

## DOCA/NaCl-induced chronic kidney disease: a comparison of renal nitric oxide production in resistant and susceptible rat strains

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**Erdely A, Freshour G, Tain Y-L, Engels K, Baylis C.** DOCA/NaCl-induced chronic kidney disease: a comparison of renal nitric oxide production in resistant and susceptible rat strains. *Am J Physiol Renal Physiol* 292: F192–F196, 2007. First published August 8, 2006; doi:10.1152/ajprenal.00146.2006.—Recent studies show nitric oxide (NO) deficiency is both a cause and consequence of chronic kidney disease (CKD). Reduced renal neuronal NO synthase (nNOS) abundance and activity parallel development of CKD with different models in the Sprague-Dawley (SD) rats, whereas Wistar Furth (WF) rats are protected against CKD and show preserved renal NO production. In this study, we compared renal NO in response to DOCA/salt-induced injury between the WF and SD. Studies were conducted on sham WF ( $n = 6$ ) and SD ( $n = 6$ ) and uninephrectomized (UNX)+75 mg DOCA+1% NaCl (WF  $n = 9$ ; SD  $n = 10$ ) rats followed for 5 wk. Kidneys were harvested for Western blot, NOS activity, and histology. Other measurements included creatinine clearance and 24-h total NO production and urinary protein excretion. Absolute values of kidney weight were lower in WF than SD rats that showed similar percent increases with UNX+DOCA/NaCl. Proteinuria and decreased creatinine clearance were present in the SD but not the WF rats following UNX+DOCA/NaCl. Glomerular injury was mild in the WF compared with SD rats that showed many globally damaged glomeruli. Although renal nNOS abundance was decreased in both strains (higher baseline in WF), soluble NOS activity was maintained in the WF but significantly reduced in the SD rats. Renal endothelial NOS abundance and membrane NOS activity were unaffected by treatment. In summary, WF rats showed resistance to UNX+DOCA/NaCl-induced CKD with maintained renal NO production despite mild reduction in nNOS abundance. Further studies are needed to evaluate how WF rats maintain renal NO production despite similar changes in abundance as the vulnerable SD strain.

neuronal nitric oxide synthase; endothelial nitric oxide synthase; nitric oxide synthase activity; glomerulosclerosis; creatinine clearance

RECENT EVIDENCE SUGGESTS that nitric oxide (NO) deficiency is both a cause and consequence of chronic kidney disease (CKD) (5). Clinical data show decreased total NO production in patients with CKD and end-stage renal disease (ESRD) (7, 21–23, 31). In animal models of CKD, renal NO deficiency is evident irrespective of the initial insult (1, 9–11, 20, 29, 30) and enhanced progression is seen with superimposed NO synthase (NOS) inhibition and protection with L-arginine supplementation (14, 16). Furthermore, chronic NOS inhibition

alone leads to hypertension, proteinuria, and renal injury (33). Within the kidney, loss of the neuronal isoform of NOS always associates with injury in multiple models of CKD (9–12, 20, 30) and correlates with level of damage and declining renal function (26).

The Wistar Furth (WF) rat is remarkably resistant to CKD induced by chronic NOS inhibition, puromycin-aminonucleoside (PAN)-induced CKD, and 5/6th renal ablation/infarction (A/I) (8, 10, 12, 13) and total NO production and renal nNOS abundance were preserved compared with the progressing Sprague-Dawley (SD) rat (10, 12). Previous work showed that WF rats are resistant to mineralocorticoid-induced CKD (27). In this study, we used the uninephrectomy (UNX) DOCA/NaCl model of CKD to compare the responses of WF and SD in terms of NO production and renal nNOS abundance.

### MATERIALS AND METHODS

Studies were conducted on two strains of male rat, SD and WF, purchased from Harlan at age 10 wk. Two groups of each strain were studied for 5 wk: WF sham ( $n = 6$ ) and 75 mg DOCA ( $n = 9$ ) and SD sham ( $n = 6$ ) and 75 mg DOCA ( $n = 10$ ). Sterile surgery, approved by the West Virginia University Animal Care and Use Committee, involved uninephrectomy of the right kidney and subcutaneous placement of a time release DOCA pellet (Innovative Research of America) on the back behind the shoulders. Sham rats were not uninephrectomized. Following surgery, rats receiving DOCA pellets were placed on 1% NaCl ad libitum in the drinking water.

