Mutagenesis of the Cleavage Site of (Pro)Renin Receptor Abrogates Angiotensin II-Induced Hypertension in Mice

Fei Wang[1*], Yanting Chen[1*], Chang-jiang Zou[1], Renfei Luo[1], and Tianxin Yang[1]

[1]Department of Internal Medicine, University of Utah and Veterans Affairs Medical Center, Salt Lake City, Utah, USA.

*, equal contribution

Running title: sPRR and AngII hypertension

Address correspondence to:
Tianxin Yang, M.D., Ph.D.
University of Utah and Veterans Affairs Medical Center
Division of Nephrology and Hypertension
30N 1900E, RM 4C224
Salt Lake City, UT 84132
Tel: 801-585-5570
Fax: 801-584-5658
Email: Tianxin.Yang@hsc.utah.edu

Methods

Mouse Experiment. Male 16 to 20-week-old $PRR^{R279V/L282V}$ mice and their littermate wild-type (WT) controls were studied. Five days prior to AngII treatment, the radiotelemetric device was implanted via catheterization of carotid artery. Blood pressure (BP) readings were taken every 5 minutes throughout the acute experiment. After the baseline blood pressure (BP) parameters were recorded, all mice were administrated with vehicle or a single bolus subcutaneous injection of Ang II at 30 μg/kg. After injection, BP was continuously recorded for 90 mins.

Immunoblotting. Heart, liver, adipose, vessel, adrenal gland, muscle were lysed and subsequently sonicated in PBS that contained 1% Triton x-100, 250 μM phenylmethanesulfonyl fluoride (PMSF), 2 mM EDTA, and 5 mM dithiothrietol (DTT) (pH 7.5). Protein concentrations were determined by using Coomassie reagent. Forty μg of proteins for each sample was denatured in boiling water for 10 min, then separated by SDS-PAGE gel, and transferred onto nitrocellulose membranes. The blots were blocked overnight with 5% nonfat dry milk in Tris-buffered saline (TBS), followed by incubation overnight with primary antibody. After being washed with TBS, blots were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody and visualized using Enhanced Chemiluminescence (ECL). Primary antibodies are as follows: rabbit anti-PRR antibody (cat no. HPA003156; Sigma), rabbit anti-LC3b antibody (cat no. Ab48394; Abcam), rabbit anti-p62 antibody (cat no. Ab109012; Abcam), rat anti-LAMP2 antibody (cat no. Ab13524; Abcam), mouse anti-β-actin antibody (cat no. A1978; Sigma).

Histological analysis. Formalin-fixed renal tissues were subsequently embedded in paraffin. Four-micrometer sections were cut from these tissue blocks and stained with Hematoxylin and Eosin (H&E) to evaluate gross morphology. For each individual animal, five non-overlapping fields of renal cortex, or outer medulla, or inner medulla were assessed under high magnification (400×). All examinations were performed independently in a blinded manner by two individuals.

Enzyme immunoassay. The angiotensinogen (AGT), angiotensin I (AngI) and aldosterone in biologic fluids were determined by using the commercially available enzyme immunoassay kits according to the manufacturer's instructions: the kits for AGT (cat no. JP27413; IBL), Ang I (cat no. S-1188; Peninsula Laboratories International), and aldosterone (cat no. Ab136933, Abcam) .

Cell experiments. As4.1 cells were cultured in DMEM medium with high glucose, sodium pyruvate, and L-Glutamine supplemented with 10% fetal bovine serum. M-1 cells were cultured in DMEM/F12 medium with 2.5 mM L-glutamine adjusted to contain 15 mM HEPES, 0.5 mM sodium pyruvate and 1.2 g/L sodium bicarbonate supplemented with 0.005 mM dexamethasone and 5% fetal bovine serum. Upon confluence, cells were serum-starved for 12 h, and then treated with 10 nM sPRR-His alone or in combination treatment with 10 μM ICG-001 (HY-14428, Med Chem Express). After 24 h treatment, the cells and medium were then harvested for analysis.

Statistical analysis. GraphPad Prism software (version 8.4) was used for data analysis Data is summarized as means ± SEM. All data points represent animals that were included in the statistical analyses. Sample sizes were determined on the basis of similar previous studies or pilot experiments. The animal experiments were performed by using regular two-way ANOVA with the Bonferroni test for multiple comparisons or by using unpaired two-tailed Student's t test for 2 comparisons. The cell experiments were performed by using regular one-way ANOVA with the Bonferroni test for multiple comparisons. A value of $P < 0.05$ was considered statistically significant.

Supplemental Figure Legend

Figure. S1. Assessment of sPRR protein abundance in various types of tissues in male 16 to 20-week-old $PRR^{R279V/L282V}$ mice and their littermate controls. Heart, liver, adipose tissue, vessel, adrenal gland, and muscle from WT and $PRR^{R279V/L282V}$ were subjected to immunoblotting analysis of sPRR abundance. Densitometry values are shown underneath the blots. *P < 0.05 vs. mutant group, **P < 0.01 vs. mutant group. N = 4 per each group. WT, wild-type; Mutant, $PRR^{R279V/L282V}$.

Figure. S2. Histological analysis and assessment of autophagic-lysosomal markers in male 16 to 20-week-old $PRR^{R279V/L282V}$ mice and their littermate controls. Formalin-fixed renal tissues from WT and $PRR^{R279V/L282V}$ mice were embedded in paraffin, followed by H&E staining to evaluate gross morphology under low (40x) and high magnification (400x) (A). Renal tissues from WT and $PRR^{R279V/L282V}$ mice were subject to immunoblotting analysis of the autophagosome markers expression (B) and lysosome marker expression (C). N = 5 per each group. WT, wild-type; Mutant, $PRR^{R279V/L282V}$.

Figure. S3. Blood pressure response of $PRR^{R279V/L282V}$ mice to acute AngII treatment. WT and $PRR^{R279V/L282V}$ mice were instrumented with radiotelemetric devices and after 5 days of recovery were treated with vehicle or AngII (n = 5 per each group). A single bolus of Ang II (30 μg/kg) or vehicle was administrated at 35 mins via subcutaneous injection. (A) systolic blood pressure (SBP), (B) mean arterial blood pressure (MAP), (C) diastolic blood pressure (DBP), and (D) heart rate (HR). The statistical significance was determined by using one-way ANOVA with the Bonferroni test for multiple comparisons. *, p <0.05 WT + AngII vs. WT + Vehicle group. #, p < 0.05 Mutant + AngII vs. Mutant + Vehicle. Data are mean ± SE. WT, wild-type; Mutant, $PRR^{R279V/L282V}$.

