ORIGINAL RESEARCH

Effect of Angiotensin II on ENaC in the Distal Convoluted Tubule and in the Cortical Collecting Duct of Mineralocorticoid Receptor Deficient Mice

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BACKGROUND: Angiotensin II stimulates epithelial Na^+ channel (ENaC) by aldosterone-independent mechanism. We now test the effect of angiotensin II on ENaC in the distal convoluted tubule (DCT) and cortical collecting duct (CCD) of wild-type (WT) and kidney-specific mineralocorticoid receptor knockout mice (KS-MR-KO).

METHODS AND RESULTS: We used electrophysiological, immunoblotting and renal-clearance methods to examine the effect of angiotensin II on ENaC in KS-MR-KO and wild-type mice. High K^+ intake stimulated ENaC in the late DCT/early connecting tubule (DCT2/CNT) and in the CCD whereas low sodium intake stimulated ENaC in the CCD but not in the DCT2/CNT. The deletion of MR abolished the stimulatory effect of high K^+ and low sodium intake on ENaC, partially inhibited ENaC in DCT2/CNT but almost abolished ENaC activity in the CCD. Application of losartan inhibited ENaC only in DCT2/CNT of both wild-type and KS-MR-KO mice but not in the CCD. Angiotensin II infusion for 3 days has a larger stimulatory effect on ENaC in the DCT2/CNT than in the CCD. Three lines of evidence indicate that angiotensin II can stimulate ENaC by MR-independent mechanism: (1) angiotensin II perfusion augmented ENaC expression in KS-MR-KO mice; (2) angiotensin II stimulated ENaC in the DCT2/CNT but to a lesser degree in the CCD in KS-MR-KO mice; (3) angiotensin II infusion augmented benzamil-induced natriuresis, increased the renal K^+ excretion and corrected hyperkalemia of KS-MR-KO mice.

CONCLUSIONS: Angiotensin II-induced stimulation of ENaC occurs mainly in the DCT2/CNT and to a lesser degree in the CCD and MR plays a dominant role in determining ENaC activity in the CCD but to a lesser degree in the DCT2/CNT.

Key Words: aldosterone ■ AT1R ■ hyperkalemia ■ hypertension

The epithelial Na^+ channel (ENaC) is expressed in the apical membrane of the late distal convoluted tubule (DCT2), connecting tubule (CNT) and cortical collecting duct (CCD).[1–4] Moreover, electrophysiology and immunostaining experiments have shown that ENaC activity/expression is larger in the late DCT2/early CNT than in the CCD of animals on a normal Na^+ diet.[1,5] Although ENaC is responsible only for the reabsorption of 1% filtered sodium load under physiological conditions, gain-of-function-mutations of ENaC are associated with hypertension.[6–8] Moreover, since Na^+ absorption by ENaC across the apical membrane generates the lumen-negative transepithelial potential which is essential for K^+ secretion through ROMK (Kir1.1),[9,10] the inhibition of ENaC impairs renal K^+ excretion and causes hyperkalemia whereas the stimulation of ENaC increases renal K^+ excretion and leads to hypokalemia.[9]

ENaC is composed of 3 subunits, ENaCα, ENaCβ, and ENaCγ, it is well established that aldosterone is the most important hormone regulating ENaC activity and stimulating the expression of ENaCα in the aldosterone-sensitive distal nephron segments.[11] The effect of

CLINICAL PERSPECTIVE

What Is New?

- Angiotensin II stimulates epithelial Na+ channel in the distal convoluted tubule by a mineralocorticoid receptor-independent mechanism.
- Type I angiotensin II receptor(AT1R) plays a role in the regulation of epithelial Na+ channel activity in the distal convoluted tubule but it is less important in the cortical collecting duct.

What Are the Clinical Implications?

- Our study provides the molecular mechanism by which the inhibition of AT1R causes hyperkalemia in hypertension patients treated with AT1R antagonist.
- Since AT1R activity stimulates K+ excretion by a mineralocorticoid receptor-independent mechanism, patients receiving both mineralocorticoid receptor and AT1R antagonists are more vulnerable for developing hyperkalemia.

Nonstandard Abbreviations and Acronyms

AngII	angiotensin II
AT1R	angiotensin II type 1 receptor
CCD	cortical collecting duct
CNT	connecting tubule
DCT	distal convoluted tubule
ENaC	epithelial sodium channel
HK	high potassium
LS	low Sodium
MR	mineralocorticoid receptor
ENaC	epithelial sodium channel
ROMK	renal outer medullary K channel
SGK1	serum-glucocorticoid-induced Kinase 1

aldosterone on ENaC is mediated by stimulation of mineralocorticoid receptors (MR).[11–14] Acute stimulation of MR activates serum-glucocorticoid-induced kinase 1 (SGK1) thereby increasing ENaC activity by inhibiting Nedd4-2-dependent ubiquitination of ENaC.[12,15–18] Also, the activation of MR increases the transcription of ENaC in the aldosterone-sensitive distal nephron segments thereby upregulating ENaC expression.[11,19]

In addition to aldosterone, previous studies have demonstrated that angiotensin II (AngII) is able to stimulate ENaC activity in the aldosterone-sensitive distal nephron segments by an aldosterone-independent mechanism and that the effect of AngII is mediated by type I angiotensin II receptor (AT1R) since the inhibition of AT1R abolishes the effect of AngII on ENaC.[20–25]

Although both MR and AT1R are expressed in the aldosterone-sensitive distal nephron segments including DCT2 and CCD,[26] the finding that ENaC activity was largely inhibited in the CCD but not significantly affected in the DCT2/CNT of aldosterone synthase-deficient mice suggests that the regulation of ENaC by MR and AT1R may be different between these 2 segments.[5] We now hypothesize that AT1R plays a more important role in stimulating ENaC by an MR-independent mechanism in the DCT2/CNT than in the CCD. The hypothesis is tested by examining the effect of the endogenous aldosterone pathway and AngII pathway on ENaC activity using a renal tubule-specific MR-deficient mouse model.[27]

METHODS

The corresponding author will make the data, methods used in the analysis, and materials used to conduct the research available to any researcher for purposes of reproducing the results or replicating the procedures. The authors also declare that the procedures for animal study were in accordance with institutional guidelines.

Animals and Tubule Preparation

The generation of kidney-specific mineralocorticoid receptor knock-out (KS-MR-KO) mice were described previously,[27] and they were bred at New York Medical College for the experiments. Briefly, mice expressing Pax8-rtTA and tet-on LC-1 transgene were crossed with *mr*-floxed mice to generate inducible KS-MR-KO. MR deletion was performed in 8-week-old male and/or female mice homozygous for floxed *mr*-gene and heterozygous for Pax8-rtTA/LC-1 transgene by providing doxycycline (5 mg/mL, 2% sucrose) in the drinking water for 2 weeks. This was followed by at least 2 additional weeks without doxycycline treatment, before performing experiments. Littermate mice of the same age and genetic background drinking 2% sucrose were used as wild-type (WT). Ear DNA was polymerase chain reaction-amplified with the following primers: *mr*-flox7 (5′-CTGGAGATCTGAACTCCAGGCT) and *mr*-flox8 (5′-CCTAGAGTTCCTGAGCTGCTGA) and *mr*-flox10 (5′-TAGAAACACTTCGTAAAGTAGAGCT) yielding a 335 bp product from the floxed *mr*- gene and a 285 bp product from the wild-type allele; Pax8rtTA forward 5′-CCATGTCTAGACTGGACAAGA-3′ and Pax8rtTA reverse 5′-CAGAAAGTCTTGCCATGACT-3′ which yields a 220 bp product; and LC1-CRE forward 5′-TTTCCCGCAGAACCTGAAGATG-3′ and reverse 5′-TCACCGGCATCAACGTTTTCTT-3′ which yields a 190 bp product. To determine the efficacy of the MR deletion, we used quantitative real-time polymerase chain reaction as described previously.[28]

Because KS-MR-KO mice could not tolerate well with high K^+ (HK) or low sodium, the mice were fed with control diet (n=9, 0.4% NaCl and 1% KCl), with HK diet (n=6, 5% KCl) for 3 days, or LS diet (n=6, <0.01% NaCl) for 5 days and had free access to water. HK diet (catalog no#TD.110866) and LS diet (catalog no#TD.90228) were purchased from Harlan Laboratory (Madison, WI). To study the effect of AngII infusion on ENaC, we used a no-pressure dose of AngII (200 ng/min per kg BW) for continuous perfusion through a subcutaneously installed osmotic pump (Alzet, Palo Cupertino, CA). We followed the method described previously to prepare the DCT and CCD for the experiments.[29–32]

