine (Ultravision LPValue Detection System, Thermo Scientific) (36). Slides were counterstained with methyl green (n = 4 sections/group, 15–20 images). Images were captured using a Nikon 55i microscope with DS-Fi1 5-Meg Color C digital camera (Nikon,

Fig. 4. Impact of DOCA-induced hypertension on RBF and GFR in the HSRA model at week 18. A: measurement of RBF. DOCA + 1% NaCl-treated groups demonstrated significantly lower RBF compared with placebo-treated groups. RBF for the placebo+S and placebo+UNX8 groups were significantly elevated compared with the placebo+C group. B: RBF normalized to kidney weight (KW). The DOCA+S group exhibited the largest reduction in RBF followed by the DOCA+UNX8 and DOCA+C groups.

Histological analysis. Kidneys and hearts were fixed in 10% buffered formalin, embedded in paraffin, cut into 4-μm sections, and stained with hematoxylin and eosin as well as Masson’s trichrome.

Western blot analysis and ELISA. Gene expression differences were evaluated using SYBR green dye chemistry with Bio-Rad CFX96 (n = 6/group) on RNA isolated from a piece of kidney that contained both the cortex and medulla (week 18). RNA quality was assessed by an Experion Automated Electrophoresis System (RNA quality indicator > 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 [transforming growth factor-β]-α-lactin] and proinflammatory factors [transforming growth factor-β-actin] and proinflammatory factors [transforming growth factor-β]. Urinary excretion rates of nephrin and podocalyxin were determined by ELISA (Exocell, Philadelphia, PA) per the manufacturer’s instructions.

Renal Blood Flow (ml/min)

Renal Blood Flow (ml/min/gKW)

Glomerular Filtration Rate (μl/min)

Glomerular Filtration Rate (μl/min/gKW)

Fig. 4. Impact of DOCA-induced hypertension on RBF and GFR in the HSRA model at week 18. A: measurement of RBF. DOCA + 1% NaCl-treated groups demonstrated significantly lower RBF compared with placebo-treated groups. RBF for the placebo+S and placebo+UNX8 groups were significantly elevated compared with the placebo+C group. B: RBF normalized to kidney weight (KW). The DOCA+S group exhibited the largest reduction in RBF followed by the DOCA+UNX8 and DOCA+C groups. C: measurement of GFR. GFR for DOCA+S and DOCA+UNX8 groups was significantly decreased compared with placebo+S and placebo+UNX8 groups. D: GFR normalized to KW. The DOCA+S group exhibited the largest reduction in GFR compared with the DOCA+UNX8 group, with both groups significantly lower than the DOCA+C group. *P < 0.05, DOCA-treated groups compared with their respective placebo-treated group; †P < 0.05, placebo+S and placebo+UNX8 groups compared with the placebo+C group; ‡P < 0.05, DOCA+S and DOCA+UNX8 groups compared with each other and the DOCA+C group.
Melville, NY) and analyzed using Nis-Elements image-analysis software (version 3.03, Nikon Instruments).

Statistical analysis. The comparison of experimental data (proteinuria, BP, etc.) from six groups of HSRA animals (DOCA+S, DOCA+C, DOCA+UNX8, placebo+S, placebo+C, and placebo+UNX groups) was evaluated by one-way or two-way ANOVA followed by either Dunnett’s or Bonferroni procedures (GraphPad Prism 6, La Jolla, CA). P values of <0.05 were considered to be statistically significant. All data are presented as means ± SE.

RESULTS

Mean arterial pressure (MAP) steadily increased in all three groups of DOCA-treated animals from weeks 13 to 18 (Fig. 2A). DOCA+S rats exhibited a more rapid increase in MAP compared with either DOCA+UNX8 or DOCA+C rats, starting at week 15 (2 wk posttreatment). By week 18, the DOCA+S group demonstrated the highest MAP (192 ± 5 mmHg) followed by the DOCA+UNX8 (173 ± 3 mmHg) and DOCA+C (154 ± 3 mmHg) groups. BP for all three placebo-treated groups remained essentially unchanged and ranged between 100 and 110 mmHg. Proteinuria increased before MAP for DOCA+S animals (week 14), but the increase in proteinuria was concurrent with MAP for DOCA+UNX8 animals. The DOCA+C group did not 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 group exhibited the most severe proteinuria (205 ± 3 mg/24 h), which was 2.5 times more than that observed for the DOCA+UNX8 group (76 ± 3 mg/24 h). All other groups demonstrated no significant change in proteinuria from weeks 13 to 18, as all stayed below ~10 mg/24 h.

