Vitamin E reduces glomerulosclerosis, restores renal neuronal NOS, and suppresses oxidative stress in the 5/6 nephrectomized rat

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CHRONIC KIDNEY DISEASE (CKD) is accompanied by oxidative stress and nitric oxide (NO) deficiency. The major ROS is superoxide, and in kidney this is mainly generated by NADPH oxidase (6). NO deficiency in CKD has many causes, including inactivation of NO by oxidative stress and inhibition of nitric oxide synthase (NOS) enzyme activity by increased levels of endogenous inhibitors; in kidney, there is reduced neuronal nitric oxide synthase (nNOS) enzyme abundance/activity (3, 21, 28). Because superoxide and NO have counterbalancing actions and reciprocally reduce each other’s bioavailability, an imbalance of NO and superoxide may shift the kidney toward a state of superoxide dominance, causing renal vasoconstriction, enhanced tubular sodium reabsorption, and CKD progression (21). Therefore, antioxidants have been considered for prevention of CKD progression by reducing oxidative stress and/or preserving NO bioavailability.

Vitamin E is a potent, naturally occurring lipid-soluble antioxidant that scavenges ROS and lipid peroxyl radicals. The most active and predominant form of vitamin E is α-tocopherol, and this has been used therapeutically in many conditions, although the impact of vitamin E on CKD progression is controversial (1, 4, 5, 10, 12, 17, 23, 27). In this study, we investigate the impact of the 5/6 renal ablation model on renal nNOS abundance and also whether the protective effects of vitamin E therapy are associated with preservation of renal nNOS abundance and reduction in NADPH-dependent superoxide generation. The endogenous NOS inhibitor asymmetric dimethylarginine (ADMA), generated by type I protein arginine methyltransferase (PRMT) and metabolized by dimethylarginine dimethylaminohydrolase (DDAH), is increased in CKD (3). In this study, we also determined the impact of vitamin E treatment on the circulating level of ADMA and on PRMT1 abundance as well as activity and enzyme expression.

MATERIALS AND METHODS

Studies were conducted on 17 male Sprague-Dawley rats (12 wk old) purchased from Harlan (Indianapolis, IN). Rats were kept under standard conditions and fed rat chow and water ad libitum. All rats had baseline metabolic cage measurements and were subjected to either sham surgery or 5/6 nephrectomy (NX); 5/6 NX was performed under isoflurane general anesthesia using the full sterile technique. By the retroperitoneal approach, two poles of the left kidney were removed, and then 1 wk later the right kidney was removed. All rats were assigned to the following groups at the time of the first surgery: group 1 (sham, n = 6), sham-operated rats kept on regular rat chow (powdered) and tap water; group 2 (5/6 NX, n = 6), 5/6 NX rats maintained on regular rat chow and tap water; and group 3 (5/6 NX + Vit E, n = 5), 5/6 NX rats treated with vitamin E supplementation (Sigma Diagnostics, St. Louis, MO) of regular rat chow containing 5,000 IU α-tocopherol/kg chow, begun at the polectomy surgery.

In all rats, 24 h urine was collected in a metabolic cage for measurement of protein by the Bradford method and total NO production (from NOx = NO2− + NO3−) by Griess reaction every other week after surgery. Rats were followed for 15 wk after surgery and then killed. At death, blood pressure was measured via the abdominal aorta, and then an aortic blood sample was collected for measurement of plasma creatinine, ADMA, and NOx levels. The left kidney remnant was perfused blood free with cold PBS and removed, a
section was placed in 10% buffered formalin for pathology, and the remainder was separated into cortex and medulla, flash-frozen in liquid nitrogen, and stored at −80°C for analysis of NOS protein and superoxide. Plasma and urine creatinine levels were measured by HPLC as described by us previously (9).