Figure. S4. Physiological parameters in WT and $PRR^{R279V/L282V}$ mice during chronic AngII infusion. WT and $PRR^{R279V/L282V}$ mice were chronically infused with vehicle or AngII as described in Fig. 2. At the end of the experiment, animals were placed in metabolic cages and following parameters

were collected. (A) Body weight, (B) Heart weight, (C) Urine output, and (D) Urinary sodium excretion. The statistical significance was determined by using one-way ANOVA with the Bonferroni test for multiple comparisons, and the p values were indicated in the figure. Data are mean ± SE. WT, wild-type; Mutant, $PRR^{R279V/L282V}$.

Figure. S5. Renin enzyme activity assay on biological fluid and renal samples. Plasma, urine, tissue lysates of the renal cortex and inner medulla samples from WT and PRRR279V/L282V mice following vehicle or AngII treatment were collected at the end of the experiment (day 14), and subjected to renin enzyme activity assay for assessment of renin activity (A&C&E&G), active renin content (B&D&F&H). The statistical significance was determined by using two-way ANOVA with the Bonferroni test for multiple comparisons, and the p values were indicated in the figure. Data are mean ± SE. WT, wild-type; Mutant, PRRR279V/L282V.

Figure. S6. ELISA measurement of prorenin/renin in WT and $PRR^{R279V/L282V}$ mice during chronic AngII infusion. Samples were collected at the end of the experiment (day 14) (A) Plasma prorenin/renin, (B) Urinary prorenin/renin excretion, (C) Renal cortical prorenin/renin content, and (D) Renal medullary prorenin/renin content. The statistical significance was determined by using two-way ANOVA with the Bonferroni test for multiple comparisons, and the p values were indicated in the figure. N =5 per group. Data are mean ± SE. WT, wild-type; Mutant, $PRR^{R279V/L282V}$.

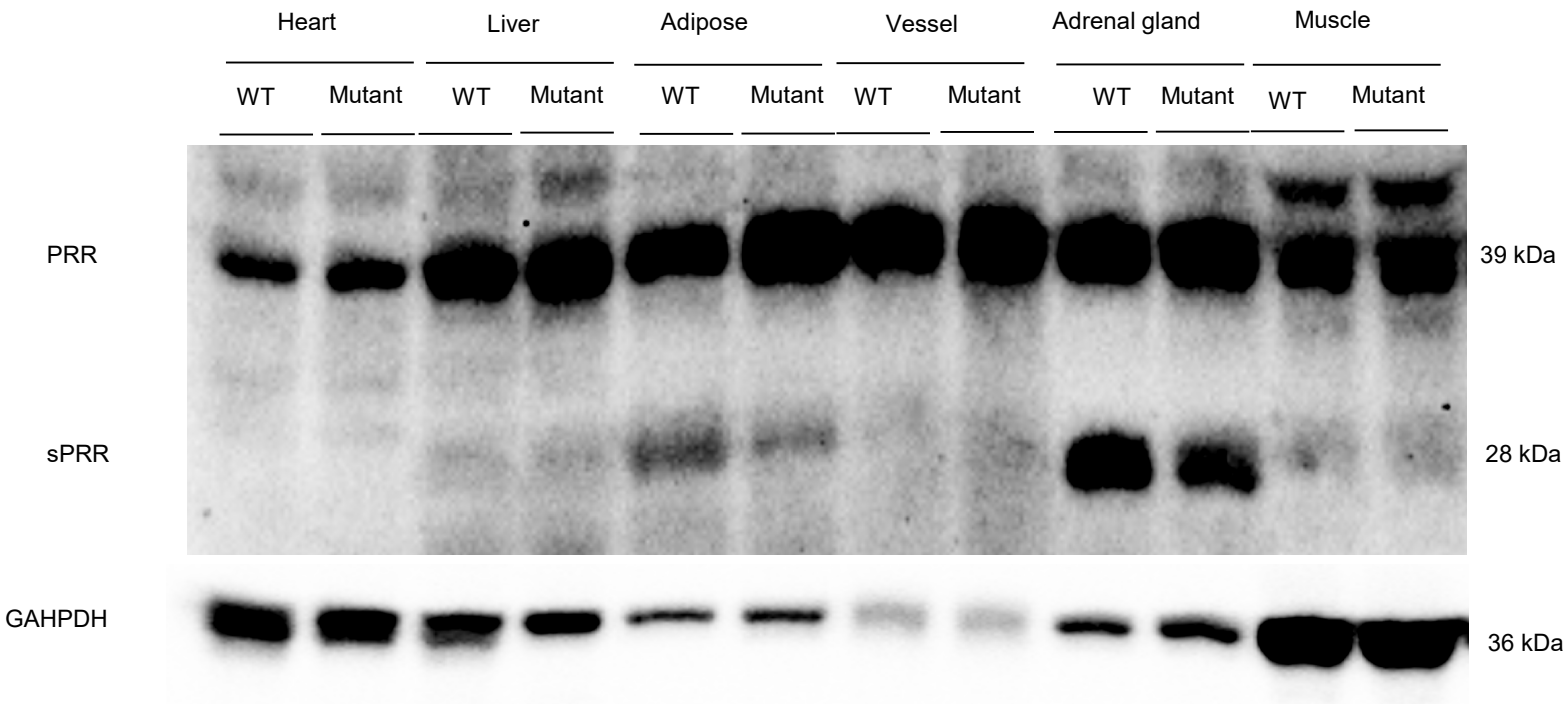
Figure. S7. ELISA measurement of AGT, aldosterone, and AngI in WT and $PRR^{R279V/L282V}$ mice during chronic AngII infusion. Samples were collected at the end of the experiment (day 14) (A) Plasma AGT, (B) Urinary AGT excretion, (C) Plasma aldosterone, (D) Plasma AngI, (E) Urinary AngI excretion, (F) Renal cortical AngI content, and (G) Renal medullary AngI content. The statistical significance was determined by using two-way ANOVA with the Bonferroni test for multiple comparisons, and the p values were indicated in the figure. N =5 per group. Data are mean ± SE. WT, wild-type; Mutant, $PRR^{R279V/L282V}$.

