

Proximal Tubule-Specific Deletion of the NHE3 (Na⁺/H⁺ exchanger 3) in The Kidney Attenuates Angiotensin II-Induced Hypertension in Mice

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Short Title: *Role of proximal tubule NHE3 in ANG II-induced hypertension*

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ONLINE SUPPLEMENT

Supplemental Methods and Materials

Animals

Adult male and female wild-type (WT), genetically modified, proximal tubule-specific knockout of NHE3 (PT-*Nhe3*^{-/-}) mice were used in all experimental protocols in the present study, as approved by the Institutional Animal Care and User Committee of the University of Mississippi Medical Center.

Wild-type mice. As both original *Nhe3*^{loxlox} ^{1,2} and iL1-SGLT2-Cre mouse strains were generated and back-crossed to several generations on the C57BL/6J genetic background, ^{1,3} both male and female age-matched C57BL/6J mice were used as wild-type controls.

Proximal tubule-specific *Nhe3*^{-/-} mice (PT-*Nhe3*^{-/-}) mice. PT-*Nhe3*^{-/-} mice were generated in this laboratory using the gold standard *SGLT2-Cre/Nhe3*^{loxlox} approach, as described previously. ¹⁻³ *Nhe3*^{loxlox} mice were generated on the C57BL/6J genetic background by Li *et al.* and breeding pairs of these mice were provided by Dr. Manoocher Soleimani of the University of Cincinnati. ² iL1-Sglt2-Cre transgenic mouse strain was generated on the C57BL/6J genetic background by and provided by Rubera *et al.* ³ Heterozygous male (or female) *Nhe3*^{loxlox} mice were mated to female (or male) iL1-sglt2 promoter-driven Cre mice to generate homozygous male and female PT-*Nhe3*^{-/-} mice for the present study. ¹⁻³

Experimental Protocols

Measurement of basal systolic, diastolic and mean arterial blood pressure, glomerular filtration rate (GFR), proximal tubule Na⁺ reabsorption, and urinary Na⁺ excretion in adult male and female PT-*Nhe3*^{-/-} mice. Systolic (SBP), diastolic (DBP) and mean arterial blood pressure (MAP) were measured in 2 groups (n=8-15 per group) of conscious adult male and female WT and PT-*Nhe3*^{-/-} mice using both the implanted telemetry (Data Science International) and noninvasive tail-cuff approaches using Visitech Blood Pressure Analysis Systems BP-200 Series II (Visitech, NC). ⁴⁻⁶ The whole-kidney GFR was measured in 2 groups (n=8-10 per group) of male and female WT and PT-*Nhe3*^{-/-} mice using the transdermal GFR monitoring approach with fluorescein-labeled sinistrin, FITC-sinistrin, as described. ^{7,8} The whole-kidney proximal tubule Na⁺ reabsorption and 24 h urinary Na⁺ and K⁺ excretion was measured in 2 groups (n=8-12 per group) of male and female WT and PT-*Nhe3*^{-/-} mice using metabolic cage and the noninvasive lithium clearance technique as we described previously. ^{1,5,6,9}

Induction of ANG II-induced hypertension in adult male and female WT and PT-Nhe3^{-/-} mice. Six groups (n=5-12 per group) of adult male and female WT and PT-Nhe3^{-/-} mice were studied. Group 1 served as time-control without treatment; Group 2 were infused with a high pressor (1.5 mg/kg/day, *i.p.*)¹⁰ or a slow pressor dose of ANG II via an osmotic minipump (0.5 mg/kg/day, *i.p.*) supplemented with a 2% NaCl diet for 2 weeks;⁴ and Group 3 were treated with ANG II as in Group 2, but concurrently treated with the AT₁ receptor blocker losartan (20 mg/kg/day, *p.o.*, in drinking water).⁴ SBP, DBP, and MAP were measured at basal and weekly after treatments with or without ANG II, 2% high salt diet, or losartan.

