Antidiuretic Action of Collecting Duct (Pro)Renin Receptor Downstream of Vasopressin/EP4 Receptor

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Running title: Function of collecting duct PRR

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METHODS

Immunofluorescence staining. The tissues were fixed in 10% neutral buffered formalin for 24 h and then embedded in paraffin. After deparaffinization, thin sections (4 μm) were processed for double-labeling with immunofluorescence. The slides were blocked in 1% BSA for 1 h and were then co-incubated with Rabbit anti-PRR antibody (Abcam, Cambridge, MA) which was against residues 335-350 in the C terminus (termed anti-PRR-C antibody), Goat anti-AQP2 antibody (Santa Cruz, Dallas, Texas) at 4°C for overnight. After washing off the primary antibody, sections were incubated for 1 h at room temperature with Donkey anti-goat-IgG-FITC (Santa Cruz, Dallas, TX) and Donkey anti-Rabbit IgG- TRITC (Life Technologies, Grand island, NY).

Generation of mice with collecting duct-specific PRR gene deficiency. Mice with conditional deletion of PRR in the CD was generated by genetic crosses between PRR floxed mice[(1)] and AQP2-Cre mice [(2)]. Since the PRR gene is on X-chromosome, male mice heterozygous for floxed PRR and for AQP2-Cre (termed CD PRR KO) presumably carry complete deletion of PRR in the CD. Only male CD PRR KO mice were used in the present study. The DNA recombination was assessed in microdissected nephron segments including glomerulus (Glom), proximal convoluted tubule (PCT), cortical thick ascending limb (cTAL), distal convoluted tubule (DCT), cortical collecting duct (CCD) from CD PRR KO mice as previously described [(3)]. Male 12-wk-old CD PPR KO mice and their littermate floxed control mice were subjected to WD treatment and sample collections as described above for rats except that mice were dehydrated for 24 h.

Renin activity assay. The urine, plasma and medium were diluted with the EIA buffer, the renal cortex and inner medulla were homogenized in 2.6 mM EDTA, 3.4 mM hydroxyquinoline, 5

mM ammonium acetate, 200 μM PMSF, and 0.256 μM dimercaprol. The homogenates were centrifuged at 4,000 rpm at 4°C for 30 min and the supernatant was harvested. Renin activity in plasma, urine, tissue homogenates and medium was determined by the delta value of the AngI generation using an ELISA kit from the sample incubating at 4°C and 37°C, respectively. Active renin concentration was determined with excessive angiotensinogen, and total renin concentration with excessive angiotensinogen plus trypsinization. The prorenin concentration was calculated from subtracting the total renin concentration with active renin concentration.

qRT-PCR. Total RNA was isolated from renal tissues and reverse transcribed to cDNA. Oligonucleotides were designed using Primer3 software (available at http://bioinfo.ut.ee/primer3-0.4.0). Primers of renin: 5'- cctctctgggcactcttgtt-3' (sense) and 5'-atgtctactccccgctcctc-3' (antisense); primers for GAPDH: 5'-gtcttcactaccatggagaagg-3' (sense) and 5'-tcatggatgaccttggccag-3' (antisense).

Immunoblotting. Renal tissues were lysed and subsequently sonicated. Protein concentrations were determined by using Coomassie reagent. Forty μg of protein for each sample was denatured in boiling water, then separated by SDS-PAGE, and transferred onto nitrocellulose membranes. Blots were blocked 1h with 5% nonfat dry milk in Tris-buffered saline (TBS), followed by incubation for overnight with anti-PRR antibody. After washing with TBS, blots were incubated with goat anti-rabbit/mouse horseradish peroxidase (HRP) -conjugated secondary antibody and visualized using enhanced chemiluminescence (ECL). The blots were quantitated by using Imagepro-plus.

