INCREASED REACTIVE OXYGEN SPECIES CONTRIBUTES TO KIDNEY INJURY IN MINERALOCORTICOID HYPERTENSIVE RATS

L. JIN¹, R.A. BESWICK², T. YAMAMOTO⁵, T. PALMER¹, T.A. TAYLOR⁴, J.S. POLLOCK³,4, D.M. POLLOCK³, M.W. BRANDS¹,3, R.C. WEBB¹,3

¹Department of Physiology, Medical College of Georgia, Augusta, GA
²Department of Physiology, University of Michigan, Ann Arbor, MI
³Vascular Biology Center, Medical College of Georgia, Augusta, GA
⁴Department of Pharmacology and Toxicology, Medical College of Georgia, Augusta, GA
⁵First Department of Medicine, Hamamatsu University School of Medicine, Hamamatsu, Japan

Hypertension is associated with increased reactive oxygen species (ROS). Renal ROS production and their effects on renal function have never been investigated in mineralocorticoid hypertensive rats. In this study we hypothesized that increased ROS production in kidneys from deoxycorticosterone (DOCA)-salt rats contributes to adverse renal morphological changes and impaired renal function in DOCA-salt hypertensive rats. We also determined whether ROS-induced renal injury was dependent on blood pressure. DOCA-salt hypertensive rats exhibited a marked increase in blood pressure, renal ROS production, glomerular and tubular lesions, and microalbuminuria compared to sham rats. Treatment of DOCA-salt hypertensive rats with apocynin for 28 days resulted in attenuation of systolic blood pressure and improvement of renal morphology. Renal superoxide level in DOCA-salt rats was 215% of sham-operated rats and it was significantly decreased to 140% with apocynin treatment. Urinary protein level was decreased from 27 ± 3 mg/day in DOCA-salt hypertensive rats to 9 ± 2 mg/day. 28 days of Vitamin E treatment also reduced renal injury in regard to urinary protein level and renal morphology but had no effect on blood pressure in DOCA-salt rats. Increased urinary 8-isoprostane, a marker for oxidative stress, in DOCA-salt hypertensive rats (55 ± 8 ng/day) was diminished by vitamin E treatment (24 ± 6 ng/day). These data suggest that renal injury characteristic of mineralocorticoid hypertension is associated with oxidative stress and is partly independent of blood pressure.

Key Words: vitamin E, apocynin, kidney injury, mineralocorticoid hypertension.
INTRODUCTION

Reactive oxygen species (ROS) are involved in the regulation of physiological functions such as vascular tone, inflammation and cell growth or apoptosis (1, 2). Considerable evidence implicate that hypertension is associated with increased ROS formation. Excessive ROS production in the kidney has been reported in different hypertensive animal models, including angiotensin II-induced hypertensive rats, N-omega-nitro-L-arginine-induced hypertensive rats (3), Dahl salt-sensitive hypertensive rats (4), and spontaneously hypertensive rats (5). Progressive renal injury is observed in these animal models of hypertension.

Studies have shown that NADPH oxidase is a major source of ROS in vasculature (6, 7). Previously, we also reported that treatment with an NADPH oxidase inhibitor significantly reduced superoxide production in aorta from deoxycorticosterone (DOCA-salt) hypertensive rats (8). Inhibitors of xanthine oxidase and nitric oxide (NO) synthase did not alter ROS generation. In addition, NADPH oxidase subunit p22phox mRNA expression was increased, indicating that NADPH oxidase is responsible for increased ROS production in rat aorta. Moreover, DOCA-salt hypertensive rats exhibited significantly increased renal monocyte/macrophage infiltration compared to normotensive rats (9). Treatment with antioxidants attenuated this inflammatory response. However, whether local ROS are increased in the kidney and whether renal injury is mediated by ROS in DOCA-salt rats, a hypertensive rat model with low renin-angiotensin level, remains to be elucidated.

This study tests the hypothesis that excess ROS production contributes significantly to renal injury and hemodynamic changes in the DOCA-salt hypertensive rats. Chronic antioxidant treatment will attenuate renal ROS production and renal injury in the DOCA-salt hypertensive rats. Furthermore, we hypothesized that the adverse effect of ROS on renal function is partly independent of blood pressure. A pharmacological approach utilizing apocynin, an inhibitor of NADPH oxidase, was used to investigate the source of renal ROS in the intact rats (via drinking water) (9, 10). In addition, vitamin E (via mixed food) was used to determine whether ROS-induced renal injury is independent of blood pressure since it has been reported that vitamin E reduces ROS levels without changing blood pressure in hypertensive rats (3, 11).

