Proximal tubule microvilli remodeling and albuminuria in the Ren2 transgenic rat

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Departments of ¹Internal Medicine and ²Medical Pharmacology and Physiology and Divisions of ³Endocrinology and ⁴Nephrology, School of Medicine, and ⁵College of Veterinary Medicine, University of Missouri, and ⁶Harry S. Truman Veterans Affairs Medical Center, Columbia, Missouri; and ⁷Hypertension and Vascular Disease Unit, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, North Carolina

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Hayden MR, Chowdhury NA, Cooper SA, Whaley-Connell A, Habibi J, Witte L, Wiedmeyer C, Manrique CM, Lastra G, Ferrario C, Stump C, Sowers JR. Proximal tubule microvilli remodeling and albuminuria in the Ren2 transgenic rat. Am J Physiol Renal Physiol 292: F861–F867, 2007. First published October 10, 2006; doi:10.1152/ajprenal.00252.2006.—TG(mRen2)27 (Ren2) transgenic rats overexpress the mouse renin gene, with subsequent elevated tissue angiotensin II, hypertension, and nephropathy. The proximal tubule cell (PTC) is responsible for the reabsorption of 5–8 g of glomerular filtered albumin each day. Excess filtered albumin may contribute to PTC damage and tubulointerstitial disease. This investigation examined the role of angiotensin II-induced oxidative stress in PTC structural remodeling: whether such changes could be modified with in vivo treatment with angiotensin type 1 receptor (AT₁R) blockade (valsartan) or superoxide dismutase/catalase mimetic (tempol). Male Ren2 (6–7 wk old) and age-matched Sprague-Dawley rats were treated with valsartan (30 mg/kg), tempol (1 mmol/l), or placebo for 3 wk. Systolic blood pressure, albuminuria, N-acetyl-β-glucosaminidase, and kidney tissue malondialdehyde (MDA) were measured, and ×60,000 transmission electron microscopy images were used to assess PTC microvilli structure. There were significant differences in systolic blood pressure, albuminuria, lipid peroxidation (MDA and nitrotyrosine staining), and PTC structure in Ren2 vs. Sprague-Dawley rats (each P < 0.05). Increased mean diameter of PTC microvilli in the placebreated Ren2 rats (P < 0.05) correlated strongly with albuminuria (r² = 0.83) and moderately with MDA (r² = 0.49), and there was an increase in the ratio of abnormal forms of microvilli in placebo-treated Ren2 rats compared with Sprague-Dawley control rats (P < 0.05). AT₁R blockade, but not tempol treatment, abrogated albuminuria and N-acetyl-β-glucosaminidase; both therapies corrected abnormalities in oxidative stress and PTC microvilli remodeling. These data indicate that PTC structural damage in the Ren2 rat is related to the oxidative stress response to angiotensin II and/or albuminuria.

angiotensin II; malondialdehyde; proximal tubule cell; TG(mRen2)27 transgenic rat

CHRONIC KIDNEY DISEASE is increasing worldwide (15, 27). In the United States, it is estimated that 372,000 persons have end-stage renal disease, and ~11% of adults are in the earlier stages of chronic kidney disease (15). Principal causes of chronic kidney disease leading to end-stage renal disease are type 2 diabetes mellitus and hypertension (HTN) (15, 27, 29, 31). Proteinuria is emerging as a major mediator of progressive renal disease leading to renal interstitial fibrosis and progressing renal injury (15, 29). The concept has been developed that excess protein filtered through the glomerulus exerts direct toxicity on epithelial cells of the kidney proximal tubule (3, 5, 8, 28). Excessive filtered protein, particularly albumin, is thought to exert direct proinflammatory and profibrotic effects on tubular epithelial cells (3, 5, 8, 13, 21, 29) and cause renal interstitial fibrosis (5, 8, 13).

Renal proximal tubular epithelial cells (PTC) are important for the reabsorption of most of the glomerular physiologically filtered albumin (8). Approximately 5–8 g of albumin pass through the glomerular filtration barrier daily, yet only ~30 mg or less appear in the urine (8). Reabsorption of filtrate in the PTC occurs primarily by clathrin- and receptor-mediated endocytosis (3, 5, 8, 13, 28, 35). Microvilli cover the apical portion of the PTC and provide the surface responsible for receptor-mediated endocytosis of albumin. Receptors that mediate this endocytosis include megalin and cubulin. Cytoskeletal remodeling involving actin- and myosin-mediated contraction facilitates the formation of endocytic vesicles (3, 5, 8, 13, 28, 35). Albumin is dissociated from these receptors and transported to lysosomes for degradation to amino acids, which are released through the basolateral membranes and absorbed into interstitial capillaries.