The increased BP observed in DOCA-treated groups was corroborated by increased cardiac hypertrophy compared with placebo-treated groups (Fig. 3A). Despite the DOCA+S group demonstrating a greater rise in MAP compared with the DOCA+UNX8 group, there was no significant difference in the heart weight-to-body weight ratio (3.8 ± 0.1 and 3.8 ± 0.1, respectively). However, both groups were significantly higher compared with the DOCA+C group (3.3 ± 0.1). There were no significant differences in the heart weight-to-body weight ratio observed between the three placebo-treated 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. 3, B and C). The DOCA+S group (2.4 ± 0.2%) exhibited the greatest amount of cardiac fibrosis compared with all other groups followed by the DOCA+UNX8 (1.8 ± 0.1%) and DOCA+C (1.4 ± 0.2%) groups, which were statistically higher than their respective placebo-treated groups (average: ~1%).

Fig. 5. Impact of DOCA-induced hypertension on kidney hypertrophy and glomerular injury parameters in the HSRA model. A: time-course measurements of nephrin at weeks 13, 15, and 18. B: time-course measurements of podocalyxin at weeks 13, 15, and 18. C: KW-to-body weight ratio at week 18. The DOCA+S group demonstrated the greatest kidney hypertrophy compared with the DOCA+UNX8 and DOCA+C groups. D: semiquantitative 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-treated groups. *P < 0.05, DOCA-treated groups compared with placebo-treated groups; †P < 0.05, DOCA+S and DOCA+UNX8 groups compared with each other and the DOCA+C group; ‡P < 0.05, placebo+S and placebo+UNX8 groups compared with the placebo+C group.
Renal hemodynamic measurements were performed at the end of the study (week 18) to investigate the impact of increased systemic hypertension on RBF and GFR. RBF (not corrected for kidney weight) was significantly increased in placebo/H11001S and placebo/H11001UNX8 rats compared with placebo/H11001C rats, but when corrected for differences in kidney weight, there were smaller differences between groups (Fig. 4A and B). RBF (not corrected for kidney weight) was similar in DOCA/H11001S rats (7.6 ± 0.3 µl/min) compared with DOCA/H11001C rats (7.8 ± 0.3 µl/min), whereas it was modestly increased in DOCA+UNX8 rats (9.0 ± 0.5 µl/min; Fig. 4A). However, when each DOCA-treated group was compared with its placebo-treated control group, the DOCA/S group exhibited the largest decrease in RBF (−40.7%) followed by the DOCA+UNX8 group (−18.3%) and DOCA/C group (−13.4%). When RBF was analyzed to correct for kidney weight, the DOCA/S group (2.9 ± 0.32 µl·min⁻¹·g kidney weight⁻¹) demonstrated a twofold reduction compared with the DOCA/C group (6.1 ± 0.45 µl·min⁻¹·g kidney weight⁻¹; Fig. 4B). The DOCA+UNX8 group (4.1 ± 0.45 µl·min⁻¹·g kidney weight⁻¹) demonstrated the most fibrosis (cortex + medulla) compared with other DOCA-treated groups. Medulla fibrosis was more prominent than in the cortex. Real-time PCR of kidney injury markers [kidney injury molecule (Kim)-1 and neutrophil gelatinase-associated lipocalin (Ngal)] confirmed the histological findings of increased tubular injury and fibrosis. *P < 0.05, DOCA-treated groups compared with placebo-treated groups; † P < 0.05, DOCA+S group compared with DOCA+C and DOCA+UNX8 groups.