Isolation of DCT for the Patch-Clamp Experiments

After mice were euthanized by CO_2 inhalation plus cervical dislocation, the abdomen was opened to expose the left kidney, we perfused the left kidney with 2 mL L-15 medium (Life Technology) containing type 2 collagenase (250 unit/mL). The collagenase-perfused kidney was then removed for further dissection. The renal cortex was separated and cut into small pieces for additional incubation in collagenase-containing L-15 media for 30 to 50 minutes at 37°C. The tissue was then washed 3 times with fresh L-15 medium and transferred to an ice-cold chamber for dissection. The isolated tubules were placed on a small cover glass coated with poly-lysine and the cover glass was placed on a chamber mounted on an inverted microscope. The protocol for using mice was approved by New York Medical College independent animal use committee. We performed the patch-clamp experiments in split-open tubule for studying ENaC activity in the last 100 μm DCT before the transition between the DCT and CNT. Since the diameter of the DCT2 is normally larger than the CNT, this anatomic characterization has been used to determine the end of the DCT or the start of the CNT. Figure 1 is an image of an isolated DCT2/CNT with plain view (A) or with Na-Cl Cotranspoter (NCC) staining (B). It is apparent that NCC staining was clearly detected in the DCT. Moreover, the diameter of the CNT directly connected with DCT2 (indicated by an arrow) was smaller than those of the DCT2. However, it was not always obvious to identify the beginning of the CNT since the early portion of the CNT seems to have some morphological characteristic of DCT2. It was likely that some experiments were actually performed in the early CNT. Thus, we have referred that the study was performed in the DCT2/CNT. Also, Figure 1C and 1D show an image of an isolated tubule from DCT1 to CNT with a plain view or with ENaCα staining. It is apparent that ENaCα staining is visible from DCT2 to CNT and that ENaCα staining seems more robust in the DCT2 than in the CNT.

Whole-cell recording

We used an Axon 200A patch-clamp amplifier to record the amiloride-sensitive Na^+ currents with the perforated whole-cell recording which were low-pass filtered at 1 KHz, digitized by an Axon interface (Digidata 1440A). Data were collected with gap-free protocol and analyzed using the pClamp software system 9.0 (Axon). After forming a high resistance seal (>2 GΩ), the membrane capacitance was monitored until the whole-cell patch configuration was formed. The membrane capacity (which was used as an index of cell sizes) was also calculated by reading the compensation pF used to offset the membrane capacity induced by forming whole-cell membrane configuration. We have also checked the seal resistances after each experiment to make sure it was stable. ENaC currents were determined by adding amiloride (10 or 100 μmol/L) in the bath solution. Since both doses of amiloride have achieved the same level of inhibition of ENaC, we pooled the results. The pipette solution contained 125 mmol/L K-gluconate, 15 mmol/L KCl, 2 mmol/L MgATP, 1 mmol/L EGTA, and 10 mmol/L HEPES (pH 7.4), whereas the bath solution contained 130 mmol/L Na-gluconate, 10 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L $CaCl_2$, 2 mmol/L $MgCl_2$, and 5 mmol/L HEPES (pH 7.4). We filled the tip of the pipette first with the above solution without amphotericin B and pipette was then back-filled with the solution containing amphotericin B (20 μg/0.1 mL).

Procedures for Renal Clearance

Animal (n=5 for each group) were anesthetized by 2% to 4% isoflurane through an inhaling mask. The mice were placed on a heated small blanket to maintain body temperature at 37°C. The trachea was cannulated to clear any mucus that may be produced during the experiment. A carotid artery was catheterized with PE10 tubing for blood collection, jugular vein was also cannulated for intravenous infusion. The bladder was exposed and catheterized via a suprapubic incision with a 10-cm piece of PE-10 tubing for urine collections. After completion of surgery, isotonic saline was given intravenously for 4 hours (0.2–0.3 mL/1 hr and total 0.8–1.2 mL 0.9% saline) to replace surgical fluid losses and to maintain hemodynamics. Urine collections started 1 hour after infusion of 0.3 mL saline and a total of 6 collections (every 30 minutes) were performed (2 for controls and 4 for experiments). Benzamil concentration for renal clearance is 5 mg/kg BW. After renal clearance experiment, the mice were euthanized by intravenous somnasol.
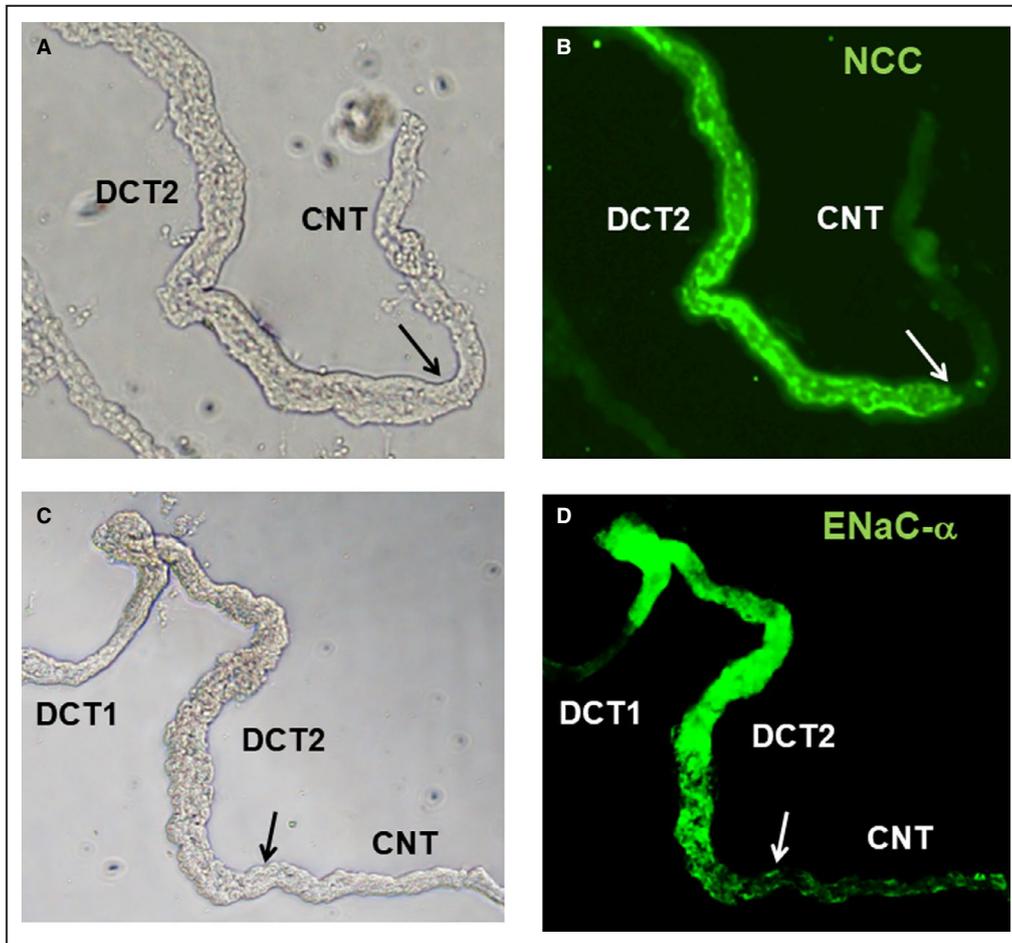
Figure 1. An image of an isolated tubule of the late distal convoluted tubule/connecting tubule.
A microscopic image shows an isolated distal convoluted tubule/connecting tubule with plain view (**A**) and with green fluorescence immunostaining of Na-Cl cotransporter (**B**). A microscopic image shows an isolated tubule from early distal convoluted tubule (DCT1), DCT2 to connecting tubule with a plain view (**C**) or with green fluorescence immunostaining of epithelial Na$^+$ channel-α subunit (**D**). The transition between DCT2 and connecting tubule is indicated by an arrow. Note that the diameter of the connecting tubule connected to the DCT2 is slightly smaller than the DCT2. The patch-clamp experiments were performed in the late DCT2 and early portion of connecting tubule. CNT indicates connecting tubule; DCT, distal convoluted tubule; ENaCα, epithelial Na$^+$ channel-α subunit; and NCC, Na-Cl cotransporter.

Immunoblotting

Whole kidney protein extract was obtained from frozen kidney (n=5 for each group) homogenized in a buffer containing 250 mmol/L sucrose, 50 mmol/L Tris-HCl pH 7.5, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L dithiothreitol (DTT) supplemented with phosphatase and protease inhibitor cocktails (Sigma). Protein (40–60 μg) was separated on 4% to 12% (wt/vol) Tris-Glycine gel (Thermo Fisher Scientific) and transferred to nitrocellulose membrane. The membranes were incubated 1 hour with LI-COR blocking buffer (PBS) and then incubated overnight at 4°C with anti-ENaC-α (Catalog # SPC-403, 1:1000, StressMarq), anti-ENaC-β (catalog# SPC-404, 1:1000, StressMarq) and anti-ENaC-γ subunit (Catalog # SPC-405, 1:1000, StressMarq).

An Odyssey infrared imaging system (LI-COR) was used to capture the images at a wavelength of 680 or 800 nmol/L.