The mechanism by which exposure of excessive albumin leads to PTC injury and the morphological and functional nature of this injury remain poorly understood. Compensatory hypertrophy of PTC in response to renal damage caused by HTN, hypoxia, and metabolic abnormalities appears to be an early process in the progression to tubular atrophy and tubulointerstitial fibrosis (18, 38). This hypertrophic process may be followed by apoptosis and other mechanisms leading to loss of PTC and/or microvilli (18, 20, 37, 38). There is accumulating evidence that the renal renin-angiotensin system (RAS) plays a pivotal role in PTC maladaptive responses to various types of renal injury (4, 9, 16, 17). PTC produce and secrete angiotensin II (ANG II) into the lumen, where its concentration (~6–10 nmol/l) is ~10 times higher than in plasma (17). ANG II (AT₁R and AT₂R) receptors are located on basolateral and luminal sides of the PTC (4, 9, 16). The critical location of ANG II receptors and the high concentration of ANG II suggest an autocrine/paracrine role for the RAS in the PTC. Furthermore, in pathophysiological conditions of albuminuria, the costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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as in type 2 diabetes mellitus or HTN, treatment with blockers of the AT1R reduces the progression of renal disease (15, 27, 29, 43), as well as urine albumin loss (36). However, the precise mechanisms by which ANG II mediates renal tubular damage remain poorly understood.

Excessive albuminuria has been reported to induce ANG II production and increase ANG II uptake by AT1R-mediated process (17). Increased renal ANG II has been reported in systemically ANG II-infused rats and in the transgenic Tg(mRen2)27 (Ren2) rat (19, 22, 41). The Ren2 rat overexpresses ANG II in tissues and develops HTN, insulin resistance, and albuminuria (2, 22, 36). Investigations conducted in this and other hypertensive, insulin-resistant, proteinuric rodent models have shown that ANG II-induced oxidative stress contributes to development of insulin resistance, HTN, albuminuria, and renal injury (2, 22, 36) and that these effects can be abrogated by AT1R blockade and antioxidant therapy (2, 26, 30, 36). Accordingly, in the present investigation, we hypothesized that 1) the Ren2 rat would manifest oxidative stress-mediated PTC injury in relation to albuminuria and 2) treatment of this model of ANG II overexpression in vivo with an AT1R blocker or a cell-permeable SOD/catalase mimetic (1, 32) would attenuate this ANG II-induced oxidative stress-mediated PTC injury.

MATERIALS AND METHODS

Animals and treatments. Male Ren2 rats and Sprague-Dawley (SD) controls (SDC) were obtained at 6 wk of age and fed rat chow throughout the investigation. They were randomly assigned to valsartan-treated (Ren2-V and SDV), tempol-treated (Ren2-T and SDT), and placebo-treated (Ren2-C and SDC) groups. On the basis of previous work (2), the treated animals received valsartan (30 mg·kg⁻¹·day⁻¹) or tempol (1 mmol/l) in drinking water for 21 days before they were killed. All protocols were approved by the University of Missouri and Harry S. Truman Veterans Affairs Medical Center Animal Care and Use Committees and housed/harvested in accordance with National Institutes of Health guidelines.

Determination of body weight, systolic blood pressure, albuminuria, and N-acetyl-β-D-glucosaminidase. Restraint conditioning was initiated on the day of initial systolic blood pressure (SBP) measurement; SBP was measured in triplicate, on separate occasions throughout the day, using the tail-cuff method (Student Oscillometric Recorder, Harvard Systems), on day 19 or 20 of treatment before the animal was killed (2, 36). Body weight was also obtained at these time points.

A commercially available kit (Nephrat, Exocell, Philadelphia, PA) was used to measure urine albumin. Albumin was measured in urine collected over a 24-h period at the end of treatment and normalized to creatinine. Urine creatinine was measured using the Jaffe reaction on an automated chemistry analyzer (model AU400, Olympus America, Dallas, TX). Results are reported as a ratio of urine albumin to creatinine (mg/mg).

