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Abstract—Activation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase by angiotensin II is integral to the formation of oxidative stress in the vasculature and the kidney. 3-Hydroxy-3-methylglutaryl-coenzyme A reductase inhibition is associated with reductions of oxidative stress in the vasculature and kidney and associated decreases in albuminuria. Effects of 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibition on oxidative stress in the kidney and filtration barrier integrity are poorly understood. To investigate, we used transgenic TG(mRen2)27 (Ren2) rats, which harbor the mouse renin transgene and renin-angiotensin system activation, and an immortalized murine podocyte cell line. We treated young, male Ren2 and Sprague-Dawley rats with rosuvastatin (20 mg/kg IP) or placebo for 21 days. Compared with controls, we observed increases in systolic blood pressure, albuminuria, renal NADPH oxidase activity, and 3-nitrotryosine staining, with reductions in the rosuvastatin-treated Ren2. Structural changes on light and transmission electron microscopy, consistent with periarteriolar fibrosis and podocyte foot-process effacement, were attenuated with statin treatment. Nephrin expression was diminished in the Ren2 kidney and trended to normalize with statin treatment. Angiotensin II–dependent increases in podocyte NADPH oxidase activity and subunit expression (NOX2, NOX4, Rac, and p22phox) and reactive oxygen species generation were decreased after in vitro statin treatment. These data support a role for increased NADPH oxidase activity and subunit expression with resultant reactive oxygen species formation in the kidney and podocyte. Furthermore, statin attenuation of NADPH oxidase activation and reactive oxygen species formation in the kidney/podocyte seems to play roles in the abrogation of oxidative stress-induced filtration barrier injury and consequent albuminuria. (*Hypertension*. 2008;51[part 2]:474-480.)

Key Words: angiotensin II ■ albuminuria ■ glomerular filtration barrier ■ transgenic Ren2 rat ■ rosuvastatin

Renin-angiotensin system (RAS) activation and subsequently elevated angiotensin II (Ang II) exert the pressor, proliferative, profibrotic, and proinflammatory actions.[1–3] Activation of tissue reduced nicotinamide-adenine dinucleotide phosphate (NADPH) oxidase seems to contribute to deleterious actions, such as oxidative stress and endothelial dysfunction manifesting as hypertension, albuminuria, and progressive glomerular dysfunction, that may ultimately lead to chronic kidney disease.[1,4] There is accumulating evidence that tissue-based RAS further modulates cell growth, metabolism, and tissue remodeling.[5,6]

Evidence for a local RAS in the glomerulus raises the prospect of NADPH oxidase–induced podocyte and filtration barrier injury.[7,8] Furthermore, in vitro protein exposure, mechanical stretch, and glomerular hypertension enhance tissue Ang II production, which may potentiate the impact of elevated blood pressure on glomerular injury manifesting as albuminuria.[9,10] Previous work related to the pathogenesis of albuminuria delineated abnormalities such as basement membrane thickening, loss of the slit-pore diaphragm integrity, and widening of the podocyte foot process base width.[11] Recent evidence characterized foot process effacement and loss of the slit-pore diaphragm as critical in decreasing filtration barrier integrity.[12]

3-Hydroxy-3-methylglutaryl (HMG)-coenzyme A (CoA) reductase inhibitors (statins) exert beneficial actions on oxidative stress and endothelial dysfunction independent of their cholesterol-lowering properties. Many of the actions of statins are thought to be mediated by decreasing reactive oxygen species (ROS) formation in various tissues.[13] ROS derived from NADPH oxidase activation have been shown to play a critical role in hypertrophy, fibrosis, and remodeling in the heart and vasculature.[14–16] NADPH oxidase is a multicomponent enzyme complex that is composed of the

membrane-bound heterodimer gp91[phox] (phox indicates phagocytic oxidase; NOX2) and its homologue NOX4; p22[phox]; the cytosolic regulatory subunits p40[phox], p47[phox], and p67[phox]; and the small GTP-binding protein, Rac1.[14,15] Statins reduce cellular levels of isoprenoids and inhibit the subsequent isoprenylation of small G proteins such as Rac.[17,18]

Accordingly, we evaluated the impact of statin treatment on increases in NADPH oxidase activity and contemporaneously on the glomerular filtration barrier and podocyte integrity. We used the transgenic TG(mRen2)27 (Ren2) rat with the mouse renin transgene and RAS activation, as well as an immortalized murine podocyte cell line. We hypothesized that Ang II stimulation of NADPH oxidase would contribute to glomerular filtration barrier remodeling and the podocyte generation of ROS. We further hypothesized that the beneficial effects of statin therapy would be mediated in part through reductions in NADPH oxidase activation and generation of ROS.

Materials and Methods
Animals and Treatments
All of the animal procedures were approved by the institutional animal care and use committees at the University of Missouri Harry S. Truman Veterans' Affairs Medical Center and housed in accordance with National Institutes of Health guidelines. Ren2 (5- to 6-week–old) and age-matched Sprague-Dawley (SD) male rats were randomly assigned to placebo-treated control (Ren2-C and SD-C, respectively; n=6 each) or rosuvastatin (AstraZeneca) treatment groups (Ren2-RSV and SD-RSV, respectively; n=4 each). Rosuvastatin (20 mg/kg per day) in saline or an equal volume of saline was administered intraperitoneally to the rosuvastatin or control groups, respectively, for 21 days.

Systolic Blood Pressure and Albuminuria
Details of systolic blood pressure (SBP) and albuminuria are available in a data supplement available online at http://hyper.ahajournals.org. Briefly, SBP was determined at the end of rosuvastatin treatment on days 19 or 20 using the tail cuff after acclimatization and restraint conditioning for 48 hours on each group of rats (SDC, n=6; SD-RSV, n=4; Ren2-C, n=6; Ren2-RSV, n=4).[12–16] In addition, albuminuria from each group of rats (SDC, n=6; SD-RSV, n=4; Ren2-C, N=6; Ren2-RSV, n=4) was determined at the beginning, middle, and end of treatment.[12]

Transmission Electron Microscopy Methods
Details of the transmission electron microscopy (TEM) in this study are available in the data supplement. Briefly, kidney cortical tissue from each group of rats (SDC, n=6; SD-RSV, n=4; Ren2-C, n=6; Ren2-RSV, n=4) was prepared as described previously,[12,14,19] and 3 glomeruli per rat were evaluated with five 10-k and 60-k images per glomeruli. Five measurements were performed for each variable per image, including basement membrane thickness, slit pore diameter and number per 100 μm, and foot-process base width.