Figure. S8. Assessment of renal expression of sodium transporters in WT and $PRR^{R279V/L282V}$ mice during chronic AngII infusion. Renal cortex from these animals was subjected to immunoblotting analysis of NHE3, NKCC2, and NCC. N = 5 per group. WT, wild-type; Mutant, $PRR^{R279V/L282V}$.

Figure. S9. In vitro assessment of ENaC activity. Confluent primary IMCD cells from WT or $PRR^{R279V/L282V}$ mice on Snapwells were treated with vehicle or 1 μM AngII for 24 h. Amiloride-sensitive transepithelial Na^+ transport, an index of ENaC activity, was recorded by Ussing chamber technique. N = 4 per each group. The statistical significance was determined by using two-way ANOVA with the Bonferroni test for multiple comparisons, and the p values were indicated in the figure. Data are mean ± SE. WT, wild-type; Mutant, $PRR^{R279V/L282V}$.

Figure. S10. The role and mechanism of sPRR in regulation of renin expression in cultured As4.1 and M-1 cells. The confluent cells were pretreated for 1 h with the β-catenin inhibitor ICG-001 at 10 μM and then treated for 24h with 10 nM sPRR-His. At the end of the experiment, cells and/or the media were harvested for qRT-PCR analysis of renin mRNA, enzyme activity-based assay for renin activity, active renin content, and total renin content, and ELISA measurement of prorenin/renin content. (A) Medium total prorenin/renin content in As4.1 cells. (B) Cellular renin activity in As4.1 cells. (C) Cellular active renin content in As4.1 cells. (D) Medium total prorenin/renin content in M-1 cells. (E) Cellular renin activity in M-1 cells. (F) Cellular active renin content in M-1 cells. N = 5-8 per group. Data are mean ± SE.

Figure S1



	Heart		Liver		Adipose		Vessel		Adrenal gland		Muscle	
	WT	Mutant	WT	Mutant	WT	Mutant	WT	Mutant	WT	Mutant	WT	Mutant
PRR/GAPDH	1.00 ± 0.01	1.39 ± 0.04*	4.32 ± 0.05	4.32 ± 0.06	4.69 ± 0.04	8.47 ± 0.46*	7.94 ± 0.04	7.85 ± 0.05	5.09 ± 0.16	6.01 ± 0.11*	0.56 ± 0.03	.61 ± 0.01
sPRR/GAPDH	N/A	N/A	N/A	N/A	3.68 ± 0.23	1.32 ± 0.16**	N/A	N/A	5.01 ± 0.28	2.36 ± 0.248**	N/A	N/A

Figure S2

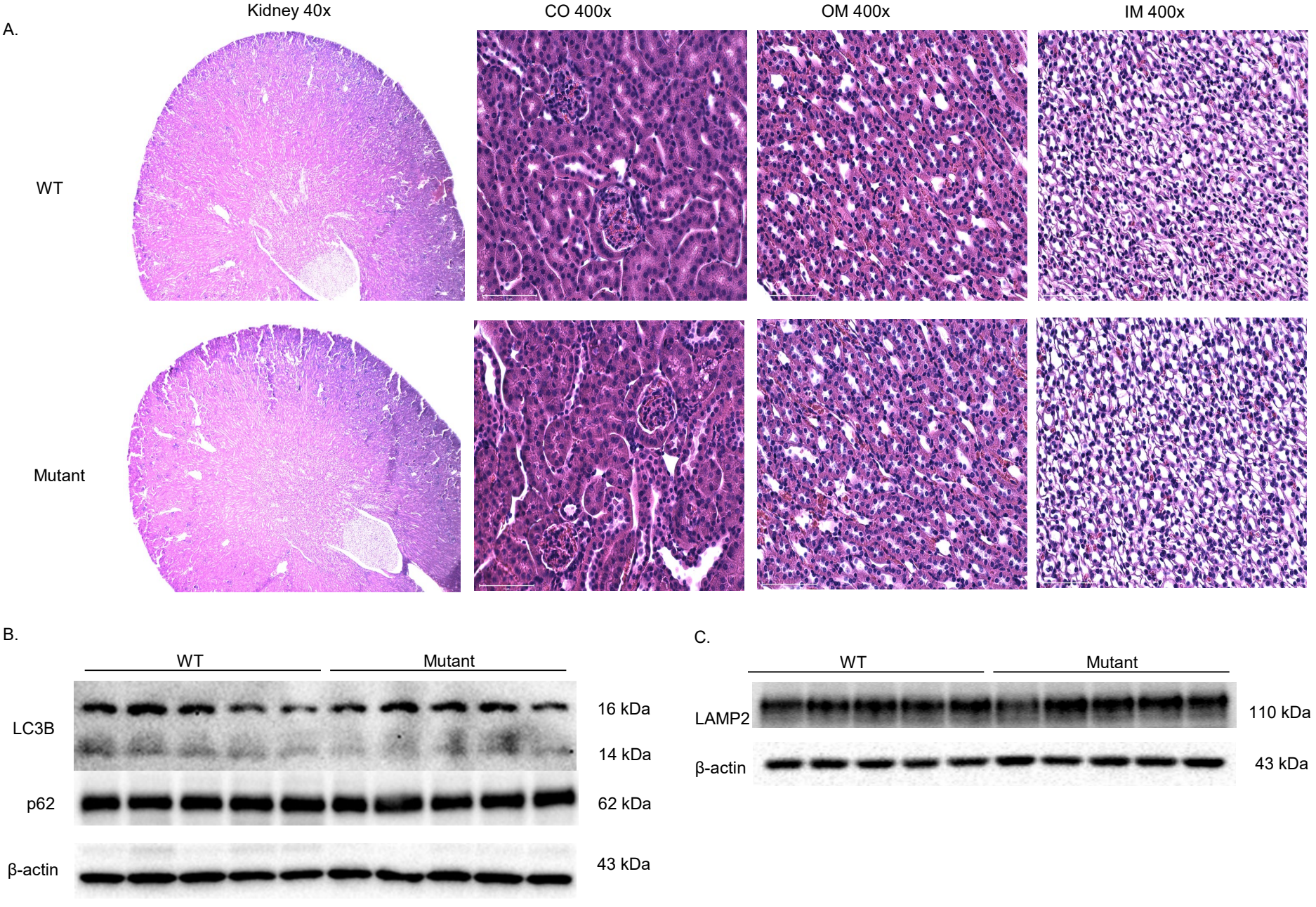

A.
Kidney 40x
CO 400x
OM 400x
IM 400x
WT
Mutant
B.
WT
Mutant
LC3B
16 kDa
14 kDa
p62
62 kDa
β-actin
43 kDa
C.
WT
Mutant
LAMP2
110 kDa
β-actin
43 kDa