Fig. 6. Semiquantitative measurement of tubular injury, renal fibrosis, and kidney injury markers at week 18. A: semiquantitative measurement of tubular injury. Tubules were evaluated for the 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-treated groups, with the DOCA+S group exhibiting the most severe injury. B: tubulointerstitial fibrosis in the kidney cortex and medulla. The DOCA+S group demonstrated the most fibrosis (cortex + medulla) compared with other DOCA-treated groups. Medulla fibrosis was more prominent than in the cortex. C: real-time PCR of kidney injury markers [kidney injury molecule (Kim)-1 and neutrophil gelatinase-associated lipocalin (Ngal)]. D: Western blot analysis of α-actin and NGAL. A total of three gels were evaluated (for a total n = 6 samples/group). The gel most representative of the average of the calculated protein-to-GAPDH ratio is shown. Both real-time PCR and Western blot analysis confirmed the histological findings of increased tubular injury and fibrosis. *P < 0.05, DOCA-treated groups compared with placebo-treated groups; † P < 0.05, DOCA+S group compared with DOCA+C and DOCA+UNX8 groups.

Fig. 7. Representative images of the renal cortex and medulla stained with Masson’s trichrome at ×10 magnification.

AJP-Renal Physiol • doi:10.1152/ajprenal.00555.2015 • www.ajprenal.org
kidney weight$^{-1}$) was significantly lower than the DOCA+C group, but not to the same degree as the DOCA+S group. The DOCA+S group exhibited the largest decrease in RBF (−62.3%) followed by the DOCA+UNX8 group (−37.0%) and DOCA+C group (−13.4%) compared with each placebo-treated group, which was a larger decline than observed for uncorrected RBF values.

GFR (not corrected for kidney weight) was significantly higher in the placebo+S (+1.8-fold) and placebo+UNX8 (+1.7-fold) groups compared with the placebo+C group (Fig. 4C). DOCA+S and DOCA+UNX8 animals exhibited a significant decline in GFR after DOCA-NaCl treatment, whereas DOCA+C animals exhibited a modest increase in GFR compared with placebo-treated control animals (−38.0%, −26.2%, and +25.7%, respectively). While there were clear differences between each DOCA-treated group and its respective placebo-treated control group, no significant differences in GFR (uncorrected) between the DOCA-treated groups were observed. In contrast, when GFR was corrected for differences in kidney weight, there were no differences among the placebo-treated groups, whereas the DOCA+S group exhibited a significant decline in renal function (600 ± 42 µl·min$^{-1}$·g kidney weight$^{-1}$) relative to the DOCA+UNX8 group (963 ± 36 µl·min$^{-1}$·g kidney weight$^{-1}$) and DOCA+C group (1,484 ± 37.0%$^{1}$). DOCA$^{-}$S and DOCA$^{-}$UNX8 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 weeks 13, 15, and 18 (Fig. 5, A and 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 with all other groups. At week 18, the DOCA+UNX8 group exhibited a significant increase (−15-fold) in nephrin compared with the DOCA+C group, whereas the DOCA+S was significantly higher than both groups (3.5- 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 groups (compared with other groups) occurred earlier, at weeks 13 and 15, respectively.

In addition to the examination of urinary biomarkers, renal injury was assessed by the determination of renal hypertrophy and histological examination. There was a slight but significant difference in the kidney weight-to-body weight ratio between

Fig. 8. Measurement of inflammatory cell infiltration and inflammatory markers at week 18. A: quantification of macrophage infiltration (CD68). The DOCA+S group exhibited the largest number of infiltrating macrophages. B: quantification of T cell infiltration (CD43). The DOCA+S and DOCA+UNX8 groups demonstrated significantly more T cell infiltration compared with the DOCA+C and placebo-treated groups. C: heat map of real-time PCR data of important inflammatory factors. For DOCA-treated groups, almost all inflammatory-related genes were significantly upregulated (red) compared with placebo-treated groups (green). Ccr, chemokine (C-C motif) receptor; Cxcl, chemokine (C-X-C motif) ligand; Il, interleukin; Tgf, transforming growth factor; Tlr, Toll-like receptor. D: representative Western blot analysis of CD68 and TGF-$\beta$. A total of three gels were evaluated (for a total n = 6/group). The gel most representative of the average calculated protein-to-GAPDH ratio is shown. Both real-time PCR and Western blot analysis was consistent with the immunohistological findings. *P < 0.05, DOCA-treated groups compared with placebo-treated groups; †P < 0.05, DOCA+S group compared with DOCA+C and DOCA+UNX8 groups.