Immunofluorescent Studies
Details of the immunofluorescent studies are available in the data supplement. Briefly, harvested kidney cortical tissue from each group of rats (SDC, n=4; SD-RSV, n=4; Ren2-C, n=4; Ren2-RSV, n=4) was prepared[13–16] and evaluated for nephrin C-17, gp91[phox] (NOX2), and Rac1 antibody, as well as to assess 3-nitrotyrosine content, a marker for peroxynitrite formation.[13–15]

Western Blot Analysis
Details of the Western blot analysis are available in the data supplement. Briefly, kidney cortical tissue from each group of rats

(SDC, n=4; SD-RSV, n=4; Ren2-C, n=4; Ren2-RSV, n=4) was used to evaluate nephrin with 2 antibodies (N-20 and C-17); total protein was stained with amido-black staining to normalize the data.

Measurement of NADPH Oxidase Activity
NADPH oxidase activity was determined in plasma membrane fractions from each group of rats (SDC, n=6; SD-RSV, n=4; Ren2-C, n=6; Ren2-RSV, n=4) and podocyte cell lysates.[15–17]

Light Microscopy
Harvested kidney cortical tissue from each group of rats (SDC, n=4; SD-RSV, n=4; Ren2-C, n=4; Ren2-RSV, n=4) were stained with Verhoeff-van Gieson, which is specific for fibrosis and stains elastin (black), nuclei (blue black), collagen (red), and connective tissue (yellow).[14,15,19]

Podocyte Cell Culture
Details of cell culture are available in the data supplement. Briefly, a dose-response curve was established for Ang II stimulation of NADPH oxidase activity in podocyte cell lysates using a time-dependency assay, and rosuvastatin inhibition was based on previous experiments.[17] In addition, superoxide generation in podocyte cell lysates was evaluated using oxidative fluorescent dihydroethidium staining.[17]

RNA Extraction and Subunit Expression by Real-Time PCR
Details of the determination of RNA extraction from podocyte cell lysates are available in the data supplement. RNA was extracted from podocytes using RNeasy Mini kits (Qiagen), and 100 ng of starting cDNA was used for Real-Time PCR with the following primers: p22[phox] and NOX4, gp91[phox] (NOX2; 5′-CCA ACT GGG ATA ACG AGT trichloroacetic acid (TCA)-3′) and reverse (5′-GAG AGT TTC AGC CAA GGC TTC-3′) and Rac1.[20] Expression levels were normalized using 18S as the control.

Statistical Analysis
All of the values are expressed as mean±SE. Statistical analyses were performed in SPSS 13.0 (SPSS Inc) using ANOVA with Fisher's least significant difference as appropriate and Student's *t* test for paired analysis. Significance was accepted as $P<0.05$.

Results
Effect of Rosuvastatin on SBP and Albuminuria
As measured at the end of the treatment period, there were increases in SBP in Ren2 (207.4±1.3 mm Hg) when compared with SD controls (156.0±9.8 mm Hg; $P<0.05$) without reductions in rosuvastatin-treated animals (202±4.5 mm Hg and 159.7±9.7 mm Hg, respectively; Figure 1A). Albuminuria was increased in the Ren2 rats at 5 weeks (0.15±0.05 mg/mg), 7 to 8 weeks (0.33±0.05 mg/mg), and significantly at 9 weeks (0.47±0.08 mg/mg; $P<0.05$) when compared with SD controls at 5 weeks (0.12±0.02 mg/mg), 7 to 8 weeks (0.14±0.04 mg/mg), and 9 weeks (0.11±0.01 mg/mg; Figure 1B). There were improvements in the rosuvastatin-treated Ren2 rats at 5 weeks (0.17±0.03 mg/mg), 7 to 8 weeks (0.18±0.04 mg/mg), and significantly at 9 weeks (0.26±0.04 mg/mg; $P<0.05$).

### Effect of Rosuvastatin on Glomerular Remodeling
Glomerular filtration barrier structural integrity was measured by TEM and light microscopy with Verhoeff-van Gieson and nephrin immunostaining. TEM images at 10 000 and 60 000 (Figure 2A) were used to evaluate 4 criteria for
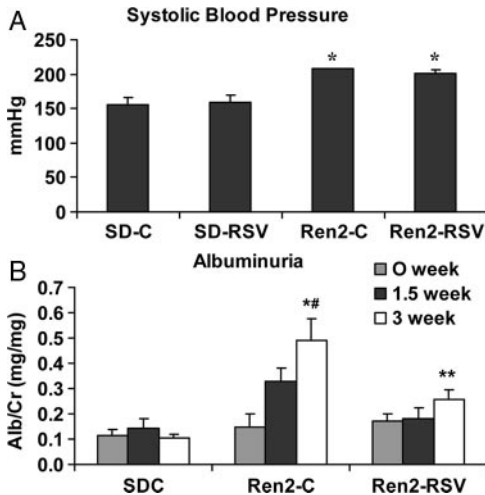
Figure 1. SBP and albuminuria in transgenic Ren2 rats. A, SBP in Ren2 rats at the end of treatment period. B, Albuminuria in the Ren2 rat as measured at the beginning of treatment (6 weeks of age or 0 weeks of treatment), middle of treatment period (7 to 8 weeks of age or 1.5 weeks of treatment), and end of treatment period (9 weeks of age or 3 weeks of treatment). *$P<0.05$ vs age-matched SD-C (n=6); #$P<0.05$ when Ren2-C (n=6) 3 week is compared with 0 weeks; **$P<0.05$ when Ren2-RSVs (n=4) are compared with age-matched SD-C rats.

filtration barrier integrity as described above. Significant changes in all 4 of the variables were observed when comparing Ren2 to SD glomeruli, and these changes were attenuated with rosuvastatin treatment (Figure 2B).

