NOX4/H_2O_2/mTORC1 pathway in salt-induced hypertension and kidney injury.

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Running title: NOX4-mTORC1 in salt-induced hypertension

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Material and Methods:

Mammalian cell culture and treatment

NRK-52E cells were cultured in DMEM (11995; Thermo Fisher) supplemented with 10% FBS in the presence of 5% CO_2. Starvation medium was DMEM (10313021; Thermo Fisher) without addition of serum and glutamine. For starvation, the growth medium was removed, and 70% confluent monolayers were washed thrice with starvation media and incubated for 24 h before adding H_2O_2, LY294002 (CS-9901S), and rapamycin (R-5000 Rapamycin). Cells were pretreated with inhibitor for 30 min followed by H_2O_2 treatment for 30 min. Protein extraction was performed by addition of 2X Laemmli sample buffer followed by brief sonication.

Western Blot

Western blot was performed as described previously[1]. Briefly, blotting experiments were performed with 30 µg of protein obtained from a renal tissue homogenate. Proteins were separated on a 10–20% SDS-PAGE gel (Bio-Rad), transferred to a polyvinylidene fluoride membrane (PVDF), blocked with 5% bovine albumin serum for 1 h at room temperature, and incubated overnight with primary antibody at 4°C. The list of primary antibodies used to study the components of mTOR pathway were PTEN (1:1000; CS-.9188S), TSC2 (1:1000; CS-4308) , $pS6^{S235/236}$ (1:1000; CS-2211), S6 (1:1000; CS-2317), $pAKT^{S473}$ (1:1000; CS-4060S), $pAKT^{T308}$ (1:1000; CS-13038S), AKT (1:1000; CS-2920S), $S6K1^{S371}$ (1:1000; CS-9208P), $S6K1^{T389}$ (1:1000; CS-9205), S6K1 (1:1000; CS-2708P), $4E\text{-}BP1^{T37/46}$ (1:1000; CS-2855S), 4E-BP1 (1:1000; CS-9644), $pAMPK^{T172}$ (1:1000; CS-2535T), AMPK (1:1000; CS-2532S), GAPDH (1:1000; AF5718), β-ACTIN (1:1000; SC-47778), NOX4 (1:500; Ab133303). Bound primary antibodies were detected with a horseradish peroxidase-labeled secondary antibody and visualized by chemiluminescence. A ChemiDox XRS+ imaging system (BioRad) was used for chemiluminescence detection of bands, and Image Laboratory software (version 5.2). was used for quantification by densitometry. The activity of mTORC1 and mTORC2 was determined by normalizing the $pS6^{S235/236}$ and $pAKT^{S473}$ by endogenous S6 and AKT, respectively.

Experimental animals: Male $SS^{Nox4\text{-}/\text{-}}$ rats[2] were obtained as weanlings from colonies maintained at the Medical College of Wisconsin. All experiments were performed in age-matched control and experimental animals. The $SS^{Nox4\text{-}/\text{-}}$ breeders and weanlings were maintained on purified AIN-76A rodent diet (Dyets, Bethelem, PA) containing 0.4% NaCl. At 10 wk of age, the salt content of the diet was increased to 4.0% NaCl for the remaining 21 day of the study; all experimental measurements were made throughout the study period. For mTORC1 pathway analysis, 6 wk male $SS^{Nox4\text{-}/\text{-}}$ and SS rats were fed a 4.0% NaCl for 21 days. Another group of $SS^{Nox4\text{-}/\text{-}}$ and SS rats were maintained on the 0.4% NaCl diet for 3 wk of the study; so experimental measurements in these rats were also made at 9 wk of age. All the animals were provided water and food ad libitum, and the protocols were approved by Medical College of Wisconsin Institutional Animal Care and Use Committee.

Protocols for preparation of rapamycin, chronic measurement of arterial blood pressure and urine collection.