N-acetyl-β-D-glucosaminidase (NAG), a 140-kDa lysosomal enzyme, is present in high concentrations in PTC but is not typically excreted into the urine by normal intact PTC (25). Thus increased β-NAG in the urine is a potential marker for PTC injury (25). Urine excretion of β-NAG was determined by colorimetric assay (Roche Diagnostics, Indianapolis, IN). Urine samples were first fractionated on a gel filtration column for removal of NAG inhibitors and then assayed for NAG activity via a colorimetric assay on an automated chemistry analyzer (model AU400, Olympus America). β-NAG excretion is expressed as millinits of NAG activity.

Tissue malondialdehyde. To measure lipid peroxidation, tissue malondialdehyde (MDA) levels were determined as a surrogate end marker for oxidative stress (36). Butylated hydroxytoluene (5 mM) was added to kidney cortical tissue (100 mg) to prevent new lipid peroxidation. Samples were then homogenized in a buffer solution (0.25 M sucrose, 0.5 mM EDTA, 50 mM HEPES, and protease inhibitors) on ice and centrifuged at 4°C at 15,000 rpm for 10 min. The supernatant was collected for MDA and o-phthalaldehyde-derivative protein assays. A spectrophotometric assay kit (model MDA-586, OxisResearch Biotech, Portland, OR) was used to measure free MDA in supernatant (200 μl). Total protein was measured by o-phthalaldehyde-fluorometric assay using a fluorometer (model FLX-800, BioTek, Winooski, VT).

Immunostaining of PTC with nitrotyrosine. Nitrotyrosine staining was used to corroborate levels of lipid peroxidation in the PTC. Sections of kidney were deparaffinized and rehydrated, and epitopes were retrieved in citrate buffer. Endogenous peroxidases were quenched with 3% H2O2, and nonspecific binding sites were blocked with avidin and biotin and, finally, with protein block (Dako, Carpentry, CA). Sections were incubated with a 1:200 dilution of primary rabbit polyclonal anti-nitrotyrosine antibody (catalog no. AB5411, Chemicon) and then washed, incubated with the secondary antibodies, linked, and labeled (streptavidin: LSAB+ kit; catalog no. K0690, Dako) for 30 min each. After several rinses with distilled water, diaminobenzidine (catalog no. K3466, Dako) was applied for 10 min. Sections were again rinsed with distilled water, stained with hematoxylin for 1 min, rehydrated, and mounted with a permanent medium. Slides were evaluated under a bright-field microscope (model 50i, Nikon), and ×40 images were captured with a Cool Snap camera. Images were analyzed and signal intensities were measured with MetaVue (Boye Scientific, Gary Summit, MO).

Transmission electron microscopy methods. Renal cortical tissue from excised kidneys was thinly sliced and placed immediately in primary electron microscopy fixative (2% glutaraldehyde and 2% paraformaldehyde) in 0.1 M sodium cacodylate buffer, pH 7.35. A laboratory microwave oven (model 3440, Pelco) was utilized for secondary fixation, with acetone dehydration and Epon-Spurr resin infiltration. Specimens were placed on a rocker overnight at room temperature, embedded the following morning, and polymerized at 60°C for 24 h. A microtome (Ultracut UCT, Leica) with a 45° diamond knife (Diatome) was used to prepare thin (85-nm) sections. The specimens were stained with 5% uranyl acetate and Sato’s triple-lead stain. A transmission electron microscope (model 1200-EX, JEOL) was utilized to view all renal samples.

Three glomerular/proximal tubule fields were randomly chosen per rat to obtain three ×60,000 images per kidney containing cross-sectional views of the microvilli. PTC were visualized adjacent to glomeruli and identified on the basis of their continuous microvilli at the apical surface. Five (450 × 450 nm) images were cropped out of each ×60,000 image and analyzed using Image J (a public domain Java image-processing program, National Institutes of Health). The microvilli were viewed in cross section in each cropped image, and the diameter of the microvilli was measured and recorded in a blinded fashion (38). Additionally, abnormal forms, as defined by a diameter >100 nm, were counted per field (see Fig. 3). The value of 100 nm was chosen as a value approximately >2 standard deviations above the upper limit of 76.6–86.9 nm of normal proximal tubules observed in control animals.

RNA isolation and reverse transcriptase-real-time polymerase chain reaction. RNA was isolated from renal tissue and measured for angiotensinogen, renin, angiotensin-converting enzyme (ACE), ACE2, nephrilysin, AT1-R, and the mas receptor using the TRIzol reagent (GIBCO Invitrogen, Carlsbad, CA) as previously reported (34).