Figure S3

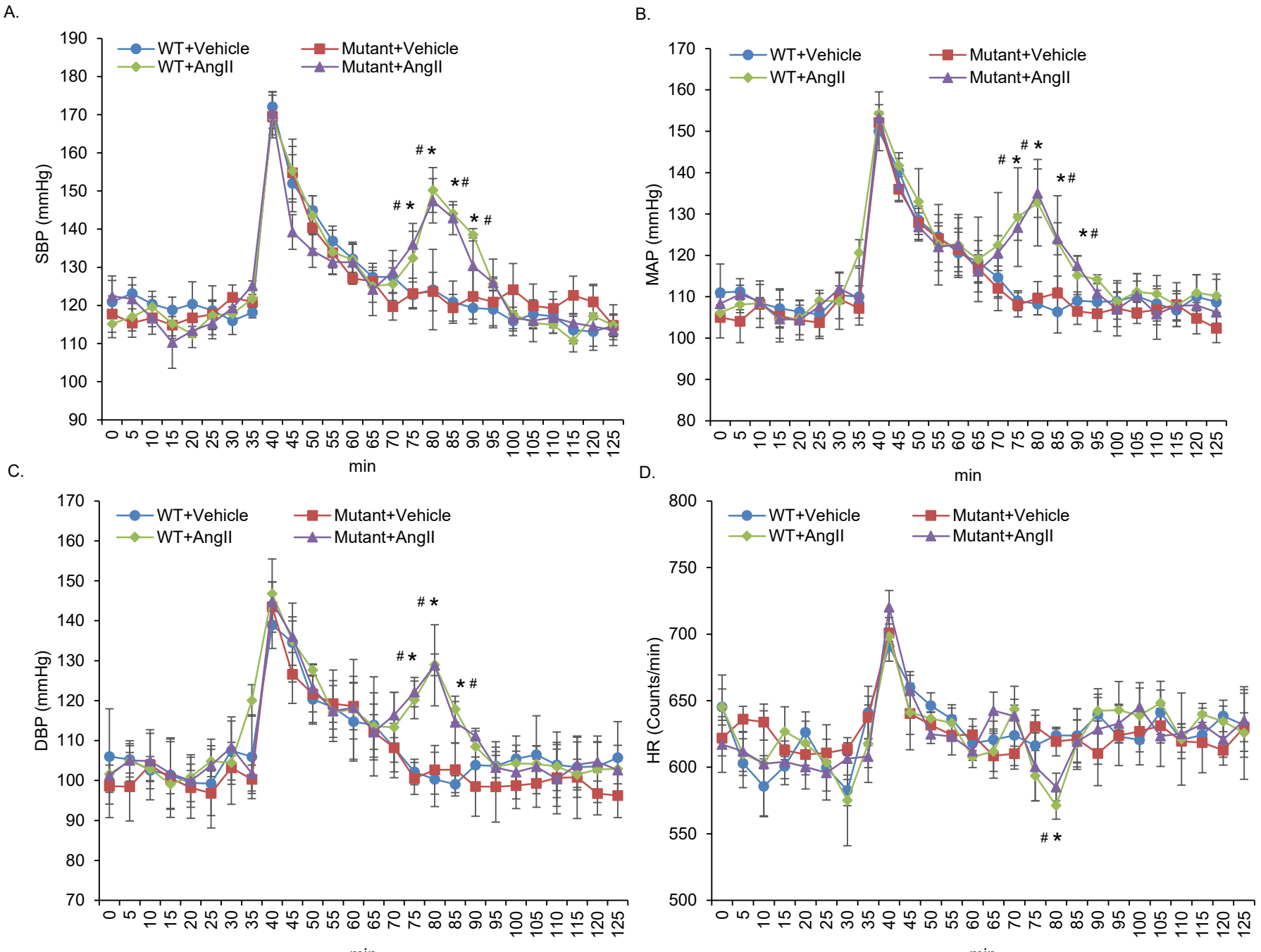

A.
SBP (mmHg)
WT+Vehicle
Mutant+Vehicle
WT+AngII
Mutant+AngII
min
B.
MAP (mmHg)
min
C.
DBP (mmHg)
min
D.
HR (Counts/min)
min