Rapamycin (i.p., 1.5 mg/kg/day) was administered chronically to $SS^{Nox4\text{-}/\text{-}}$ rats fed a 0.4% NaCl diet for 4 days followed by 4.0% NaCl diet for 3 wk (21 days). Rapamycin (R-5000

rapamycin) was dissolved in 100% ethanol, stored at -20°C and diluted freshly in a vehicle solution containing 5% Tween 80, 5% PEG 400 (Sigma, St. Louis, MO) and 4% ethanol just before injection. One day prior to surgery, 9-week-old rats were placed in metabolic cages for 24 h collection of urine to measure albumin, protein, and creatinine. The following day radiotelemetry catheters and transmitters were surgically implanted and rats were given 5-7 days to recover before recording blood pressure. As we have described[1], baseline heart rate (HR), systolic, blood pressure (SBP), diastolic blood pressure (DBP), and mean arterial pressure (MAP) were recorded 24 h/day for three days without rapamycin followed by another four days during which rats were treated with vehicle or rapamycin while they continued to be maintained on the 0.4% salt diet then switched to 4% NaCl diet for 21 day. Daily body weight was measured to adjust the daily dose of rapamycin. On the final day of the 4.0% NaCl diet period, rats were placed in a metabolic cage for a 24 h urine collection. Rats were not acclimatized in metabolic cages prior to placing in metabolic cages. Average food intake for 21 day was determined by taking the total food consumed over the experimental period and divided it by the total number of days. Albumin was quantified with an Albumin Blue 580 (Molecular Probes) fluorescence assay. Protein was quantified with Weichselbaum's biuret reagent on an ACE autoanalyzer (Alfa Wassermann).

Immunohistochemistry and Histology analyses of kidneys

Kidney sections from vehicle or rapamycin treated SS$^{Nox4-/-}$ were obtained and immunostained with antibodies against T lymphocytes (Anti-human CD3; 1:100; DAKO), macrophages (CD68 1:100, Santa Cruz Biotechnology). Biotinylated goat anti-rabbit secondary antibody (1:500, Santa Cruz Biotechnology) was used for development with avidin-biotinylated HRP complex (Vectastain ABC Elite kit, Vector Laboratories) followed by counterstaining with hematoxylin and mounted for image capturing. For background control, tissues were incubated with blocking buffer in the absence of primary antibody. Once processed the kidney images were captured with a Nikon Ni-E Motorized Upright microscope equipped with DS-QiMc camera and NIS-Element software. The investigator analyzing the images was blinded to the treatment conditions of the rats. Twenty fields (each field equal to 0.56 mm^2) were randomly selected for each renal cortex and outer medulla and the numbers of immunolabeled cells were counted using an automated counting method[1].

As described previously[1], additional whole kidney sections from the experimental groups were cut in 4µm thick slices, stained with Gomori's trichrome and imaged on a Nikon Ni-E Motorized Upright microscope at a resolution of 0.68 µm/px. To assess renal tubular injury for quantification, regions of interest were drawn over the medulla of each kidney. Color filters were manually defined for each kidney to select for area of medullary protein casts within the regions of interest. Glomerular injury was assessed on these kidneys and were scored based on a 0-4 injury index.

Quantitative Polymerase Chain Reaction

Tissue was collected from treated or nontreated SS$^{Nox4-/-}$ rats fed a 4.0% NaCl diet for day 21. The outer medulla was dissected from the cortex, and samples of both regions were snap-frozen in liquid nitrogen and stored at −80°C. Total RNA was extracted from both the renal cortex and the outer medulla tissue using Trizol reagent. The quality and quantity of each sample was determined by spectrophotometry (Nanodrop). Two micrograms of total RNA was reverse-transcribed by random hexamer primers into cDNA (Thermo

Scientific Revert Aid First Strand cDNA synthesis kit) and real time polymerase chain reaction analysis performed using 8 to 10 ng total RNA as we have described using SYBR green chemistry on an ABI Prism 7900HT (Applied Biosystems). All primer sequences are given below.