Statistical analysis. Values are means ± SE. Statistical analyses were performed in SPSS 13.0 (SPSS, Chicago IL). Abnormal forms were evaluated via nonparametric binomial testing, with SDC values as the test parameter and a cut point of 100 as a value approximately >2 standard deviations above the upper limit of 76.6–86.9 nm of normal proximal tubules. All other variables were evaluated paramet-
rically via ANOVA with Fisher’s least squared differences, as appropriate. Data from PTC measurements were then plotted against final SBP, albuminuria, and MDA and then natural log (ln) transformed as appropriate for linear regression analysis. 

$P < 0.05$ was considered statistically significant.

RESULTS

SBP, albuminuria, and $\beta$-NAG. SBP was significantly higher in Ren2-C ($n = 6, 192 \pm 5.8 \text{ mmHg}$) than in SDC ($n = 4, 123 \pm 5.5 \text{ mmHg}$) rats ($P < 0.05$; Table 1) and was significantly lower in Ren2-V rats ($n = 6, 147 \pm 6.1 \text{ mmHg}$, $P < 0.05$) but was not improved in Ren2-T rats ($n = 4, 188 \pm 8.4 \text{ mmHg}$, $P > 0.05$). Consistent with prior observations that the Ren2 rat is insulin resistant, measured glucose levels were higher in the Ren2 than in the SD rat ($P < 0.05$). Similarly, the albumin-to-creatinine ratio (Table 1) was higher in Ren2-C than in SDC rats (0.27 $\pm$ 0.02 vs. 0.06 $\pm$ 0.01 mg/mg, $P < 0.05$) and was decreased with valsartan (0.05 $\pm$ 0.02 mg/mg, $P < 0.05$), but not with tempol (0.31 $\pm$ 0.07 mg/mg, $P > 0.05$). $\beta$-NAG was not significantly elevated in Ren2-C compared with SDC rats. However, significant reductions were seen with valsartan treatment (Table 1).

Oxidative stress. As marker of lipid peroxidation, MDA levels were elevated in Ren2-C compared with SDC kidney cortical tissue (0.56 $\pm$ 0.17 vs. 0.10 $\pm$ 0.01 mg MDA/mg).

Table 1. Experimental parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SDC</th>
<th>SDV</th>
<th>SDT</th>
<th>Ren2-C</th>
<th>Ren2-V</th>
<th>Ren2-T</th>
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<tr>
<td>Weight, g</td>
<td>365.5±4.4</td>
<td>347.5±11.5</td>
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<td>350.8±13.6</td>
<td>388.2±23.7</td>
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<tr>
<td>SBP, mmHg</td>
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<td>117.5±3.2</td>
<td>100.8±3.9</td>
<td>192.5±5.8*</td>
<td>146.8±6.1†</td>
<td>188±8.4</td>
</tr>
<tr>
<td>Albumin/Cr, mg/mg</td>
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<td>0.07±0.02</td>
<td>0.06±0.02</td>
<td>0.27±0.02*</td>
<td>0.05±0.02†</td>
<td>0.31±0.07</td>
</tr>
<tr>
<td>$\beta$-NAG, mU</td>
<td>0.24±0.01</td>
<td>0.16±0.05‡</td>
<td>0.23±0.02</td>
<td>0.24±0.04</td>
<td>0.16±0.05‡</td>
<td>0.28±0.03</td>
</tr>
<tr>
<td>Kidney MD, $\mu$M/mg</td>
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<td>0.19±0.05</td>
<td>0.20±0.01</td>
<td>0.56±0.17*</td>
<td>0.10±0.01†</td>
<td>0.22±0.01†</td>
</tr>
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</table>

*Values are means ± SE. SDC, Sprague-Dawley (SD) control; SDV, valsartan-treated SD; SDT, Tempol-treated SD; Ren2-C, placebo-treated TG(mRen 2)27 (Ren2); Ren2-V, valsartan-treated Ren2; Ren2-T, tempol-treated Ren2; SBP, systolic blood pressure; Alb/Cr, albumin-to-creatinine ratio; $\beta$-NAG, N-acetyl-$\beta$-D-glucosaminidase; MDA, malondialdehyde. $*P < 0.05$ vs. SDC. $†P < 0.05$ vs. Ren2-C. $‡P < 0.05$ vs. respective control (SDC or Ren2-C).