Twenty-four-hour urine collections were made to determine total protein,  $\text{NO}_3 + \text{NO}_2$  (NOx), and creatinine excretion. All rats were placed on a low-NOx diet (ICN AIN 76C) 2 days before placement in metabolic cages. Total urine protein was determined using the Bradford assay and urinary NOx was measured via the Greiss reaction (25). Creatinine was measured by HPLC (12). Blood was collected (3–5 ml), and tissues were perfused with cold phosphate-buffered saline, harvested onto dry ice, and then snap-frozen in liquid nitrogen. Plasma NOx and creatinine were determined as above. When the left kidney was harvested, a longitudinal section of the cortex was sliced and placed into a cassette and immediately immersed in 10% formalin for histology. The medulla was dissected away from the remaining cortex, and tissues were snap-frozen and stored at  $-80^\circ\text{C}$  to be used for Western blot and NOS activity measurements.

NOS activity was measured from the conversion of L-[ $^3\text{H}$ ]arginine to L-[ $^3\text{H}$ ]citrulline in kidney cortex as described previously (32). Briefly, tissues were homogenized in iced homogenization buffer,

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Table 1. Terminal data for WF and SD sham and rats treated with UNX+DOCA/NaCl for 5 wk

	BW, g	Left. KW, g	Left KW/100 g BW	PCr, mg/dl	Ccr, ml·min <sup>-1</sup> ·g KW <sup>-1</sup>	UNOxV, μmol·24 h <sup>-1</sup> ·100 g BW <sup>-1</sup>	UpV, mg·24 h <sup>-1</sup> ·100 g BW <sup>-1</sup>
WF sham	287±13	0.92±0.06	0.32±0.01	0.22±0.01	2.20±0.49	1.31±0.09	4.31±0.45
SD sham	459±7*	1.8±0.09*	0.39±0.02*	0.26±0.02*	1.40±0.12	1.15±0.10	9.52±1.44*
WF 75 mg	280±11	1.78±0.14†	0.63±0.04†	0.24±0.02	1.71±0.17	1.74±0.19†	2.79±0.20†
SD 75 mg	429±9*†	3.80±0.21*†	0.88±0.04*†	0.39±0.04*†	0.77±0.10*†	1.12±0.06*	42.66±8.66*†

Values are means ± SE. WF, Wistar-Furth; SD, Sprague-Dawley; KW, kidney weight; BW, body weight; PCr and Ccr, plasma creatinine and creatinine clearance, respectively; UNOxV, total NO production; UpV, total urinary protein excretion. \**P* < 0.05 for SD vs. WF. †*P* < 0.05 for Sham vs. 75 mg.

ultracentrifuged, and both soluble [contains predominantly the neuronal (n)NOS] and membrane fractions [contains mostly endothelial (e)NOS] were used for assay. Samples were run in triplicate at baseline and duplicate in the presence of nonselective NOS inhibitors, *N*<sup>G</sup>-monomethyl-L-arginine (L-NMMA; 5 mM), *N*<sup>ω</sup>-nitro-L-arginine methyl ester (L-NAME; 10 mM), and the calcium chelator trifluoperazine (TFP; 2 mM). Data are expressed as picomoles of L-[<sup>3</sup>H]arginine converted to L-[<sup>3</sup>H]citrulline per minute per milligram of protein (pmol citrulline·min<sup>-1</sup>·mg protein<sup>-1</sup>) minus any activity not inhibited by the NOS inhibitor cocktail and adjusted for background.

For Western blot analysis, nNOS was detected with a rabbit polyclonal antibody (17) [1:5,000 dilution, 1-h incubation; secondary antibody, goat, anti-rabbit IgG-horseradish peroxidase (HRP), Bio-Rad; 1:3,000 dilution, 1 h]. Membranes were stripped and reprobed for eNOS (mouse, monoclonal antibody, Transduction Labs, 1:250 dilution, 1 h; secondary antibody goat, anti-mouse IgG-HRP conjugate, Transduction Labs, 1:2,000 dilution, 1 h). Bands of interest were visualized using ECL reagent and quantitated by densitometry, as integrated optical density (IOD) after subtraction of background. The IOD was factored for ponceau red staining to correct for any variations in total protein loading and for an internal standard (eNOS = 10 μg bovine aortic endothelial cell lysate; nNOS = 1 μg of rat cerebellar homogenate) to allow comparison between different membranes. Additional details have been published previously (30).