AJP-Renal Physiol • doi:10.1152/ajprenal.00555.2015 • www.ajprenal.org
the placebo+S (5.3 ± 0.21) and placebo+UNX8 (4.9 ± 0.13) groups, and both were significantly greater (~1.5-fold) compared with the placebo+C group (3.2 ± 0.02; Fig. 5C). Kidneys from DOCA+S rats exhibited significant renal hypertrophy (+1.3-fold) compared with DOCA+UNX8 rats (Fig. 5C). The DOCA+S group exhibited the largest increase in the kidney weight-to-body weight ratio (+92.6%) followed by the DOCA+UNX8 group (+66.5%) and DOCA+C group (+51.6%) compared with their respective placebo-treated control groups.

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

Tubular injury (including atrophy, vacuolization, and the presence of protein casts) was greatest in the DOCA+S group (3.6 ± 0.1) followed by the DOCA+UNX8 group (2.7 ± 0.2) and DOCA+C group (1.7 ± 0.2; Figs. 6–7), with very little injury or fibrosis in the placebo-treated groups. For the DOCA-treated groups, there was a significant increase in both cortical and medullary fibrosis (compared with placebo-treated groups), consistent with the observed differences in tubular injury (Fig. 6, A and B). The degree of tubular injury and fibrosis were confirmed by the measurement of kidney injury markers via real-time PCR (kidney injury molecule-1 and Ngal) and Western blot analysis (α-actin and TGF-β) using whole kidneys (Fig. 6, C and D). In the cortical region, kidneys from both DOCA+S (10.1 ± 0.7%) and DOCA+UNX8 (9.2 ± 1.3%) groups exhibited a similar level of fibrosis, but each was 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 with the DOCA+UNX8 group (11.5 ± 2.6%; Fig. 7).

No differences in macrophage or T cell infiltration were observed among groups in the absence of DOCA-NaCl treatment. Tubulointerstitial macrophage infiltration (CD68-positive cells) was significantly increased (+17-fold) in kidneys from DOCA+S animals compared with placebo-treated animals and +4.6- or +1.7-fold compared with DOCA+C and DOCA+UNX8 animals, respectively (Fig. 8A). Kidneys from DOCA+S and DOCA+UNX8 animals also demonstrated an increase in T cell infiltration (CD43-positive cells) compared with animals from all other groups (Fig. 8B). However, there was no difference in T cell infiltration between DOCA+C and DOCA+UNX8 groups (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 [chemokine (C-C motif) receptor 2 (Ccr2), IL-6 (Il6), Tgfb1, and Tgfb2] and Western blot analysis (CD68 and TGF-β; Fig. 8, C and D). Kidneys from the DOCA+S group displayed the highest level of a number of inflammatory factors (e.g., Ccr2, Il6, Tgfb1, and Tgfb2) compared with all

![Fig. 9. Representative immunohistochemistry images (week 18) of macrophage (CD68) and T cell (CD43) infiltration at ×20 magnification.](http://ajprenal.physiology.org/)

AJP-Renal Physiol • doi:10.1152/ajprenal.00555.2015 • www.ajprenal.org
other groups, including the DOCA+UNX8 and DOCA+C groups.

DISCUSSION

It is well established that hypertension can promote kidney injury and impaired renal function (8, 27), but there are 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 single-kidney individuals. Previously, we developed and characterized the age-related changes (over 18 mo) in cardiovascular and renal physiology in the HSRA model (35). We observed that congenital single-kidney animals are born with significantly fewer nephrons (vs. comparable individual kidneys in two-kidney control animals), and while these rats develop significant hypertrophy at birth, there are no obvious histological changes compared with two-kidney control rats. In fact, there is essentially no change in BP, renal injury (proteinuria), or renal function between these groups until 5 mo of age (35). After mo 5, congenital single-kidney animals exhibit a significant degree of kidney injury and gradual decline in renal function compared with both control and uninephrectomized animal groups 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 animals, and animals with uninephrectomy in the same strain during a period of time when there are no obvious baseline differences in cardiovascular and renal function (less than 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 compared with other experimental approaches, such as ANG II and N-nitro-L-arginine methyl ester (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 BP 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 BP on kidney injury in the HSRA model.