Statistical analysis. Data is summarized as means + SE. All data points, animals, and humans were included in the statistical analyses. Sample sizes were determined on the basis of similar

previous studies or pilot experiments and no test of normality or power analysis was performed. Statistical analysis for animal and cell cultures experiments was performed by using ANOVA with the Bonferroni test for multiple comparisons or by paired or unpaired Student' t-test for two comparisons. The correlation between urine osmolality and urine sPRR concentrations in humans was analyzed by using Spearman correlation analysis using SPSS 19 (IBM). $p<0.05$ was considered statistically significant.

References

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2. Nelson, R.D., Stricklett, P., Gustafson, C., Stevens, A., Ausiello, D., Brown, D., and Kohan, D.E. 1998. Expression of an AQP2 Cre recombinase transgene in kidney and male reproductive system of transgenic mice. *Am J Physiol* 275:C216-226.
3. Zhang, H., Zhang, A., Kohan, D.E., Nelson, R.D., Gonzalez, F.J., and Yang, T. 2005. Collecting duct-specific deletion of peroxisome proliferator-activated receptor gamma blocks thiazolidinedione-induced fluid retention. *Proc Natl Acad Sci U S A* 102:9406-9411.

Figure Legends

Supplemental Fig. 1. Immunostaining analysis of renal cortical PRR in dehydrated rats. Rats were water deprived for 48 h or had free access to water. Immunostaining analysis of renal cortical PRR was performed by using anti-PRR-C antibody. Red, PRR; green, AQP2.

Supplemental Fig. 2. Immunostaining analysis of renal medullary PRR in dehydrated rats. Rats were water deprived for 48 h or had free access to water. Immunostaining analysis of renal medullary PRR was performed by using anti-PRR-C antibody. Red, PRR; green, AQP2.

Supplemental Fig. 3. Measurement of renin levels during antidiuresis and after EP_4 antagonism. Renin activity assay was performed to determine renin activity and prorenin content in plasma, the cortex, the inner medulla, and urine in Control, WD, and WD + ONO rats. (A) Plasma prorenin concentration (n = 4 rats per group). (B) Renal cortical prorenin concentration (n = 4 rats per group). (C) Renal inner medullary prorenin concentration (n = 4 rats per group). (D) Urinary prorenin excretion (n = 4 rats per group). (E) Plasma renin activity (n = 4 rats per group). (F) Renal cortical renin activity (n = 4 rats per group). (G) Renal inner medullary renin activity (n = 4 rats per group). (H) Urinary renin activity (n = 4 rats per group). Data are mean $\pm$ SE.

Supplemental Fig. 4. Assessment of renin levels by ELISA and qRT-PCR. ELISA was performed to determine prorenin/renin content in plasma, the cortex, the inner medulla, and urine in Control, WD, and WD + ONO rats. Renin mRNA in the tissues was determined by qRT-PCR and normalized by GAPDH. (A) Plasma prorenin/renin concentration (n = 5 rats per group). (B) Renal cortical prorenin/renin concentration (n = 5 rats per group). (C) Renal inner medullary prorenin/renin concentration (n = 5 rats per group). (D) Urinary prorenin/renin excretion (n = 5 rats per group). (E) Renal cortical renin mRNA expression (n = 5 rats per group). (F) Renal inner medullary renin mRNA expression (n = 5 rats per group). Data are mean $\pm$ SE.

Supplemental Fig. 5. Validation of CD-specific KO of PRR. (A) PCR validation of DNA recombination in microdissected nephron segments. Various nephron segments were microdissected from CD PRR KO mice and subjected to PCR analysis of DNA recombination. The 1381-bp band denotes the floxed allele and the 381-bp band denotes the recombined allele. (B) Confirmation of PRR deletion at protein level. Renal medulla including outer and inner medulla from the two genotypes was subjected to immunoblotting analysis of PRR (n = 4 mice per group). The same blot in Fig. 9A was stripped and reprobed with anti-PRR-C antibody. The same β-actin data in Fig.9A was used to normalize PRR densitometry. Data are mean $\pm$ SE.