METHODS

1. Animal preparation

Experiments were conducted on male Sprague-Dawley rats (Harlan, 200 g) and all procedures were approved by the institutional animal care and use committee. Rats were anesthetized with an intramuscular injection of 100 mg/kg ketamine/20 mg/kg xylazine (The Butler Company), and a Silastic sheet (Dow Corning) containing DOCA (Sigma; 200 mg/kg body weight) was inserted subcutaneously via midscapular incision. Right uninephrectomy was performed via flank incision.
Sham-operated rats underwent uninephrectomy with implantation of a Silastic sheet without DOCA. The period of DOCA or sham treatment was 28 days.

One set of experiments was designed to determine the effect of apocynin. The rats were divided into three groups: 1) sham-operated rats received tap water; 2) DOCA-treated rats received 1% NaCl and 0.1% KCl in tap water; and 3) DOCA-treated rats received apocynin (1.5 mmol/L) in their drinking water in addition to the 1% NaCl and 0.1% KCl during the 28 day treatment period. The doses of apocynin used in this study were based on previous reports showing efficacy with respect to proposed biological activity (8, 9). Another set of experiments was designed to determine the effect of vitamin E. DOCA-salt treated rats received vitamin E (α-tocopherol, 200 mg/kg body weight) mixed with 0.1 g vanilla yogurt (Dannon) for 28 days. The sham-operated rats and another group of DOCA-salt rats were fed with 0.1 g yogurt without vitamin E.

Systolic blood pressures were measured once a week by the tail cuff method (pneumatic transducer). At seven day intervals after implantation, DOCA-treated rats were killed to characterize renal ROS production (see below). In some experiments, rats were housed individually in metabolic cages with free access to food and respective drinking water (day 26 following implantation). Urine was collected for measurement of urinary albumin and 8-isoprostane. On day 28 after implantation, rats were anesthetized with ketamine/xylazine cocktail and the kidneys were carefully removed and placed in cold physiological salt solution (PSS; composition in mmol/L; NaCl 130, KCl 4.7, KH₂PO₄ 1.18, MgSO₄·7H₂O 1.17, NaHCO₃ 14.9, dextrose 5.5, EDTA .26, CaCl₂ 1.6). Kidneys were sectioned along the sagittal plane at 2-3 mm thickness and placed on ice until lucigenin analysis for ROS production.

2. Measurement of renal superoxide production by lucigenin assay and 8-isoprostane

Lucigenin chemiluminescence was used to measure superoxide production. In recent studies, 5 μmol/L lucigenin has been shown to correlate well with electron spin resonance as a quantitative measure of superoxide production (12, 13). Details of this assay have been published previously (14). Renal sections were allowed to equilibrate for 30 minutes at 37 °C. Scintillation vials containing 2 ml PSS with 5 μmol/L lucigenin (Sigma Chemical Co.) were placed into a scintillation counter (Beckman LS 6000IC) and switched to out-of-coincidence mode and background counted. After dark adaptation, background counts were recorded and renal sections were added to the vial. Scintillation counts were recorded every minute for twenty minutes and counts between the 15-20 minute intervals were averaged. Tissue sections were then dried in an oven for 24 hours and results were expressed as counts above background per mg of dried tissue.

8-Isoprostane, a marker for oxidative stress, is produced via the random oxidation of tissue phospholipids by oxygen radicals (15). Urinary 8-isoprostane was analyzed by an enzyme-linked immunosorbent assay (EIA) kit according to the manufacturer’s instruction (Cayman).

3. Measurement of urinary protein excretion

Protein concentration in the urine was measured by standard Bradford assay (BioRad) and by a competitive EIA assay (Nephrat; Exocell, Inc.).

4. Examination of renal injury by morphologic evaluation

For conventional morphology, kidneys from each treatment group were fixed overnight with 3% paraformaldehyde and paraffin embedded. Paraffin-embedded sections in the sagittal plane were stained with periodic acid-Schiff stain (PAS) and Masson’s trichrome stain. The presence of glomerular lesions was evaluated in at least 30 glomeruli/section. Morphologic evaluation was
conducted in a blind fashion using light microscopy, as previously reported (16). The severity of glomerular hypertrophy, glomerulosclerosis, glomerular necrosis, fibroid necrosis and proliferative glomerular lesions was calculated semiquantitatively using a 0 to 3 scale (0, normal or almost normal; 1, mild; 2, moderate; 3, severe) in each glomerulus. The level of tubulointerstitial lesions was also graded semiquantitatively at 100x magnification and the mean score was obtained from out of more than 16 fields per sample.