Gene symbol	Primer name	Primer sequence
CYBB	Nox2 FK	CCACTCCACGTTGAACAGAT
	Nox2 RK	CTCAACCAGAATTCGAAGACAAC
Ncf2	p67 5 race fw	AGCAGAAGAGCAGTTAGCATTGG
	p67 5 race rv	TGCTTTCCATGGCCTTGTC
Ccl2	CCL2 F1	TCCACCACTATGCAGGTCTC
	CCL2 R1	GGGCATTAACTGCATCTGGCT
Mmp9	Mmp9 F2	AGCCGACGTCACTGTAACTG
	Mmp9 R2	AACAGGCTGTACCCTTGGTC
Col1a1	ratcol1a1-rt-s	GGACATGGGGTTCTCGGACATA
	ratcol1a1-rt-s	GCACAGGCCCTCAAAAACAA
a-Sma	a-Sma F1	TTCGTGACTACTGCTGAGCG
	a-Sma R1	CTGTCAGCAATGCCTGGGTA
Kim-1	Qiagen	Rn_havcr1_1_SG,QT00185983

Statistical Methods Data are presented as mean values ± standard error mean. A two-way analysis of variance (ANOVA) for repeated measures test was used for blood pressure analysis. For multiple groups, statistical significance was analyzed by one-way ANOVA (Tukey's post hoc multiple comparison test). Student's t-test was used to compare between the two treatments. Paired t-test was used to compare the effect of treatments on body weight of SS rats fed a 4.0% NaCl diet. $P<0.05$ was considered significant.

1. Kumar V, Wollner C, Kurth T, Bukowy JD, Cowley AW, Jr. Inhibition of mammalian target of rapamycin complex 1 attenuates salt-induced hypertension and kidney injury in dahl salt-sensitive rats. *Hypertension*. 2017;70:813-821
2. Cowley AW, Jr., Yang C, Zheleznova NN, Staruschenko A, Kurth T, Rein L, Kumar V, Sadovnikov K, Dayton A, Hoffman M, Ryan RP, Skelton MM, Salehpour F, Ranji M, Geurts A. Evidence of the importance of nox4 in production of hypertension in dahl salt-sensitive rats. *Hypertension*. 2016;67:440-450