The pressure-natriuresis response to ANG II-induced, male and female hypertensive WT and PT-Nhe3^{-/-} mice. The pressure-natriuresis response was studied in 2 groups (n=8 per group) of male and female WT and PT-Nhe3^{-/-} mice, infused with the slow pressor dose of ANG II, 0.5 mg/kg/day, *i.p.*, and fed a 2% NaCl diet, to determine whether ANG II infusion impairs the pressure-natriuresis response and whether deletion of NHE3 selectively in the proximal tubules attenuates ANG II-induced hypertension by improving the pressure-natriuresis response.¹

Effect of pharmacological inhibition of NHE3 in the proximal tubule of the kidney by an orally absorbable NHE3 inhibitor on angiotensin II-induced hypertension in C57BL/6J mice. Five groups (n=5-15 per groups) of adult male C57BL/6J mice were used to test whether the pharmacological inhibition of NHE3 mainly in the proximal tubules of the kidney attenuates ANG II-induced hypertension. Group 1 served as time-controls. Group 2 were treated with an orally active, readily absorbable NHE3 inhibitor AVE0657 alone (a generous gift from Sanofi-Aventis; 20 mg/kg/day, *p.o.*) to inhibit NHE3 selectively in the kidney, primarily in the proximal tubules and less in the loop of Henle. The dose of AVE0657 used in the present study was based on the blood pressure and 24 h urinary Na⁺ excretory responses from preliminary dose-dependent studies using 5, 10, and 20 mg/kg/day, *p.o.* Group 3 were infused with a slow pressor dose of ANG II (0.5 mg/kg/day, *i.p.*, via minipump) and fed a 2% NaCl diet to slowly and moderately induce ANG II-dependent hypertension in 2 weeks.⁴ Group 4 were infused with ANG II as in Group 3, but treated with AVE0657 for 2 weeks as in Group 2. Group 5 were treated as in Group 4, but adding losartan (20 mg/kg/day, *p.o.*) to block AT₁ receptors. SBP, DBP, and MAP and 24 h fecal and urinary sodium excretion were determined at basal and weekly after treatments with ANG II, 2% high salt diet, AVE0657 or losartan.

Induction of L-NAME-induced hypertension in male and female hypertensive WT and PT-Nhe3^{-/-} mice. To determine whether genetic deletion of NHE3 selectively in the proximal tubules of the kidney prevents L-NAME-induced hypertension, 4 groups (n=10 per group) of adult male and female WT and PT-Nhe3^{-/-} mice were treated without or with the non-selective nitric oxide synthase inhibitor L-NAME (50 mg/kg/day, *p.o.*) for 4 weeks. SBP, DBP, and MAP, and 24 h urinary Na⁺ and K⁺ excretion were determined before and weekly after L-NAME treatment.

Statistical analysis

All results are presented as mean \pm SEM. The differences between different groups of male and female WT and PT-*Nhe3*^{-/-} mice in all responses including SBP, DBP and MAP, 24 h urinary and fecal Na⁺ excretion, the hypertensive responses to ANG II or L-NAME, and the pressure-natriuretic response to ANG II were first analyzed using one-way ANOVA in all groups or treatments, followed by Student's unpaired t test if a significant response between groups or treatments of WT and PT-*Nhe3*^{-/-} mice was detected. The significance of statistical differences between responses were set $P < 0.05$.