NOS, PRMT1, DDAH1, and DDAH2 protein abundances were detected by Western blot. Details for detection of the NOS have been published previously (25). Briefly, samples (200 μg of kidney cortex and 100 μg of kidney medulla) were loaded on a 7.5% polyacrylamide gel and separated by electrophoresis (200 volts, 60 min). The nNOS-α was detected with a rabbit polyclonal antibody (1:10,000 dilution, 1 h incubation; see Ref. 18) followed by a secondary donkey anti-rabbit IgG-horseradish peroxidase (HRP) antibody (1:3,000 dilution, 1 h incubation; Bio-Rad). Membranes were stripped and reprobed for endothelial (e) NOS with a mouse monoclonal antibody (1:250 dilution, 1 h incubation; Transduction Laboratories) and a secondary goat anti-mouse IgG-HRP antibody (1:2,000 dilution, 1 h incubation; Transduction Laboratories). For PRMT1 and DDAH, we loaded 200 μg of kidney cortex and used 12% gels with an otherwise identical protocol to that used for the NOS. For DDAH, we used a goat anti-rat DDAH1 antibody (1:500 overnight incubation; Santa Cruz) and a goat anti-rat DDAH2 antibody (1:100 overnight incubation; Santa Cruz), followed by a secondary donkey anti-goat antibody (1:2,000 dilution, 1 h incubation; Santa Cruz). For PRMT1, we used a rabbit anti-PRMT1 antibody (1:2,000 dilution, overnight incubation; Upstate) and a goat anti-rabbit antibody (1:3,000, 1 h incubation; Bio-Rad). For DDAH2 peptide competition, 200 μg of kidney cortex and kidney medulla were loaded on a 12% polyacrylamide gel and separated by electrophoresis. The membrane was separated into identical halves: one for control and the other for peptide competition. For peptide competition, 100 μg of neutralizing peptide (Santa Cruz) were incubated with 5 μg of the DDAH2 antibody (20×) overnight at 4°C. The antibody-peptide mixture was centrifuged (11,000 rpm) for 10 min. The supernatant was collected, diluted in blocking solution (1:100), mixed well, and used for DDAH2 detection as described above. Bands of interest were visualized using enhanced chemiluminescence reagent and quantified by densitometry (VersaDoc imaging system and Quantity One Analysis software; Bio-Rad) as integrated optical density (IOD) after subtraction of background. The IOD was factored for Quantity One Analysis software; Bio-Rad) 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. The protein abundance was represented as IOD/Ponceau Red/Std.

ADMA levels were measured in plasma using reverse-phase HPLC with the Waters AccQ-Fluor fluorescent reagent kit. Samples were prepared by mixing 100 μl of plasma with 5 μl of 23.8 μM N-omega-propyl-l-arginine as internal standard (Cayman, Ann Arbor, MI) and 350 μl of borate buffer (pH = 9). This was placed on an unconditioned Waters Oasis MCX column and then washed with 1 ml borate buffer, 3 x 1 ml H2O, and 1 ml methanol. The sample was eluted with 1 ml NH4OH-H2O-methanol (10:40:50), dried under nitrogen gas, and then reconstituted with 50 μl H2O. Recovery was ~85%. Sample (50 μl) was injected on a Luna 150 × 3 mm C18 reversed-phase column (Phenomenex, Torrance, CA). A flow rate of 1.3 ml/min was attained with a PerkinElmer Series 200 Pump, and intensity was measured using a series 200 fluorescent detector with excitation 250 nm and emission 395 nm (PerkinElmer Life and Analytical Sciences, Shelton, CT). Standards contained concentrations of ADMA in the range of 0.156–5 μM. This method was adapted from Heresztyn et al. (14).

Renal DDAH activity was measured by a colorimetric assay measuring the rate of citrulline production, as optimized by us recently (unpublished observation). Briefly, after incubation with urease for 15 min, 100 μl (2 mg) of kidney homogenate were incubated with 1 mM ADMA for 45 min at 37°C. After deproteinization, supernatant was incubated with color mixture at 60°C for 110 min. The absorbance was measured by spectrophotometry at 466 nm. The DDAH activity was represented as micromolar citrulline formation per gram protein per minute at 37°C.

NADPH-dependent superoxide production was measured by electron spin resonance (ESR) spectroscopy with hydroxylamine spin probe 1-hydroxy-3-carboxypropyrolidine (CPH). Membrane samples from kidneys were prepared as described previously (13), and 10 μg of protein were added to 1 mM CPH, 200 μM NADPH, and 0.1 mM diethylenetriaminepentaacetic acid in a total volume of 100 μl of Chelex-treated PBS. In duplicate samples, NADPH was omitted. Samples were placed in 50 μl glass capillaries (Corning, New York, NY). The ESR spectra were recorded using an EMX ESR spectrometer (Bruker) and a super-high-Q microwave cavity. Superoxide formation was assayed as NADPH-dependent, superoxide dismutase-inhibitable formation of 3-carboxypropyrolidine.

Pathology was performed on 5-μm sections of formalin-fixed kidney, blocked in paraffin wax, and stained with periodic acid-Schiff. The level of renal injury was assessed on a blinded basis by determining the sclerotic damage to glomeruli using the 0 to 4+ scale, where 1+ injury involved <25% damage to the glomerulus, 2+ = 25–50% injury, 3+ = 51–75% damage, and 4+ = 76–100% damage. Data were represented as percentage of damaged glomeruli (n = 100), showing any level of injury (scale 1+ to 4+). The total numbers of damaged glomeruli including all levels of injury were also measured and represented as total percentage of damaged glomeruli (n = 100).