5. Statistical analysis

Data were presented as mean ± standard error of the mean (SEM). Statistically significant differences among groups were tested by one-way of variance or analysis of variance with repeated measures followed by post hoc testing (Tukey’s multiple range test or Student’s t test as appropriate). The Newman-Keuls correction was used during multiple testing. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Blood pressure and renal superoxide production in DOCA-salt rats

Systolic blood pressure increased significantly in DOCA-salt treated rats compared to that in sham-operated rats during 28 days of treatment (Fig. 1A). There was no significant difference in superoxide production in DOCA or sham operated rat kidneys during weeks 1-3 of treatment. However, superoxide production was significantly increased at week 4 compared to sham operated rats ($1804 ± 206$ in DOCA rats vs. $964 ± 136$ counts/min/mg in sham rats, Fig. 1B).

Effect of apocynin on blood pressure and renal superoxide production

Treatment of DOCA-salt hypertensive rats with the NADPH oxidase inhibitor, apocynin, significantly decreased systolic blood pressure compared to rats treated with DOCA alone during all 4 weeks (Fig. 2A). Chronic treatment with apocynin also significantly decreased renal superoxide production compared to rats treated with DOCA alone at week 4 ($1804 ± 206$ counts/min/mg in DOCA rats vs. $1169 ± 210$ counts/min/mg in apocynin-treated DOCA rats, Fig. 2B). There was no significant difference in renal superoxide production in apocynin-treated rats compared to that in sham rats. Chronic administration of antioxidants to sham animals resulted in a slight but insignificant decrease in renal superoxide production in apocynin treated sham rats compared to that in sham rats ($837 ± 117$ vs. $964 ± 136$ counts/min/mg, respectively). Moreover, there was no significant difference in systolic blood pressure of the apocynin-treated sham groups compared to sham-operated values. These data suggest that NADPH oxidase is responsible for the increased superoxide production in kidneys from DOCA-salt treated rats.

Effect of apocynin on urinary protein excretion and renal morphology

Urinary protein excretion is an index of renal injury. In DOCA-salt rats, urinary excretion of albumin was significantly increased compared to that in sham-operated
rats (27 ± 3 vs. 8 ± 0.3 mg/day, respectively; Fig. 3). Treatment with apocynin significantly reduced albumin excretion (9 ± 2 mg/day) in DOCA-salt rats.

Table 1 lists results of morphological examination of the kidneys from the various rat groups. Glomerular hypertrophy, glomerulosclerosis, proliferative glomerular lesions, and tubulointerstitial fibrosis were significantly greater in DOCA-salt hypertensive rats compared to sham rats. Treatment with apocynin caused a significant decrease in glomerular hypertrophy, glomerulosclerosis and tubulointerstitial fibrosis compared to rats treated with DOCA-salt alone (Tab. 1).

Effect of vitamin E on blood pressure and renal oxidative stress

Studies have shown that vitamin E treatment in hypertensive rats decreases ROS levels but has no effect on the elevated blood pressure (3, 11). To determine whether ROS-induced renal injury is dependent on high blood pressure, a group of DOCA-salt rats received vitamin E therapy for 28 days. Our data show that vitamin E had no significant effect on the blood pressure of DOCA-salt rats.
during the 4 week treatment (Fig. 4A). Renal ROS production was determined by measuring urinary 8-isoprostane levels, a marker for oxidative stress. The results suggest that 8-isoprostane was significantly increased in DOCA-salt rats at week 4 (55 ± 8 ng/day for DOCA-salt rats vs. 14 ± 3 ng/day for sham rats). This increase in 8-isoprostane was reduced by vitamin E treatment (Fig. 4B; 24 ± 6 ng/day for vitamin E-treated DOCA-salt rats).

**Effect of vitamin E on urinary protein excretion and renal morphology changes**

Similar to the experiments with apocynin, vitamin E treatment markedly decreased albumin excretion in DOCA-salt rats (Fig. 5; 13 ± 6, 71 ± 11, and 16 ± 4 mg/day for sham, DOCA-salt and vitamin E-treated DOCA-salt rats,
respectively). Furthermore, vitamin E decreased the development of glomerular hypertrophy, glomerulosclerosis, proliferative glomerular lesions and tubulointerstitial fibrosis in DOCA-salt hypertensive rats (Tab. 2). In addition, interstitial mononuclear cell infiltration, tubular cast and glomerular tuft adhesion

\[ \text{Table 1. Morphological analysis of kidneys from DOCA-salt hypertensive rats.} \]