Fig. S1

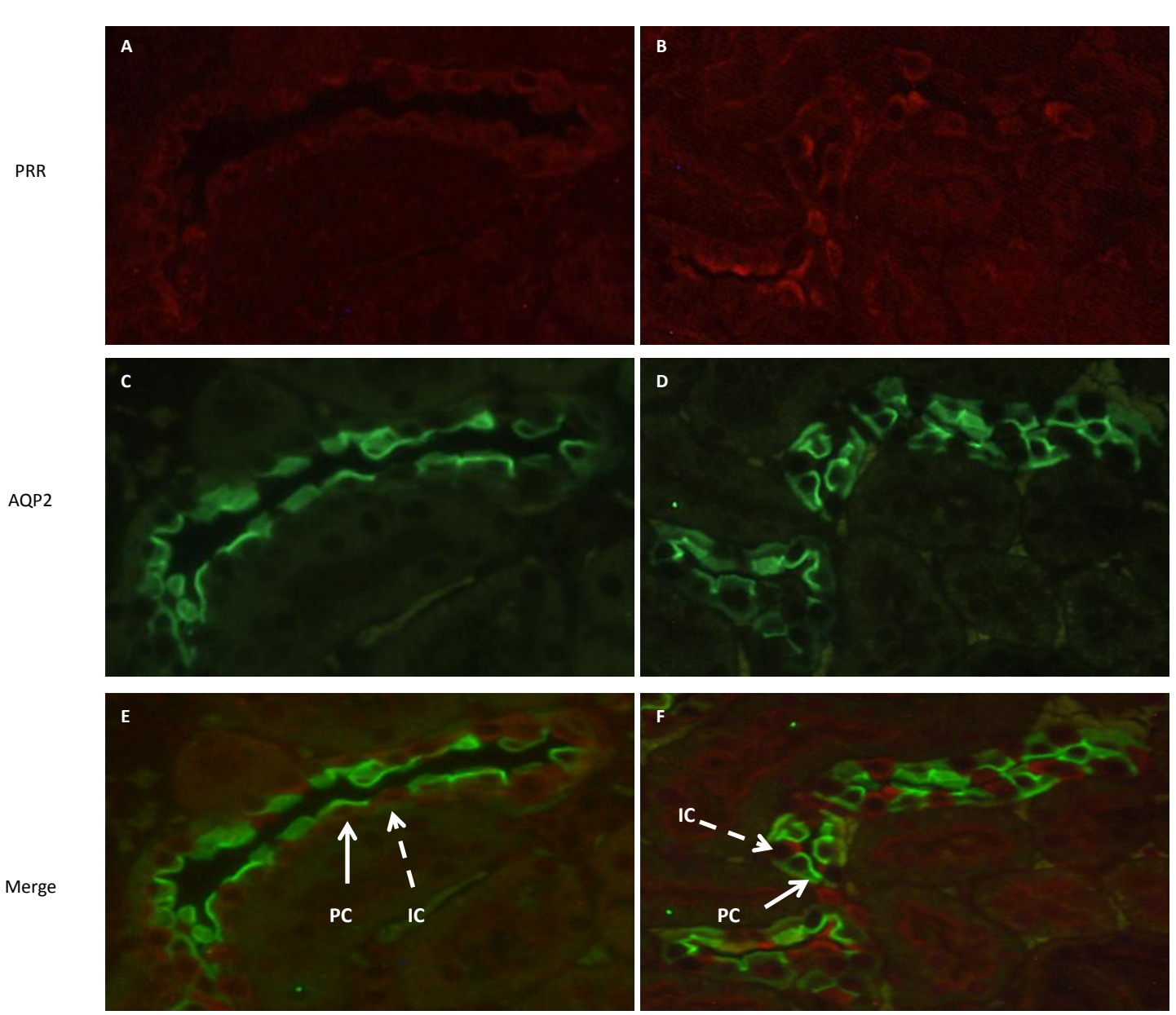