Statistical analysis. Results are presented as means ± SE. Parametric data were analyzed by unpaired t-test and one-way ANOVA. Histological (nonparametric) data were analyzed by Mann-Whitney U-test. P < 0.05 was considered statistically significant.

RESULTS

As shown in Table 1, 5/6 NX + Vit E group had higher body weight (similar to shams) vs. 5/6 NX group at 15 wk after renal mass reduction, whereas the body weights were similar between all three groups before surgery (sham: 429 ± 13 g; 5/6 NX: 435 ± 10 g; 5/6 NX + Vit E: 449 ± 5 g). Left kidney weights and the ratio of kidney weight to body weight were higher in 5/6 NX rats than in shams because of compensatory hypertrophy, whereas values were intermediate in 5/6 NX + Vit E group and were not different from sham. Rats with 5/6 NX remained normotensive, and blood pressure was similar in all groups. Plasma creatinine and urine protein excretion were increased similarly, and 24-h creatinine clearance was similarly reduced, in both 5/6 NX groups compared with sham (Table 1). As shown in Fig. 1, however, the appearance of proteinuria was delayed by ~2 wk in the 5/6 NX + Vit E group (week 6) compared with the 5/6 NX rats (week 4). The total number of damaged glomeruli (all levels of injury) was 10 ± 2% in shams, which is normal for this strain and age (8, 25), and greater in both 5/6 NX groups (5/6 NX: 40 ± 3%, P < 0.01; 5/6 NX + Vit E: 24 ± 2%, P < 0.01). The total injury was also greater in the untreated 5/6 NX group vs. the 5/6

Table 1. Measurements at 15 wk following surgery

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>5/6 NX</th>
<th>5/6 NX + Vit E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body wt, g</td>
<td>533 ± 12</td>
<td>492 ± 15</td>
<td>535 ± 8*</td>
</tr>
<tr>
<td>Kidney wt, g</td>
<td>1.50 ± 0.07</td>
<td>2.40 ± 0.14*</td>
<td>2.12 ± 0.43</td>
</tr>
<tr>
<td>Blood pressure, mmHg</td>
<td>91 ± 3</td>
<td>82 ± 8</td>
<td>81 ± 5</td>
</tr>
<tr>
<td>Plasma creatinine, mg/dl</td>
<td>0.38 ± 0.02</td>
<td>2.10 ± 0.62*</td>
<td>1.80 ± 0.41*</td>
</tr>
<tr>
<td>CCC/body wt, ml/min⁻¹·kg</td>
<td>6.5 ± 0.4</td>
<td>1.5 ± 0.3*</td>
<td>1.5 ± 0.2*</td>
</tr>
<tr>
<td>Proteinuria, mg/24 h</td>
<td>38 ± 5.7</td>
<td>72 ± 15*</td>
<td>100 ± 19*</td>
</tr>
</tbody>
</table>

Values are means ± SE. CCC, 24-h clearance of creatinine; NX, nephrectomy; Vit E, vitamin E. P < 0.05 vs. sham (*) and vs. 5/6 NX (†).
NX + Vit E \((P = 0.01)\). As shown in Fig. 2, there were more damaged glomeruli at 1+, 2+, and 4+ levels of severity in the 5/6 NX group vs. sham, whereas only the 2+ injury severity was greater in the 5/6 NX + Vit E group vs. sham. In general, the severity of injury was intermediate in the 5/6 NX + Vit E between shams and 5/6 NX but was significantly less \((P < 0.05)\) compared with 5/6 NX at the 4+ level. The 5/6 NX group also showed increased renal cortex NADPH-dependent superoxide production vs. sham (Fig. 3), which was completely prevented by vitamin E therapy.

As shown in Fig. 4, the 24-h UNOxV fell in all groups with time, and at 14 wk after surgery there was a greater reduction in both the 5/6 NX groups vs. shams, suggesting that CKD contributed to the reduced total NO independent of the age effect. This was supported by the lower plasma NOx in both NX groups (expressed as a ratio factored for creatinine to eliminate an effect of reduced renal clearance) of 0.12 ± 0.02, 5/6 NX & 0.11 ± 0.01, 5/6 NX + Vit E vs. 0.31 ± 0.06 for shams (both \(P = 0.01\) vs. sham).