Figure S4

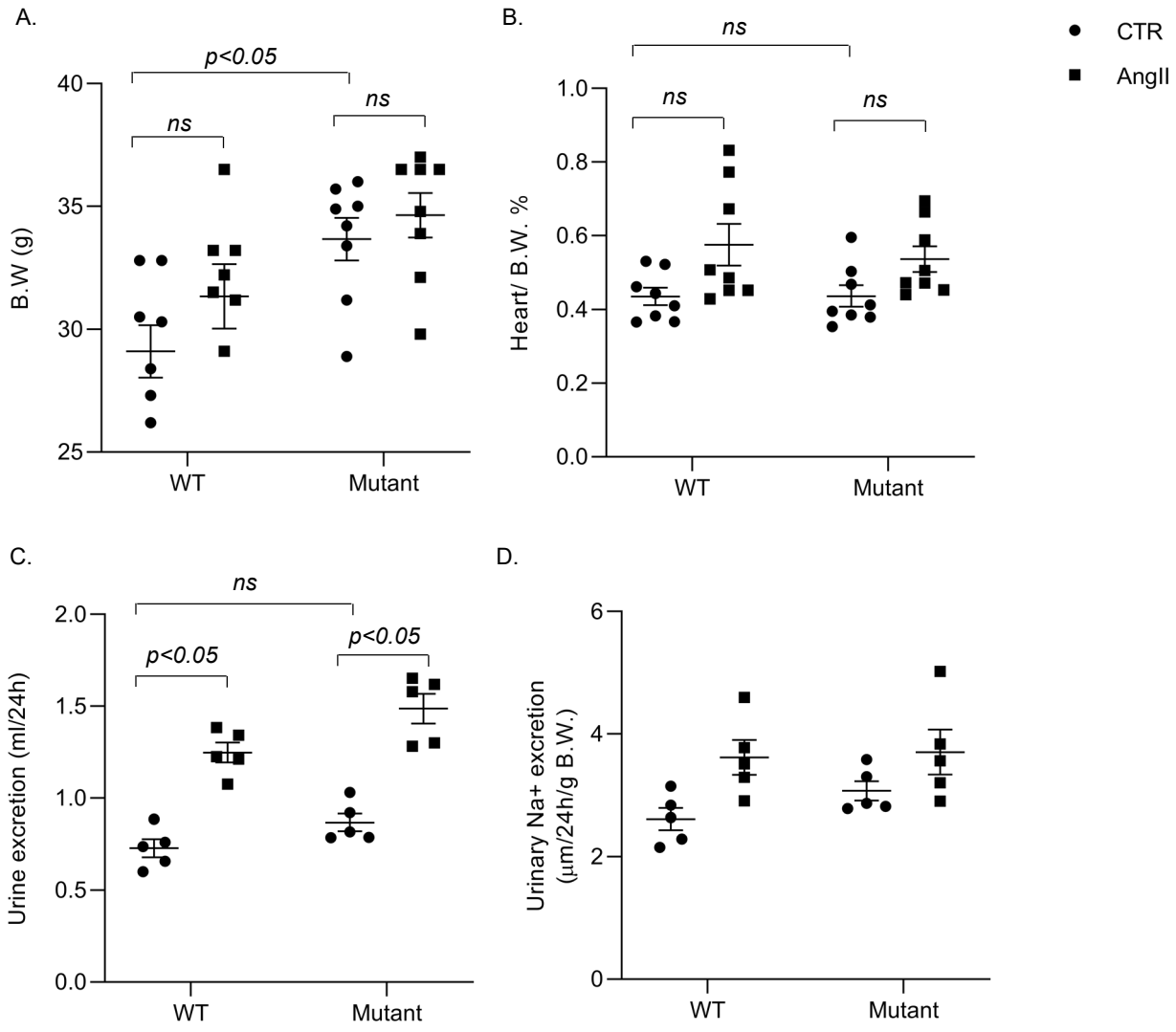

A.
B.
C.
D.
CTR
AngII
p<0.05
ns
B.W (g)
Heart/ B.W. %
Urine excretion (ml/24h)
Urinary Na+ excretion (µm/24h/g B.W.)
WT
Mutant

Figure S5

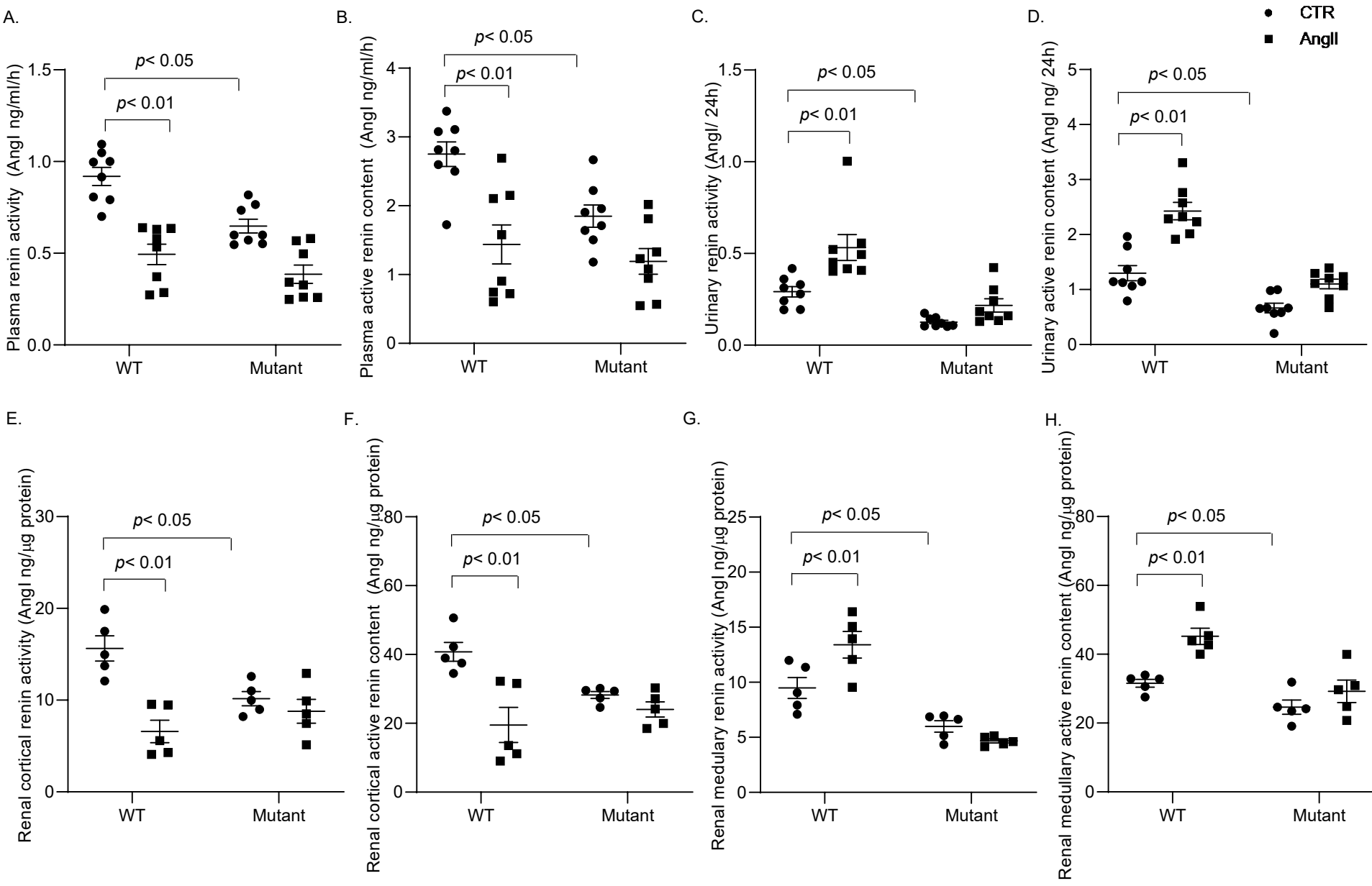

A.
B.
C.
D.
CTR
AngII
Plasma renin activity (AngI ng/ml/h)
Plasma active renin content (AngI ng/ml/h)
Urinary renin activity (AngI/ 24h)
Urinary active renin content (AngI ng/ 24h)
p< 0.05
p< 0.01
WT
Mutant
E.
F.
G.
H.
Renal cortical renin activity (AngI ng/µg protein)
Renal cortical active renin content (AngI ng/µg protein)
Renal medulary renin activity (AngI ng/µg protein)
Renal medullary active renin content (AngI ng/µg protein)