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Supplemental Figures

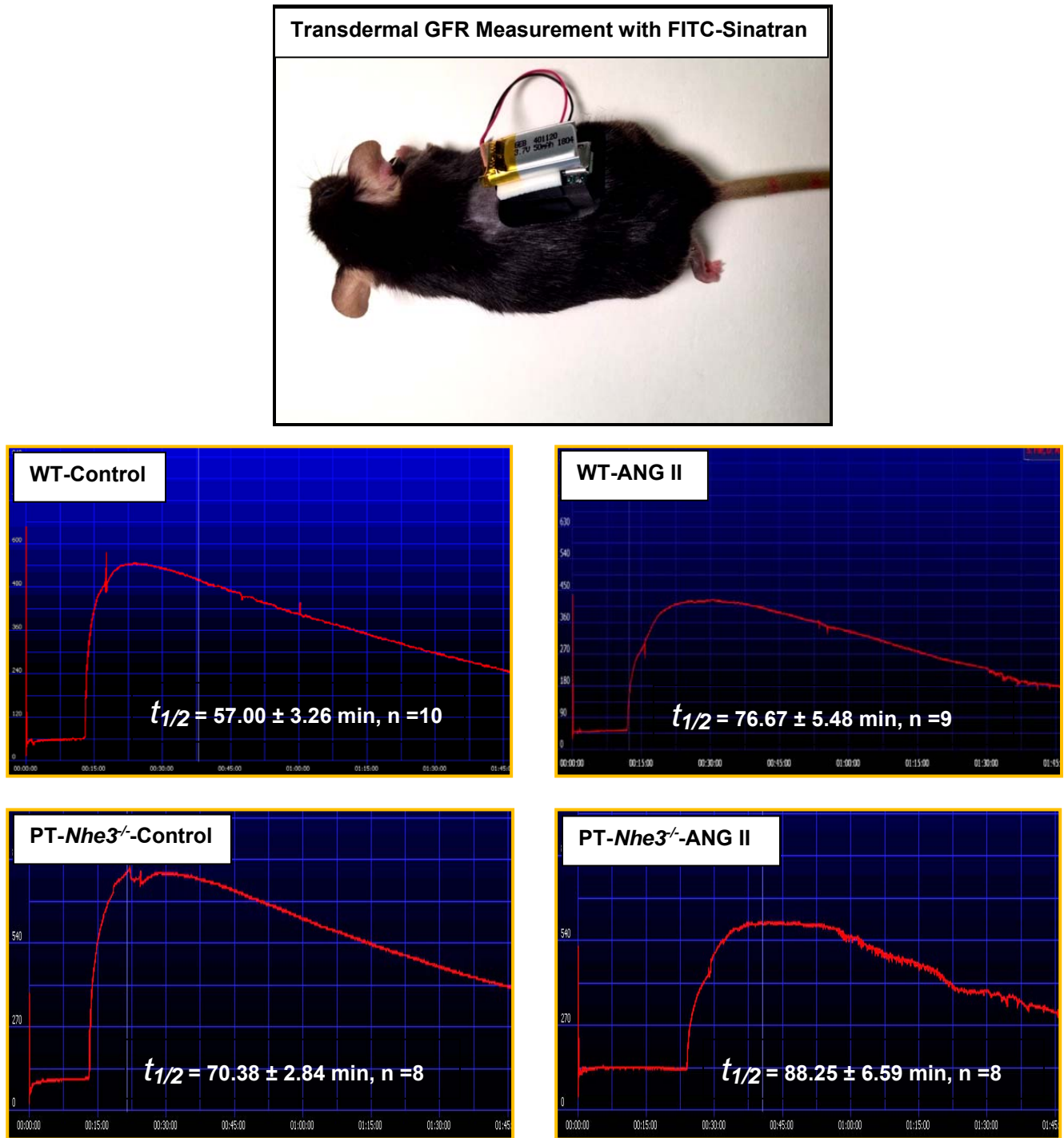


Figure S1. Measurement glomerular filtration rate (GFR) in control and angiotensin II-infused WT and PT-*Nhe3*^{-/-} mice using transdermal GFR device with FITC-Sinistrin (10 mg/100 g body wt., i.v.). FITC-Sinistrin was dissolved in sterilized 0.9% saline as a stock solution of 10 mg/1 mL. Mice were anesthetized with pentobarbital (50 mg/kg, i.p.)

and FITC-Sinistrin was injected directly into the left jugular vein via a fine catheter, whereas blood FITC levels were continuously monitored for 2 h.

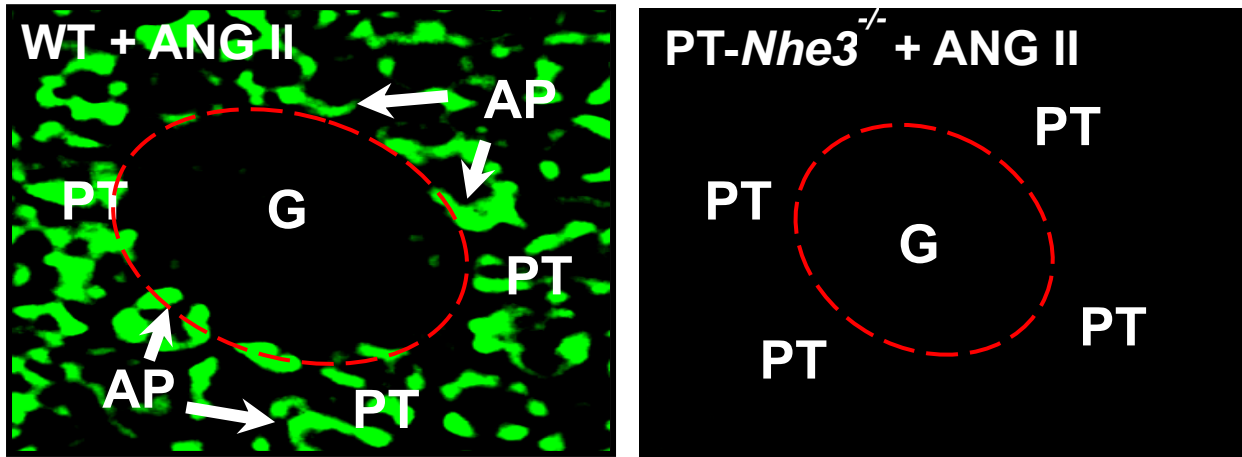


Figure S2. Effect of a slow pressor dose of ANG II infusion, 0.5 mg/kg/day, i.p., via an osmotic minipump for 2 weeks significantly increases NHE3 proteins in apical membranes in the proximal tubules of the kidney in a representative WT mouse (left panel), compared with a representative PT-*Nhe3*^{-/-} mouse (right panel). G, glomerulus. PT, proximal tubule.