Histology was performed on kidneys fixed in 10% formalin and then embedded in paraffin wax. Five-micrometer sections were cut and stained (PAS with HE counterstain) and examined, blinded for level of glomerulosclerosis. Statistics were unpaired Student's *t*-test, repeated-measures ANOVA, and least squares means. All data are expressed as means ± SE.

## RESULTS

WF rats had ~50% smaller kidneys compared with SD and body weight (BW) was ~60% less. Following UNX+DOCA/NaCl, both strains showed approximate doubling of kidney weight (KW) and KW/BW was also increased but rose more in SD due to a reduced BW in the treatment group (Table 1). At baseline, WF rats had lower total protein excretion than SD and after 5 wk of UNX+DOCA/NaCl SD developed significant proteinuria while WF showed none (Table 1). The PCr was lower in WF than SD in shams, although the 24-h Ccr was not quite statistically significantly higher. UNX+DOCA/NaCl resulted in decreased Ccr in SD but preservation in WF (Table 1). Blood pressure (BP) was lower in WF vs. SD at baseline (WF 67 ± 4, SD 76 ± 3; *P* < 0.05 vs. WF) and increased in both strains with UNX+DOCA/NaCl by ~10 mmHg (WF 76 ± 3, SD 101 ± 5; *P* < 0.05 vs. WF). We recognize that these values, obtained immediately after laparotomy and under general anesthesia, are unlikely to reflect those seen in the conscious state and their major value is the demonstration of a maintained, lower BP in WF vs. SD with UNX+DOCA/NaCl.

As a result of UNX+DOCA/NaCl, SD developed marked glomerulosclerosis compared with shams (39 ± 7 vs. 7 ± 1,

*P* < 0.01) with a high proportion of globally damaged glomeruli (Fig. 1, *bottom*). In WF, the baseline glomerular injury was less than SD (2 ± 1; *P* < 0.01 vs. SD) and increased to 8 ± 2% (equal to the SD sham value) with only mild segmental damage.

Total NO production (UNOxV) was increased 5 wk following UNX+DOCA/NaCl in the WF but unchanged in the SD at either dose as shown in Table 1. Plasma NOx was unchanged in either the WF (sham 13 ± 1 μM; 75 mg DOCA 11 ± 1) or SD (sham 10 ± 2; 75 mg DOCA 12 ± 1) as was PNOx factored for PCr (Sham WF 0.69 ± 0.07, SD 0.33 ± 0.05; 75 mg DOCA WF 0.54 ± 0.06, SD 0.36 ± 0.05), although the absolute level was higher in the WF (*P* < 0.05). Cortical nNOS abundance was reduced with UNX+DOCA/NaCl treatment in both strains (Fig. 2, *top*), although WF had significantly higher absolute values compared with SD in sham (0.073 ± 0.013 vs. 0.031 ± 0.009; *P* < 0.05) and 75 mg (0.030 ± 0.007 vs. 0.010 ± 0.003; *P* < 0.05). Cortical NOS activity in the soluble fraction was unchanged in the WF but reduced in SD with UNX+DOCA/NaCl compared with sham (Fig. 3, *bottom*). Cortical eNOS abundance (Fig. 2) and membrane NOS activity were unaffected by treatment in either strain, although activity in both soluble (Fig. 3, *top*) and membrane fractions (Fig. 3, *bottom*) was higher in SD than WF as seen previously (10, 12).

We measured NOS protein abundance (but not NOS activity due to limited tissue) for medulla and as shown in Fig. 4, nNOS protein declines with DOCA salt while eNOS remains unchanged and there are no strain differences.