Figure S6

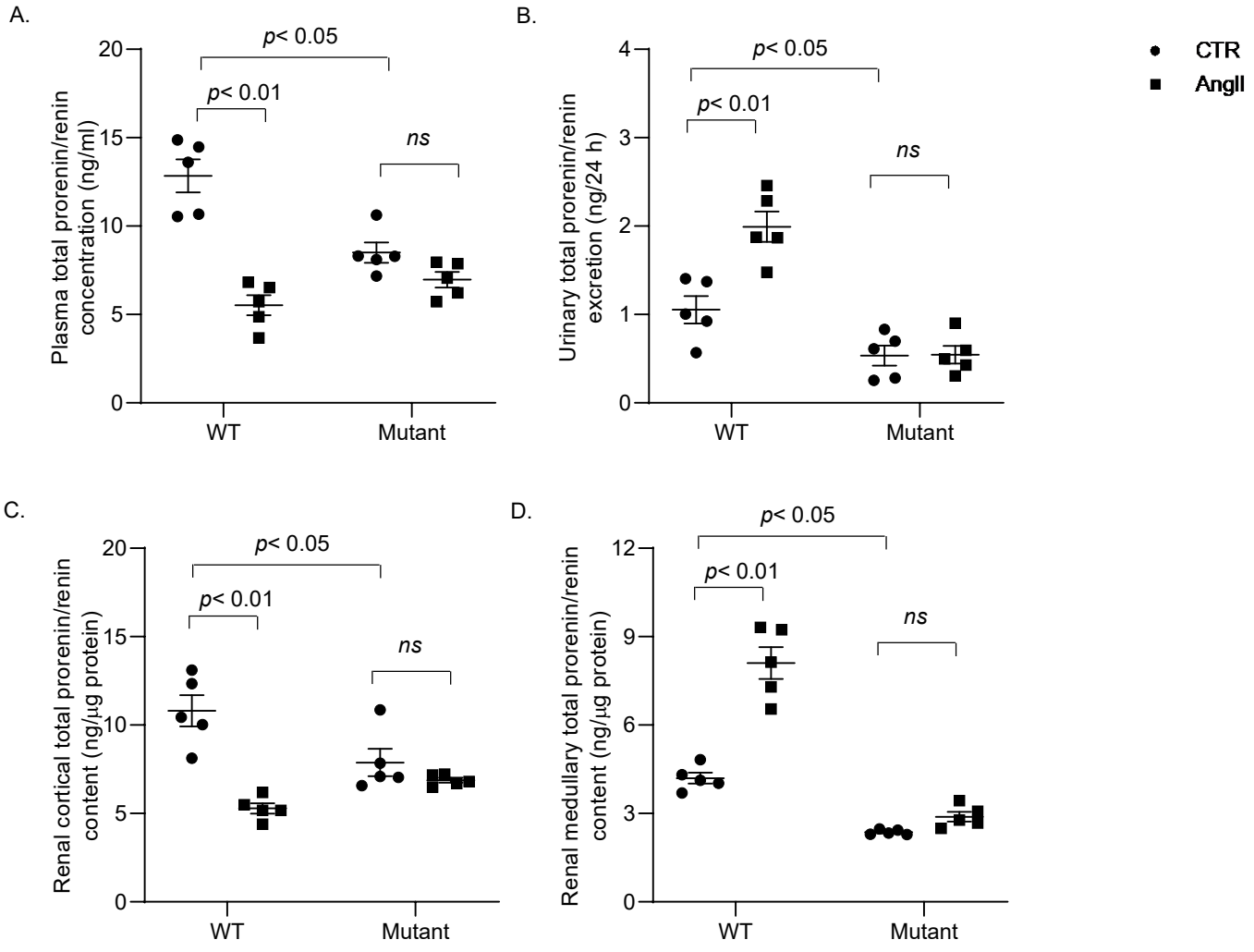

A.
B.
C.
D.
CTR
AngII
p< 0.05
p< 0.01
ns
Plasma total prorenin/renin concentration (ng/ml)
Urinary total prorenin/renin excretion (ng/24 h)
Renal cortical total prorenin/renin content (ng/µg protein)
Renal medullary total prorenin/renin content (ng/µg protein)
WT
Mutant
0
5
10
15
20
1
2
3
4
6
9
12