There were several major findings of this study, including that 1) DOCA+S animals demonstrated the most prominent increase in BP 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 with HSRA+UNX8 animals (and all other groups); 3) kidney injury in DOCA-treated animals was more pronounced in the medullary region (vs. the cortical region) and characterized by greater fibrosis, protein casts, and activation of immune genes, with this difference being greatest in DOCA+S animals; and 4) despite a significant rise in BP 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 BP between groups before week 15 (2 wk posttreatment). However, renal injury (demonstrated by proteinuria and increased nephrin and podocalyxin) was significantly greater in the DOCA+S group compared with all other groups during the same period. The most obvious explanation for this findings relates to single-kidney animals having fewer nephrons and the inability of these kidneys to deal with increased water and salt retention compared with DOCA+UNX8 animals (Fig. 10) (8). This is consistent with our previous study, which found that single nephron GFR in single-kidney rats was significantly elevated over two-kidney control rats as well as our current finding of hyperfiltration (elevated total GFR) in either placebo+S and placebo+UNX8 rats compared with placebo+C rats. In support of this conclusion, studies have revealed that humans with fewer 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 present study, volume expansion likely increased the degree of hyperfiltration during the initial phase of DOCA-NaCl treatment. Subsequently, this resulted in accelerated

Fig. 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 kidneys 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, and tubulointerstitial fibrosis, culminating in reduced renal function and elevated BP (35). This finding is similarly observed in HSRA-C animals subjected to uninephrectomy, but changes in renal function and BP 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 a rapid decline in renal function, volume expansion, and greater susceptibility to hypertension compared with uninephrectomy (red arrows).
glomerular injury, reduced RBF, and GFR at only 18 wk of age (5 wk posttreatment), whereas a similar degree of injury was previously observed at much a later time (greater than month 12) in the absence of hypertension (Fig. 10) (35). Thus, the rapid decline in renal function exhibited by HSRA rats with a single kidney leads to a cycle of increased volume expansion, hyperfiltration, and injury as well as elevated BP compared with other DOCA-treated groups (Fig. 10).

The renal hemodynamic data (RBF and GFR) were presented as both uncorrected and corrected for differences in kidney weight. 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 was observed only when using GFR corrected for kidney weight ($r = -0.88$ 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 groups but that congenital solitary kidney rats exhibited a greater reduction in RBF and GFR with increased systemic BP than would be expected based on incremental loss of nephrons (approximately $\geq 20\%$) 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 group exhibit a more substantial decrease in renal function considering a relatively modest difference in nephron numbers.

Histological analysis demonstrated 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 the 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 $O_2$ 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 Wilms tumor protein 1, glial cell line-derived neurotrophic factor, Ret protooncogene, etc.) (for a review, see Ref. 31). These types of models exhibit numerous abnormalities of the urogenital system and/or skeletal system, but the incidence of unilateral or bilateral agenesis is variable and typically below the incidence (50–75%) observed in the HSRA model. To our knowledge, none of the studies using these models have directly compared solitary rats with two-kidney control rats and nephrectomized rats in the same study. There are two reported models of unilateral or bilateral agenesis in the rat, including the unilateral urogenital anomalies rat ($<50\%$) (1, 2) and August $\times$ Copenhagen Irish (ACI) 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 rat has been used as a control strain (nornotensive and not susceptible for renal injury) for genetic studies, along with the Fawn hooded hypertensive rat to identify loci controlling hypertension and renal injury (34). The ACI strain is one of the eight progenitor strains used to derive the National Institutes of Health heterogeneous stock 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 rat (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-kidney 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-NNAME) could impact and/or modulate susceptibility toward kidney injury in single-kidney individuals.

**REFERENCES**


10. Benz K, Amann K.
11. Fujikura T.
23. Lane PW, Birkenmeier CS.
22. Nangaku M.
18. Morgan WC.