There were fewer slit-pores in Ren2 rats (4.3 ± 0.2 slit pores/100 μm; $P<0.05$) versus SD control rats (5.0 ± 0.1 slit pores/100 μm), improved with rosuvastatin treatment in Ren2 and SD rats (5.1 ± 0.1 and 5.8 ± 0.1 slit pores per 100 μm, respectively; each $P<0.05$; Figure 2B). Similarly, slit-pore diameter was less in Ren2 glomeruli (25.0 ± 1.5 nm) than in SD (34.3 ± 1.3 nm; $P<0.05$). However, rosuvastatin treatment did not improve slit-pore diameter in the Ren2 or SD rats (23.9 ± 1.9 nm and 29.3 ± 2.5 nm, respectively; each $P>0.05$). Increases in podocyte foot process base width paralleled the loss of the slit-pore number and diameter in the Ren2 rats (208.2 ± 9.7 nm) when compared with SD controls (159.6 ± 5.5 nm) that improved with rosuvastatin treatment in both Ren2 and SD rats (140.6 ± 4.9 nm and 131.8 ± 5.7 nm, respectively; each $P<0.05$). Interestingly, basement membrane thickness was not greater in the Ren2 (98.7 ± 1.9 nm) when compared with SD (95.3 ± 2.6 nm) glomeruli ($P>0.05$), with no rosuvastatin treatment effect in either Ren2 or SD rats (102.6 ± 2.9 nm and 99.9 ± 5.9 nm; each $P>0.05$).
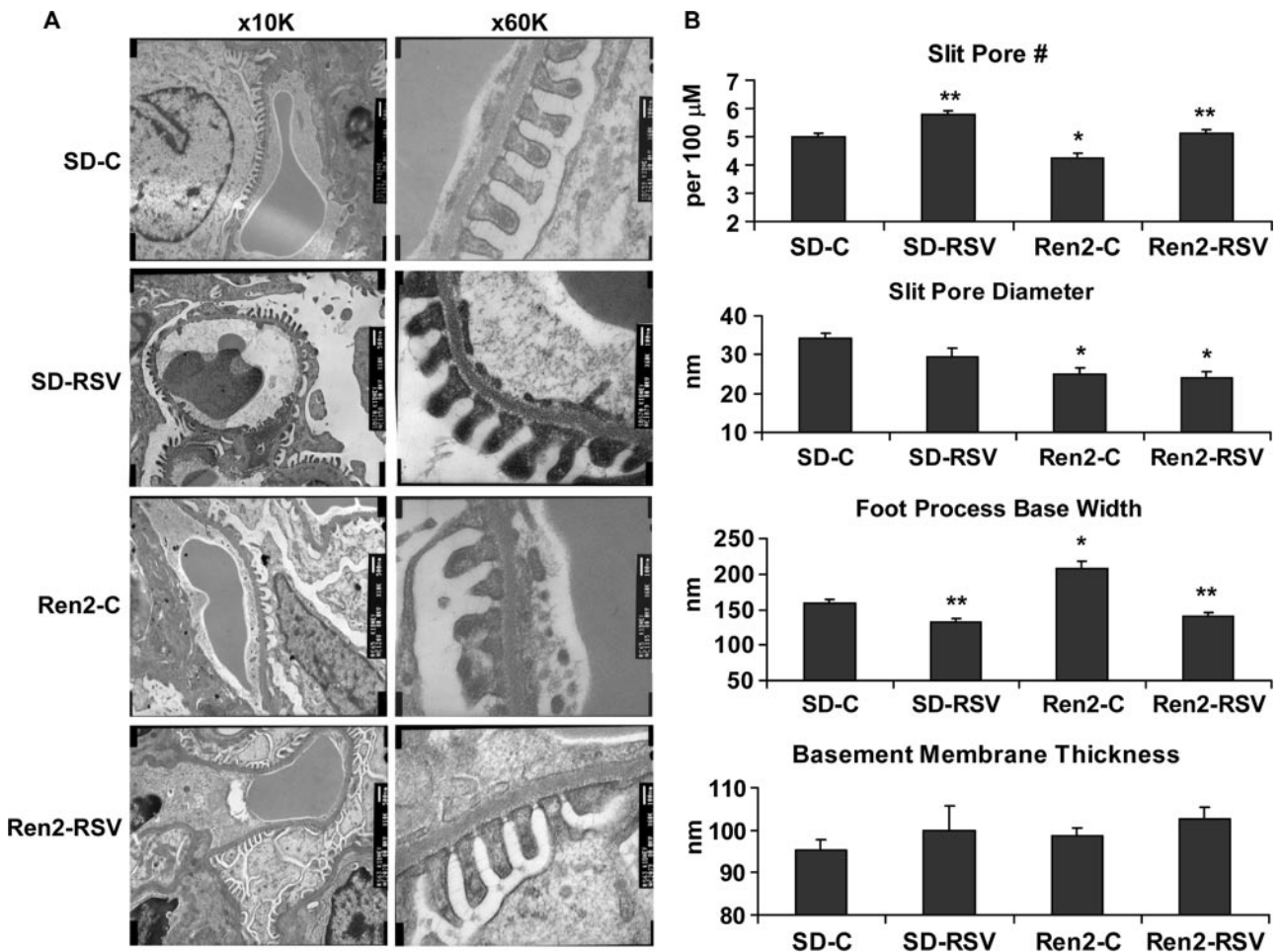
Figure 2. Rosuvastatin improves indices of podocyte foot-process effacement on TEM. A, Representative TEM images at ×10 000 (left panel) and ×60 000 (right panel). B, Indices for glomerular filtration barrier integrity. *$P<0.05$ when Ren2-Cs (n=6) are compared with age-matched SD-Cs (n=6); **$P<0.05$ when Ren2-RSVs (n=4) or SD-RSVs (n=4) are compared with age-matched controls.
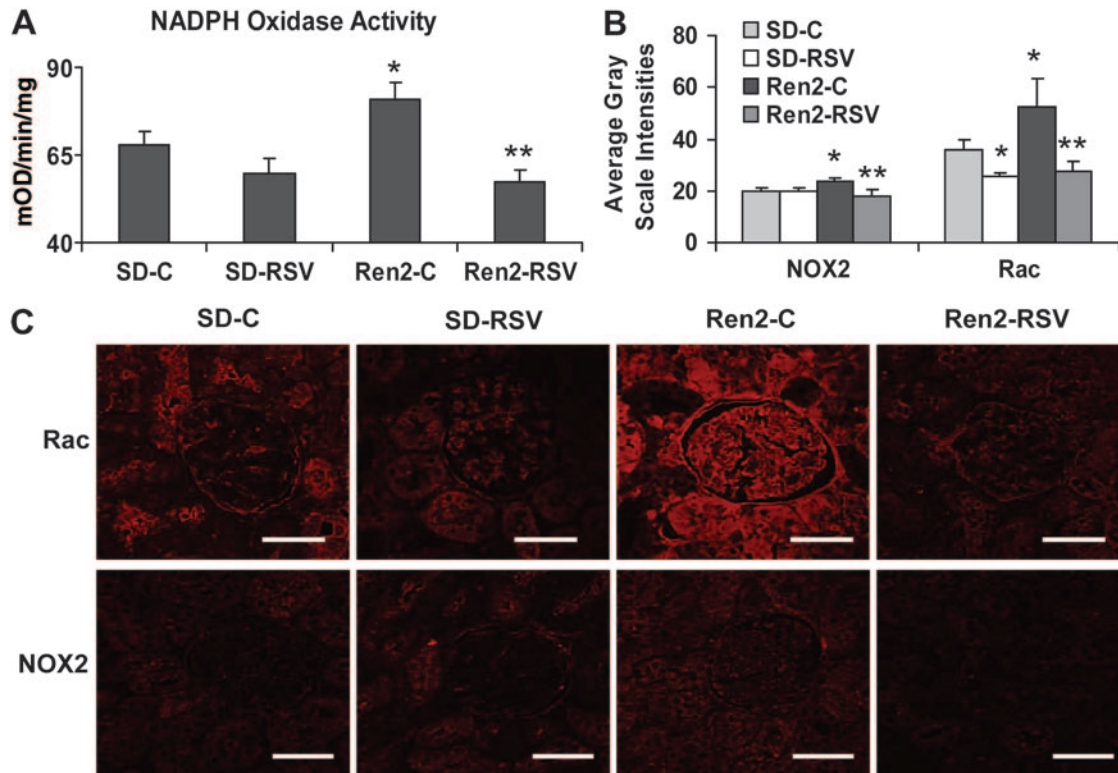
Figure 3. Rosuvastatin attenuation of NADPH oxidase in the transgenic Ren2 rat. A, Kidney cortical tissue NADPH oxidase activity in the Ren2 rat. B, Grayscale intensity measures of NADPH oxidase subunit expression for C. C, Representative images of glomerular sections immunostained for NADPH oxidase subunits NOX2 and Rac. (scale bar=50 μmol/L). *$P<0.05$ when Ren2-Cs (n=6) are compared with age-matched SD-Cs (n=6); **$P<0.05$ when Ren2-RSVs (n=4) or SD-RSVs (n=4) are compared with age-matched controls.