Figure S7

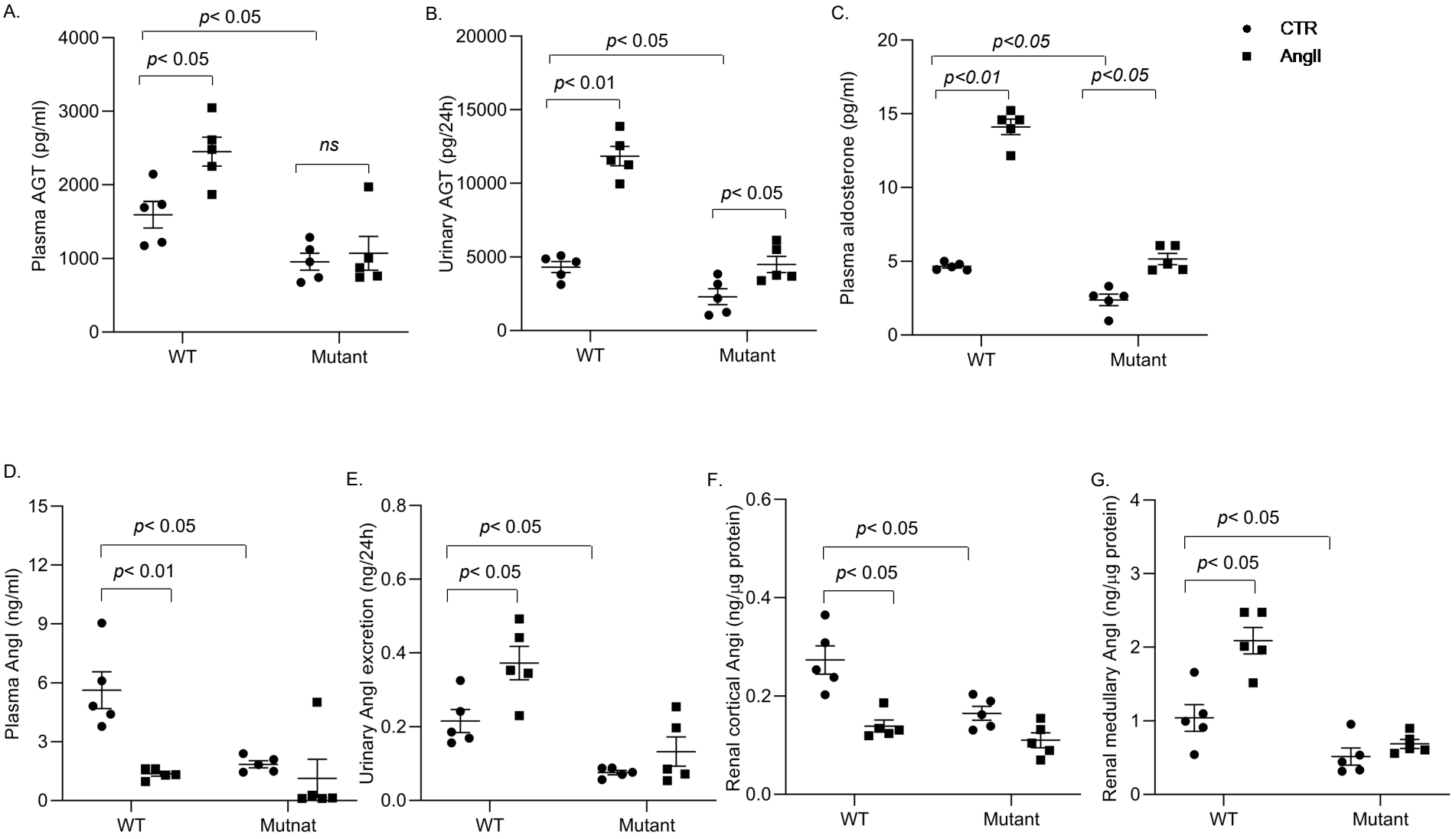

A.
Plasma AGT (pg/ml)
4000
3000
2000
1000
0
p< 0.05
p< 0.05
ns
WT
Mutant
B.
Urinary AGT (pg/24h)
20000
15000
10000
5000
0
p< 0.05
p< 0.01
p< 0.05
WT
Mutant
C.
Plasma aldosterone (pg/ml)
20
15
10
5
0
p<0.05
p<0.01
p<0.05
WT
Mutant
CTR
AngII
D.
Plasma Angl (ng/ml)
15
12
9
6
3
0
p< 0.05
p< 0.01
WT
Mutnat
E.
Urinary Angl excretion (ng/24h)
0.8
0.6
0.4
0.2
0.0
p< 0.05
p< 0.05
WT
Mutant
F.
Renal cortical Angi (ng/μg protein)
0.6
0.4
0.2
0.0
p< 0.05
p< 0.05
WT
Mutant
G.
Renal medullary Angl (ng/μg protein)
4
3
2
1
0
p< 0.05
p< 0.05
WT
Mutant

Figure S8

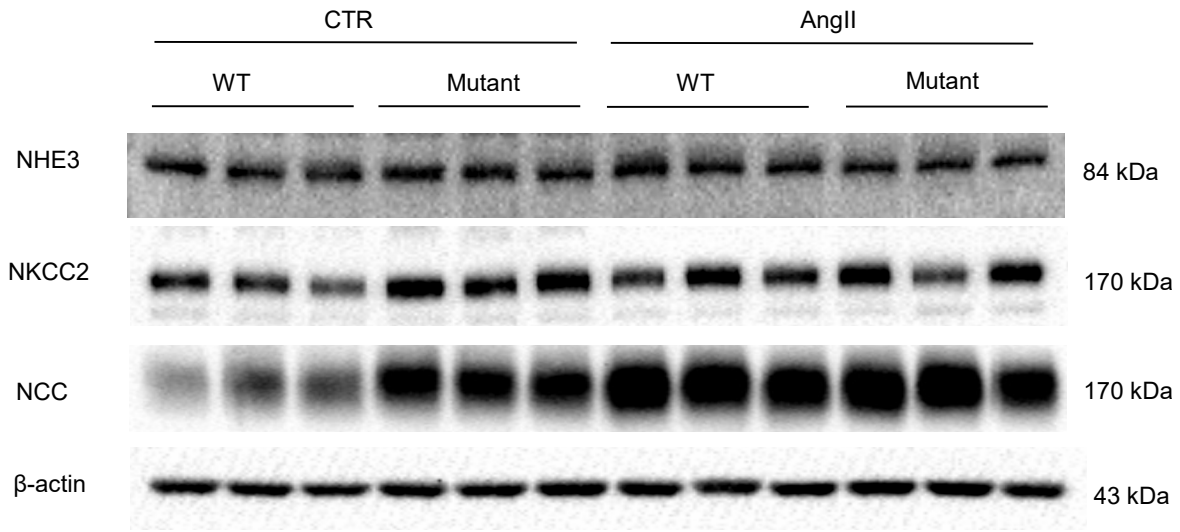

CTR
AngII
WT
Mutant
WT
Mutant
NHE3
84 kDa
NKCC2
170 kDa
NCC
170 kDa
β-actin
43 kDa