Immunostaining

Isolated DCT/CNT tubules were placed on a small cover glass coated with poly-D-lysine (Sigma-Aldrich), and then were fixed with 4% paraformaldehyde in PBS for 30 minutes. We used 0.2% Triton-X100 in PBS containing 1% BSA to permeabilize tubular cells for 15 minutes. Unspecific binding sites were blocked with 5% donkey serum for 30 minutes at room temperature. Tubules were incubated with rabbit primary antibody of NCC (1:200) or ENaCα (1:500) for overnight at 4°C, washed with PBS, and incubated with
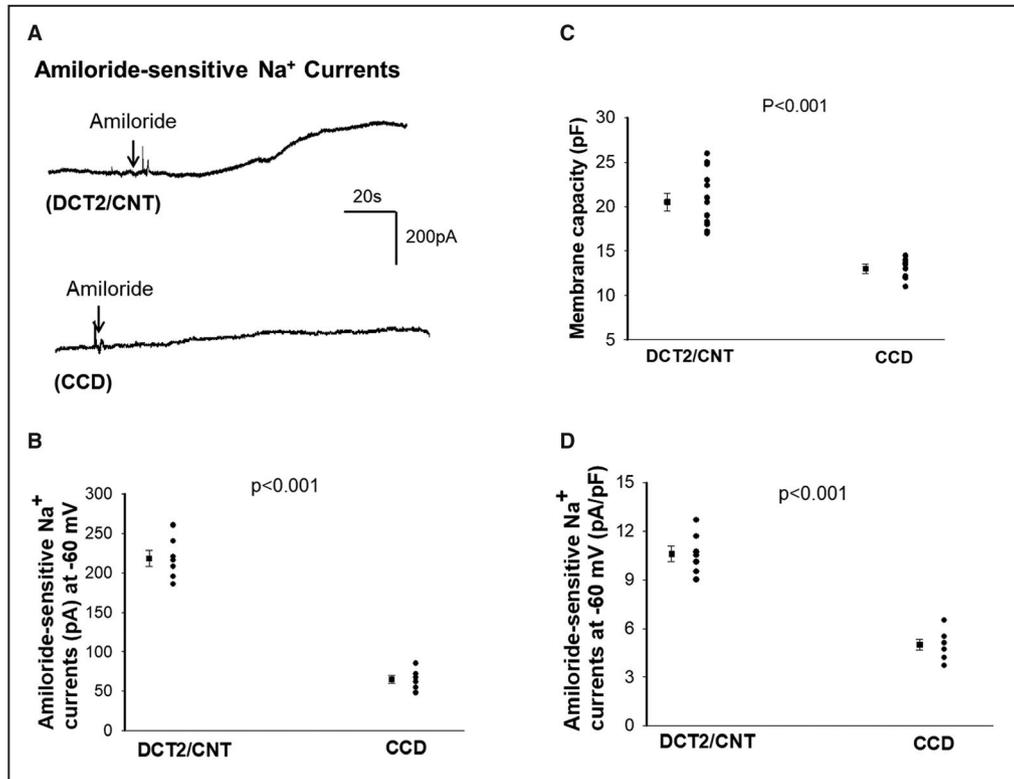
Figure 2. Epithelial Na⁺ channel in the late distal convoluted tubule/connecting tubule is larger than in the cortical collecting duct.
A, A typical whole-cell recording showing epithelial Na⁺ channel currents in the distal convoluted tubule/connecting tubule and in the cortical collecting duct, respectively. The currents were measured at −60 mV with the whole-cell recording. **B**, A scatter plot shows each data point and mean value of epithelial Na⁺ channel currents measured at −60 mV. **C**, A scatter plot shows each data point and mean value of the membrane capacity (pF) in the distal convoluted tubule2/connecting tubule and cortical collecting duct, respectively. **D**, A scatter plot shows the normalized epithelial Na⁺ channel density (pA/pF) for the distal convoluted tubule2/connecting tubule and cortical collecting duct. T-test was used for determining the significance between 2 groups. CCD indicates cortical collecting duct; DCT2-CNT, distal convoluted tubule2/connecting tubule.

Alexa Fluor 488-conjugated donkey anti-rabbit secondary antibody (1:200, Invitrogen) for 1 hour at room temperature.

Materials

Amiloride and benzamil were purchased from Sigma–Aldrich (St. Louis, MO). We have obtained NCC and GAPDH antibodies from StreeMarq and Cell Signaling, respectively.

Statistical Analysis

We used software of SigmaPlot to conduct the statistical analysis. For analyzing the values between 2 groups we used t test and for comparisons of the values within the same group we used paired t test. We used one-way ANOVA for analyzing results of ≥2 groups, and Holm-Sidak test was used as post-hoc analysis. $P < 0.05$ were considered statistically significant. Data are presented as mean±SEM.

RESULTS

We first examined the ENaC activity in the DCT2/CNT and CCD of the wild-type (WT) mice on a control diet (0.4% Na⁺ and 1% K⁺). Figure 2A is a recording of whole-cell Na⁺ currents (at −60 mV) conducted in DCT2/CNT and in the CCD, respectively. Figure 2B is a scatter plot showing each individual data point and the mean value of the amiloride-sensitive Na⁺ currents measured at −60 mV from DCT2/CNT and CCD. We confirmed the previous report that the amiloride-sensitive Na⁺ currents in the DCT2 (218±10 pA, n=7) was significantly larger than in the CCD (65±5 pA, n=6).[5] Considering the possibility that large Na⁺ currents in the DCT2/CNT may be because of the large cell membrane size of DCT2/CNT, we measured the cell capacitance between DCT2/CNT and CCD with the whole cell recording. Figure 2C is a scatter plot summarizing the experimental results showing the mean capacity of the DCT2/CNT (20.5±0.5 pF, n=13) was larger than that in the CCD (13.0±0.5 pF,
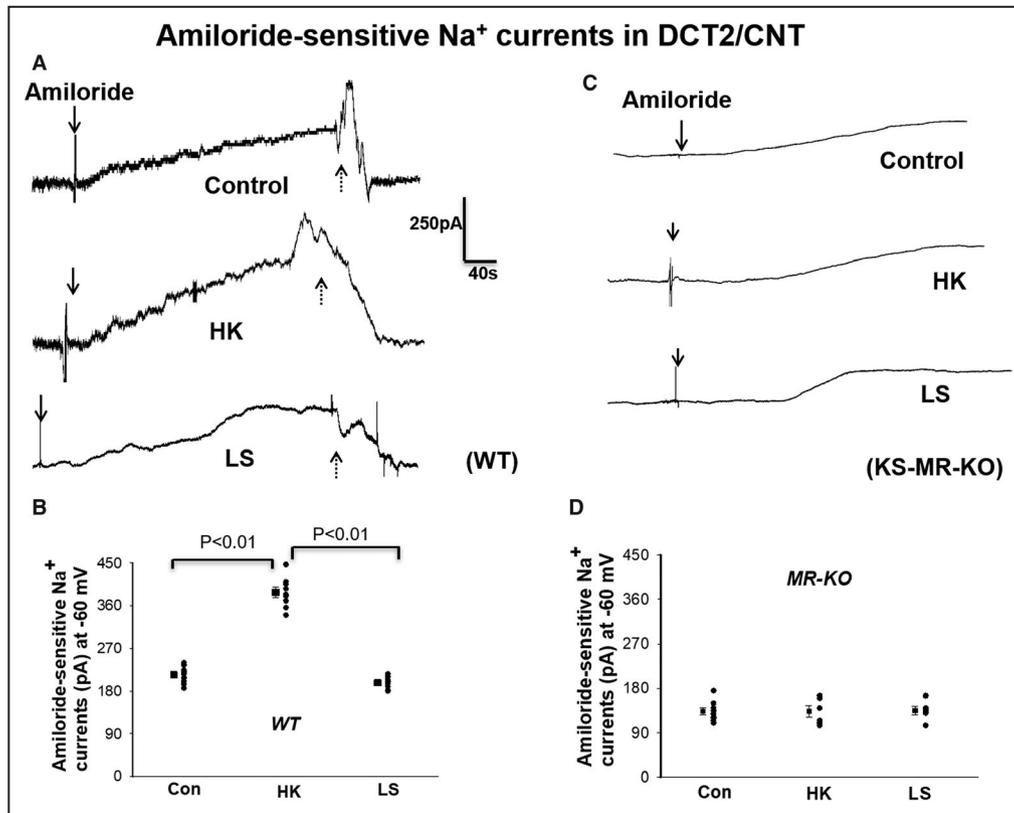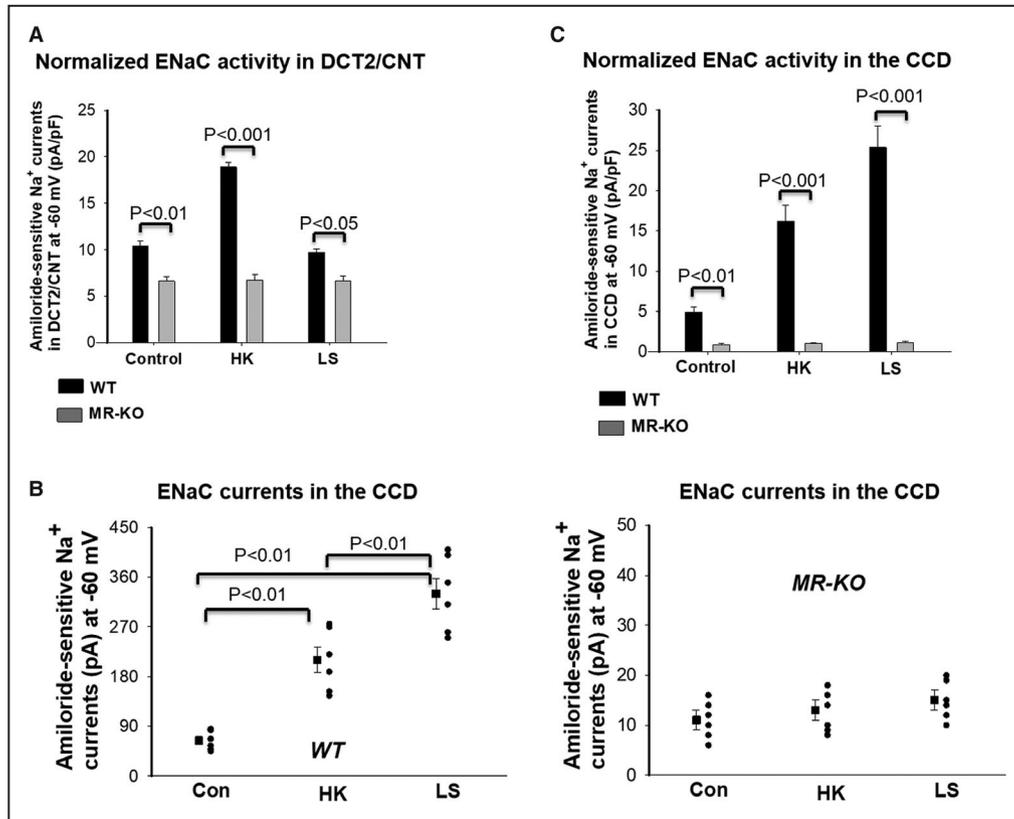
Figure 3. Deletion of mineralocorticoid receptor blunted but not abolished epithelial Na$^+$ channel activity in the late distal convoluted tubule2/connecting tubule.

A, A set of amiloride-sensitive Na$^+$ current traces measured with whole-cell recording at −60 mV in the distal convoluted tubule2/connecting tubule of the wild-type mice on control, high K$^+$ (3 days) or low sodium diets (5 days), respectively. **B**, A scatter plot shows the mean value and each data point of the above experiments. **C**, A set of amiloride-sensitive Na$^+$ current traces measured at −60 mV in the distal convoluted tubule2/connecting tubule of the kidney-specific mineralocorticoid receptor knockout mice on control, high K$^+$ (3 days) or low sodium diets (5 days), respectively. **D**, A scatter plot shows the mean value and each data point of the above experiments. The pipette solution contains 125 mmol/L K-gluconate, 15 mmol/L KCl, 2 mmol/L MgATP, 1 mmol/L EGTA, and 10 mmol/L N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES, pH 7.4). The bath solution contains 130 mmol/L Na-gluconate, 10 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L CaCl$_2$, 2 mmol/L MgCl$_2$, and 5 mmol/L HEPES (pH 7.4). Addition of amiloride (10 μmol/L) is indicated by solid arrows and the washout is indicated by a dotted arrow. One-way ANOVA test was used for the statistical analysis. HK indicates high K$^+$; KS-MR-KO, kidney-specific mineralocorticoid receptor knockout; LS, low sodium; and WT, wild-type.

n=9). However, the normalized Na$^+$ currents density in the DCT2/CNT (10.6±0.5 pA/pF, n=7) was significantly larger than in the CCD (5.0±0.34 pA/pF, n=6). Thus, the results indicate that ENaC density in the DCT2/CNT is higher than in the CCD.

Since aldosterone plays a key role in the regulation of ENaC, we then examined effects of aldosterone on ENaC in the DCT2/CNT by measuring the amiloride-sensitive whole-cell Na$^+$ current in the mice on a high K$^+$ diet (5% KCl, HK) for 3 days or a low sodium diet (<0.01% Na$^+$, LS) for 5 days to stimulate the endogenous aldosterone. Figure 3A is a set of recordings showing amiloride (10 μmol/L)-sensitive Na$^+$ current measured with whole-cell recording in the DCT2/CNT of the WT mice on control, HK and

LS diets, respectively. It is apparent that HK intake increased ENaC current of the DCT2 whereas LS intake did not stimulate ENaC activity in comparison with the control value. Figure 3B is a scatter plot summarizing results of 9 experiments showing the individual data point and the mean value measured at −60 mV. HK intake increased amiloride-sensitive Na$^+$ currents from 214±6 pA to 388±11 pA but LS intake did not significantly alter ENaC currents (198 ±4 pA) in comparison with control value. These results were consistent with the previous report that LS diet had no significant effect on ENaC currents in DCT2.[5] To determine whether the stimulatory effect of HK on EnaC in the DCT2/CNT depended on MR, we next examined EnaC currents in the DCT2/

Figure 4. Deletion of mineralocorticoid receptor largely abolished epithelial Na⁺ channel activity in the cortical collecting duct.

A, A bar graph summarizes the normalized epithelial Na$^+$ channel activity (pA/pF) in the late distal convoluted tubule2/connecting tubule of the wild-type mice and kidney-specific mineralocorticoid receptor knockout mice on control, high K$^+$ (3 days) or low sodium diets for 5 days, respectively. T-test was used for determining the significance between 2 groups. **B**, A scatter plot shows the mean value and each data point of amiloride-sensitive Na$^+$ currents measured with whole-cell recording at −60 mV in the CCD of the wild-type mice (Left) and kidney-specific mineralocorticoid receptor knockout mice (right) on control, high K$^+$ (3 days) or low sodium diets (5 days), respectively. One-way ANOVA test was used for the statistical analysis. **C**, A bar graph summarizes the normalized epithelial Na$^+$ channel activity (pA/pF) in the CCD of the wild-type mice and kidney-specific mineralocorticoid receptor knockout mice on control, high K$^+$ (3 days) or low sodium diets for 5 days, respectively. T-test was used for determining the significance between 2 groups. The amiloride-sensitive Na$^+$ currents were measured at −60 mV with whole-cell recording. CCD indicates cortical collecting duct; DCT2-CNT, distal convoluted tubule2/connecting tubule; ENaC, epithelial Na$^+$ channel; HK, high K$^+$; LS, low sodium; and MR-KO, mineralocorticoid receptor knockout.

CNT of KS-MR-KO mice on control, HK or LS diets. Figure 3C is a set of recordings showing amiloride-sensitive Na$^+$ currents measured in the DCT2/CNT of KS-MR-KO mice on different diets. Results of the experiments are summarized in a scatter plot (Figure 3D) showing the mean value and each data point measured at −60 mV. The deletion of MR significantly reduced the amiloride-sensitive Na$^+$ currents in the DCT2/CNT of the mice on control diet (134±7 pA, n=9), HK diet (133±12 pA, n=6), and LS diet (135±9 pA, n=6). Moreover, it is apparent that the effect of HK on ENaC was completely absent in KS-MR-KO mice, suggesting that the stimulatory effect of HK on ENaC was dependent on MR presence.

Although the effect of HK on ENaC was absent in the DCT2/CNT of KS-MR-KO mice, the deletion of

MR only partially decreased ENaC current. Figure 4A is a bar graph showing the normalized ENaC density in the DCT2/CNT of WT after the consideration of the cell membrane capacity (control, 10.4±0.5 pA/pF; HK 18.9±0.6 pA/pF; LS 9.7±0.4 pA/pF, n=9) and KS-MR-KO mice (control, 6.6±0.5 pA/pF; HK 6.7±0.6 pA/pF; LS 6.6±0.6 pA/pF, n=6–9). The data have suggested that a large portion of ENaC activity in the DCT2/CNT was regulated by MR-independent mechanism.

After showing that MR was only partially responsible for regulating ENaC in the DCT2/CNT, we next examined the ENaC activity in the CCD of WT and KS-MR-KO mice using whole-cell recording. Figure 4B is a scatter plot summarizing results of 6 experiments showing the individual data point and the mean value of ENaC currents measured at −60 mV. HK intake

increased amiloride-sensitive Na$^+$ currents from 64±7 pA to 210±23 pA and LS intake further increased ENaC currents (330 ±28 pA). The deletion of MR not only decreased ENaC currents (11 ±2 pA, n=6) under control conditions but also abolished the effect of HK (13 ±2 pA, n=6) and LS (15 ±2 pA, n=6) on ENaC current in the CCD. Considering the fact that principal cell size was smaller than those in the DCT2/CNT, we presented data in pA/pF to make a comparison with DCT2/CNT. Figure 4C is a bar graph showing that HK intake significantly increased ENaC currents in the CCD from 4.9±0.6 pA/pF (n=6) to 16.2±2 pA/pF (n=6) and that LS intake further increased ENaC currents in the CCD to 25.4±2.6 pA/pF (n=6), a value

was significantly larger than ENaC currents of the mice on HK diet. The deletion of MR not only largely eliminated ENaC currents (0.85± 0.2 pA/pF, n=6) but also abolished the effect of HK or LS diets on ENaC activity in the CCD (HK, 1.0±0.2 pA/pF; LS, 1.1±0.2 pA/pF, n=6). These results suggest that MR plays a dominant role in determining the ENaC activity in the CCD in comparison with the DCT2/CNT and is indispensable for effects of either HK or LS on ENaC in the CCD.

Because the deletion of MR only partially decreased ENaC currents in the DCT2/CNT, we have speculated whether AT1R may play a role in determining ENaC activity in the DCT2/CNT. Thus, we used the whole-cell
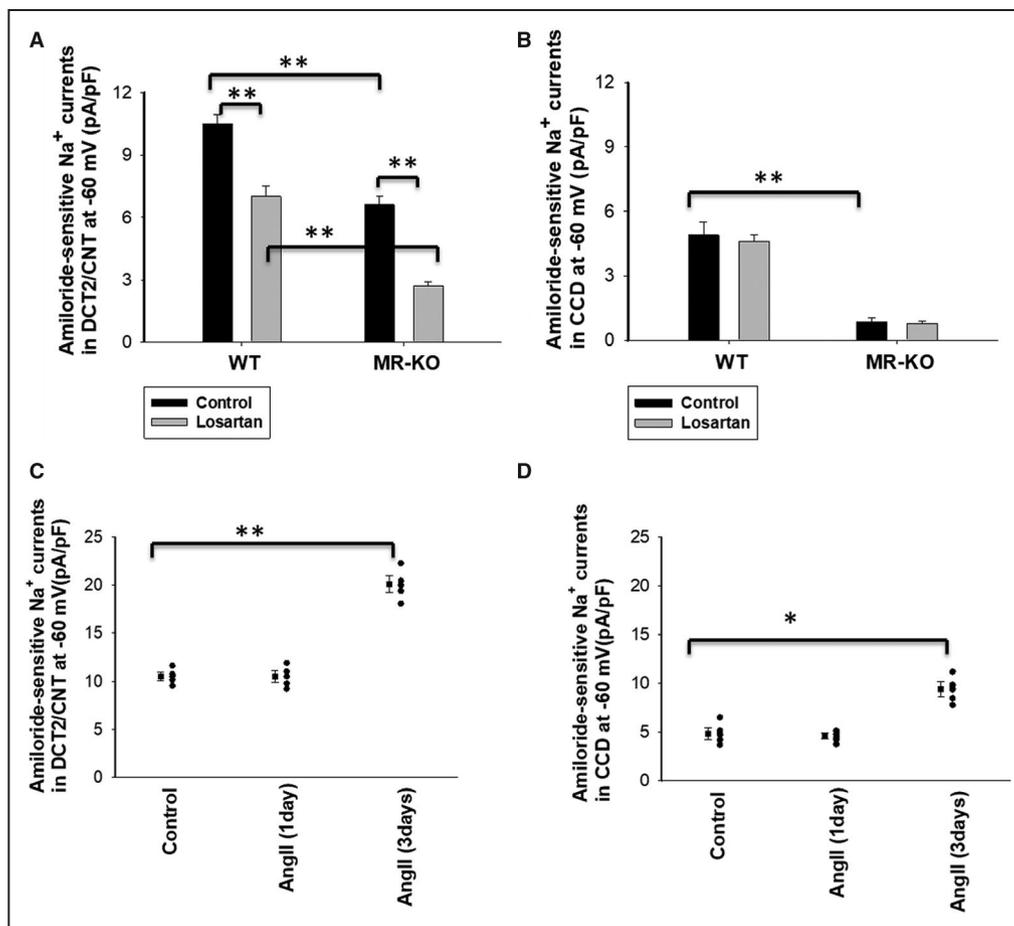

Figure 5. Inhibition of type I angiotensin II receptor decreases epithelial Na$^+$ channel in the late distal convoluted tubule/connecting tubule.

A bar graph summarizes the results of amiloride-sensitive Na$^+$ currents measured at −60 mV in the distal convoluted tubule2/connecting tubule (**A**) or in the cortical collecting duct (**B**) of the wild-type mice and kidney-specific mineralocorticoid receptor knockout mice with losartan or vehicle infusion for 3 days. T-test was used for determining the significance between 2 groups. Losartan was infused (4 μg/min per kg BW) by an osmotic pump installed subcutaneously. A scatter plot shows individual values and mean value of amiloride-sensitive Na$^+$ currents in the distal convoluted tubule2/connecting tubule (**C**) and in the cortical collecting duct (**D**) of the mice treated with angiotensin II for 1 and 3 days, respectively. The experiments were performed with whole-cell recording and the currents were measured at −60 mV. Single asterisk and double asterisks indicate that P value is <0.05 and 0.01, respectively. One-way ANOVA test was used for the statistical analysis. AngII indicates angiotensin II; MR-KO, mineralocorticoid receptor knockout; and WT, wild-type.

recording to examine the ENaC currents in the DCT2/ CNT and in the CCD of the WT and KS-MR-KO mice treated with vehicle or losartan (4 µg/min per kg BW) which was infused continuously for 3 days through a subcutaneously installed osmotic pump. The results are summarized in Figure 5A showing that the inhibition of AT1R decreased the ENaC currents in the DCT2/CNT of the WT mice from 10.5± 0.5pA/pF to 7.0±0.5 pA/pA pA (n=5) and in KS-MR-KO mice from 6.6±0.4 to 2.7±0.2 pA/pF (n=5). This finding provides a direct evidence that AT1R plays a role in determining the ENaC activity in the DCT2/CNT under control conditions. In contrast, the inhibition of AT1R had no significant effect on the ENaC currents in the CCD of WT (4.9± 0.6 pA/pF versus 4.6±0.3 pA/pF, n=5) and MR-KO mice (0.84±0.2 pA/pF versus 0.77 ±0.1 pA/pF, n=5) (Figure 5B). These results suggest that AT1R was

not essential for maintaining ENaC activity in the CCD under control conditions.

To further examine the role of AngII in regulating ENaC activity, we examined the ENaC activity by measuring amiloride-sensitive Na^+ currents in the DCT2/ CNT and in the CCD of the mice treated with vehicle or AngII perfusion (200 ng/min per kg, a non-pressor dose) by an osmotic pump for 1 or 3 days. Results of each experiment are summarized in a scatter plot for the DCT2/CNT (Figure 5C) and for the CCD (Figure 5D). Although AngII infusion for 1 day did not significantly change the amiloride-sensitive Na^+ currents in the DCT2/CNT (Vehicle, 10.5±0.44 pA, AngII, 10.5±0.6 pA), AngII infusion for 3 days significantly increased ENaC currents (20.1±0.9 pA, n=5). Similar results were observed in the CCD; AngII infusion for 1 day did not affect ENaC currents (4.85±0.6 pA, AngII,
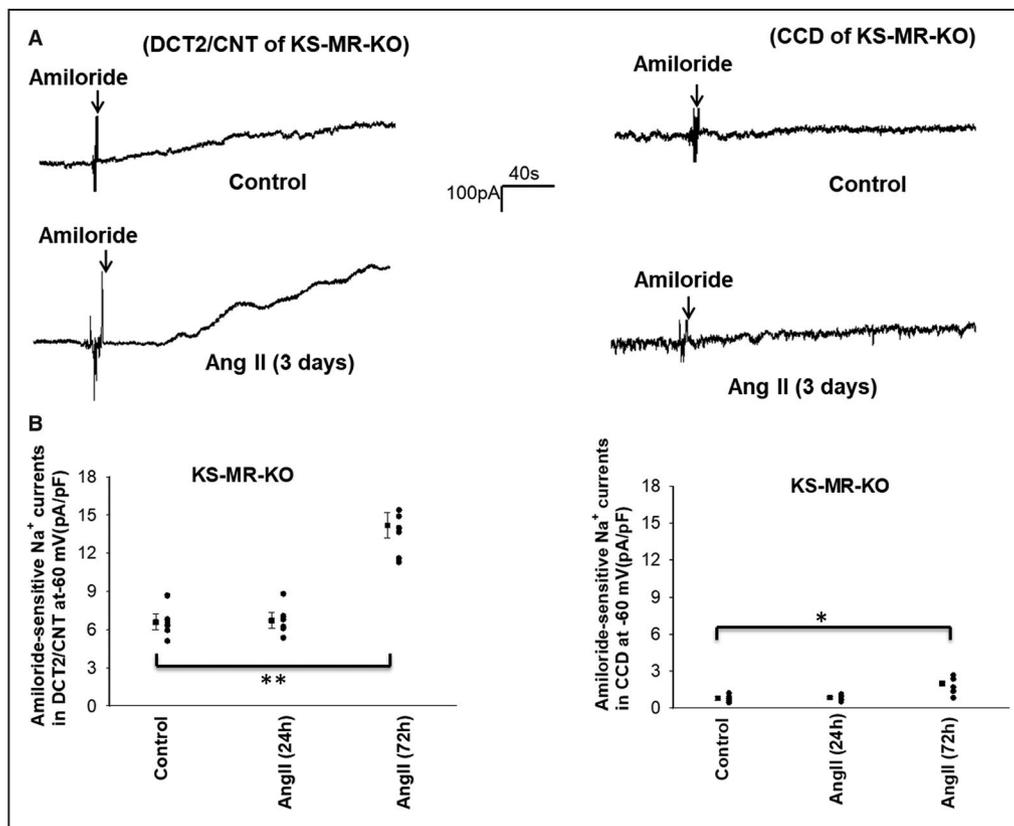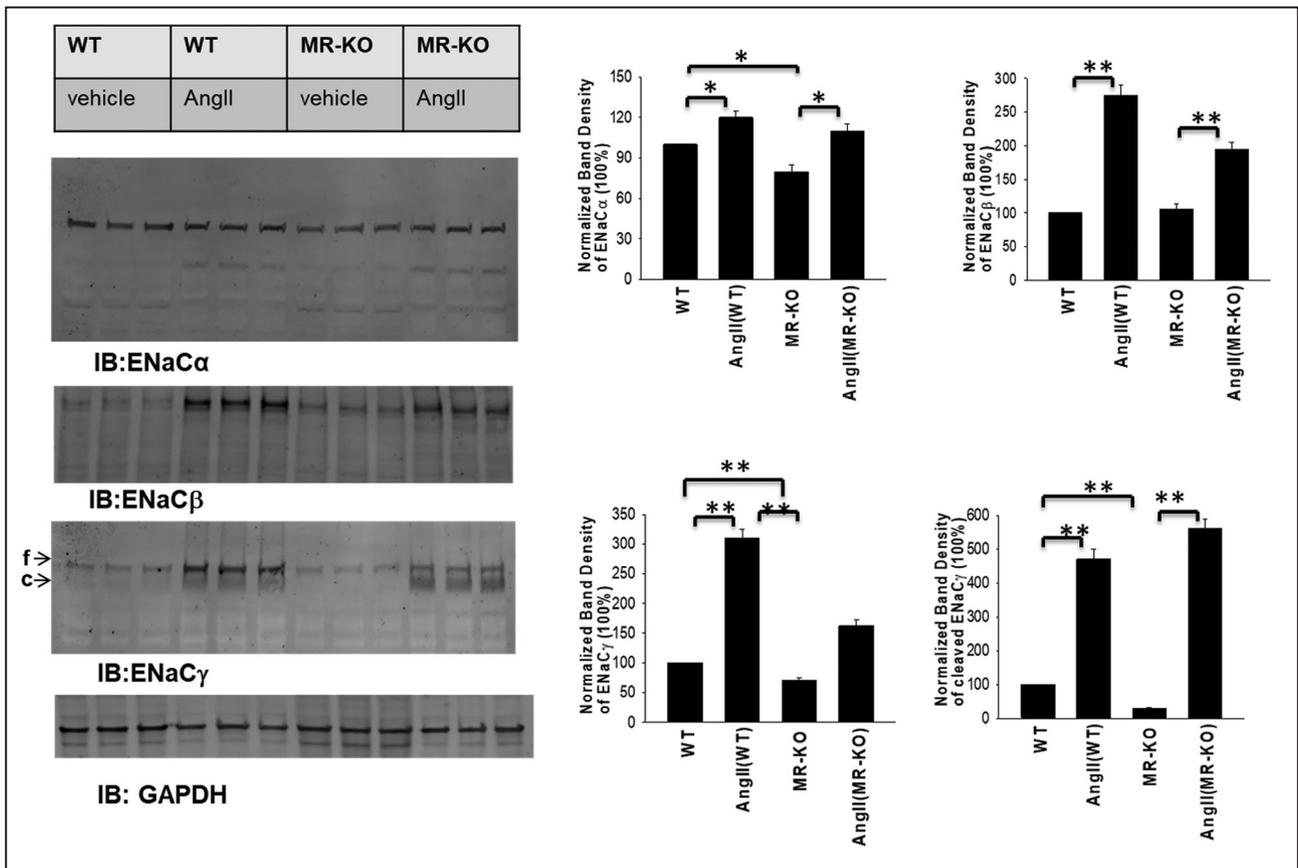

Figure 6. Angiotensin II stimulates epithelial Na^+ channel in the distal convoluted tubule of kidney-specific mineralocorticoid receptor knockout mice.
A, A set of recordings shows the amiloride-sensitive whole-cell Na^+ currents in the late distal convoluted tubule2/connecting tubule and in the cortical collecting duct of kidney-specific mineralocorticoid receptor knockout mice in vehicle and angiotensin II infusion for 3 days. Angiotensin II was infused continually through a subcutaneous osmotic pump. The epithelial Na^+ channel currents were measured at –60 mV. **B**, A scatter plot shows mean value and each data point of experiments in which amiloride-sensitive whole-cell Na^+ currents were measured at –60 mV in the distal convoluted tubule and the cortical collecting duct of kidney-specific mineralocorticoid receptor knockout mice treated with vehicle or angiotensin II for 1 or 3 days. Single asterisk and double asterisks indicate that P value is <0.05 and 0.01, respectively. One-way ANOVA test was used for the statistical analysis. AngII indicates angiotensin II; CCD, cortical collecting duct; DCT2-CNT, distal convoluted tubule2/connecting tubule; ENaC, epithelial Na^+ channel; and KS-MR-KO, kidney-specific mineralocorticoid receptor knockout.

4.6±0.3 pA, n=5). However, AngII infusion for 3 days increased the ENaC current to 9.4±0.8 pA (n=8). After demonstrating that the stimulation of AT1R increased ENaC activity in the DCT2/CNT and the CCD, we next examined whether MR was involved in mediating the effect of AngII on ENaC.

Thus, we next examined ENaC currents in the DCT2/CNT and in the CCD of KS-MR-KO mice treated with vehicle or AngII, respectively. Figure 6A is a set of traces showing amiloride-sensitive whole-cell Na$^+$ currents in the DCT2/CNT and in the CCD of KS-MR-KO mice. Figure 6B is a scatter plot summarizing each data point and the mean value of ENaC currents in the DCT2/CNT (left panel) and in the CCD (right panel) of the vehicle-treated and AngII-treated mice. Like WT mice, AngII infusion for 1 day had no significant effect on ENaC in the DCT2 (6.6±0.6 versus 6.73±0.6 pA/pF, n=6) nor in the CCD (0.8±0.15 versus 0.85±0.1 pA/pF, n=6). However, AngII infusion for 3 days caused a larger increase of ENaC currents in the DCT2/CNT (14.2±1 pA/pF, n=6) than in the CCD (2±0.23 pA/pF, n=6), indicating that AngII

was able to stimulate ENaC activity in the DCT2/CNT but to a lesser degree in the CCD by an MR-independent mechanism. Since AngII perfusion for 3 days increased ENaC currents in both WT and KS-MR-KO mice, we examined whether AngII stimulated ENaC expression in the mice receiving AngII infusion for 3 days. Figure 7 is a western blot showing that AngII infusion for 3 days increased ENaC expression in WT and KS-MR-KO mice (n=5). From the inspection of Figure 7, It is apparent that the deletion of MR decreased the expression of total ENaCα (80±5% of control value), full-length ENaCγ (70±5% of control value), and cleaved ENaCγ (30±3% of control value). Moreover, infusion of AngII for 3 days modestly but significantly increased the expression of ENaCα (120±5% of the control), robustly increased the expression of ENaCβ (275±15% of the control value), full-length ENaCγ (310±15%) and cleaved ENaCγ (470±30%) in WT mice. Also, AngII infusion also increased the expression of all ENaC subunits in KS-MR-KO mice (ENaCα, 110±5%; ENaCβ, 195±10%; full-length ENaCγ, 160±10%; cleaved ENaCγ,

Figure 7. Angiotensin II perfusion stimulates epithelial Na$^+$ channel expression.
A western blot shows the expression of epithelial Na$^+$ channel subunits in wild-type and the kidney-specific mineralocorticoid receptor knockout mice with or without AngII treatment. Full-length and cleaved epithelial Na+ channel-gamma subunits are indicated by an arrow. The normalized band density of epithelial Na$^+$ channel expression is summarized in a set of bar graph (right panel). T-test was used for determining the significance between 2 groups. Single asterisk and double asterisks indicate that P value is <0.05 and 0.01, respectively. AngII indicates angiotensin II; ENaC, epithelial Na$^+$ channel; MR-KO, mineralocorticoid receptor knockout; and WT, wild-type.

560±30% of the control value). These data have strongly indicated that AngII is able to stimulate ENaC expression by an MR-independent mechanism.

Since AngII infusion increased ENaC currents and expression in KS-MR-KO mice, it is conceivable that AngII should augment the benzamil-induced natriuresis in KS-MR-KO mice treated with AngII. Thus, we used renal clearance experiments to examine the effect of benzamil (5 mg/kg body weight) on urinary Na^+ excretion (E_{Na}). Figure 8A summarizes results from each individual experiment (5 mice for each group) and Figure 8B is a scatter graph showing the delta value of benzamil-induced net Na^+ excretion (before and after benzamil). From the inspection of Figure 8A, it is apparent that benzamil-induced natriuresis in KS-MR-KO mice treated with AngII (0.62±0.04 to 2.56±0.16 µEq/

min per 100 g BW) was significantly larger than in untreated mice (1.0±0.06 to 1.62±0.06 µEq/min per 100 g BW). Thus, AngII infusion for 3 days is able to stimulate ENaC activity by an MR-independent mechanism. Moreover, AngII infusion significantly decreased the basal level of renal Na^+ excretion (0.62±0.04 versus 1.0±0.06 µEq/min per 100 g BW), indicating that AngII enhanced renal Na^+ absorption.

Because AngII stimulates ENaC activity, it is conceivable that AngII should also stimulate renal K^+ excretion in KS-MR-KO mice. Thus, we have also used the renal clearance method to examine the effect of benzamil on renal K^+ excretion (E_K) in untreated KS-MR-KO mice and the mice treated with AngII infusion for 3 days. Figure 8C is a line graph demonstrating the results of each experiment, the mean values and
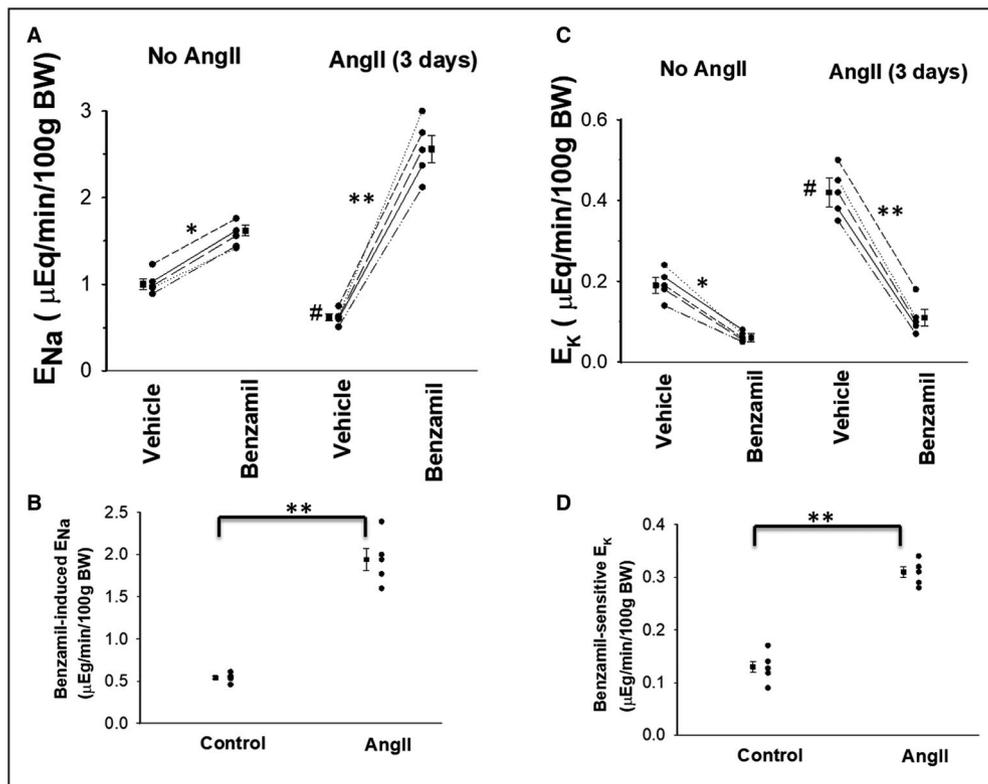
Figure 8. Angiotensin II (AngII) perfusion augments benzamil-sensitive renal Na^+ excretion and increases renal K^+ excretion.

A, A line graph summarizes each individual data point and mean value of experiments in which renal clearance was used to examine benzamil (5 mg/kg body weight)-induced renal Na^+ excretion in the kidney-specific mineralocorticoid receptor knockout mice treated with vehicle (No AngII) and AngII infusion for 3 days. **B**, A scatter plot shows the difference of net Na^+ excretion before and after benzamil (delta value) in kidney-specific mineralocorticoid receptor knockout mice treated with vehicle or AngII for 3 days. **C**, A line graph summarizes each individual data point and mean value of experiments in which renal clearance was used to examine benzamil-induced reduction of renal K^+ excretion (E_K) in kidney-specific mineralocorticoid receptor knockout mice treated with vehicle or AngII infusion for 3 days. **D**, A scatter plot shows the difference of basal renal K^+ excretion (E_K) before and after benzamil (delta value) in kidney-specific mineralocorticoid receptor knockout mice treated with vehicle or AngII for 3 days. Benzamil concentration for renal clearance: 5 mg/kg BW. Single asterisk and double asterisks indicate that P value is <0.05 and 0.01, respectively. Paired t test was used for analyzing data in (**A** and **C**) whereas t test was used for analyzing results in (**B** and **D**). AngII indicates angiotensin II; and E_{Na}, Na^+ excretion.
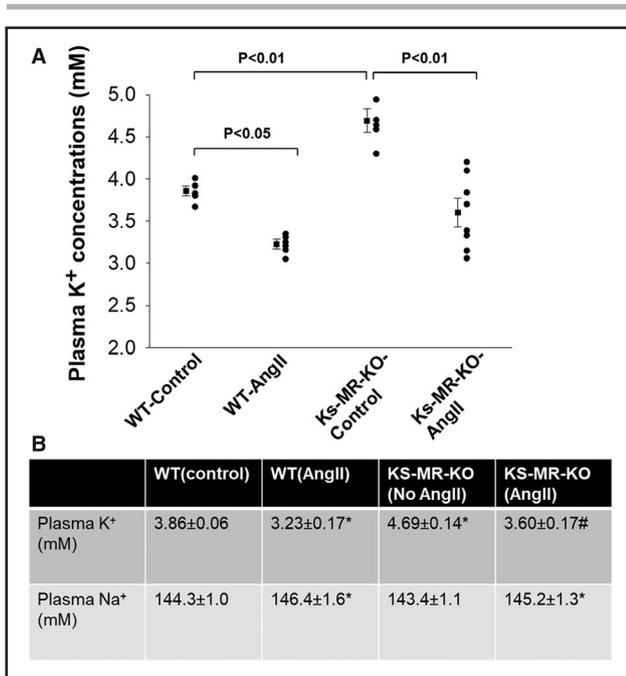
Figure 9. Angiotensin II (AngII) infusion decreased the plasma K$^+$ concentrations in wild-type and kidney-specific mineralocorticoid receptor knockout mice.
A, A scatter plot summarizes the results of experiments in which plasma K$^+$ levels were measured in the wild-type and the kidney-specific mineralocorticoid receptor knockout mice treated with AngII infusion (200 ng/min/kg BW) for 3 days. **B**, A table shows the mean value and statistical information of above experiments. Asterisks indicate that P value is <0.05 in comparison with the wild-type mice (no AngII). # indicates the significant difference in comparison with corresponding control (kidney-specific mineralocorticoid receptor knockout, no AngII). T-test was used for determining the significance between 2 groups. KS-MR-KO indicates kidney-specific mineralocorticoid receptor knockout; and WT, wild-type.

statistical information from a total of 5 mice for each group. Figure 8D is a scatter plot showing delta values of benzamil-induced net K$^+$ excretion (before and after benzamil). The K$^+$ excretion under basal (vehicle) conditions was significantly smaller (0.19±0.02 μEq/min per 100 g BW) in untreated mice than in AngII-treated mice (0.42±0.03 μEq/min per 100 g BW). Also, the benzamil-induced net K$^+$ excretion in AngII-treated mice (0.31±0.01 μEq/min per 100 g BW) was significantly larger than vehicle-treated mice (0.13±0.01 μEq/min per 100 g BW), suggesting that AngII is able to stimulate the net renal K$^+$ excretion in KS-MR-KO mice.

After observing that AngII perfusion for 3 days stimulated ENaC and enhanced renal K$^+$ excretion in both WT and KS-MR-KO mice, we have speculated that infusion of AngII should decrease the plasma K$^+$ concentration. This possibility has been examined by measuring plasma K$^+$ in WT and KS-MR-KO mice treated with AngII for 3 days. Figure 9A is a scatter plot showing each data point and mean

value of plasma K$^+$ in vehicle or AngII-treated WT mice or KS-MR-KO mice and statistical information is summarized in a table (Figure 9B). AngII infusion for 3 days significantly decreased plasma K$^+$ in WT mice from 3.86±0.06 to 3.23±0.06 mmol/L (n=6). While plasma K$^+$ in KS-MR-KO mice was significantly higher (4.69±0.14 mmol/L) than in WT mice, KS-MR-KO mice treated with AngII for 3 days decreased plasma K$^+$ to 3.60±0.17 mmol/L (n=8), a value was not significantly different from the corresponding WT mice. Thus, data have strongly indicated that AngII is able to stimulate ENaC and renal K$^+$ secretion by MR-independent mechanism.

DISCUSSION

Our present study has confirmed the previous reports that AT1R plays a role in stimulating ENaC and in facilitating renal K$^+$ secretion by an aldosterone-independent pathway.[20,22–24] However, the major finding of our study is to demonstrate that AT1R plays a more important role in regulating ENaC activity by an MR-independent mechanism in the DCT2/CNT than in the CCD under physiological conditions. This notion is supported by several lines of evidence: (1) The deletion of MR largely abolished ENaC activity in the CCD but only partially blunted ENaC activity in the DCT2/CNT; (2) The inhibition of AT1R with losartan significantly decreased amiloride-sensitive Na$^+$ currents in the DCT2/CNT but it had no significant effect on ENaC currents in the CCD under control conditions; (3) Only the combined inhibition of MR and AT1R was able to largely inhibit ENaC activity in the DCT2/CNT; (4) Losartan treatment decreased amiloride-sensitive Na$^+$ currents in the DCT2/CNT of KS-MR-KO mice to the same degree as those in WT mice. Thus, data have strongly suggested that MR plays a dominant role in determining ENaC activity in the CCD and that AT1R is constitutively involved in determining the ENaC activity in the DCT2/CNT but it may not be involved in determining the basal ENaC activity in the CCD under control conditions. However, a previous study had reported that losartan treatment further enhanced the spironolactone-induced inhibition of ENaC activity in the CCD of the mice on Na$^+$-deficient diet.[22] Thus, it is possible that AT1R may play a role in synergizing the effect of aldosterone on ENaC in the CCD during Na$^+$ restriction.

The notion that AT1R stimulates ENaC activity in the DCT2/CNT by an MR-independent mechanism is also supported by 2 additional findings. First, AngII infusion (at no pressure dose) for 3 days had a robust stimulatory effect on ENaC in the DCT2/CNT of KS-MR-KO mice. Second, renal clearance experiments have demonstrated that benzamil-sensitive renal Na$^+$

absorption was significantly augmented in KS-MR-KO mice treated with AngII in comparison with vehicle-treated mice. Although the effect of AngII on benzamil-sensitive renal Na+ absorption was the result of the stimulation of ENaC not only in the DCT2/CNT but also in the CCD, ENaC activity in the DCT2/CNT should be mainly responsible for increasing Na+ absorption in KS-MR-KO mice. This view was based on the fact that AngII- induced increase of ENaC currents in the CCD of KS-MR-KO mice was modest and significantly smaller than those in the DCT2/CNT.

We have also confirmed the previous report that ENaC currents in the DCT2/CNT under physiological conditions were larger than in the CCD,[5] suggesting that the DCT2/CNT plays an important role in mediating Na+ absorption and K+ excretion under control conditions. We speculate that larger amiloride-sensitive Na+ currents in the DCT2/CNT than in the CCD were because both MR and AT1R are involved in determining basal ENaC activity in the DCT2/CNT whereas only MR is mainly responsible for determining ENaC activity in the CCD. This notion was supported by the finding that the magnitude of the MR-deletion induced inhibition of ENaC was different between the DCT2/CNT and CCD. While ENaC currents were almost absent in the CCD of KS-MR-KO mice, amiloride-sensitive Na+ currents in the DCT2/CNT were only partially decreased, suggesting that MR was only partially responsible for regulating ENaC activity in the DCT2/CNT. The observation that the amiloride-sensitive Na+ currents were still detected in the DCT2/CNT of the KS-MR-KO mice treated with losartan suggests either incomplete inhibition of AT1R or that factors other than MR and AT1R may be also involved in the regulation of ENaC activity in the DCT2/CNT. In this regard, previous studies have shown that vasopressin and insulin/insulin-like-growth factor are involved in regulating ENaC activity.[33–36] However, AT1R and MR should be 2 major players for determining ENaC activity in the DCT2/CNT under physiological conditions since the remaining ENaC activity in the DCT of KS-MR-KO mice treated with losartan was relatively small.

We have also confirmed previous observations that low Na+ intake did not increase amiloride-sensitive Na+ currents in the DCT2/CNT,[5] although it robustly increased ENaC currents in the CCD. Since increasing dietary K+ intake was able to stimulate ENaC in the DCT2/CNT, it was unlikely that the lack of stimulatory effects of low Na+ intake on ENaC was because of the saturation of MR in the DCT2/CNT. One possibility is that low Na+ intake might selectively increase the ENaC expression/transcription in the CCD but it has little effect on ENaC expression/transcription in the DCT2/CNT, whereas HK intake stimulates ENaC equally in the DCT2/CNT and CCD. Further

experiments are required to examine the effect of low Na+ intake on ENaC expression/transcription in the DCT2/CNT and in the CCD.

We and others have demonstrated that acute application of AngII activated ENaC in the CCD.[21,24,37] However, AngII infusion for 1 day did not significantly increase amiloride-sensitive Na+ currents. In contrast, ENaC currents in the DCT2/CNT and the CCD were significantly increased in the mice treated with AngII for 3 days. It is possible that AngII may stimulate ENaC by a short-term and a long-term mechanism: acute application of AngII- induced stimulation of ENaC is mainly achieved by activating existing ENaC channels whereas a prolonged treatment of AngII increases the expression of ENaC thereby increasing amiloride-sensitive Na+ currents. In this regard, we and others have shown that acute AngII-induced stimulation of ENaC was induced by increasing superoxide products, suggesting that the effect of AngII on ENaC may be transient.[21,24] On the other hand, prolonged AngII application has been shown to increase the expression of all 3 ENaC subunits.[20] Furthermore, our present experiment has demonstrated that AngII-induced stimulation of ENaC was not dependent on MR because AngII infusion was still able to stimulate ENaC expression in KS-MR-KO mice.

We confirmed the previous finding that KS-MR-KO mice had a higher plasma K+ levels than their corresponding WT as the consequence of decreased ENaC activity.[27] However, even in the absence of MR, the mice were able to maintain the plasma K+ concentrations in a relative normal range, suggesting that KS-MR-KO mice were able to excrete K+ by an MR-independent mechanism. A previous study has demonstrated that the deletion of MR activated the renin-angiotensin II system since MR-deficient mice increased plasma renin and angiotensin II concentrations by 50-folds[14] and Loffing et al[23] have reported losartan treatment decreased renal K+ excretion in aldosterone synthase deficient mice. Thus, it is conceivable that the activation of the renin-angiotensin II system may be responsible for preventing severe hyperkalemia in KS-MR-KO mice. This notion is also confirmed by the finding that infusion of AngII for 3 days significantly increased amiloride-sensitive renal K+ excretion thereby completely correcting hyperkalemia in the KS-MR-KO mice and causing hypokalemia in the WT mice. Considering the fact that AngII stimulates ENaC in the DCT2/CNT more than in the CCD, enhancing K+ excretion in the DCT should be mainly responsible for augmenting renal K+ excretion. The finding that AngII perfusion can correct high plasma K+ in KS-MR-KO mice also suggests the possibility that ENaC activity in the DCT2/CNT plays a more important role in mediating ENaC-dependent K+ excretion than in the CCD under physiological conditions. This speculation is also supported

by the previous report that deletion of ENaC in the collecting duct did not affect Na and K$^+$ homeostasis.[38]

Perspectives

The role of aldosterone-MR in stimulating ENaC activity is well established.[2,19,39–41] Also, several studies have demonstrated that acute stimulation of AT1R was able to stimulate ENaC.[20,21,24,37] The physiological significance of the present study is to demonstrate that AT1R plays an important role in maintaining ENaC activity in the late DCT2/CNT by an MR-independent mechanism. Moreover, we have provided the evidence that the regulation of ENaC by AngII-AT1R pathway mainly occurs in the DCT2/CNT and to a lesser degree in the CCD. In contrast, MR plays a dominant role in determining ENaC activity in the CCD but to a lesser degree in the DCT2/CNT.

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Disclosures

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