There were attendant decreases in nephrin by immunostaining and Western analysis in the Ren2 rats (19.5 ± 1.6 average grayscale intensities; 0.24 ± 0.07 arbitrary units) when compared with SD controls (27.9 ± 4.5 average grayscale intensities; 0.431 ± 0.090 arbitrary units; each $P<0.05$) with a trend to improvement in the rosuvastatin-treated Ren2 rats (21.7 ± 3.6 average grayscale intensities and 0.32 ± 0.04; each $P>0.05$; Figure S1A through S1E).

To evaluate the effects of rosuvastatin on glomerular remodeling, we morphometrically evaluated periarteriolar fibrosis in Verhoeff-van Gieson–stained sections of the kidney. There was substantial periarteriolar fibrosis in the adventitia of Ren2 rats ($0.65\pm0.01\%$ area fibrosis) when compared with SD controls ($0.39\pm0.01\%$ area fibrosis; $P<0.05$) that was improved in the rosuvastatin-treated Ren2 rats ($0.41\pm0.01\%$ area fibrosis; $P<0.05$; Figure S2A and S2B). Interestingly, there were decreases in the percent area in the media of the Ren2 rats ($0.28\pm0.01\%$ area) compared with SD controls ($0.45\pm0.01\%$ area; $P<0.05$), which improved in the rosuvastatin-treated Ren2 rats ($0.47\pm0.01\%$ area; $P<0.05$).

Effect of Ang II and Rosuvastatin on NADPH Oxidase Activity and Subunits

There were expected increases in NADPH oxidase activity in the Ren2 controls (80.9 ± 0.1 mOD/mg per minute) when compared with SD controls (67.9 ± 0.2 mOD/mg per minute; $P<0.05$) that improved with rosuvastatin treatment in the Ren2 rats (57.1 ± 0.1 mOD/mg per minute; $P<0.05$; Figure

3A). Similarly, there were dose-dependent increases in Ang II stimulation of NADPH oxidase activity in podocytes with a maximum occurring at 10 nm (Figure 4A). The increase in NADPH oxidase activity induced with 10 nM of Ang II (1.15 ± 0.03 mOD/mg per minute; $P<0.05$) was completely prevented with rosuvastatin treatment (0.94 ± 0.04 mOD/mg per minute; $P<0.05$; Figure 4B).