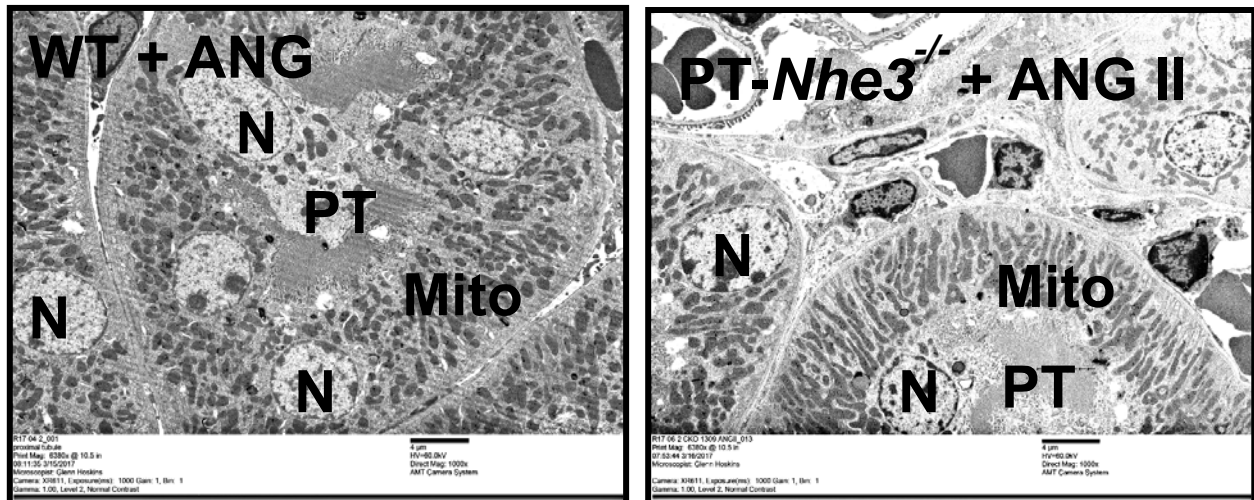


Figure S3. High resolution electron micrographs showing the ultrastructures a representative proximal tubule (PT) in an ANG II-infused WT and a PT-*Nhe3*^{-/-} mouse, respectively. Print Magnification: 6380 X. The size, orientation, lumen, brush border membranes, mitochondria (Mito), cell nuclei (N) are comparable between WT and PT-*Nhe3*^{-/-} mouse. This suggests that the antihypertensive effect of PT-specific NHE3 deletion is unlikely due to the structural responses of the proximal tubules.

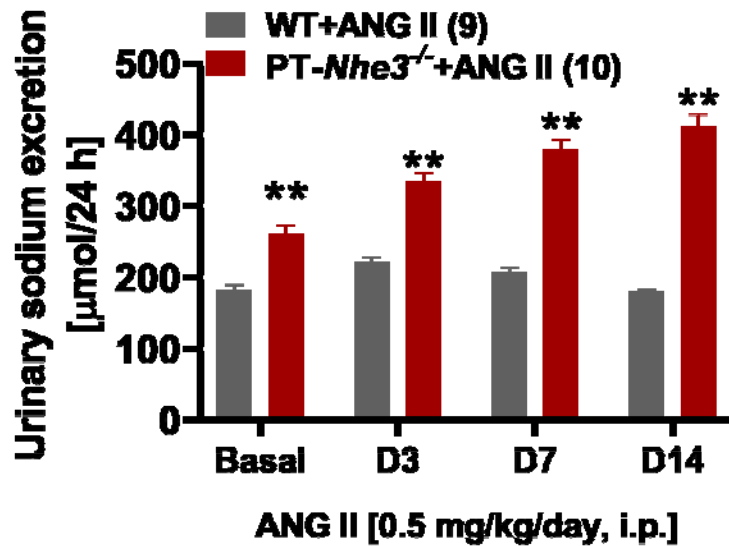


Figure S4. Time-dependent 24 h urinary Na^+ excretory responses to a slow pressor dose of ANG II infusion, 0.5 mg/kg/day, i.p., via osmotic minipump for 2 weeks in adult male WT and PT-*Nhe3*^{-/-} mice. ** $P < 0.01$ vs. WT. These data suggest that the pressure-natriuresis response was impaired in ANG II-infused WT, and improved in ANG II-infused PT-*Nhe3*^{-/-} mice.