By Western blot, we found no difference in eNOS abundance in renal cortex and medulla among three groups (Fig. 5A). Further, there was no difference in nNOS abundance in renal medulla; however, renal cortex nNOS abundance was lower in the 5/6 NX group than sham, and this reduction was prevented by vitamin E therapy (Fig. 5B).

Both 5/6 NX groups showed similar increases in plasma ADMA concentration (5/6 NX: 0.35 ± 0.06 and 5/6 NX + Vit E: 0.28 ± 0.05 \(\mu\)M) vs. sham (0.17 ± 0.02 \(\mu\)M, both \(P < 0.05\)). Renal cortex PRMT1 abundance was higher in both 5/6 NX group vs. sham (Fig. 6A). We found no difference in DDAH1 and DDAH2 abundance in renal cortex among the three groups (Fig. 6, B and C), but, in contrast, renal cortex DDAH activity was lower in both 5/6 NX groups than sham (both \(P < 0.05\)) and was not influenced by vitamin E treatment (sham: 0.41 ± 0.01; 5/6 NX: 0.34 ± 0.02; and 5/6 NX + Vit E: 0.36 ± 0.02 \(\mu\)M citrulline formation/g protein \(^{-1}\) min \(^{-1}\)). For the DDAH2 Western blot, many nonspecific bands were detected. In the presence of neutralizing peptide, however, only the DDAH2 band (≈30 kDa) was competed (Fig. 7).
DISCUSSION

The main novel finding in this study is that long-term vitamin E administration prevents the increased NADPH-dependent superoxide generation and reduction in renal cortical nNOS abundance in the 5/6 NX model of CKD. Because both reduced renal nNOS and increased renal superoxide are viewed as pathogenic in progression of CKD, this likely accounts for the protection against structural damage seen with vitamin E supplementation.

This is the first time we have used the 5/6 NX model of CKD. In this model, the right kidney is removed, and the two poles of the left kidney are cut off, leaving a true 1/6 remnant. With 5/6 NX, the rat remains relatively normotensive, whereas in the 5/6 A/I model (which involves infarction of 2/3rds of the left kidney, leaving large amounts of scar tissue) blood pressure increases rapidly and exacerbates the CKD due, at least in part, to greater activity of the renin, angiotensin, aldosterone system (15). In the present study, we found no difference in blood pressure between shams or 5/6 NX groups although we only obtained a terminal blood pressure under anesthesia, which may not reflect the awake values. However, Griffin et al. (11) used telemetry for conscious blood pressure measurement and also reported that rats with 5/6 NX remained normotensive (15 wk). Another factor that could contribute to the lack of hypertension in the 5/6 NX model vs. the 5/6 A/I is that medullary nNOS is unchanged with NX but fell with A/I (25). There is considerable evidence to suggest that loss of medullary NO can lead to salt-sensitive hypertension (26).

The abundance of eNOS in renal cortex was unchanged by 5/6 NX vs. control. In other CKD models, renal eNOS abundance is increased, reduced, and unchanged depending on the primary insult, which suggests that the eNOS varies secondary to the injury (3). In contrast, we observe that the renal cortex nNOS abundance decreased in 5/6 NX-induced CKD as we previously reported, in 5/6 A/I, accelerated 5/6th A/I (high sodium and protein intake), chronic glomerulonephritis, chronic puromycin aminonucleoside-induced nephrosis, diabetic nephropathy, and aging (3). This reinforces our hypothesis that renal cortex nNOS abundance is a primary marker of renal injury (25).

The treatment arm of this study involved antioxidant therapy with vitamin E (α-tocopherol) given in the diet (5,000 IU/kg chow). In previous studies, this dose has been shown to significantly increase plasma α-tocopherol levels in 5/6 NX rats at 6 wk and in the aging rat after 9 mo of treatment (23, 30). We observed that the treatment with vitamin E significantly reduced the degree of kidney structural damage in the 5/6 NX rats. This is in agreement with earlier studies that showed a reduction in glomerulosclerosis with vitamin E therapy in 5/6 NX rats (12, 27). However, there was no improvement in renal function, as assessed by 24-h creatinine clearance, and this also agrees with other observations where vitamin E did not improve renal function in the renal mass reduction models after 2–3 wk (4, 12). Although slightly delayed in appearance, the proteinuria was not reduced by vitamin E despite the concurrent reduction in severity and extent of glomerulosclerosis. This perhaps reflects the relatively mild protective effect of vitamin E at this time point. Nevertheless, prevention of structural damage is of benefit, and long-term (9 mo) high-dose vitamin E supplements were able
to improve structure, decrease proteinuria, and improve function (23). Importantly, this was achieved without increased mortality. It should be noted, however, that a meta-analysis of 136,000 subjects in 19 clinical trials suggested that high-dosage (≥400 IU/day) vitamin E supplements increased all-cause mortality in humans (20), limiting the utility of this therapy in humans.