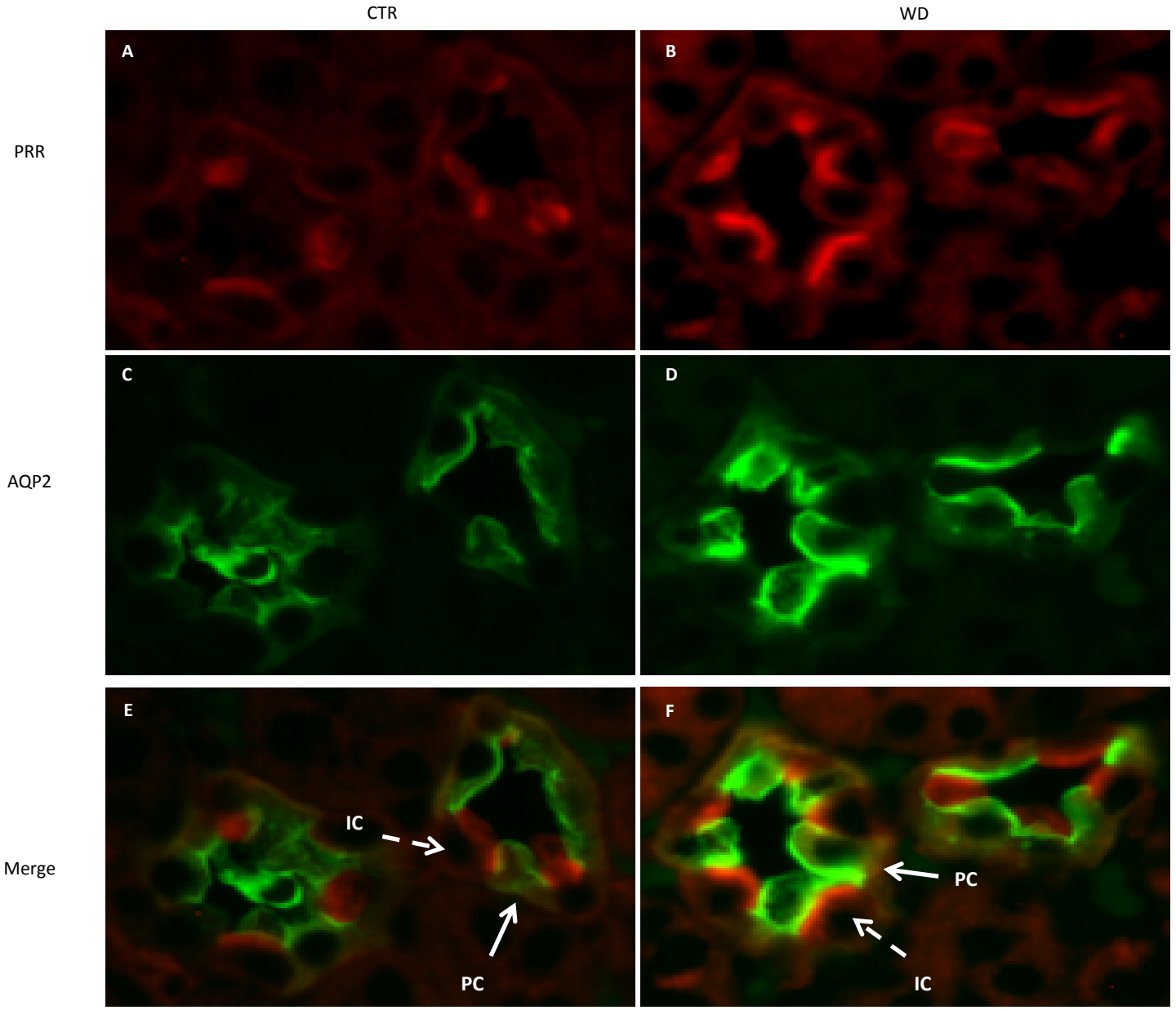