Fig. 1. A: lipid peroxidation in nitrotyrosine-immuno-stained cross sections of proximal tubule cells (PTC). SDC, Sprague-Dawley (SD) control; Ren2-C, TG(mRen 2)27 (Ren2) control; Ren2-V, valsartan-treated Ren2; Ren2-T, tempol-treated Ren2. B: gray-scale measures of intensity of PTC images in A. $*P < 0.05$ vs. SDC. $**P < 0.05$ vs. Ren2-C.
protein, \( P < 0.05 \) and decreased in Ren2-V and Ren2-T tissue (0.10 ± 0.01 and 0.22 ± 0.12 \( \mu \)g MDA/mg protein, respectively, \( P < 0.05 \); Table 1). Similar to MDA, there were significant increases in gray-scale intensity measures of nitrotyrosine, another marker of lipid peroxidation, on PTC cross sections in Ren2-C compared with SDC tissue (53.2 ± 5.6 vs. 20.6 ± 3.6 arbitrary units, \( P < 0.05 \)), which were decreased in Ren2-V and Ren2-T tissue (22.2 ± 1.8 and 32.4 ± 5 arbitrary units, respectively, each \( P < 0.05 \); Fig. 1).

**Ultrastructural analysis.** The diameters of PTC microvilli were measured in cross section and compared between treatment groups (Figs. 2 and 3). The Ren2-C group exhibited a significantly increased mean microvilli diameter (86.94 ± 0.25 nm; Fig. 3A) associated with albuminuria (\( r^2 = 0.83 \)) and levels of MDA (\( r^2 = 0.49 \); Fig. 3B) compared with the SDC group (76.58 ± 0.19 nm, \( P < 0.05 \)). Treatment with valsartan (Ren2-V) and tempol (Ren2-T) reduced the mean diameter (75.85 ± 0.14 and 80.57 ± 0.22 nm, respectively, \( P < 0.05 \)).
Even with the exclusion of abnormal forms from diameter analyses, the relations among the groups remained significant (data not shown). Thus the presence of abnormal forms alone did not account for the diameter increase in the Ren2-C animals. The number of abnormal forms as a ratio of abnormal forms to total number of microvilli was increased in the Ren2-C compared with the SDC group (0.14 ± 0.01 vs. 0.03 ± 0.01, \( P < 0.05 \); Fig. 4B). The Ren2-V and Ren2-T groups demonstrated a significant reduction in the number of abnormal forms (0.03 ± 0.002 and 0.05 ± 0.01, respectively, \( P < 0.05 \)).

**Tissue mRNA for the RAS.** There were expected increases in mRNA transcripts for AT1R (1.79 ± 0.20) and mas receptor (2.43 ± 0.04) in Ren2-C compared with SDC rats (\( P < 0.05 \)) and decreases with AT1R blockade (1.28 ± 0.18 and 1.38 ± 0.13, respectively; \( P < 0.05 \)) and also tempol treatment (mas receptor: 1.87 ± 0.16; \( P < 0.05 \)). Furthermore, there were significant improvements with AT1R blockade in the Ren2-C mRNA transcripts of ACE2 (1.80 ± 0.20), renin (5.41 ± 1.10), angiotensinogen (1.43 ± 0.11), and nephrilysin (1.61 ± 0.07, each \( P < 0.05 \)). Similar improvements were seen with tempol treatment in ACE2 (1.97 ± 0.18), nephrilysin (1.81 ± 0.22), and angiotensinogen (1.57 ± 0.1) mRNA transcripts (each \( P < 0.05 \)).

**DISCUSSION**

This investigation demonstrated that the Ren2 rat, which manifests enhanced tissue RAS (2, 22, 36, 41), develops renal tubular structural abnormalities in conjunction with development of HTN and albuminuria. Transmission electron microscopy revealed substantial microvilli abnormalities in the proximal tubules of the Ren2 rat compared with age-matched SD littermates. These abnormalities were generally characterized by increased mean diameter and increased numbers of abnormal microvilli structures. Furthermore, the extent of the abnormalities of the Ren2 microvilli was directly related to quantitative increases in urinary albumin. These observations are consistent with prior reports that albuminuria may cause PTC injury (3, 5, 8, 13, 28).