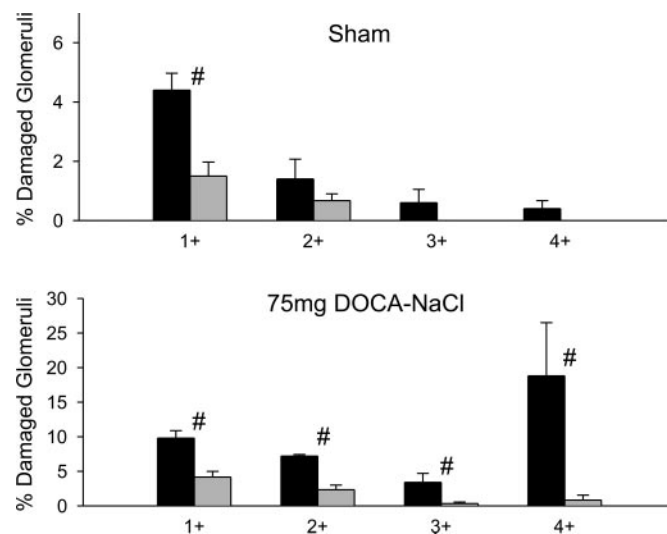
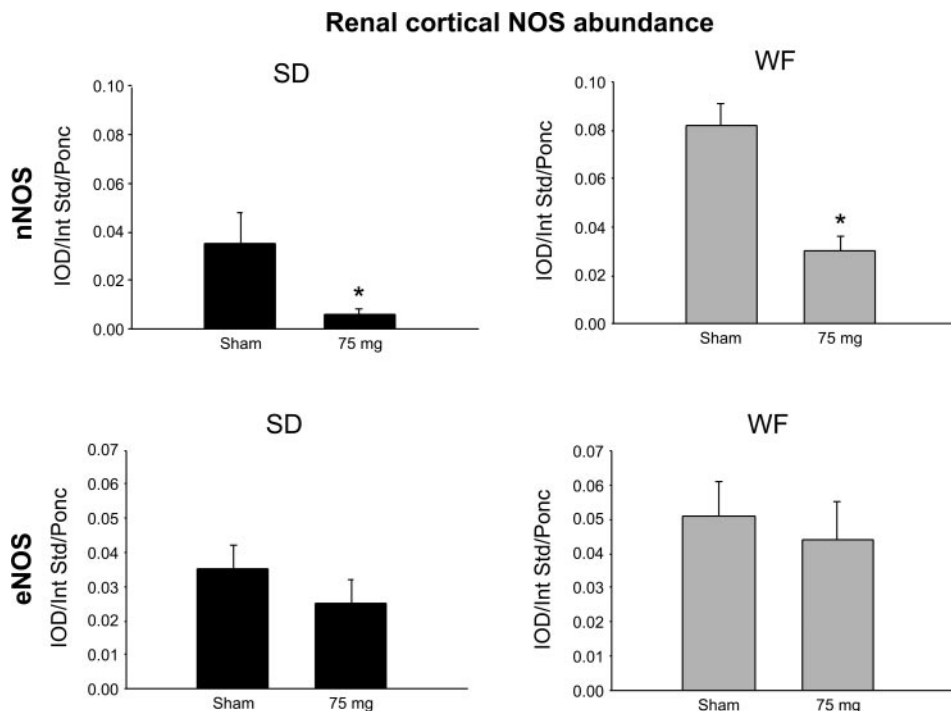


Fig. 1. Percent damaged glomeruli in WF (gray bars) and SD (black bars) according to severity of glomerular injury (1+ <25%; 2+ 25–50%; 3+ 51–75%; 4+ >75%). #*P* < 0.05 for Sprague-Dawley (SD) vs. Wistar Furth (WF).

Fig. 2. *Top left and right:* relative abundance of cortical neuronal nitric oxide synthase abundance (nNOS) in SD (black bars) and WF (gray bars). *Bottom left and right:* relative abundance of cortical endothelial nitric oxide synthase abundance (eNOS). \* $P < 0.05$  for Sham vs. 75 mg. IOD, integrated optical density.



**DISCUSSION**

Our findings confirm earlier observations that WF rats are resistant to mineralocorticoid-induced CKD (27). The WF is

also resistant to other forms of CKD (8, 10, 12) suggesting that this is not due to an isolated difference in the mineralocorticoid system of WF, but rather reflects a generalized resistance to progression of CKD. Our recent observations suggest that there is a strong correlation between the development of progressive injury during experimental CKD (irrespective of primary cause) and the reduction in abundance of renal cortical nNOS protein (9–12, 26, 30). The present study reinforces this relationship, demonstrating that the tendency to develop progressive CKD varies inversely with the level of preservation of renal cortical nNOS in the UNX+DOCA/NaCl model of CKD.