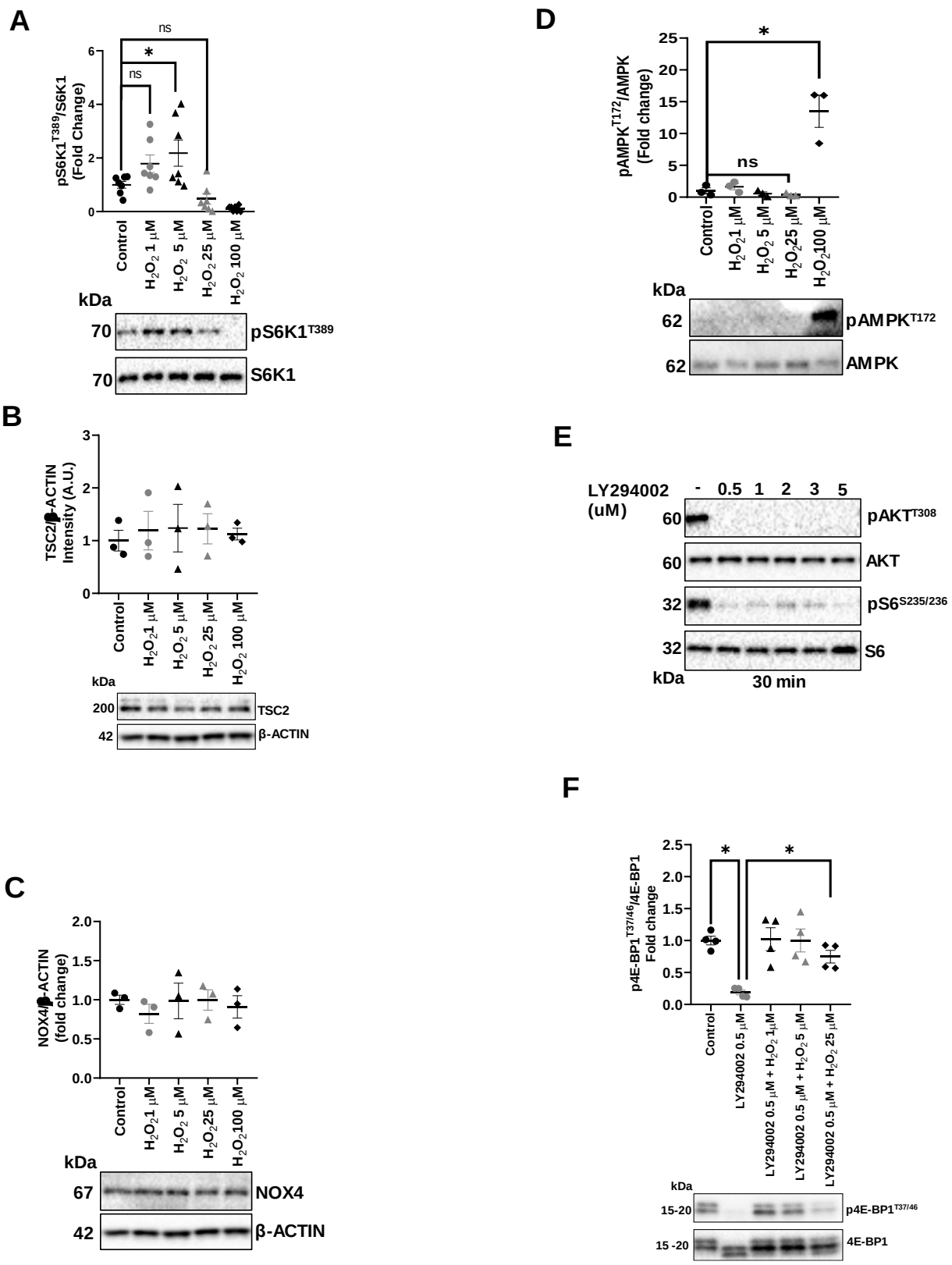
A
pS6K1T389/S6K1 (Fold Change)
ns
*
ns
Control
H2O2 1 μM
H2O2 5 μM
H2O2 25 μM
H2O2 100 μM
kDa
70
pS6K1T389
70
S6K1
B
TSC2/β-ACTIN Intensity (A.U.)
Control
H2O2 1 μM
H2O2 5 μM
H2O2 25 μM
H2O2 100 μM
kDa
200
TSC2
42
β-ACTIN
C
NOX4/β-ACTIN (fold change)
Control
H2O2 1 μM
H2O2 5 μM
H2O2 25 μM
H2O2 100 μM
kDa
67
NOX4
42
β-ACTIN
D
pAMPKT172/AMPK (Fold change)
*
ns
Control
H2O2 1 μM
H2O2 5 μM
H2O2 25 μM
H2O2 100 μM
kDa
62
pAMPKT172
62
AMPK
E
LY294002 (uM)
- 0.5 1 2 3 5
60
pAKTT308
60
AKT
32
pS6S235/236
32
S6
kDa
30 min
F
p4E-BP1T37/46/4E-BP1 Fold change
*
*
Control
LY294002 0.5 μM
LY294002 0.5 μM + H2O2 1μM
LY294002 0.5 μM + H2O2 5 μM
LY294002 0.5 μM + H2O2 25 μM
kDa
15-20
p4E-BP1T37/46
15 -20
4E-BP1