Fig.S2



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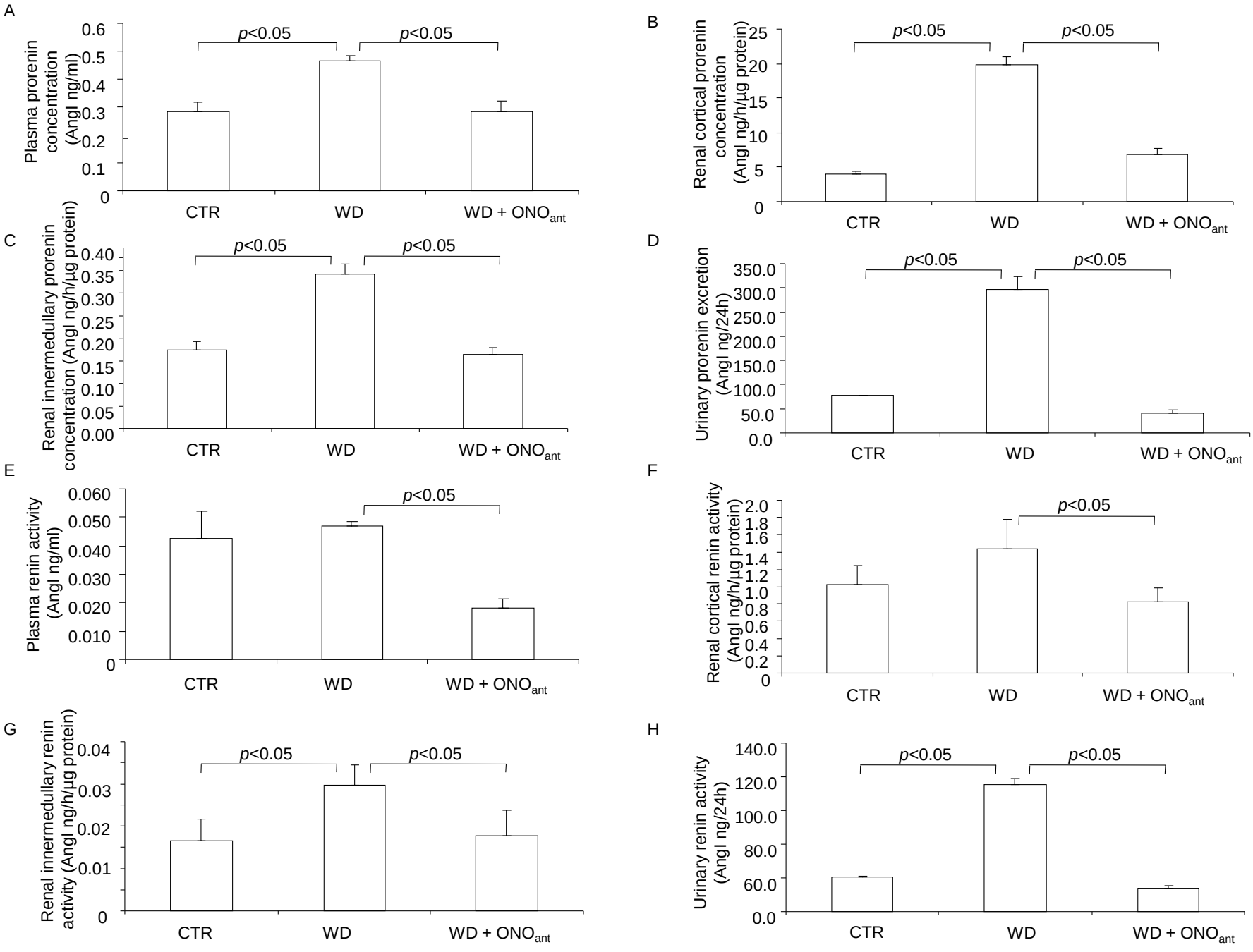