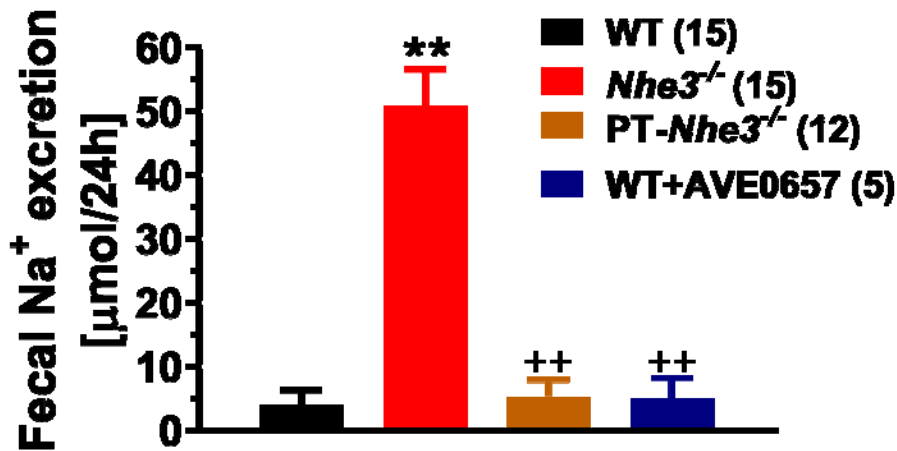


Figure S5. Effect of treatment with an orally absorbable NHE3 inhibitor AVE0657, 20 mg/kg/day, p.o., for 2 weeks on 24 h fecal Na^+ excretion in male adult C57BL/6J mice, which is compared with those of WT control, global *Nhe3*^{-/-}, or PT-*Nhe3*^{-/-} mice. There are no differences in 24 h fecal Na^+ excretion between WT mice treated with or without AVE0657 or PT-*Nhe3*^{-/-} mice. However, 24 h fecal Na^+ excretion was 10-time higher in *Nhe3*^{-/-} than WT mice. These data show that unlike global *Nhe3*^{-/-} mice which have severe salt wasting from the small intestines, the absorbable NHE3 inhibitor AVE0657 does not inhibit NHE3 in small intestines and causes salt wasting in feces. This excludes the possibility that AVE0657 attenuates ANG II-induced hypertension in part by increasing 24 h fecal Na^+ excretion and salt wasting from small intestines. ** $P < 0.01$ vs. WT control mice; ++ $P < 0.01$ vs. global *Nhe3*^{-/-} mice.

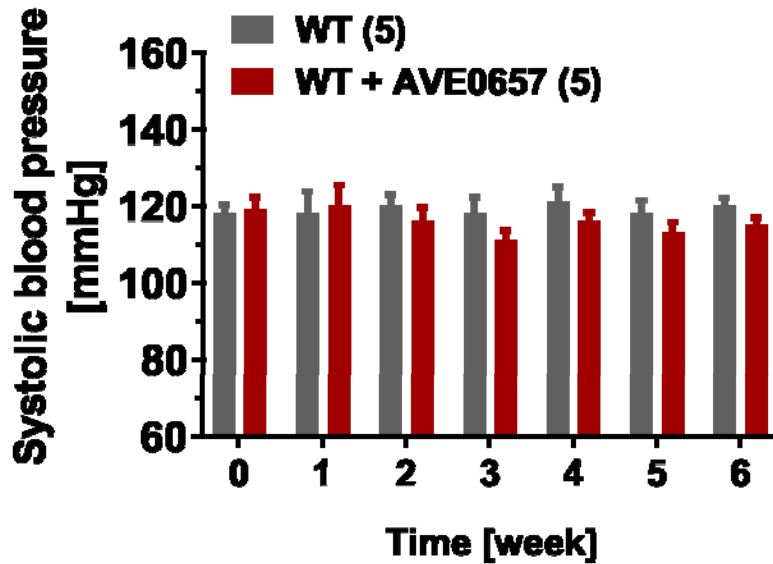


Figure S6. Effect of treatment with an orally absorbable NHE3 inhibitor AVE0657, 10 mg/kg/day, p.o., in drinking water alone for 6 weeks slightly, but not significantly, decreased systolic blood pressure after 2 weeks in male adult C57BL/6J mice. This suggests that a higher dose of AVE0657, 20 mg/kg/day, p.o., is required to attenuate the development of ANG II-induced hypertension in WT mice.