Figure S1: A-D, starved NRK-52E cells were treated with different concentration of H_2O_2 for 30 min and pS6K1^{T389}, S6K1, TSC2, NOX4, pAMPK^{T172}, AMPK, and β-ACTIN protein levels were determined by Western blot. Representative immunoblot is shown below each graph. **E**, Cells were treated with indicated concentration of LY294002 for 30 min and the effect of this drug on PI3K/AKT and mTORC1 pathway was determined by measuring pAKT^{T308} and pS6$^{S235/236}$. Representative membranes are shown. **F,** starved cells were pretreated with LY294002 with indicted concentration before addition of H_2O_2 (1, 5, and 25 µM) for 30 min. Pretreatment with LY294002 inhibited p4E-BP1$^{T37/46}$/4E-BP1 and addition of H_2O_2 reversed p4E-BP1$^{T37/46}$/4E-BP in pretreated cells. Analysis of variance (ANOVA) with Tukey's post hoc test was used for multiple comparisons. * P<0.05.

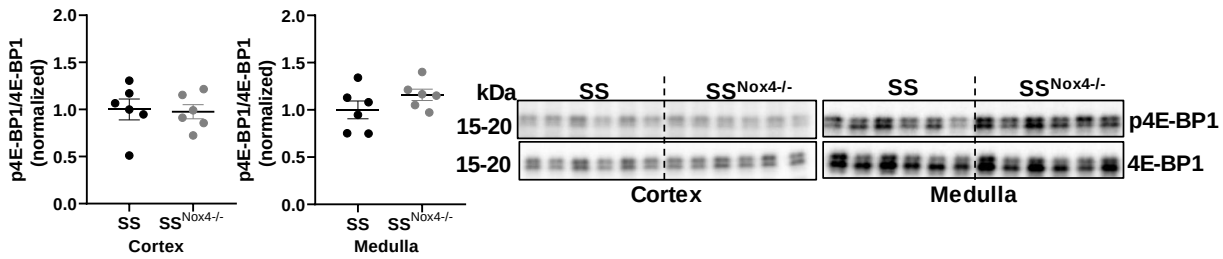

Figure S2: p4E-BP1$^{T37/46}$/4E-BP1 was determined in the cortex and medulla of SS (n=6; black closed circle) and SS$^{Nox4-/-}$ (n=6; gray closed circle) rats fed a 0.4% NaCl diet after weaning by Western blot. Representative immunoblots are shown.