Similar to enzyme activity, there were increases in NADPH oxidase subunits in the kidney cortex of the Ren2 rats and in podocytes. There were increases in NOX2 and Rac in the Ren2 rats (23.5 ± 1.4 and 54.9 ± 10.9 average grayscale intensities, respectively; each $P<0.05$) compared with SD controls (19.6 ± 1.5 and 31.7 ± 3.6 average grayscale intensities, respectively; each $P<0.05$) improved with rosuvastatin treatment (18.1 ± 2.1 and 27.2 ± 4.0 average grayscale intensities, respectively; each $P<0.05$; Figure 3B and 3C). Similarly, increases were seen in podocytes with Ang II stimulation of NOX2 and Rac mRNA expression (3.75 ± 1.30 and 3.34 ± 0.76 average grayscale intensities, respectively; each $P<0.05$) relative to controls that were reversed with rosuvastatin (0.71 ± 0.08 and 1.16 ± 0.30 average grayscale intensities, respectively; Figure 4D). There were similar increases in mRNA expression of NOX4 and p22 in podocytes after Ang II stimulation (3.09 ± 0.61 and 2.79 ± 0.68 average grayscale intensities relative to control, respectively; each $P<0.05$) reversed with rosuvastatin treatment (1.17 ± 0.30 and 0.99 ± 0.23 average grayscale intensities, respectively; each $P<0.05$; Figure 4D).
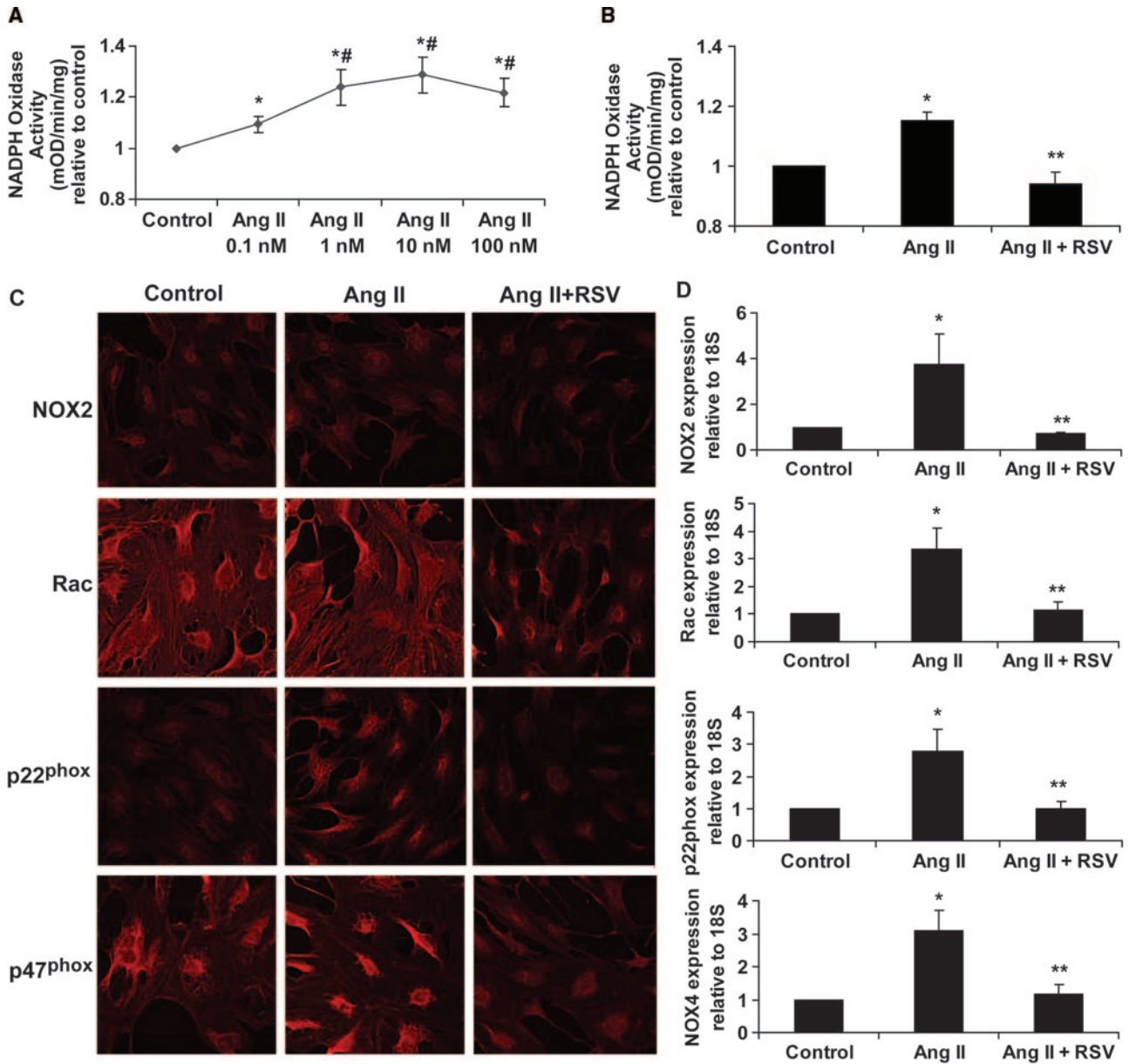
Figure 4. Rosuvastatin attenuation of Ang II stimulation of NADPH oxidase in podocytes. A, Dose response for Ang II stimulation of NADPH oxidase activity (n=6). B, Rosuvastatin reversal of Ang II stimulation of NADPH oxidase activity (n=7). C, Representative immunostaining of NADPH oxidase subunits in podocyte cell culture. D, mRNA expression of NADPH oxidase subunits in podocyte cell culture. *$P<0.05$ when Ang II was compared with control; for NOX4, Rac1 and p22[phox] (n=14) and NOX2 (n=12). **$P<0.05$ when compared with Ang II stimulation alone; each (n=8).

Effect of Ang II and Rosuvastatin on Oxidative Stress Markers

To ascertain whether rosuvastatin affects oxidative stress, we measured 3-nitrotyrosine as a marker of peroxynitrite formation in the Ren2 and dihydroethidium in podocytes as a marker of superoxide anion generation. Kidney tissue nitrotyrosine content was increased in the Ren2 (26.3 ± 1.7 average grayscale intensities) when compared with SD rats (12.9 ± 1.4 average grayscale intensities; $P<0.05$) reversed with rosuvastatin treatment in the Ren2 rats (15.4 ± 1.1 average grayscale intensities; $P<0.05$; Figure S3A and S3B). Similarly, there were increases in superoxide anion generation after Ang II stimulation (24.5 ± 4.6 average grayscale intensities; $P<0.05$) that were reversed with rosuvastatin

(35.4 ± 4.8 average grayscale intensities; $P<0.05$; Figure S3C and S3D).

Discussion

This investigation addressed the impact of HMG-CoA reductase inhibitors (statins) on NADPH oxidase activation and subunit expression, oxidative stress, and resultant glomerular filtration barrier and podocyte injury. Filtration barrier/podocyte integrity was evaluated in the transgenic Ren2 rat, an animal model with the mouse renin transgene and RAS activation, shown previously to demonstrate enhanced renal tissue oxidative stress and albuminuria.[12,19] Data from the current investigation indicate that statin therapy has renoprotective effects, as demonstrated by reductions in albuminuria,

oxidative stress, and improvements in filtration barrier indices, as well as basement membrane thickening and podocyte foot process effacement. Findings from this study are consistent with our previous findings in the heart and other reports that statins decrease NADPH oxidase–related ROS species generation through inhibition of cytosolic small-molecular-weight G proteins, such as Rac, and the membrane subunits of NADPH oxidase.[14,18,21]

The filtration barrier is composed of endothelium, basement membrane, and visceral epithelial cells called podocytes. Increasing evidence supports podocyte regulation of various glomerular functions, including basement membrane turnover, maintenance of the filtration barrier, regulation of the ultrafiltration coefficient, and mechanical support of the glomerular tuft.[22] Intraglomerular capillaries are continuously exposed to elevated hydrostatic pressure gradients and are susceptible to the effects of NADPH oxidase activation and oxidative stress.[21,23] Thus, the findings of periarteriolar and glomerular fibrosis, in addition to podocyte foot-process effacement, independent of changes in the basement membrane, in an animal model of NADPH oxidase activation support a role for ROS-mediated podocyte injury and albuminuria. Furthermore, the reductions in fibrosis and foot-process effacement with in vivo statin therapy suggest a role for statin reductions in NADPH oxidase and ROS. This supports the notion that statins may exert a renoprotective effect in maintaining the integrity of the podocyte and filtration barrier.