<table>
<thead>
<tr>
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<th>Sham</th>
<th>DOCA</th>
<th>DOCA-Apocynin</th>
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<td>Glomerular hypertrophy</td>
<td>0.06</td>
<td>0.05*</td>
<td>0.20‡</td>
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<tr>
<td>Glomerulosclerosis</td>
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<td>Proiferative</td>
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<td>0.50±</td>
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<td>Glomerular lesions</td>
<td>0.07*</td>
<td>0.07*</td>
<td>0.09‡</td>
</tr>
<tr>
<td>Tubulointerstitial</td>
<td>0.13±</td>
<td>0.75±</td>
<td>0.40‡</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>0.03</td>
<td>0.21*</td>
<td>0.07‡</td>
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</table>

0=normal
1=mild
2=moderate
3=severe

* $P < 0.05$, DOCA vs. sham
† $P < 0.05$, DOCA vs. DOCA-apocynin
‡ $P < 0.05$, DOCA-apocynin vs. sham

\[ \text{Fig. 3. Effect of apocynin on urinary protein excretion. Rats were housed individually in metabolic cages and urine was collected in the last two days of treatment. Urinary protein excretion was remarkably increased in DOCA-salt rats and apocynin treatment reduced the protein excretion in DOCA-salt rats. n=5, * } P < 0.05 \text{ versus sham, † } P < 0.05 \text{ versus DOCA-salt treated only.} \]
to Bowman’s capsule were decreased in kidneys from vitamin E treated DOCA-salt rats compared to those from DOCA-salt treated rats (Fig. 6).

**DISCUSSION**

It has been shown that ROS production is significantly increased in DOCA-salt hypertensive rat aorta (9, 18, 19). However, renal ROS production and the source of renal ROS remain unclear. The current study was conducted to examine whether renal superoxide production is increased in the DOCA-salt hypertensive rats and is dependent on increased NADPH oxidase activity. We used two different methods...
(lucigenin assay and 8-isoprostane EIA assay) to determine renal superoxide production. Our data consistently demonstrate that the levels of ROS were remarkably increased in kidneys from DOCA-salt rats when compared to those from sham-operated rats. Renal ROS production was blocked by treatment with the NADPH oxidase inhibitor, apocynin, suggesting that NADPH oxidase is mainly responsible for increased ROS production. Several previous studies also provide

<table>
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<th>DOCA-Vitamin E</th>
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<td>Glomerular</td>
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<td>Hypertrophy</td>
<td>0.16</td>
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<td>0.20†‡</td>
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<td></td>
<td>0.16</td>
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<td>0.15†</td>
</tr>
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<td>0.71±</td>
</tr>
<tr>
<td>Glomerular lesions</td>
<td>0.09</td>
<td>0.18*</td>
<td>0.20†‡</td>
</tr>
<tr>
<td>Tubulointerstitial</td>
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<td>0.86±</td>
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<td>Fibrosis</td>
<td>0.16</td>
<td>0.17*</td>
<td>0.28</td>
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</table>

0=normal
1=mild
2=moderate
3=severe

* P < 0.05, DOCA vs. sham
† P < 0.05, DOCA vs. DOCA-vitamin E
‡ P < 0.05, DOCA-vitamin E vs. sham
strong evidence that NADPH oxidase is involved in the formation of ROS within the kidney. For example, Shiose et al. recently found a novel superoxide producing NADPH oxidase subunit, which is expressed exclusively within the fetal and adult
human kidney (20). Additionally, Zou et al. found that incubation of renal homogenates with NADH caused an increase in NADPH oxidase activity (21).

Previous studies examining the relationship between proteinuric glomerulopathies and tubulointerstitial fibrosis suggest that proteins accumulate within the tubular lumen due to changes in glomerular permeability. This, in turn, triggers an inflammatory reaction that contributes to renal structural damage and progression of renal disease (22). Furthermore, the progression of tubulointerstitial injury has been shown to correlate with the development of renal failure in both diabetic and non-diabetic rats (23-27). We did not observe any significant tubulointerstitial fibrosis until the fourth week of DOCA-salt treatment, which is correlated with a significant increase in renal superoxide production (Fig. 1B). The mechanism for tubulointerstitial injury remains speculative, but oxidant-mediated injury to tubular cells has been hypothesized to play a critical role (28-31). In support of this possibility, this study showed that treatment with antioxidants blunts tubulointerstitial fibrosis in the DOCA-salt hypertensive rats, further strengthening our initial hypothesis that excess ROS exacerbate renal tubulointerstitial fibrosis leading to kidney injury.