Our rationale for use of vitamin E therapy was based on its known antioxidant properties, and the protective effects on CKD progression are presumably by decreasing oxidative stress. Vitamin E acts as a scavenger of ROS/reactive nitrogen species and lipid peroxyl radicals, and most studies on CKD have measured lipid peroxidation products or antioxidant enzymes (12, 23, 27, 30). Lipid peroxidation is certainly a marker of redox imbalance because of excess superoxide but could be associated with either decreased or increased NO bioavailability. Measurement of antioxidant enzyme activity/abundance can be misleading since both decreased (presumably primary) and elevated (presumably secondary, compensatory) changes have been correlated to oxidative stress in CKD (22). Because NADPH oxidase is the major source of ROS in kidney (6), we used NADPH-dependent superoxide production as a marker of oxidative stress. A previous study reported that renal NADPH oxidase expression increased in 5/6 NX rats (29), and our data confirm this. The novel finding in the present study is that vitamin E treatment completely prevents this increased kidney cortex NADPH oxidase-dependent superoxide production in 5/6 NX rats. Although conventional wisdom holds that vitamin E exerts its antioxidant effects by scavenging harmful radicals, it also inhibits protein kinase C-dependent events, which include NADPH oxidase assembly (2, 5). As pointed out by Vaziri (28), the best strategy for prevention of oxidative stress is to identify and inhibit the source of the ROS. Thus,
situations where increased NADPH oxidase is the source of damaging ROS, vitamin E is likely to be highly effective. It is also worth pointing out that the deleterious effects of high-dose vitamin E in clinical studies probably relate to its non-radical-scavenging actions (28).

In addition to reducing NADPH oxidase-dependent superoxide production, vitamin E also reverses the decline in renal nNOS abundance. We have repeatedly found that development of renal structural injury in diverse models of CKD is associated with decreases in the renal cortex nNOS abundance (3). Here we again see an association between injury and renal cortex nNOS abundance in yet another model of CKD, reinforcing our earlier suggestion that cortex nNOS abundance is a marker of renal injury (25). Because in this study vitamin E not only reduces renal cortex superoxide production but also preserves nNOS abundance (and presumably activity), vitamin E helps to restore the local balance between NO and superoxide in kidney. We cannot tell whether vitamin E has a direct action on the nNOS or whether this is a consequence of the injury prevention.

We used plasma and urine NOx levels as indexes of total NO production, and vitamin E did not reverse the reduction in total NO production resulting from 5/6 NX. In contrast to vitamin E, tempol increased total NO production in this model (29), which may be because of tempol-induced inactivation of superoxide, which cannot be directly achieved by vitamin E. Despite the lack of vitamin E effect on total NO production, renal cortical nNOS was restored. It is important to note, however, that renal NO production represents just a small fraction of total NO, and we have several times observed dissociation between total and renal NO production in different forms of CKD (7–9). In fact, it is likely that a persistent decline in overall NO generation will occur despite vitamin E therapy since the elevation in plasma ADMA concentration seen in 5/6 NX rats was not prevented by vitamin E.

The lack of reduction of plasma ADMA by vitamin E was unexpected since decreasing ROS should boost DDAH activity and thus lower ADMA (3, 16). Vitamin E was reported to lower ADMA levels in CKD patients (24). However, the renal DDAH enzymes play an important role in ADMA degradation, and in this 5/6 NX model it is possible that such severe reduction in renal mass irreversibly impaired DDAH breakdown. Indeed, our data suggest increased ADMA production (secondary to increased PRMT1 abundance) and decreased ADMA degradation (secondary to decreased DDAH activity), which was not prevented by vitamin E therapy. Matsuguma and colleagues (19) have recently reported increased renal PRMT1 gene expression at 12 wk after 5/6 NX, although they also reported decreased DDAH1 and -2 protein expression. In contrast to our finding that DDAH1 and -2 protein abundance was unaltered in 5/6 NX rats, however, we do observe decreased renal DDAH activity.

In conclusion, long-term vitamin E therapy reduces structural damage in rats subjected to 5/6 NX, and protection is associated with a direct action to inhibit NADPH oxidase-dependent superoxide production. As with other models, renal cortex nNOS abundance was reduced with injury in 5/6 NX rats and was preserved by vitamin E therapy. The renoprotective effect of vitamin E is likely via both reducing superoxide production and preserving renal NO generation. However, vitamin E had no effect on plasma ADMA levels and renal ADMA-related enzymes.

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