Figure S9

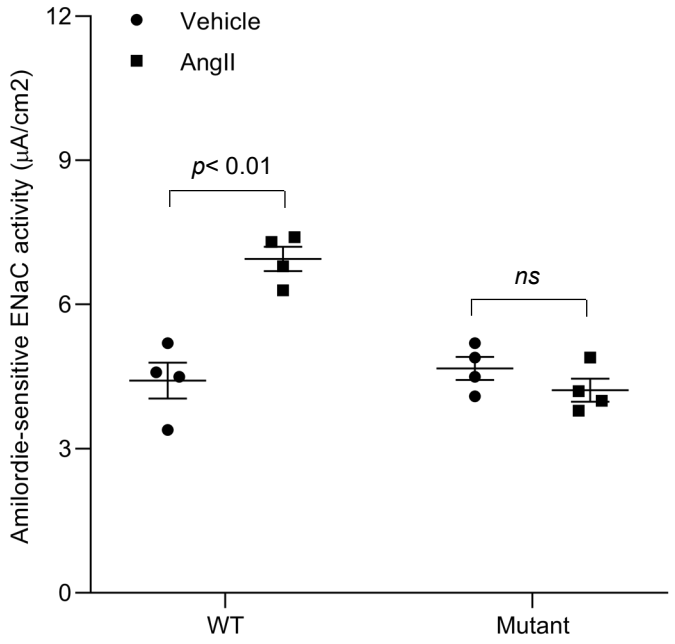
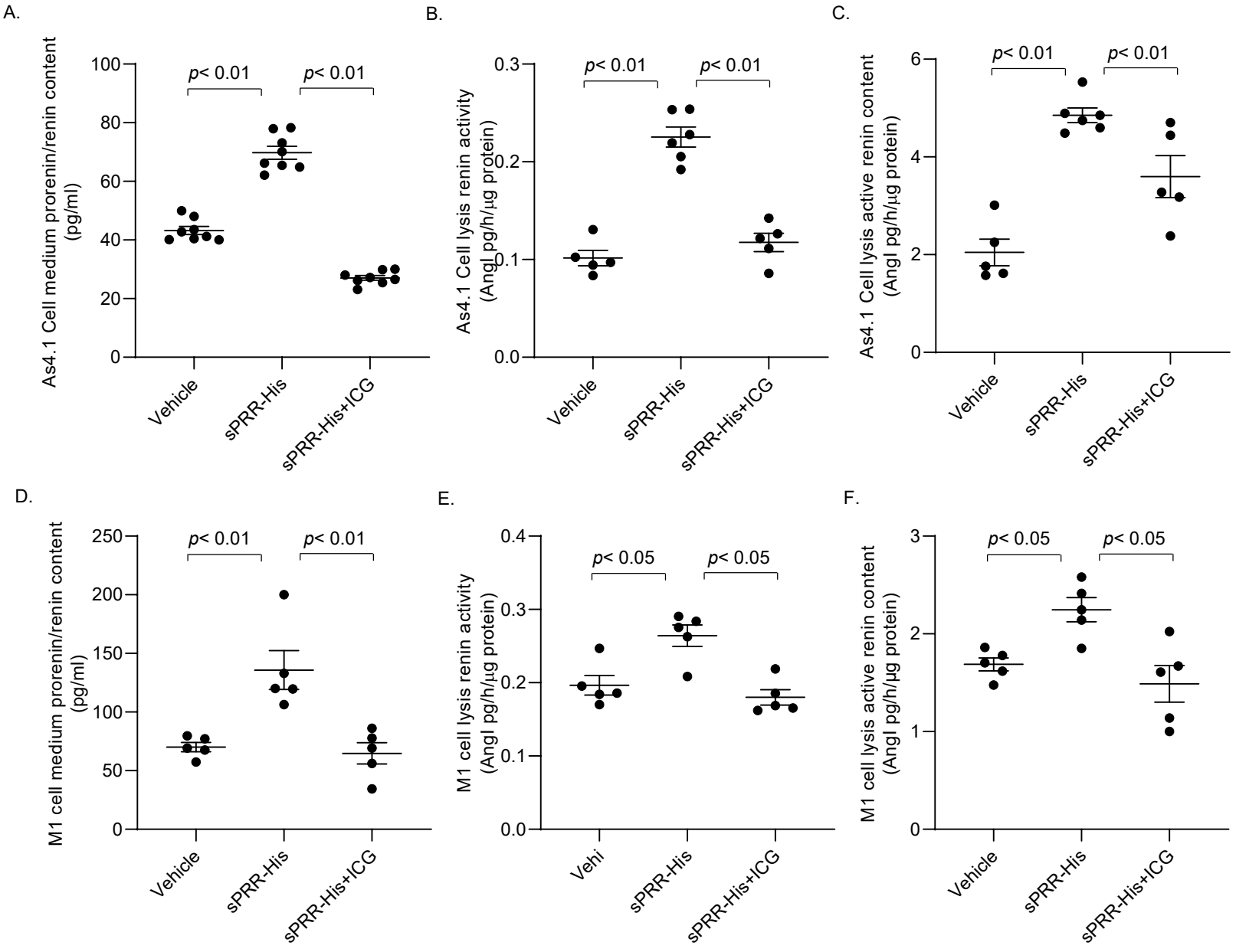

Vehicle
AngII
Amilordie-sensitive ENaC activity (μA/cm2)
12
9
6
3
0
p< 0.01
ns
WT
Mutant

Figure S10


A.
As4.1 Cell medium prorenin/renin content (pg/ml)
p< 0.01
p< 0.01
Vehicle
sPRR-His
sPRR-His+ICG
B.
As4.1 Cell lysis renin activity (Angl pg/h/μg protein)
p< 0.01
p< 0.01
Vehicle
sPRR-His
sPRR-His+ICG
C.
As4.1 Cell lysis active renin content (Angl pg/h/μg protein)
p< 0.01
p< 0.01
Vehicle
sPRR-His
sPRR-His+ICG
D.
M1 cell medium prorenin/renin content (pg/ml)
p< 0.01
p< 0.01
Vehicle
sPRR-His
sPRR-His+ICG
E.
M1 cell lysis renin activity (Angl pg/h/μg protein)
p< 0.05
p< 0.05
Vehi
sPRR-His
sPRR-His+ICG
F.
M1 cell lysis active renin content (Angl pg/h/μg protein)
p< 0.05
p< 0.05
Vehicle
sPRR-His
sPRR-His+ICG