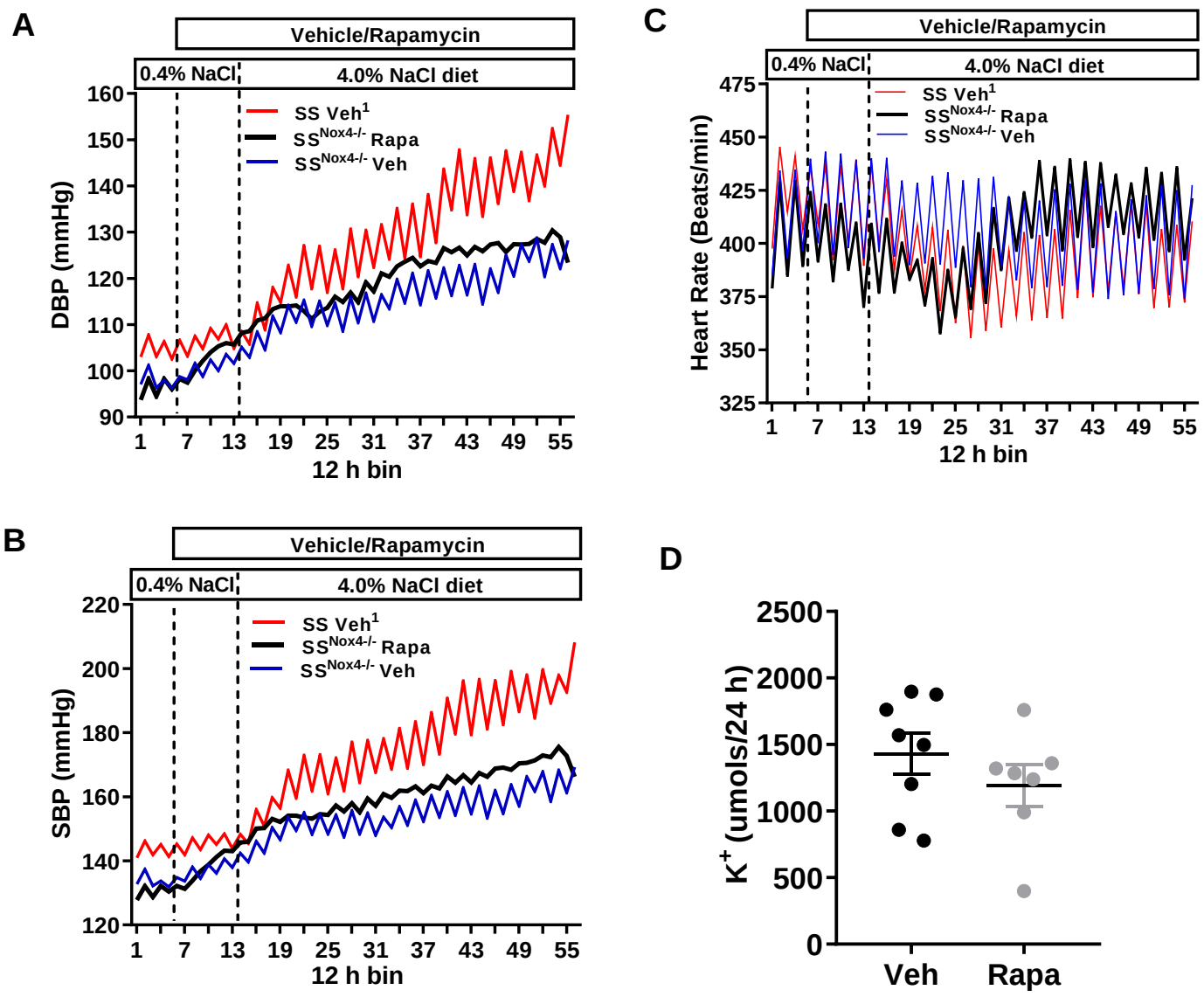

Figure S3: Diastolic blood pressure (DBP), systolic blood pressure (SBP) and heart rate (HR) were continuously measured for three days without vehicle (close circle; n=8) or rapamycin (open circle; n=8) and four day with vehicle or rapamycin on 0.4% salt followed by 21 days of vehicle or rapamycin treatment after the diet was switched to 4.0% NaCl diet. **A-C,** DBP, SBP and HR were averaged in 12 h bins to correspond to the day (6am-6pm) and night (6pm- 6am) cycles represent SS$^{Nox4-/-}$ rats treated with vehicle or rapamycin. **D**, steady-state potassium excretion at day 21 of the 4.0% NaCl diet.

