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

Adam Whaley-Connell, DO, MSPH1,3,4, Javad Habibi, PhD1,3, Ravi Nistala, MD1,3, Shawna A. Cooper, MS1,3, Poorna R Karuparthi, MD1,3, Melvin R Hayden, MD1,3, Nathan Rehmer1,3, Vincent G DeMarco, PhD1,3, Bradley T Andresen, PhD1,3, Yongzhong Wei, MD1,3, Carlos Ferrario, MD5, James R Sowers, MD1,2,3,4

University of Missouri School of Medicine Department of Internal Medicine1, Medical Pharmacology and Physiology2, Diabetes and Cardiovascular Lab3, and Harry S Truman VA Medical Center4, and Wake Forest University School of Medicine5

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

Funding: This research was supported by NIH (R01 HL73101-01A1) (JRS) and (P01 HL-51952) (CF), the Veterans Affairs Merit System (0018) (JRS), and Investigator Initiated Grant from AstraZeneca

Corresponding Author:
Adam Whaley-Connell, DO, MSPH
Assistant Professor of Medicine
University of Missouri-Columbia School of Medicine
Department of Internal Medicine, Division of Nephrology
MA436, DC043.0
One Hospital Dr
Columbia, MO 65212
Phone(573)882-7992
Fax(573)884-4820
whaleyconnella@health.missouri.edu


**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 rosvastatin (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 rosvastatin 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 gp91phox (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 previously 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).
cellular assays were normalized to total protein content, determined via Bradford assay. Superoxide (O$_2^-$) 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$^{\text{phox}}$ and NOX4, gp91$^{\text{phox}}$ (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$^{\text{phox}}$ and NOX4, SybrGreen system for p22$^{\text{phox}}$, 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.

**Funding:** This research was supported by NIH (R01 HL73101-01A1) (JRS) and (P01 HL-51952) (CF), the Veterans Affairs Merit System (0018) (JRS), and Investigator Initiated Grant from AstraZeneca

**References**


**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

A: SD-C  SD-RSV  Ren2-C  Ren2-RSV
B: SD-C  SD-RSV  Ren2-C  Ren2-RSV
C: SD-C  SD-RSV  Ren2-C  Ren2-RSV

D: arbitrary units

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

Average Gray Scale Intensities

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

*
Figure S2

A:

SD-C

Ren2-C

B:

SD-C

Ren2-C

Perivascular Fibrosis

Normalized Area

SD-C

SD-RSV

Ren2-C

Ren2-RSV

A:

B:

SD-C

Ren2-C

SD-RSV

Ren2-RSV

0 0.25 0.5 0.75

Adventitia Media Lumen

Perivascular Fibrosis

SD-C

SD-RSV

Ren2-C

Ren2-RSV

* * ** **
Figure S3

A: SD-C vs SD-RSV

B: Average Grey Scale Intensities

C: Control vs Ang II vs Ang II + RSV

D: Average Grey Scale Intensities