A
Plasma prorenin concentration (AngI ng/ml)
0.6
0.5
0.4
0.3
0.2
0.1
0
p<0.05
p<0.05
CTR
WD
WD + ONOant
B
Renal cortical prorenin concentration (AngI ng/h/µg protein)
25
20
15
10
5
0
p<0.05
p<0.05
CTR
WD
WD + ONOant
C
Renal innermedullary prorenin concentration (AngI ng/h/µg protein)
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
p<0.05
p<0.05
CTR
WD
WD + ONOant
D
Urinary prorenin excretion (AngI ng/24h)
350.0
300.0
250.0
200.0
150.0
100.0
50.0
0.0
p<0.05
p<0.05
CTR
WD
WD + ONOant
E
Plasma renin activity (AngI ng/ml)
0.060
0.050
0.040
0.030
0.020
0.010
0
p<0.05
CTR
WD
WD + ONOant
F
Renal cortical renin activity (AngI ng/h/µg protein)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
p<0.05
CTR
WD
WD + ONOant
G
Renal innermedullary renin activity (AngI ng/h/µg protein)
0.04
0.03
0.02
0.01
0
p<0.05
p<0.05
CTR
WD
WD + ONOant
H
Urinary renin activity (AngI ng/24h)
140.0
120.0
100.0
80.0
60.0
0.0
p<0.05
p<0.05
CTR
WD
WD + ONOant

Fig. S4

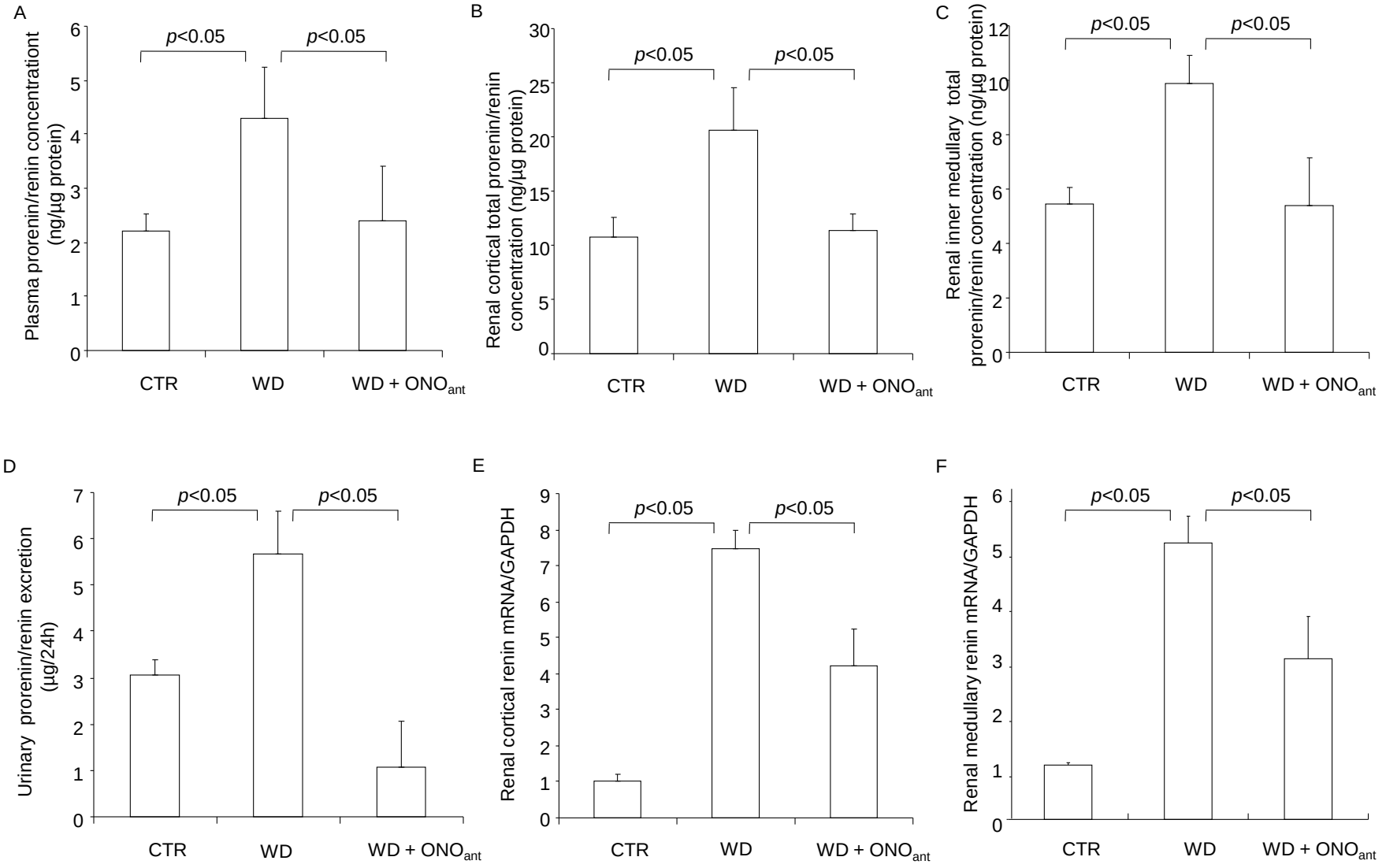
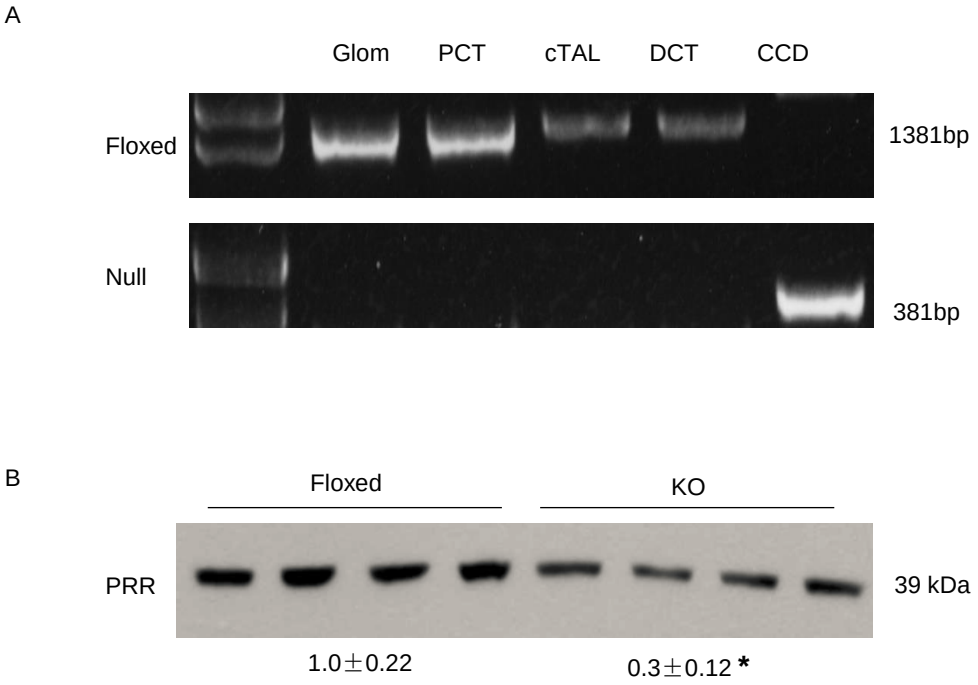

A
Plasma prorenin/renin concentrationt (ng/µg protein)
p<0.05
p<0.05
CTR
WD
WD + ONOant
B
Renal cortical total prorenin/renin concentration (ng/µg protein)
p<0.05
p<0.05
CTR
WD
WD + ONOant
C
Renal inner medullary total prorenin/renin concentration (ng/µg protein)
p<0.05
p<0.05
CTR
WD
WD + ONOant
D
Urinary prorenin/renin excretion (µg/24h)
p<0.05
p<0.05
CTR
WD
WD + ONOant
E
Renal cortical renin mRNA/GAPDH
p<0.05
p<0.05
CTR
WD
WD + ONOant
F
Renal medullary renin mRNA/GAPDH
p<0.05
p<0.05
CTR
WD
WD + ONOant

Fig. S5


A
Glom
PCT
cTAL
DCT
CCD
Floxed
1381bp
Null
381bp
B
Floxed
KO
PRR
39 kDa
1.0±0.22
0.3±0.12 *