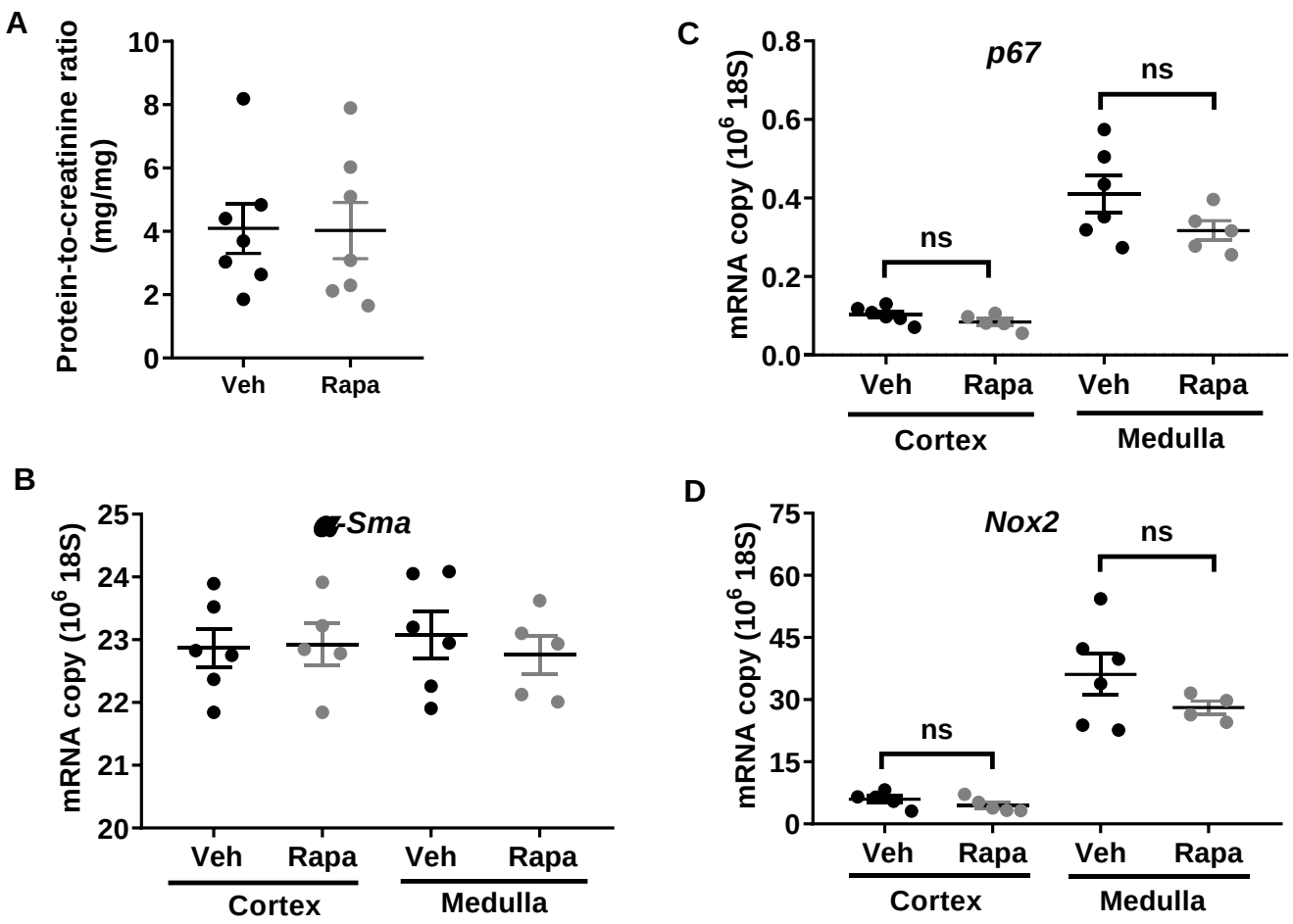

Figure S4: A, protein to creatinine was determined in urine samples of SS$^{Nox4-/-}$ rats treated with vehicle (n=8) or rapamycin (n=7) collected for 24 hours on the last day of the 4.0 % NaCl. qRT-PCR of genes **B,** *α-Sma*, **C**, *p67*, and **D**, *Nox2* from cortex and medulla of vehicle or rapamycin treated SS$^{Nox4-/-}$ rats. * P<0.05, ns; non-significant.