Our understanding of PTC damage in response to albuminuria or other insults (i.e., hypoxia and metabolic abnormalities) is very limited. However, the transmission electron microscopy abnormalities in the Ren2 rat are potentially consistent with loss of the cytoskeletal integrity necessary for maintenance of erect tubules (20, 37, 38). Indeed, it has been previously observed that actin and myosin microfilaments undergo depolymerization, fragmentation, and basolateral retraction from the apical region of the microvilli in ischemia and HTN injury.
(20, 37, 39). This loss of cytoskeletal integrity likely contributes to flaccid/folded microvilli. This alteration, in conjunction with apoptosis/necrosis, could account for the loss of microvilli and widening of remaining microvillar structures observed in this study. Finally, the observations that oxidative stress increased in the Ren2 rats, as measured by MDA and confirmed with staining of nitrotyrosine in the PTC, and that in vivo treatment with valsartan and tempol abrogated the microvilli structural abnormalities may suggest a role for oxidative stress as a common converging process mediating PTC injury in response to ANG II and albumin excess in the Ren2 rat.

As previously observed in skeletal (2) and cardiovascular (34) tissue, there were increased oxidative changes (MDA) in the renal cortical tissue of the Ren2 rat. HTN (22, 41), insulin resistance (2, 12, 34), and albuminuria (36) in this rodent model (2, 12, 34) are related to excess tissue ANG II signaling through the AT1R to increase oxidative stress. The increases in lipid peroxidation, cortical tissue MDA levels, and, more specifically, nitrotyrosine staining of the PTC in the Ren2 rats suggest a role for oxidative stress in the PTC remodeling. Our finding that AT1R blockade and tempol treatment decreased renal lipid oxidation, as well as PTC microvilli structural abnormalities, provides evidence for ANG II/albmin-induced oxidative stress as a convergent pathway in the pathogenesis of PTC injury in this rodent model of tissue RAS overexpression. Furthermore, it is also important to consider the role of ACE2 and nephrilysin activity in opposing the effects of ANG II stimulation of the AT1R (7, 10, 36). We previously observed that valsartan increased expression of ACE2 and nephrilysin in renal cortical tissue of Ren2 animals (36). Here we report similar effects after in vivo treatment with tempol as well. Collectively, these observations suggest that diminishing oxidative stress improves relative ACE2 signaling in renal cortical tissue.

ANG II generates superoxide anion (O2\textsuperscript{-}) and H2O2 in cardiovascular tissue (34) and the kidney (14, 26, 36). At normal physiological concentrations, O2\textsuperscript{-} and H2O2 can act as second messengers in ANG II-mediated signaling pathways, but in excess they mediate inflammation and cellular dysfunction (33). The tubular structural alterations were related to levels of MDA and albuminuria, and damage was ameliorated by 3 wk of tempol or valsartan treatment. Tempol, an SOD/catalase mimetic, attenuates development of HTN and vascular injury via scavenging of reactive oxygen species (ROS), O2\textsuperscript{-} and H2O2 (1, 30, 32). Indeed, previous in vivo treatment with tempol has been shown to normalize vascular superoxide levels, blood pressure, and renal inflammatory responses in ANG II-infused hypertensive rats (1, 24). Although valsartan treatment abrogated HTN, albuminuria, and PTC injury, tempol exerted its protective effects on PTC, even though it did not significantly lower SBP or albuminuria. ANG II has previously been shown to selectively increase O2\textsuperscript{-} and H2O2 in PTC via NADPH oxidase activation, independent of mitochondrial O2\textsuperscript{2} production (6, 11). In PTC, the flavoprotein inhibitor diphenyleine iodium, as well as the antioxidant N-acetylcysteine, prevented membrane-bound NADPH oxidase activation and generation of ROS (6, 11). These data collectively suggest that an increase in ROS is the convergent pathway by which increased ANG II and/or albuminuria causes PTC injury.

One limitation in the interpretation of these results is the lack of increase in β-NAG in the Ren2 rat, which has recently been reported as an indicator of tubular injury. However, results from other studies have been inconclusive with regard to the contribution of β-NAG to proximal tubule injury, and the most promising studies have been in the area of diabetic nephropathy. Furthermore, the finding that β-NAG was not increased in the Ren2 rat, despite the structural changes, may reflect its relative inability to detect early tubular injury. Another possible limitation is drawing a conclusion regarding the correlation between oxidative stress (as measured by MDA) and PTC injury with an r\textsuperscript{2} of 0.49. Although the association is not as strong as the correlation with albuminuria (r\textsuperscript{2} = 0.85), there is a modest association between the level of MDA and PTC structural changes. Furthermore, our staining of the PTC with nitrotyrosine, which is specific for oxidation, does support our conclusions. However, these associations are best corroborated with in vitro studies of the impact of ROS generation on structural and functional alterations in PTC in culture (23) (40, 42).

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