In the present study, the Ren2 rat demonstrated increases in NADPH oxidase activity, subunit expression, and oxidative stress in conjunction with podocyte remodeling/effacement and loss of the slit-pore diaphragm integrity. NADPH oxidase activation was accompanied by downregulation of nephrin, an important protein necessary for maintenance of podocyte slit-pore diaphragm integrity.[22–24] The loss of nephrin, in turn, contributed to effacement of podocytes and loss of integrity of the slit-pore diaphragm, both requisites for progression of albuminuria.[23–25] Indeed, these changes occurred in the absence of basement membrane thickening, suggesting the paramount importance of changes in slit-pore integrity in the pathogenesis of albuminuria.

In parallel with increased NADPH oxidase activity, there was an increase in peroxynitrite formation, as measured by nitrotyrosine staining in Ren2 kidneys, significantly reduced by statin therapy. NADPH oxidase catalyzes the 1-electron reduction of molecular oxygen to superoxide anion, which can react to form short-lived peroxynitrite. Peroxynitrite then forms stable 3-nitrotyrosine–conjugated molecules.[14] Indeed, nitrotyrosine staining and accompanying fibrosis were particularly pronounced in the glomerulus. Oxidative stress is a known stimulus for fibrosis; ROS formation can activate redox-sensitive transcription factors, such as nuclear factor-$\kappa\beta$, which promotes collagen and other connective tissue deposition. The current observation that in vivo statin treatment significantly decreases NADPH oxidase activity and ROS levels in concert with decreases in albuminuria and periarteriolar and glomerular fibrosis is the first such report. Moreover, benefits of rosuvastatin treatment were indepen-

dent of any effects on basement membrane thickness or blood pressure.

Data in cultured podocyte cells complement our ex vivo data demonstrating that Ang II stimulates NADPH oxidase with resultant superoxide anion generation and reversed with statin treatment. Previous work suggested a role for ATP-dependent NADPH subunits $p22^{phox}$, $p47^{phox}$, $p67^{phox}$, and NOX2 in human podocytes.[26] Our data further define a role for NOX4 in the rodent podocyte. NOX4 is a homologue of NOX2 and has been defined previously in the endothelium,[27] and recent immunohistochemical data support a role for NOX4 in renal mesangial cell injury in db/db diabetic mice.[28] Current data further indicate that the NOX4 homologue is a critical component of the functional membrane NADPH oxidase enzyme in rodent podocytes and that statin treatment reduces the expression of this subunit in concert with attenuation of podocyte injury.

Perspectives

In summary, data from this investigation support a role for activation of NADPH oxidase with resultant oxidative stress and associated loss of glomerular filtration barrier/podocyte integrity, processes that are reversed with statin treatment, suggesting a potential renoprotective effect. These salutary effects of statin treatment in this investigation were mediated independent of effects on glomerular basement membrane thickening. However, in interpreting the results of this or any study that investigates blood pressure–mediated effects, measurement of SBP can be susceptible to large variability without a time course determination.

Disclosures

None.

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MS TITLE: CHBPR - Attenuation of NADPH Oxidase Activation and Glomerular Filtration Barrier Remodeling with Statin Treatment

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Running title: Angiotensin II, NADPH Oxidase, and the Podocyte

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Expanded Methods

Animals and treatments:

All animal procedures were approved by the institutional animal care and use committees at The University of Missouri, Harry S. Truman VA Medical Center and housed in accordance with NIH guidelines. Ren2 (5 to 6 week old) and age-matched SD male rats were randomly assigned to placebo treated control (Ren2-C and SD-C, respectively) (n=6 each) or rosuvastatin (AstraZeneca) treatment groups (Ren2-RSV and SD-RSV) (n=4 each). Rosuvastatin (20 mg/kg/day) in saline or an equal volume of saline was administered *in vivo* intra-peritoneally (IP) to the rosuvastatin or control groups, respectively for 21 days.

Systolic Blood Pressure (SBP) and Albuminuria:

SBP was determined at the end of treatment using the tail-cuff method (Harvard Systems, Student Oscillometric Recorder) three-times for 10-15 minutes after acclimatization and restraint conditioning for 48 hours at the end of treatment on days 19 or 20 (1-4). Urine albumin was determined as previously described (1).

Transmission Electron Microscopy (TEM) Methods:

Kidney cortical tissue was thinly sliced, placed immediately in primary EM fixative, and prepared as previously described (1,3,5). A JOEL 1200-EX transmission microscope was utilized to view all renal samples. Three glomeruli per rat were evaluated with five 10k and 60k images per glomeruli. TEM images were analyzed using ImageJ, (public domain, NIH) and analysis was adapted from previous work (1,3,5,6). 10k images were used to analyze the number of slit pores per 100 µm of glomerular basement membrane. Straight basement membrane with uniform thickness and upright

podocyte foot processes were used to rule out bias sample cuts. 100 µm was calibrated using the image legend and randomly placed on a straight glomerular basement membrane to measure the number of slit pores. 60k images were used to measure the slit pore diameter, the basement membrane thickness and foot process base width. Five measurements were performed for each variable per image.

Immunofluorescent Studies:

Harvested kidney cortical tissue was immersed and fixed in 3% paraformaldehyde and prepared as previously described (2-4). Blocks were sectioned and incubated with 1:100 dilution of primary antibodies in 10 fold diluted blocking agent, and third/fourth sections were washed and kept in the blocker. Over the course of 48 hours, a fifth, sixth, and seventh section was incubated with 1:100 nephrin C-17 (Santa Cruz, Santa Cruz, CA), 1:100 goat gp91[phox] (NOX2) (Santa Cruz), 1:100 mouse Rac1 antibody (Upstate Cell Signaling) respectively, in 10 fold diluted blocker. Other sections were incubated with 1:300 Alexa fluor rabbit anti-goat 647 (Molecular Probe, Eugene, OR) for NOX2, goat anti-mouse for Rac, and rabbit anti-goat for Nephrin in 10 fold diluted blocker except the sixth, which was stained with 1:300 of Alexa fluor goat anti-mouse 647 for 4 hours and examined using a laser confocal scanning microscope, images captured by using Laser-sharp software (Bio-Rad), and signal intensities measured with MetaVue software. To assess 3-nitrotyrosine content, kidney cortical tissue sections were de-paraffinized, rehydrated, and epitopes were retrieved in citrate buffer as preciously described (2,3,5).