The SD rat is an outbred strain and SD rats from Harlan SD are susceptible to progressive CKD. In these rats renal cortical nNOS abundance is relatively low in control, declines further

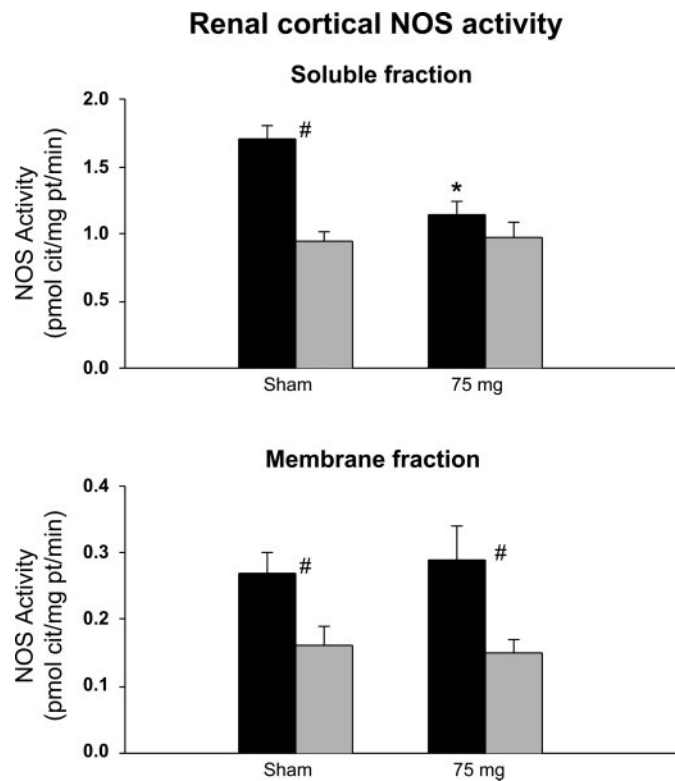


Fig. 3. NOS activity (shown as the inhibitable conversion of arginine to citrulline) measured from the soluble (*top*) and membrane (*bottom*) fraction of cortical homogenates. # $P < 0.05$  for SD vs. WF. \* $P < 0.05$  for Sham vs. 75 mg.

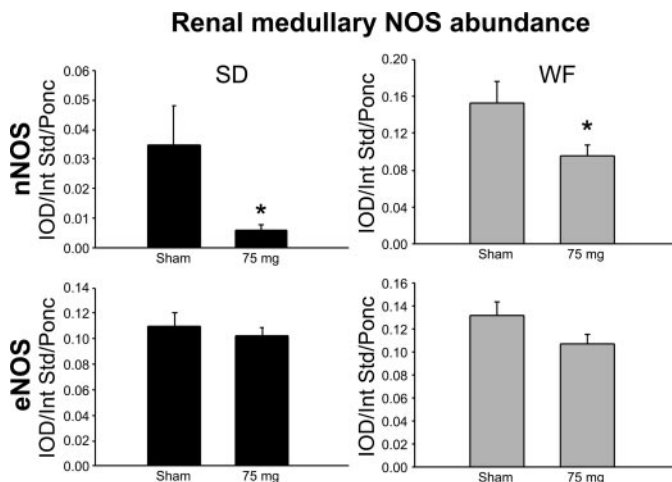


Fig. 4. *Top left and right:* relative abundance of renal medullary nNOS in SD (black bars) and WF (gray bars). *Bottom left and right:* relative abundance of kidney medullary eNOS abundance. \* $P < 0.05$  for Sham vs. 75 mg.