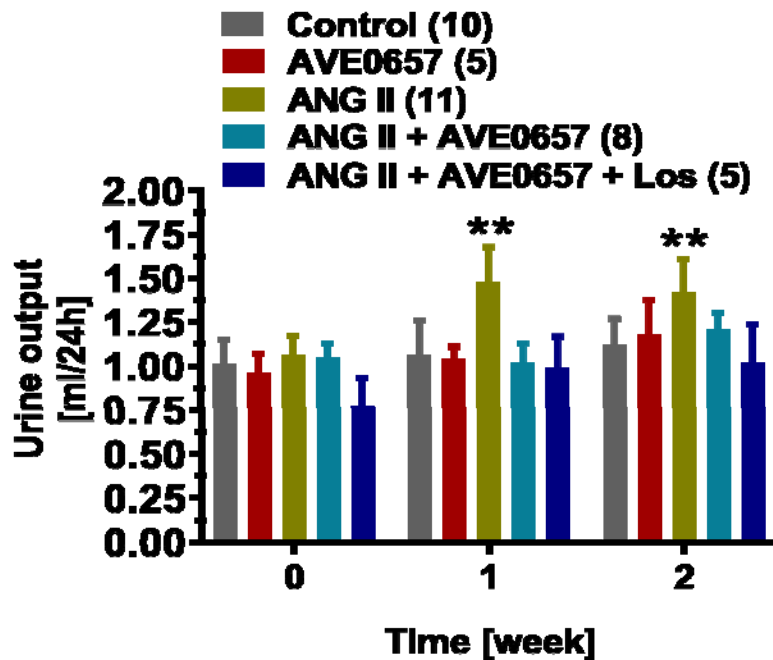


Figure S7. Effect of treatment with an orally absorbable NHE3 inhibitor AVE0657, 20 mg/kg/day, p.o., in drinking water alone for 2 weeks on 24 h urine output in adult male WT mice. AVE0657 alone or with ANG II (0.5 mg/kg/day, i.p.) or losartan (20 mg/kg/day, p.o.) had no significant effect on 24 h urine output. ** $P < 0.01$ vs. the time-control group.

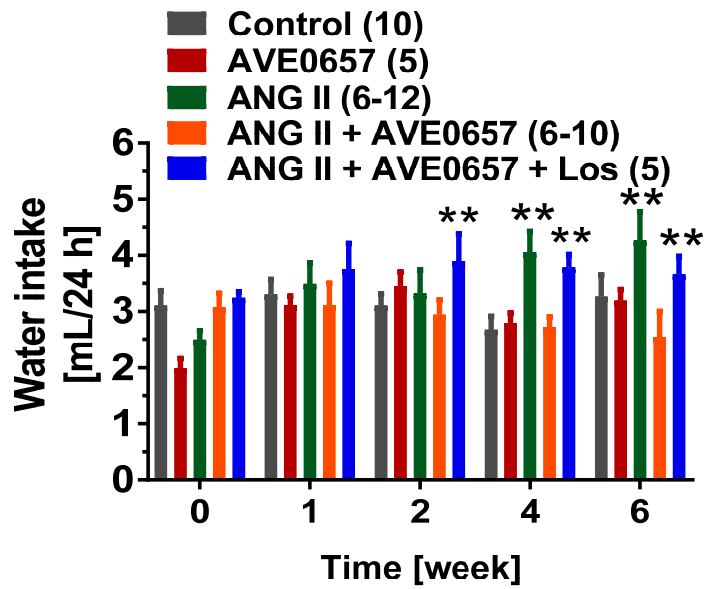


Figure S8. Effect of treatment with an orally absorbable NHE3 inhibitor AVE0657, 20 mg/kg/day, p.o., in drinking water alone for 6 weeks on 24 h water intake in adult male WT mice. AVE0657 alone had no significant effect on 24 h water intake, suggesting a Na⁺-specific effect. ***P*<0.01 vs the time-control group.