Western Blot Analysis:

Kidney cortical tissue was homogenized using a glass-on-glass Dounce homogenizer in sucrose homogenization buffer and centrifuged at 1,000 g to remove connective tissue. Protein concentrations were measured by OPA as above. 60 µg of the supernatants were analyzed via SDS-PAGE under reducing conditions on a 7% gel. The protein was transferred to a polyvinylidene difluoride (PVDF) membrane and blocked in 5% milk in TBST for 30 min. Two nephrin antibodies (Santa Cruz, N-20 and C-17) were used at 1:1000 combined to detect nephrin; an anti-goat HRP linked secondary antibody (1:5000) was used to visualize nephrin via film. The film was scanned into a computer and band density was determined using ImageJ. Total protein was stained with amido-black staining, which was used to normalize the nephrin data.

Measurement of NADPH Oxidase Activity:

NADPH oxidase activity was determined in plasma membrane fractions as previously described (3,4,7).

Light Microscopy:

Harvested kidney cortical tissue were stained with VVG (Verhoeff-van Gieson); which is specific for fibrosis and stains elastin (black), nuclei (blue black), collagen (red), and connective tissue (yellow) as previously described (2,3,5).

Podocyte Cell Culture:

Immortalized murine podocyte cells obtained from Peter Mundel, PhD, Mount Sinai School of Medicine, were cultured as previously described (7). A dose response curve was established for Ang II stimulation of NADPH oxidase activity using a time dependency assay and rosuvastatin inhibition was based on previous experiments (7). Preparation and protocols for incubations have been previously described (7). All

cellular assays were normalized to total protein content, determined via Bradford assay. Superoxide (0_2^-) generation in podocytes were evaluated by using oxidative fluorescent dihydroethidium (DHE) staining as previously described (7).

RNA extraction and subunit expression by Real-Time PCR:

Podocytes were grown, differentiated, starved overnight and treated with Ang II (10nM) or Ang II + rosuvastatin (10uM) as described above. RNA was extracted from podocytes using RNeasy Mini Kits (Qiagen, Germany). The integrity of extracted RNA was assessed visually following separation of samples on a formaldehyde gel. A ratio of 2:1 for 28S:18S was considered satisfactory. In addition, ratios of A_{260}/A_{280} of 2.0 or better indicated samples had minimal protein contamination. Samples were DNase1 treated with reagents from Invitrogen (Carlsbad, CA) and cDNA prepared using Taqman Reverse Transcription Reagents from Applied Biosystems (Foster City, CA). 100ng of starting cDNA was used for Real-Time PCR with the following primers: p22[phox] and NOX4, gp91[phox] (NOX2) (5'-CCA ACT GGG ATA ACG AGT TCA-3') and Reverse (5'-GAG AGT TTC AGC CAA GGC TTC-3') and Rac1 (8). Universal Real Time cycling parameters were used with the following exceptions: annealing temp of 70C for p22[phox] and NOX4, SybrGreen system for p22[phox], Nox4, Rac1 and NOX2 and Taqman Universal Master Mix for 18S primer/probe set. Expression levels were normalized using 18S as control. 18S levels did not change with treatments. In order to account for day-to-day and plate-to-plate variations, increases or decreases in transcript level were expressed relative to the mean of the SD control group. Measurements of fold changes were done using standard curve method.

Statistical Analysis:

All values are expressed as mean ± standard error. Statistical analyses were performed in SPSS 13.0 (SPSS Inc., Chicago IL) using ANOVA with Fisher's LSD as appropriate and student's t-test for paired analysis. Significance was accepted as $p < 0.05$.

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Figure S1: Decreased Nephrin in Transgenic Ren2 rats. A) Immunostaining of nephrin (C-17) and 4',6-diamidino-2-phenylindole (DAPI). B) Fluorescent images from panel A were merged with the transmitted images showing the glomerular structure. C) Monostaining of the paraffin embedded kidney sections of different animals which were

captured by different system for quantification of nephrin expression in the glomerular area of different treatments. D) Western analysis of nephrin. E) Average gray scale intensities measures of panel C, showing significantly smaller number in Ren2C animals. (scale bar = 50 μM) *, p <0.05 when Ren2 controls (Ren2-C) (n=4) are compared to age-matched Sprague-Dawley controls (SD-C) (n=4). (n=4 for rosuvastatin treated SD and Ren2 groups).

Figure S2: Rosuvastatin attenuation of Peri-Arteriolar Remodeling in the Transgenic Ren2 rat. (A) Representative images of Verhoeff-van Gieson (VVG), specific for fibrosis, stained sections of arterioles in kidney cortical tissue. (B) Representative images of glomeruli with increases in periglomerular and glomerular staining observed in the Ren2 control. (C) Average calculated values of percentage area of adventitia, media and the lumen of the intramural arteries in the heart. (scale bar=50 μM) *, p <0.05 when Ren2 controls (Ren2-C) (n=4) are compared to age-matched Sprague-Dawley controls (SD-C) (n=4) ; **, p <0.05 when rosuvastatin treated Ren2 rats (Ren2-RSV) (n=4) or SD (SD-RSV) (n=4) are compared to age-matched controls.