There is also evidence that high concentrations of protein in tubular fluid may cause direct renal toxicity and production of proinflammatory cytokines and vasoactive factors, which result in regional ischemia, fibroblast proliferation, increased intracellular matrix synthesis, leukocyte infiltration and glomerular lesions (32, 33). Moreover, in many inflammatory diseases, important pathological processes are linked to the ability of infiltrating leukocytes to release a complex assortment of agents that can destroy cells and dissolve connective tissue. In previous studies, we observed that kidneys from DOCA-salt hypertensive rats exhibit elevated monocyte/macrophage infiltration and increased inflammatory signal transduction precursor expression compared to sham operated kidneys (9). Additionally, apocynin treatment attenuated this infiltration. The effect of monocytes/macrophages and their ability to elicit an oxidative or respiratory burst, resulting in sequential ROS production via NADPH oxidase activation, has been addressed in many studies but still remains a controversial possibility (34-39).

Another possible mechanism of activation of NADPH oxidase is by vasoactive peptides. Emerging evidence suggests that endothelin-1 (ET-1) in an important stimulator of NADPH oxidase in different animal models of hypertension including DOCA-salt hypertensive rats, aldosterone and salt-induced hypertensive rats (40-42). Interestingly, ET-1 mRNA expression and protein synthesis is increased in the kidney of DOCA-salt rats although plasma ET-1 levels may not be increased (43-45). These findings suggest that ET-1 may be, in part, responsible for the excess superoxide production and renal injury through activation of NADPH oxidase in the kidney. The underlying molecular mechanisms of NADPH oxidase activation in kidneys of DOCA-salt rats remains to be investigated.
In the present study, since increased ROS production in kidneys from DOCA-salt was observed at week 4 while elevation of the blood pressure starts at week 1, it is likely that increased renal ROS are not the cause of hypertension. Although many studies suggested that hypertension is associated with increased oxidative stress, some evidence indicates that ROS may not be the direct cause of the elevated blood pressure. In the ET-1-induced hypertensive rat model, ROS are significantly increased in rat aorta along with enhanced NADPH oxidase activity; however, attenuation of oxidative stress using apocynin or tempol did not ameliorate hypertension (46). Further studies are needed to determine the cause and effect relationship between renal superoxide and hypertension in DOCA-salt rats.

 Nonetheless, studies have shown that ROS may have a direct effect on renal function independent of high blood pressure, although blood pressure reduction causes reno-protection. Our data showed that apocynin significantly reduced the blood pressure during the 4 week treatment with DOCA-salt. However, superoxide production was only markedly increased at week 4. In addition, apocynin completely abolished albuminuria whereas systolic blood pressure was decreased only about 50%, suggesting the beneficial effects of apocynin may not be solely dependent on the level of blood pressure. Furthermore, our observations that vitamin E reduced renal injury but not blood pressure in DOCA-salt rats suggest that ROS-induced renal injury is partly independent of elevation of blood pressure. Similarly, vitamin E therapy in DOCA-salt rats has shown to decrease ROS levels in mesenteric arteries but has no effect on blood pressure. Atarashi et al. demonstrated that decreased renal ROS levels with the antioxidant supplement alleviated renal injury of Dahl salt-sensitive rats with no change in the high blood pressure (17). Another study demonstrated that vitamin E reduced renal damage, but has no effect on blood pressure in N-omega-nitro-L-arginine-induced hypertensive rats (3). It is noteworthy that the results of vitamin E therapy are inconsistent. In some rat models of hypertension such as spontaneously hypertensive rats, vitamin E significantly reduced blood pressure (47). The controversial effects of vitamin E treatment are probably due to differences in strains of rats, period of vitamin E therapy or pathogenesis of vascular diseases.

 The difference between apocynin and vitamin E on lowering blood pressure and renal protection may be due to the additional effects of apocynin other than inhibition of NADPH oxidase. It has been reported that apocynin reduces thromboxane synthesis while stimulates prostaglandins E₂ and F₂α formation (48). Thromboxane is a potent vasoconstrictor and pro-inflammatory mediator involved in the pathogenesis of hypertension (49). Prostaglandin E₂ induces vasodilation and has been implicated in promoting renal sodium excretion (50). Therefore, by modulation of arachidonic acid metabolism, apocynin may be able to reduce blood pressure by mechanisms unrelated to inhibition of NADPH oxidase.

 In summary, our findings indicate that NADPH oxidase-dependent ROS production is increased in kidneys from DOCA-salt rats, which contributes to deleterious structural and functional renal changes. Quenching of excess ROS
with antioxidants decrease renal injury in DOCA-salt hypertensive rats; and these effects are partly independent of blood pressure change.

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Author’s address: Liming Jin, PhD, Department of Urology Johns Hopkins University, 600 N. Wolfe Street, Baltimore, MD 21287. Telephone: (410)955-0352, Facsimile: (410)614-3695.  
E-mail: ljin8@jhmi.edu