with injury, and is accompanied by a decrease in NOS activity of the cortical homogenate (soluble fraction). This is true for the 5/6 A/I, chronic glomerulonephritis, and PAN-induced models of CKD as well as the slowly evolving damage due to normal aging (10–12, 30), and we found a similar relationship in the Zucker inbred obese rat which develops type 2 diabetes (9). Also, the Harlan SD rat is highly susceptible to hypertension and CKD due to chronic NOS inhibition (33). Although of note, previous studies by Pollock and Rekitto (19) showed that the Charles River SD are relatively resistant to chronic NOS inhibition. Therefore, our general conclusions on CKD susceptibility relate to the Harlan SD only (19), although it should be noted that both Charles River and Harlan SD are reported to be susceptible to DOCA/salt-induced hypertension (2, 4). In contrast, in the resistant WF the abundance of cortical nNOS is higher than in SD under normal conditions. There was some decrease following 5/6 A/I and PAN but the absolute quantity was always greater than the SD and importantly, NOS activity was maintained in WF (but not SD) kidneys (10, 12). In the aging SD where the male is vulnerable to kidney damage, the female SD is protected, and again, the nNOS abundance and in vitro NOS activity fell in males but was preserved in the female.

Thus, the ability to conserve renal nNOS expression is associated with protection from progression of CKD. In the SD rat using the 5/6 A/I model of CKD, we found a strong inverse correlation between progression of CKD and renal cortical nNOS abundance (26). In the WF, a critical threshold of decreased abundance (and resultant activity) may exist which must be surpassed before accelerated progression will occur. In support of this possibility, high-dose chronic NOS inhibition did not produce CKD, whereas the combination of low-dose NOS inhibition with 5/6 A/I induced rapid CKD progression in the otherwise resistant WF (12). A specific role for nNOS is suggested by our preliminary observation that “selective” nNOS inhibition with 7-nitro-indazole and the nonselective NOS inhibitor, L-NAME, have equal accelerating effects in C57Bl6 mice that are otherwise resistant to ablation-induced CKD (18). In kidney cortex, nNOS is primarily located in the macula densa (MD). NO derived from nNOS in MD can dilate afferent arterioles and regulate tubuloglomerular feedback. Reduction of cortical nNOS in SD rats may cause renal vasoconstriction, decrease glomerular filtration rate, enhance tubular sodium reabsorption, and hence hastening progression of CKD. NO also has growth inhibitory actions at the glomerulus and MD-derived NO, by virtue of its proximity to mesangium, may inhibit mesangial expansion and/or matrix accumulation. Reduction in cortical nNOS could therefore also allow mesangial expansion and increased matrix. However, at present we have no information on the localization of the high abundance cortical nNOS in WF kidneys.

In the present study, we found no change in cortical eNOS abundance or membrane NOS activity in either strain 5 wk following implantation of the DOCA pellet. The eNOS response has been highly variable in all other models studied with increase, no change, and reductions being observed (5). This suggests that the eNOS response is secondary to the injury model, rather than causal, whereas the nNOS alterations appear to be primary.

Despite the marked reduction in renal nNOS protein and activity in the DOCA-treated SD, the  $U_{NOX}V$  remains un-

changed. It is important to note that  $U_{NOX}V$  is not simply a measure of renal NO production but reflects overall NO production from the entire vascular endothelium, as well as skeletal muscle, cerebellum, liver, etc; in fact, because of the relatively low NOS protein abundance in kidney, renal  $NO_x$  is likely to contribute only a few percent to the total (6). Indeed, we previously reported a dissociation between renal and total NO production in the Zucker obese diabetic rat where marked declines in renal NOS abundance and activity coexist with increased total NO production (9).

In the present study, the control groups were not uninephrectomized, whereas the DOCA-treated rats were also UNX since this is part of the model. Previous studies showed that initially following UNX alone the rapid compensatory hemodynamic response of the kidney was mediated by NO and renal NOS activity was elevated 48 h following UNX (15, 24, 28). After 1 wk following UNX, renal responses to acute NOS inhibition were similar to control two kidney rats (15, 24). Although it is unclear whether there are sustained NOS abundance/activity changes following UNX, it has been shown that urinary cGMP excretion remained elevated at 28 days post-UNX (24). Therefore, the decline in cortical NOS activity seen in the DOCA-treated SD in the present study is most likely due to the associated injury and an even greater difference is likely if the control groups were uninephrectomized.

In summary, the WF showed resistance to UNX+DOCA/NaCl-induced CKD with maintained renal NO production despite mild reduction in nNOS abundance. Further studies will be needed to evaluate how the WF rat is able to maintain renal NO production despite similar changes in abundance as seen in the vulnerable SD strain.

#### GRANTS

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