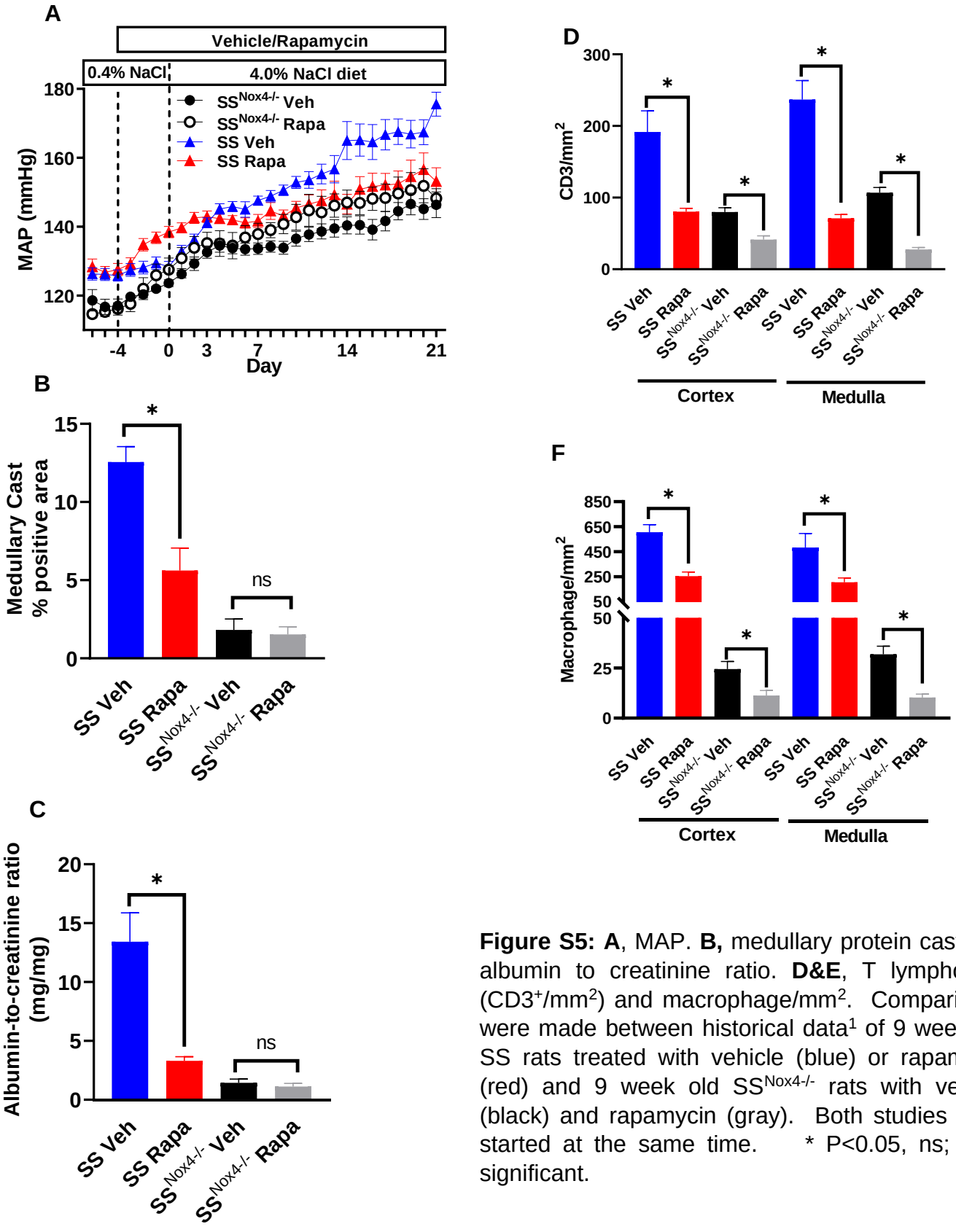

Figure S5: A, MAP. **B,** medullary protein cast. **C,** albumin to creatinine ratio. **D&E**, T lymphocyte ($CD3^+/mm^2$) and macrophage/mm^2. Comparisons were made between historical data[1] of 9 week old SS rats treated with vehicle (blue) or rapamycin (red) and 9 week old $SS^{Nox4-/-}$ rats with vehicle (black) and rapamycin (gray). Both studies were started at the same time. * P<0.05, ns; non-significant.