Figure S3: Rosuvastatin improves Oxidative Stress. (A) 3-nitrotyrosine immunostaining as a marker of peroxynitrite formation in the transgenic Ren2 rat. (B) Grey scale intensity measures of panel A. (scale bar = 50 μM) *, p <0.05 when Ren2 controls (Ren2-C) (n=4) are compared to age-matched Sprague-Dawley controls (SD-C) (n=4) ; **, p <0.05 when rosuvastatin treated Ren2 rats (Ren2-RSV) (n=4) or SD (SD-RSV) (n=4) are compared to age-matched controls. (C) Dihydroethidium immunostaining

as a marker of superoxide anion formation in podocyte cell culture (n=4). *, $p < 0.05$ when compared to control; **, $p < 0.05$ when compared to angiotensin II stimulation alone.
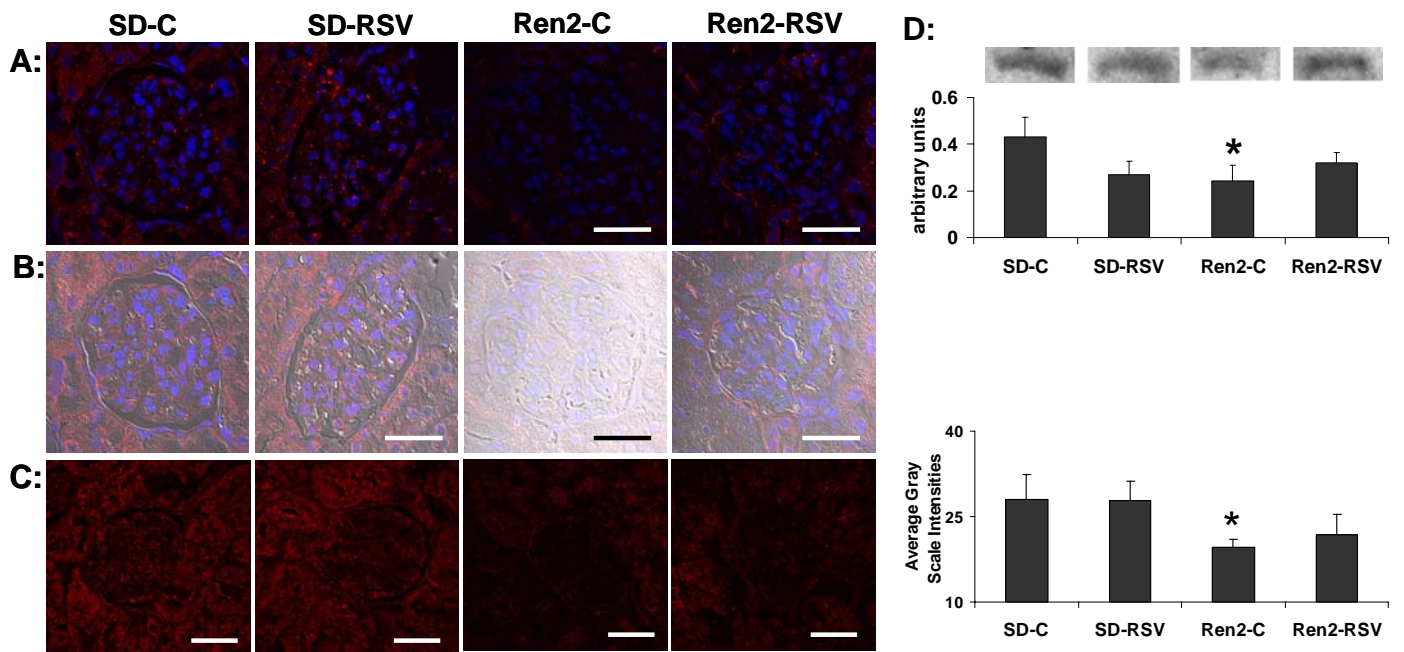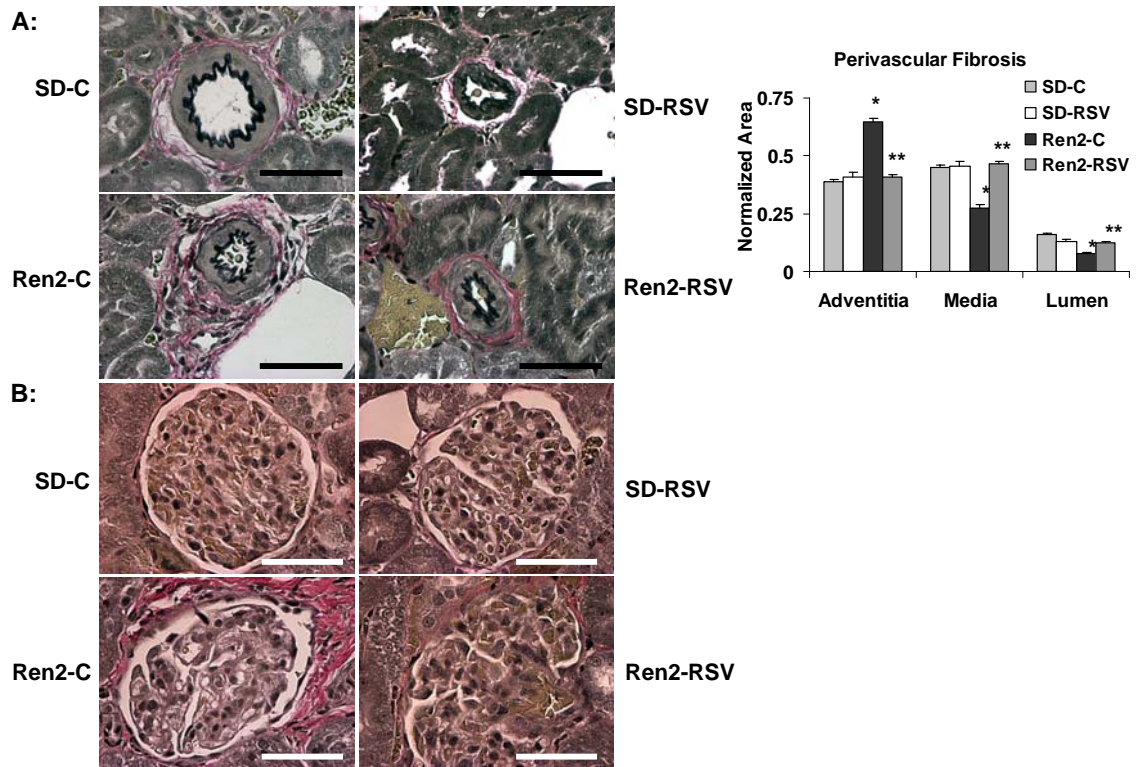
Figure S1

Figure S2

A:

SD-C   SD-RSV

Ren2-C   Ren2-RSV

Perivascular Fibrosis

- SD-C
- SD-RSV
- Ren2-C
- Ren2-RSV

Normalized Area

Adventitia   Media   Lumen

B:

SD-C   SD-RSV

Ren2-C   Ren2-RSV

Figure S3

A:

SD-C	SD-RSV
Ren2-C	Ren2-RSV

B:

C:

Control	Ang II	Ang II + RSV

D: