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## Chronic Angiotensin II infusion drives extensive aldosterone-independent ENaC activation

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### Abstract

The inability of mineralocorticoid receptor (MR) blockade to reduce hypertension associated with high Angiotensin (Ang) II suggests direct actions of Ang II to regulate tubular sodium reabsorption via the epithelial Na<sup>+</sup> channel (ENaC) in the aldosterone-sensitive distal nephron (ASDN). We used freshly isolated ASDN from mice to delineate the synergism and primacy between aldosterone and Ang II in controlling functional ENaC activity. Inhibition of MR specifically prevented the increased number of functionally active ENaC but not ENaC open probability elicited by a low sodium diet. In contrast, we found no functional role of glucocorticoid receptors (GR) in the regulation of ENaC activity by dietary salt intake. Simultaneous inhibition of MR and Ang II type 1 receptors (AT<sub>1</sub>R) ameliorated the enhanced ENaC activity caused by low dietary salt intake and produced significantly greater natriuresis than either inhibitor alone. Chronic systemic Ang II infusion induced more than two times greater increase in ENaC activity than observed during dietary sodium restriction. Importantly, ENaC activity remained greatly above control levels during maximal MR inhibition. We conclude that during variations in dietary salt intake both aldosterone and Ang II contribute complementarily to the regulation of ENaC activity in the ASDN. In contrast, in the setting of Ang II-dependent hypertension, ENaC activity is up-regulated well above the physiological range and is not effectively suppressed by inhibition of the aldosterone-MR axis. This provides a mechanistic explanation for the resistance to MR inhibition that occurs in hypertensive subjects having elevated intrarenal Ang II levels.

### Keywords

collecting duct; connecting tubule; distal nephron; renal sodium handling; hypertension

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## INTRODUCTION

Sodium reabsorption in the aldosterone-sensitive distal nephron (ASDN) is of great importance for proper renal sodium handling and maintenance of whole body sodium homeostasis<sup>1</sup>. Dysfunction of sodium transport at this site has been tightly linked to disturbances in circulating volume and blood pressure abnormalities<sup>1,2</sup>. Activity of the Epithelial Na<sup>+</sup> channel (ENaC) underlies electrogenic Na<sup>+</sup> reabsorption at ASDN, which includes the connecting tubule (CNT) and the cortical collecting duct (CCD)<sup>3-6</sup>. The relevance of ENaC function for blood pressure control in humans is unequivocally supported by the fact that genetic disorders affecting blood pressure, such as Liddle's syndrome and Type I pseudohypoaldosteronism (PHA), arise from gain-of-function and loss-of-function mutations in ENaC, respectively<sup>7-11</sup>.

ENaC activity in the ASDN is inversely related to dietary sodium intake<sup>12-14</sup>. Activation of the Renin-Angiotensin-Aldosterone system (RAAS) in response to sodium restriction increases ENaC activity and expression via aldosterone-mineralocorticoid receptor (MR) pathway<sup>3-5,9,15-17</sup>. However, the existence of the "aldosterone paradox", where elevations in circulating aldosterone in response to hypovolemia and hyperkalemia result in drastically different patterns of urinary sodium and potassium excretion<sup>18,19</sup>, points to a role for other signaling components of RAAS, most likely Angiotensin (Ang) II, in the regulation of ENaC in response to volume contraction.

Ang II is the principal effector of RAAS<sup>20,21</sup>. Increases in circulating Ang II levels induce vasoconstriction, secretion of aldosterone and elevations in systemic blood pressure<sup>22</sup>. In the kidney, Ang II promotes antinatriuresis, in part, by stimulating luminal Na<sup>+</sup> entry and tubular Na<sup>+</sup> reabsorption in the distal nephron<sup>23-25</sup>. Virtually all Ang II actions to enhance renal sodium transport are mediated by Ang II type 1 receptors (AT<sub>1</sub>R). AT<sub>1</sub>R are abundantly expressed on both apical and basolateral sides of epithelial cells along the whole length of the renal nephron from the proximal tubule to the collecting duct<sup>26,27</sup>. We<sup>28</sup> and others<sup>25,29</sup> have documented that Ang II acutely increases ENaC activity and open probability ( $P_o$ ) in freshly isolated split-opened mouse and rat ASDN. The signaling pathway involves AT<sub>1</sub>R-dependent activation of NADPH oxidase and generation of reactive oxygen species, likely superoxide and peroxide, to stimulate ENaC<sup>28</sup> and to diminish inhibitory actions of the arachidonic acid metabolites<sup>29</sup>. Of interest, acute stimulation of ENaC  $P_o$  by Ang II persists during saturation<sup>28</sup> and inhibition<sup>29</sup> of MR cascade indicating aldosterone-independent nature of this regulation<sup>19</sup>. It is also interesting that prolonged treatment of isolated murine ASDN with Ang II causes translocation of  $\alpha$ ENaC to the apical plasma membrane and increases the number of functionally active channels<sup>28</sup>. It remains unclear, whether physiologically relevant changes in Ang II levels have their own non-redundant contribution to regulation of ENaC activity in ASDN in response to changes in dietary sodium intake.

In experimental animal models of Ang II-induced hypertension<sup>30,31</sup>, intrarenal Ang II levels become much higher than those in plasma due to activation of the intrarenal and intratubular renin angiotensin system (RAS)<sup>21</sup>. Inappropriately stimulated intrarenal RAS leads to excessive sodium retention, in part, due to possible activation of ENaC in ASDN<sup>32-34</sup>. Cumulative evidence suggests that effects of elevated Ang II levels on ENaC expression and sodium reabsorption in the ASDN cannot be solely explained by Ang II-induced aldosterone secretion. Thus, mice with global knockout of the major subtype of AT<sub>1</sub> receptors, (AT<sub>1a</sub>) exhibit a marked reduction in  $\alpha$ ENaC abundance in the kidney despite slightly elevated aldosterone levels<sup>35</sup>. Furthermore, the effect of AT<sub>1</sub>R blockade on  $\alpha$ ENaC expression was not prevented by spironolactone, suggesting a direct role of the AT<sub>1</sub>R in regulation of  $\alpha$ ENaC gene expression<sup>36</sup>. Systemic infusion of Ang II increases  $\alpha$ ENaC protein abundance

in rat kidney cortex<sup>36</sup>. It is unclear, though, whether elevated circulating and intrarenal Ang II levels translate into direct changes in functional ENaC activity. Intriguingly, inhibition of MR with spironolactone in rat and mouse models of Ang II-dependent hypertension produced very mild and transient hypotensive effects<sup>32,33,37</sup>. In contrast, direct ENaC blockade with amiloride does attenuate blood pressure in Ang II infused rats<sup>34</sup>. This may indicate a dominant role of Ang II-driven aldosterone-independent ENaC activation in Ang II-infused models of hypertension<sup>38</sup>.

In the current study, we employ systemic pharmacological inhibition of MR and AT<sub>1</sub>R with direct assessment of ENaC activity using patch clamp electrophysiology in freshly isolated split-opened ASDN of mice, to test synergism and primacy in aldosterone and Ang II signals to ENaC during the physiological response to changes in dietary sodium intake and in the pathophysiology of the Ang II-induced hypertension. We found that, by controlling functional ENaC expression (number of active channels on the apical plasma membrane), the aldosterone cascade has relatively greater contribution in stimulating ENaC during conditions of dietary sodium restriction than Ang II signaling, which is responsible for regulation of ENaC  $P_o$ . In contrast, chronic Ang II infusion over-stimulates ENaC beyond the physiological range of ENaC regulation. We propose that this, in part, contributes to the excessive renal sodium conservation and hypertension in response to chronic Ang II infusion. Importantly, we report here that blockade of aldosterone-MR cascade is insufficient to effectively suppress excessively activated ENaC when Ang II levels are elevated.

## METHODS

### Reagents and animals

All chemicals and materials were from Sigma (St. Louis, MO), VWR (Radnor, PA), and Tocris (Ellisville, MO) unless noted otherwise and were at least of reagent grade. Animal use and welfare adhered to the NIH Guide for the Care and Use of Laboratory Animals following protocols reviewed and approved by the Animal Care and Use Committee of the University of Texas Health Science Center at Houston and the Tulane University School of Medicine. For experiments, male C57BL/6J mice (Charles River Laboratories, Wilmington, MA) 6–8 weeks old, were used. Animals had free access to tap water.

### Research diets and Treatments

To examine effects of salt intake, animals were provided diets containing nominally free (<0.01% Na<sup>+</sup>, TD.90228), regular (0.32% Na<sup>+</sup>, TD.7912), and high (2% Na<sup>+</sup>, TD.92034) sodium for one week. All diets were purchased from Harlan Teklad (Madison, WI, USA). Spironolactone (30 mg/kgBW), mifepristone (30 mg/kgBW), losartan (10 mg/kgBW) were added to drinking water for 3, 7, or 14 days depending on experimental design. As necessary for some protocols, mice were injected with Deoxycorticosterone acetate (DOCA) for 3 consecutive days (2.4 mg/injection/animal) prior to the experimentation similarly to what we have done previously<sup>39</sup>.

Male 6 weeks C57BL/6 mice (approximately 25 g) were infused with Ang II (Sigma) dissolved in 5% acetic acid (VWR) via subcutaneous osmotic minipumps (Alzet model 1002, Alza, Palo Alto, CA) at concentration 400ng/kgBW/min for 2 weeks closely following well-established protocols<sup>30,31</sup>.

Mice were subjected to inhaled anesthesia with isofluorane, and a catheter connected to a radiotelemetry device was inserted into the left carotid artery to monitor systolic blood pressures using telemetry (model PA-C10; Data Sciences International, St. Paul, MN) in

conscious and unrestrained conditions. After a 5-days recovery phase, baseline systolic blood pressure levels were measured, and the mice were then after subjected to a similar anesthesia for osmotic mini-pump subcutaneous implantation. Mice were infused with Ang II (400 ng/kgBW/min) for 14 days. Systolic blood pressure data were collected in sham and Ang II-infused mice and analyzed using Dataquest A.R.T, Software 4.0 (Data Sciences International).

### Electrophysiology

The procedure for isolation of the aldosterone-sensitive distal nephrons (ASDNs) suitable for electrophysiology has been described previously<sup>28,40,41</sup>. ENaC activity in principal cells was determined in cell-attached patches on the apical membrane made under voltage-clamp conditions ( $-V_p = -60\text{mV}$ ) using standard procedures (see online supplement for more details).

### Immunofluorescent microscopy

Kidneys were fixed in 10% formalin at 4°C overnight, processed for paraffin embedding, sectioning (3  $\mu\text{m}$ ), and sequentially immunostained using rabbit anti- $\alpha\text{ENaC}$  and goat anti-AQP2 antibodies as described previously<sup>42</sup>. The primary antibodies used were anti- $\alpha\text{ENaC}$  (SPC-403D; StressMarq Biosciences Inc.) at 1:400 dilution and anti-AQP2 (sc-9882, Santa Cruz Biotechnology) at 1:200 dilution for overnight and 90 minutes incubations, respectively. For immunofluorescent detection, we used donkey anti-rabbit or donkey anti-goat secondary IgG antibodies with Alexa Fluor 488 (green) or 594 (red) conjugates (Invitrogen) and counterstaining of the tissue sections for nuclei visualization with 4,6-diamidino-2-phenylindole (DAPI; D1306, Invitrogen). The immunofluorescence images were captured with a Nikon DS-Digital camera attached to a 50i Eclipse Fluorescence microscope from Nikon using objectives of 40 $\times$  and 100 $\times$  (oil immersion) magnification. Negative controls were obtained by omission of the specific primary antibody.

### Western blotting

Immediately after dissection, kidneys were frozen in liquid nitrogen and stored there for further use. Prior to experimentation the kidneys were homogenized in 3 volumes of ice-cold hypotonic lysis buffer containing 50mmol/L Tris, 1% Triton X-100, 5mmol/L EDTA (pH=7.4) supplemented with 1 mM PMSF and 2 mg/ml protease inhibitor cocktail (Complete mini, Roche Diagnostics, Germany). Homogenates were centrifuged at 1000 g for 15 min at +4 C. The supernatants were split in two parts to assess total cellular proteins and plasma membrane proteins using plasma membrane protein extraction kit (Biovision Inc, USA) following the manufacturer's protocol. Protein concentration in the homogenates was determined with a Bradford assay. The samples (25  $\mu\text{g}/\text{lane}$ ) were separated on 9% polyacrylamide gels at 150 V for 1 h 15 min and transferred to nitrocellulose membranes at 100 V for 1 h 45 min. The membranes were blocked for 1 h at RT in 5% nonfat milk in TBS-T (150 mmol/L NaCl, 50 mmol/L Tris-HCl pH=7.4, 0.1% Tween 20). Subsequently the membranes were probed with anti- $\alpha\text{ENaC}$  primary rabbit antibodies (1:1000, Stressmarq Biosciences, Canada) followed by peroxidase-conjugated goat anti-rabbit secondary antibodies (1:20000, Bio-Rad, USA) for 1 hour at RT. The membranes were re-probed with anti- $\alpha$ -tubulin (1:200, Abcam, UK) primary rabbit antibodies and peroxidase-conjugated goat anti-rabbit secondary antibodies (1:20000, Bio-Rad, USA). When total cellular  $\alpha\text{ENaC}$  expression was assessed,  $\alpha\text{ENaC}$  band intensities were normalized to the intensities of the corresponding  $\alpha$ -tubulin bands. For membrane enriched fractions, band intensities were normalized to the intensity of the first  $\alpha\text{ENaC}$  band from the first control sample. All experiments were repeated at least 3 times.

## Urinary sodium excretion

Urine was collected from bladders of sacrificed animals and frozen at  $-20^{\circ}\text{C}$  until analyzed. The time of urine sampling was the same (10AM–11AM) for all animals. Urinary sodium excretion was calculated as the ratio of urinary sodium to creatinine concentrations. Urinary sodium concentration was measured using PFP7 Flame photometer (Techne, Burlington, NJ). Urinary creatinine concentration was assessed with Hitachi 7000 HPLC System (Pleasanton, CA, USA).

## Data analysis

All summarized data were reported as mean  $\pm$  SEM. Data from different experimental sets were compared using a Student's (two-tailed) *t*-test or an One-Way ANOVA as appropriate.  $P < 0.05$  was considered significant.

## RESULTS

### ENaC activity is indistinguishable in CNT and CCD

We first performed a detailed characterization of ENaC activity in the structurally different CNT and CCD in mice kept on a regular salt intake ( $0.32\% \text{ Na}^+$ ). As illustrated in Figure 1A, we mechanically isolated bifurcations of the distal nephrons representing merging of two connecting tubules (CNT) into a cortical collecting duct (CCD). ENaC activity was surveyed using patch clamp electrophysiology in cell attached configuration within split-opened areas located up to  $100 \mu\text{m}$  above (CNT region) and  $300 \mu\text{m}$  below (CCD region) the bifurcations. To reliably assess ENaC activity ( $fNP_o$ ), the mean  $NP_o$  was corrected to a frequency of observing patches with active channels ( $f = \text{number of patches with channels} / \text{total number of patches}$ ). As summarized in Figure 1B, ENaC activity was nearly identical within the designated area. We also did not detect any differences in ENaC gating properties (open probability,  $P_o$ ) and the number of functional ENaC within a patch ( $N$ ) in both CNT and CCD (data not shown). These results suggest that ENaC activity is uniform in the cortical region of the classical ASDN representing transition of CNTs into CCDs. Thus, we did not further discriminate ENaC activity in CNT and CCD.

### Mineralocorticoid but not glucocorticoid receptors are involved in regulation of ENaC activity by aldosterone in murine ASDNs

To address the relative contribution of aldosterone-independent mechanisms, such as Ang II, in regulation of functional ENaC expression in the ASDN, we first inhibited MR with spironolactone ( $30 \text{ mg/kgBW}$  for 7 days) in mice maintained on a regular salt intake. This treatment significantly diminished but did not abolish ENaC activity ( $fNP_o$ ) from  $0.40 \pm 0.03$  ( $n=223$ ) to  $0.21 \pm 0.05$  ( $n=52$ ). To ensure that the concentration of spironolactone is sufficient for complete inhibition of MR, we injected mice with deoxycorticosterone acetate (DOCA) for 3 consecutive days prior to the assessment. Saturation of mineralocorticoid status significantly increased ENaC activity to  $0.61 \pm 0.08$  ( $n=54$ ) in ASDNs from control mice but had no effect on ENaC ( $0.23 \pm 0.04$ ,  $n=58$ ) in ASDNs from mice treated with spironolactone (supplementary Figure S1).

Elevated circulating aldosterone levels can potentially affect ENaC function via glucocorticoid receptors (GR) particularly during sodium restricted conditions. To test this possibility, we performed detailed analysis of ENaC activity in mice kept on different salt diets for one week (low,  $<0.01\%$ ; regular,  $0.32\%$ ; and high,  $2\% \text{ Na}^+$ ) in the absence and presence of systemic inhibition of GR with mifepristone ( $30 \text{ mg/kgBW}$ , for 7 days). This concentration is similar to that used previously for GR inhibition<sup>43</sup>. ENaC activity was augmented during low  $\text{Na}^+$  diet and diminished under high  $\text{Na}^+$  diet (Figure 2A, see also



Figure 3, top row for representative traces of ENaC activity). This involved changes in both functional ENaC expression (Figure 2B) and ENaC  $P_o$  (Figure 2C). As shown, mifepristone treatment fails to alter ENaC activity (Figure 2A), functional ENaC expression (Figure 2B), and ENaC  $P_o$  (Figure 2C) under all tested experimental conditions (see supplementary Table S1 for the absolute values).

Overall, we concluded that aldosterone utilizes activation of MR but not GR to modulate ENaC activity in ASDN.

### **Inhibition of MR does not abolish regulation of ENaC by systemic salt status**

We next probed how inhibition of aldosterone-MR axis affects regulation of ENaC by variations in dietary salt intake. Using a similar strategy as discussed above, mice were fed with various Na<sup>+</sup> diets for one week and were supplemented with MR inhibitor spironolactone (30 mg/kgBW). As summarized in Figure 4A, spironolactone greatly attenuated ENaC activity under low salt intake while had no significant effect of ENaC in mice kept on a high (2%) salt diet (see Figure 3, middle row for representative traces). Nevertheless, we observed modest but significant augmentation of ENaC activity when comparing high and low sodium diets in mice receiving spironolactone treatment (Figures 3, 4A). Detailed patch clamp analysis revealed that inhibition of MRs prevented changes in functional ENaC expression in response to dietary salt variations (Figure 4B, see also supplementary Table S1). Furthermore, we did not observe changes in the frequency of patches with active channels on low and high sodium diets (25% and 24%, respectively) in spironolactone treated animals. For control animals frequencies were 50% and 31%, respectively. These results indicate a dominant role of aldosterone-MR axis in regulation of functional ENaC expression in response to systemic physiological cues, such as dietary sodium variations. In contrast, we found that regulation of ENaC  $P_o$  by dietary salt intake remained intact after spironolactone treatment (Figure 4C, see also supplementary Table S1). Thus, we concluded that aldosterone-independent stimulation of ENaC activity by dietary salt restriction involves augmentation of ENaC open probability.

### **Ang II signaling is critical for increased ENaC $P_o$ by low sodium diet**

The observation that ENaC  $P_o$  responds to variations in systemic salt status in the absence of aldosterone-MR cascade (Figure 4C) argues for the involvement of additional mechanisms responsible for this regulation. We have recently documented that Ang II via activation of AT<sub>1</sub>R is capable of increasing ENaC  $P_o$  and this regulation persists even in the presence of saturated mineralocorticoid status<sup>28</sup>. Thus, we next tested whether Ang II signaling contributes to regulation of ENaC  $P_o$  by dietary salt intake. Mice were placed on different salt diets for 1 week and treated simultaneously with spironolactone and AT<sub>1</sub>R antagonist, losartan (10 mg/kgBW). We found that, in contrast to the treatment with spironolactone alone, ENaC activity was no longer significantly different in animals kept on low and high sodium diets (Figure 4D, see also Figure 3 bottom row). Absolute values are reported in supplementary Table S1. Consistently, we observed that functional ENaC expression was not different between diets with losartan having no further inhibitory effect (Figure 4E). Importantly, we found that with both MR and AT<sub>1</sub>R blockade, ENaC  $P_o$  was only slightly different in mice placed on a low and high sodium diets and this difference did not reach significance (Figure 4F). Therefore, we conclude that Ang II and aldosterone cascades contribute complementarily to stimulation of ENaC activity during dietary salt restriction. Aldosterone signaling, by specifically controlling the number of functional ENaCs, has a relatively greater contribution than Ang II, which is mainly responsible for stimulation of ENaC open probability.

### **Inhibition of aldosterone-MR and Ang II-AT<sub>1</sub>R cascades additively produces natriuresis**

We next probed if coordinated activation of ENaC activity by aldosterone and Ang II signaling cascades parallels the changes in renal sodium handling. For this, we assessed snapshot urinary sodium excretion at day 3 in mice on low sodium regimen. Systemic treatment with losartan (10 mg/kgBW, for 3 days) and spironolactone (30 mg/kgBW, for 3 days) significantly increased urinary sodium excretion (Figure 5A) but had no effect on urinary creatinine levels (Figure 5B). Importantly, combined treatment with both inhibitors caused a modestly greater natriuresis compared to that produced by either substance alone. In contrast, there were no measurable changes in urinary sodium excretion in mice kept on a high sodium diet for 3 days with any of the treatments (Figure 5C). Consistently, urinary creatinine values were also unaffected (Figure 5D). The results in Figure 5, while indicative, support the concept that aldosterone and Ang II signaling cascades stimulate ENaC activity in a complementary manner to maximally increase distal nephron sodium reabsorption specifically under conditions of dietary salt restriction. It is recognized, that changes in urinary sodium excretion during pharmacological inhibition of Ang II and aldosterone signaling pathways also reflect possible changes in activity of other Na<sup>+</sup>-transporting systems in the kidney, most notably the sodium chloride cotransporter (NCC)<sup>44,45</sup>.

### **ENaC activity is greatly augmented in Ang II-infusion induced hypertension**

Substantial experimental evidence suggests marked upregulation of intrarenal Ang II concentration during many pathological states, including hypertension<sup>30,31</sup> and diabetes mellitus<sup>46,47</sup>. Studies in both rats and mice have demonstrated that chronic Ang II infusions lead to increased urinary levels of angiotensinogen and Ang II<sup>21,38</sup>. These data suggest that augmented intratubular Ang II signaling enhances renal sodium reabsorption, in part, by over-stimulating ENaC. Thus, we directly assessed changes in blood pressure (Figure 6A) and ENaC activity (Figure 6B) in mice kept on a normal salt regimen (0.32% Na<sup>+</sup>) in response to chronic infusion of Ang II (400ng/kgBW·min for 14 days). Mice infused with Ang II displayed significant elevated systolic blood pressures compared with the vehicle infused control group after Day 3 of Ang II infusions (129±5 vs. 115±2 mm Hg; *P*=0.06). By Day 5, systolic blood pressures were augmented in mice receiving Ang II (144±4 vs. 117±1 mm Hg; *P*<0.05) and continued to increase by Day 9 (151±3 vs. 118±2 mm Hg; *P*<0.001) and Day 13 (157±9 vs. 120±1 mm Hg; *P*<0.05) compared to the control group. The time course of developing hypertension is consistent with previous reports using a similar experimental strategy<sup>30,33</sup>.

As visualized by the representative traces of ENaC activity (Figure 6B) and summarized in Figure 7A, chronic Ang II infusion leads to a robust (approximately 6 times) elevation of ENaC activity compared to that in vehicle infused animals. Of note, this elevation was twice the activity than that observed in response to strong physiological stimuli, such as dietary salt restriction (see Figures 3, 4 for comparison). Specifically, we found that Ang II infusion increases all components of functional ENaC activity: the frequency of observing patches with active channels (Figure 7B), functional ENaC expression (Figure 7C), and ENaC *P<sub>o</sub>* (Figure 7D). The absolute values of ENaC activity in vehicle infused and Ang II infused animals are reported in supplementary Table S2.

To assess relative contribution of aldosterone-dependent and -independent mechanisms in activation of ENaC activity in Ang II-infused animals, mice were provided with spironolactone for the 14 day period of the treatment. Inhibition of MR did attenuate the stimulatory effect of Ang II on ENaC (Figure 6B). However, ENaC activity remained more than 4 times higher than in control animals treated with spironolactone (Figure 7A, shown with a dash line). Inhibition of MR failed to return the functional ENaC expression to the levels observed in control animals treated with spironolactone (Figure 7C, shown with a

dash line). Furthermore, spironolactone did not diminish the frequency of observing patches with active channels (Figure 7B), and had no measurable effect on ENaC  $P_o$  in Ang II-infused mice (Figure 7D). Supplementary Table S2 contains absolute values of ENaC activity, ENaC  $P_o$  and functional ENaC expression in Ang II infused animals treated with spironolactone. We conclude that chronic elevation of Ang II levels increases ENaC activity greatly above the levels observed during routine changes in dietary salt amount. Inhibition of mineralocorticoid receptors fails to efficiently suppress ENaC activity thus suggesting a critical contribution for Ang II-mediated mechanisms independent of aldosterone.

### Regulation of plasma membrane $\alpha$ ENaC levels in Ang II-infused mice

We finally probed whether Ang II infusion results in augmentation of ENaC protein abundance at the plasma membrane. For this, we visualized the spatial subcellular  $\alpha$ ENaC distribution in kidney sections at the cortex (Figures 8A, B) and inner medulla (Figures 8C, D) regions of control and Ang II-infused mice with immunofluorescent microscopy. No fluorescent signal was detected in the absence of primary but in the presence of secondary antibodies (data not shown). Using a higher magnification, we observed that  $\alpha$ ENaC fluorescent signal displays a predominant apical distribution in the collecting ducts from kidney sections from Ang II-infused mice compared to control mice (Figures 8E, F). The  $\alpha$ ENaC immunoexpression was present in the principal cells co-stained with a specific marker AQP-2 (Figures 8G–J).

Since immunofluorescent microscopy provides only qualitative evidence of the subcellular ENaC redistribution in Ang II-infused mice, we further monitored the full-length 95 kDa  $\alpha$ ENaC in whole kidney homogenates (total cell fraction) and plasma membrane enriched fraction using a sucrose gradient-based membrane protein extraction kit (see concise methods). For the experiments, kidneys from the same animals as in Figures 6 and 7 were used. As demonstrated on the representative Western blot in Figure 9A, the membrane enriched fraction nearly lacked  $\alpha$ -tubulin staining. Importantly, we observed a shift of  $\alpha$ ENaC molecular weight suggesting that plasma membrane  $\alpha$ ENaC is glycosylated. This is consistent with previously published results that cell surface  $\alpha$ ENaC in MDCK cells is almost exclusively glycosylated<sup>48</sup>. The glycosylated  $\alpha$ ENaC in the total cell fraction was barely detectable (data not shown). This suggests that only a minor fraction of the total cell full length  $\alpha$ ENaC protein pool resides on the plasma membrane. Chronic Ang II infusion had only a tendency to increase  $\alpha$ ENaC abundance in whole kidney homogenates (Figures 9B, D), whereas plasma membrane  $\alpha$ ENaC levels were significantly higher in Ang II-treated mice (Figure 9C, E). Administration of MR inhibitor spironolactone decreased  $\alpha$ ENaC abundance in total cell (Figures 9B, D) and plasma membrane enriched fraction (Figure 9C, E) of Ang II-infused animals below the values observed in control mice. We concluded that chronic Ang II-infusion increases plasma membrane  $\alpha$ ENaC protein levels in an aldosterone-dependent manner. Furthermore, the observed augmentation of functional ENaC activity in Ang II-infused animals under spironolactone treatment (Figures 6, 7) is likely due to activation of inactive “silent” ENaCs on the apical plasma membrane and is not associated with augmented levels of the channel.

## DISCUSSION

This study was undertaken to examine how signals from two major hormones responsible for sodium homeostasis, Ang II and aldosterone, modulate ENaC activity in the distal nephron in response to systemic signals. We found that during physiological alterations in dietary sodium, aldosterone cascade plays a conclusive role by controlling the number of functionally active ENaC channels. Ang II signaling, by shaping gating properties of ENaC, has a lesser contribution. In contrast, aldosterone-independent mechanisms of ENaC



activation become dominant during the pathology of Ang II-dependent hypertension. This involves augmentation of the number of functional ENaC and ENaC  $P_o$  and cannot be effectively suppressed by inhibition of aldosterone-MR axis.

In the current study, we found that inhibition of GR with mifepristone had minor effect on ENaC activity during all tested dietary salt intakes (Figure 2) suggesting a minor role of glucocorticoid signaling mechanisms in setting basal ENaC activity during different sodium diets. In agreement, a recent study reports that systemic administration of dexamethasone, while increasing  $\alpha$ ENaC abundance in rat kidneys in an aldosterone-independent manner, fails to activate  $\text{Na}^+$  currents measured in isolated split-open collecting ducts<sup>49</sup>. We also demonstrate that maximal stimulation of mineralocorticoid signaling in DOCA-treated mice does not increase ENaC activity when MR are inhibited with spironolactone (supplementary Figure S1). This argues against the possibility that elevated circulating aldosterone levels, particularly during volume depleted states, stimulate GR to affect ENaC. Therefore, the mounting evidence points to an almost exclusive role of MR as mediators of steroid-induced ENaC activation in the ASDN during physiological responses to dietary sodium supplementations. However, over-stimulation of GR might contribute to the development of abnormal sodium retention in the distal nephron. Thus, a cumulative role of both GR and MR in stimulation of ENaC activity has been proposed in patients with Cushing syndrome and animal models with infusion of adrenocorticotrophic hormone (ACTH) as a possible mechanism of hypertension<sup>50</sup>.

Using a physiologically relevant object, split-opened murine ASDN, we demonstrate that inhibition of aldosterone-MR signaling fails to suppress regulation of functional ENaC activity by variations in dietary sodium regimen (Figures 4A–C). This unequivocally points to an important role of aldosterone-independent mechanisms for proper control of ENaC activity by systemic salt status. A previous report demonstrates that spironolactone administration blocked the increase in  $\alpha$ ENaC protein abundance in response to dietary NaCl restriction<sup>51</sup>. However, ENaC redistribution to the apical plasma membrane was not prevented by inhibition of MR<sup>51</sup>. We did not detect significant changes in the number of functionally active ENaC in spironolactone-treated mice kept on high and low sodium diet suggesting that alterations in subcellular ENaC localization do not transform to increased functional ENaC expression. We did observe, however, that regulation of ENaC gating properties (i.e.  $P_o$ ) by dietary sodium is not affected by MR inhibition. In contrast, combined pharmacological blockade of  $\text{AT}_1\text{R}$  and MR nearly abolished changes in ENaC  $P_o$  when dietary  $\text{Na}^+$  was manipulated (Figures 3, 4). This argues that Ang II cascade has non-redundant stimulatory actions on ENaC activity via control of channel gating. Consistently, we<sup>28</sup> and others<sup>29</sup> recently documented that acute stimulation of ENaC  $P_o$  by Ang II in split-opened ASDNs is independent of MR status.

Augmented circulating Ang II levels in the models of Ang II-induced hypertension result in marked decreases in renal sodium excretion and likely increases in distal nephron sodium reabsorption<sup>33</sup>. Systemic Ang II infusion also increases  $\alpha$ ENaC protein abundance in rat renal cortex<sup>36</sup>. Paradoxically, inhibition of MR with spironolactone<sup>33</sup> and eplerenone<sup>32</sup> fails to correct renal sodium retention and hypertension in response to systemic Ang II infusion. It was proposed that increased function of NCC but not ENaC could be a key mechanism in Ang II-dependent hypertension<sup>37</sup>. Using direct measurements of ENaC activity in native ASDNs with patch clamping, we demonstrate a robust excessive ENaC activation in Ang II-infused mice to the values exceeding the physiological range of ENaC regulation by dietary salt (Figure 6B). Spironolactone in the concentration sufficient to completely abolish stimulatory actions of aldosterone on ENaC (Figure S1) was ineffective to suppress ENaC to the control values. The number of active ENaC on the apical plasma membrane and ENaC  $P_o$  remained elevated upon spironolactone treatment (Figure 7). The stimulatory effect of

Ang II infusion on ENaC  $P_o$  in the presence of MR inhibition was anticipated<sup>28,29</sup> and consistent with aldosterone-independent contribution of Ang II cascade to regulation of ENaC  $P_o$  by dietary salt intake (Figure 4). Several studies suggested that Ang II might also play a direct role in regulation of ENaC expression. Thus, AT<sub>1</sub>R  $-/-$  mice have decreased  $\alpha$ ENaC abundance in the presence of elevated circulating aldosterone levels<sup>35</sup>. AT<sub>1</sub>R blockade also results in a fall in  $\alpha$ ENaC mRNA abundance, and downregulation of  $\alpha$ ENaC expression was not blocked by spironolactone<sup>36</sup>. In the current study, we probed whether the increased number of functional ENaC on the apical plasma membrane in Ang II-infused animals after spironolactone is associated with aldosterone-independent ENaC expression and/or trafficking. While there was only a tendency of increasing  $\alpha$ ENaC levels in whole kidney homogenates of Ang II infused mice, the stimulatory effect of Ang II on  $\alpha$ ENaC was significant for the plasma membrane fraction (Figure 9). Consistently, our immunofluorescent studies demonstrate accumulation of  $\alpha$ ENaC predominantly in the apical region in principal cells of Ang II-infused mice (Figure 8). Strikingly, spironolactone treatment decreased  $\alpha$ ENaC protein abundance on the plasma membrane below the control levels in Ang II infused mice (Figure 9C). Therefore, the observed elevation of functionally active ENaC with patch clamping in Ang II-infused animals after spironolactone is not due to increased protein levels but rather stimulation of “silent” channels residing on the apical plasma membrane.

The important observation of this study is that systemic infusion of Ang II increases ENaC activity in the ASDN to levels greatly exceeding those observed during physiological conditions associated with volume contraction (Figures 6B, 7). Accumulating experimental evidence suggests a critical role of intrarenal reninangiotensin system (RAS) in development of excessive sodium retention and Ang II-dependent hypertension<sup>21,52</sup>. Intrarenal synthesis of Ang II appears to be sufficient to affect sodium handling in the distal nephron, since luminal perfusion of cortical collecting ducts with Ang I stimulates apical sodium entry in an ACE-dependent manner<sup>53</sup>. In contrast to the systemic RAS where elevations in circulating Ang II inhibit renin secretion from the granular cells in the juxtaglomerular apparatus, augmented distal nephron Ang II concentration further leads to increased renin mRNA and protein levels in principal cells of CNT and CCD<sup>54,55</sup>. This feed-forward mechanism is thought to underlie inappropriate augmentation of sodium reabsorption in the distal nephron<sup>38,56</sup>. Indeed, it was recently reported that systemic infusion of Ang II results in increased urinary Angiotensinogen (AGT) levels indicating intratubular RAS activation<sup>57</sup>. In contrast, dietary sodium restriction does not lead to augmentation of urinary AGT and Ang II excretion rates in rats and thus to appreciable intratubular RAS activation<sup>58</sup>. Since principal cells express angiotensin receptors at both apical and basolateral sides, it is likely that circulating and tubular Ang II can separately regulate ENaC-mediated sodium reabsorption during different physiological and pathological conditions. Specifically, activation of basolateral receptors by renal interstitial Ang II levels will play a dominant role in regulation of ENaC open probability during dietary sodium restriction. During Ang II-dependent hypertension, the additional stimulation of intratubular RAS leads to further ENaC over-activation via mechanisms that are independent of mineralocorticoid status.

## PERSPECTIVES

It is now becoming recognized that inputs from coordinated but discrete converging signals are critical for proper control of renal sodium transport. This is known to be essential for normal blood pressure control. ENaC-mediated Na<sup>+</sup>-reabsorption in the distal nephron finalizes adjustments of renal sodium excretion to match dietary sodium intake and maintain sodium balance. The current study documents that aldosterone and Ang II signals provide integrated control of ENaC-mediated sodium reabsorption in the distal nephron. The aldosterone cascade prevails during adaptations to dietary sodium intake. In contrast,

augmentation of circulating Ang II levels leads to activation of intrarenal RAS and switches regulation of ENaC activity to a largely aldosterone-independent mode. From a clinical standpoint, increased urinary excretion rates of Ang II or its precursor AGT in the setting of essential hypertension might be useful as indicators of increased ENaC-mediated sodium absorption in the distal nephron via mechanisms independent of classic aldosterone actions. This, in turn, may predict resistance to antihypertensive strategy of MR blockade.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## NOVELTY AND SIGNIFICANCE

### What is new

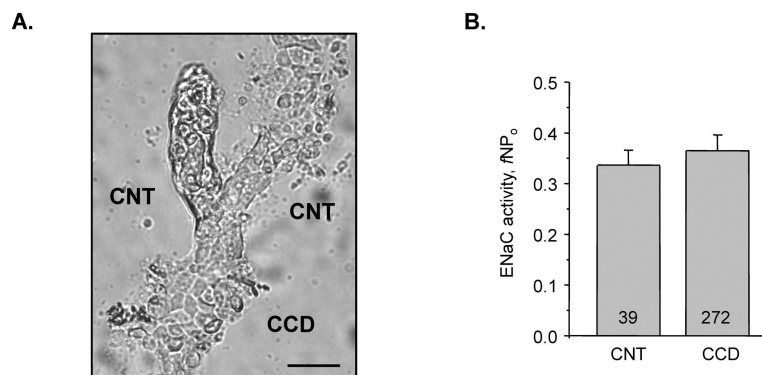
- Coordinated actions of aldosterone and Ang II cascades underlie respective increases in the number of functionally active ENaC channels and channel open probability when dietary sodium intake is reduced.
- During the pathology of Ang II-dependent hypertension, ENaC is over-active and is no longer effectively regulated by the aldosterone-MR axis.

### What is relevant

ENaC activity in the distal part of the renal nephron serves as a final regulator of urinary sodium excretion. Therefore, revealing the cellular and molecular mechanisms modulating ENaC activity during systemic inputs is directly relevant to understanding normal blood pressure control and pathophysiology of hypertension. These data provide a mechanistic explanation for the resistance to MR inhibition that occurs in hypertensive subjects having elevated intrarenal Ang II levels.

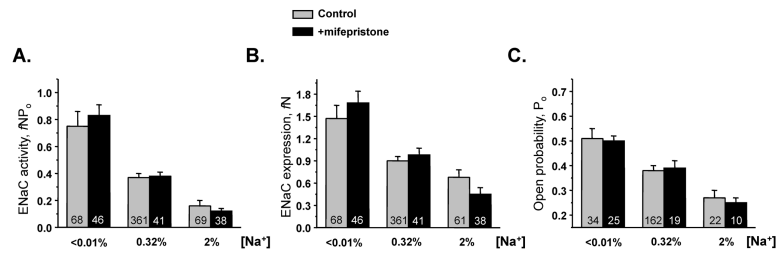
### Summary

- Direct regulation of ENaC activity by Ang II signaling has its own non-redundant role in adaptation of ENaC-mediated sodium reabsorption in the ASDN to variations in dietary salt intake.
- Chronic Ang II infusion over-stimulates ENaC beyond the physiological range of ENaC regulation contributing to the excessive renal sodium conservation and hypertension. Blockade of aldosterone-MR cascade is insufficient to suppress excessively activated ENaC when Ang II levels are elevated.



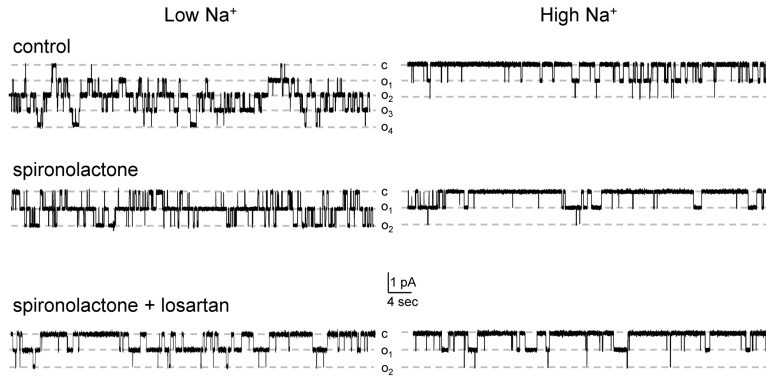
**Figure 1. ENaC activity is similar in CNT and CCD**

(A) A representative micrograph of freshly isolated murine ASDN split-opened at CCD (bottom) and at the right branch of CNT (top) used for patch clamp evaluation of ENaC activity. Scale bar is 40  $\mu\text{m}$ . (B) Summary of ENaC activity ( $fNP_o$ ) in CNT and CCD of ASDN from mice kept on a regular salt regimen (0.32%  $\text{Na}^+$ ). Here and below the number of individual patch recordings are shown.



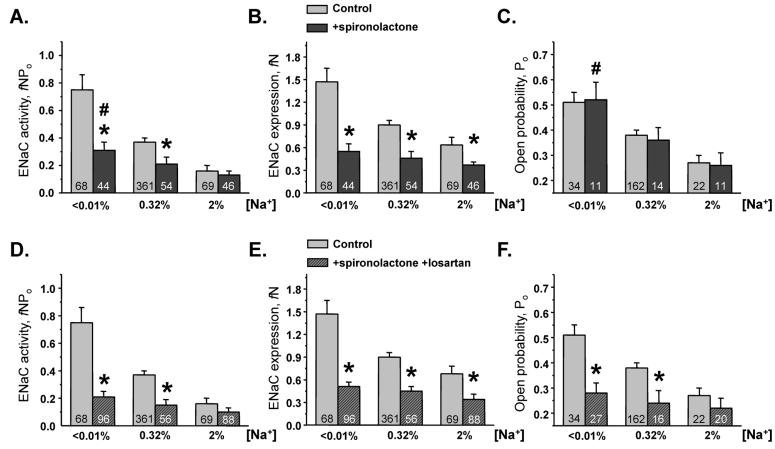
**Figure 2. Glucocorticoid receptors do not contribute to regulation of ENaC by systemic salt intake**

Summary graph of ENaC activity,  $fNP_o$  (A); functional ENaC expression,  $fN$  (B); and ENaC open probability,  $P_o$  (C) in mice kept on low (<0.01%), regular (0.32%), and high (2%) Na<sup>+</sup> diets for one week, respectively, in the control (light grey bars) and after treatment with glucocorticoid receptor (GR) antagonist, mifepristone (black bars).



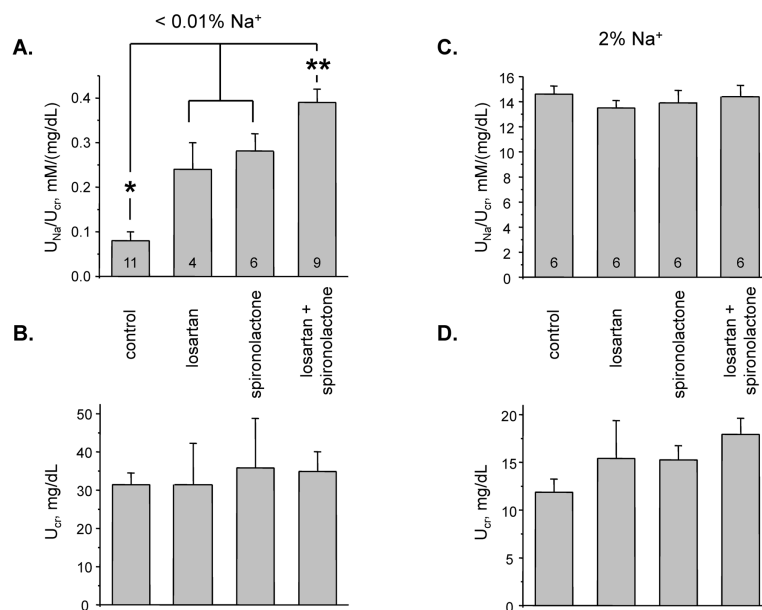
**Figure 3. Coordinated regulation of ENaC activity by aldosterone and Ang II signaling cascades**  
 Representative continuous current traces from cell-attached patches monitoring ENaC activity in mice on low and high  $\text{Na}^+$  diets in the control (top row), after treatment with MR antagonist spironolactone (middle row) and after combined treatment with spironolactone and  $\text{AT}_1\text{R}$  blocker, losartan for one week (bottom row). Patches were held at a test potential of  $V_h = -V_p = -60$  mV. Inward  $\text{Li}^+$  currents are downward. Dashed lines indicate the respective current states marked as  $o_1$  and a  $c$  denotes the closed state.





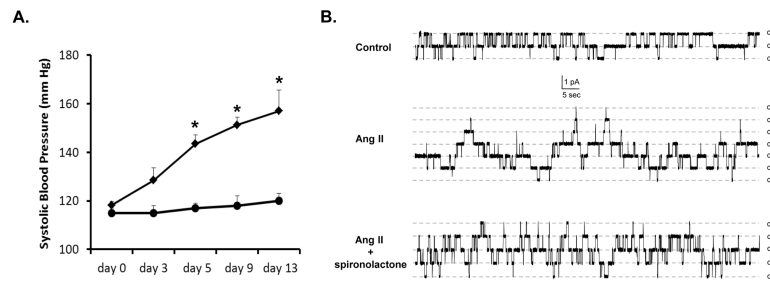
**Figure 4. Inhibition of MR and AT<sub>1</sub>R abolishes stimulation of ENaC activity by dietary sodium restriction**

Summary graphs of ENaC activity,  $fNP_o$  (A); functional ENaC expression,  $fN$  (B); and ENaC open probability,  $P_o$  (C) in mice kept on low (<math><0.01\%</math>), regular (0.32%), and high (2%) fNP\_o (D); functional ENaC expression,  $fN$  (E); and ENaC open probability,  $P_o$  (F) in mice kept on low (<math><0.01\%</math>), regular (0.32%), and high (2%) 1R blocker, losartan (shaded dark grey bars). \* -significant decrease versus respective control values.



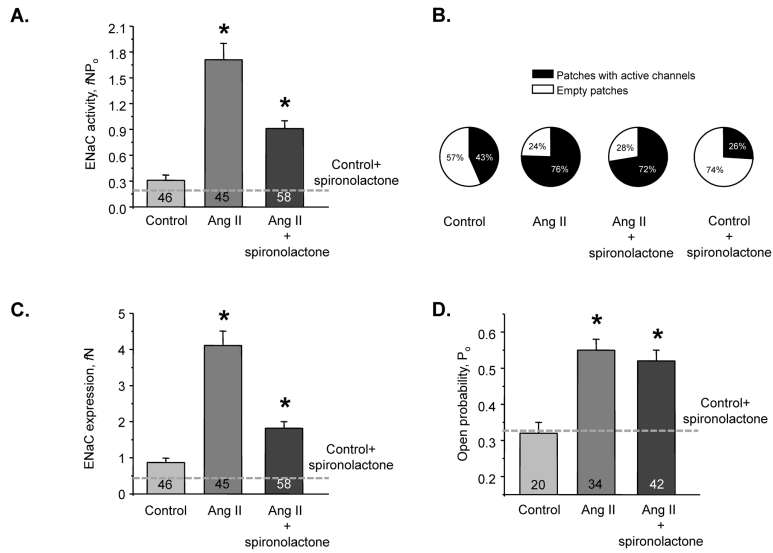
**Figure 5. Aldosterone and Ang II signaling cascades additively contribute to regulation of urinary  $\text{Na}^+$  excretion during low sodium intake**

Summary graphs of urinary  $\text{Na}^+$  excretion normalized to creatinine levels in mice kept on low (A) and high (C) sodium diets for 3 days in the control and after systemic treatment with losartan, spironolactone, losartan+spironolactone. Summary graphs of urinary creatinine levels in mice kept on low (B) and high (D) sodium diets for 3 days in the control and after systemic treatment with losartan, spironolactone and losartan+spironolactone. \* - significant increase versus control; \*\* - significant increase versus losartan and spironolactone.

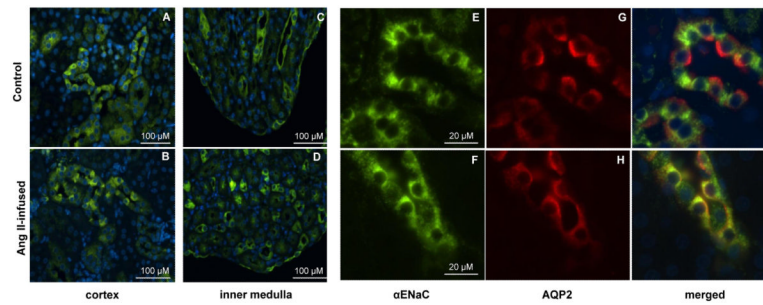


**Figure 6. Chronic Ang II infusion elevates systemic blood pressure and drastically increases functional ENaC activity**

(A) The time courses of changes in systolic blood pressure in response to chronic infusion of vehicle (circles) and 400 ng/kgBW/min of Ang II (diamonds) as determined by telemetry. \* - significant increase versus respective systolic blood pressure values in control vehicle infused mice. (B) Representative continuous current trace from cell-attached patches monitoring ENaC activity in mice after 2 weeks infusion of vehicle (top row), Ang II (middle row) and Ang II in the presence of spironolactone (bottom row). All other conditions are identical to that described in Figure 4.



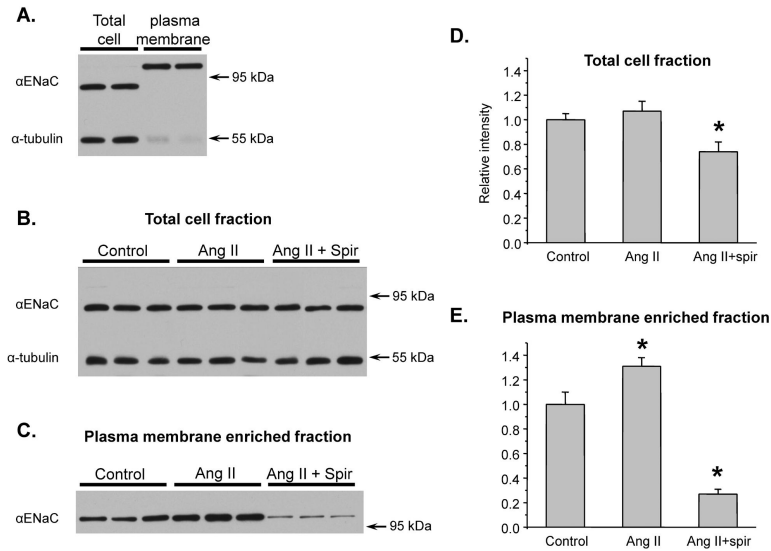
**Figure 7. MR blockade fails to restore ENaC activity during chronic Ang II infusion**  
**(A)** Summary graph of ENaC activity,  $fNP_o$ , in mice kept on regular salt intake after 2 weeks infusion of vehicle (Control), Ang II and Ang II in the presence of MR inhibition with spironolactone. Dashed line indicates the level of ENaC activity after spironolactone treatment in mice kept on regular salt intake (see also Figures 2, 3). **(B)** Pie charts representing the frequency of observing patches with active channels ( $f$ ) for the conditions described in (A). For comparison, the frequency of observing patches with active channels for condition of low sodium diet is also included. Summary graphs of functional ENaC expression **(C)** and ENaC  $P_o$  **(D)** after 2 weeks infusion of vehicle (Control), Ang II and Ang II in the presence of MR inhibition with spironolactone. Dashed line indicates the respective levels observed during regular salt intake + spironolactone. \* - significant increase versus Control.



**Figure 8. Chronic Ang II infusion promotes apical ENaC distribution in principal cells of the collecting duct**

Representative micrographs of  $\alpha$ ENaC immunofluorescent staining in 3  $\mu$ m kidney sections from control (A) and chronic Ang II-infused mice (B) in the cortex region as well as in the inner medulla regions (C, D). High resolution images of the collecting duct of a kidney section from control and Ang II-infused mice demonstrating expression of  $\alpha$ ENaC (E, F), a marker of principal cells, AQP2 (G, H) and the respective merged micrographs (I, J). Note the lack of merging colors in the apical aspect in most of the principal cells of the collecting duct from the control mouse kidney section (G) compared to the Ang II-infused mouse (J). Fluorescent signals reporting  $\alpha$ ENaC and AQP2 expression are shown with pseudocolor green and red, respectively. Nuclei are stained using 4,6-diamidino-2-phenylindole (DAPI, pseudocolor blue). Respective scale bars are also shown.





**Figure 9. Chronic Ang II infusion increases  $\alpha$ ENaC plasma membrane abundance in an aldosterone-dependent manner**

(A) Representative Western blot from whole kidney lysates (total cell fractions and membrane enriched fractions) probed with anti- $\alpha$ ENaC and anti- $\alpha$  tubulin antibodies. (B) Representative Western blot of total cell fractions from control, Ang II-infused, and Ang II-infused + spironolactone (spir) mice probed with anti- $\alpha$ ENaC and anti- $\alpha$  tubulin antibodies. Each band represents individual animal. The mice were the same as used for Figures 6 and 7. (C) Representative Western blot of plasma membrane enriched fractions from control, Ang II-infused, and Ang II-infused + spironolactone (spir) mice probed with anti- $\alpha$ ENaC and anti- $\alpha$  tubulin antibodies. All other conditions are the same as in (B). (D) Summary graph comparing total cell  $\alpha$ ENaC expression in the control, after Ang II-infusion, and after Ang II infusion + spironolactone from Western blots similar to that shown in (B). \* - significant decrease versus control. (E) Summary graph comparing  $\alpha$ ENaC expression in membrane enriched fraction in the control, after Ang II-infusion, and after Ang II infusion + spironolactone from Western blots similar to that shown in (C). \* - significant decrease versus control.