Renin–Angiotensin–Aldosterone System

Chronic Angiotensin II Infusion Drives Extensive Aldosterone-Independent Epithelial Na\(^+\) Channel Activation

Mykola Mamenko,* Oleg Zaika,* Minolfa C. Prieto, V. Behrana Jensen, Peter A. Doris, L. Gabriel Navar, Oleh Pochynyuk

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\(^+\) channel (ENaC) in the aldosterone-sensitive distal nephron. We used freshly isolated aldosterone-sensitive distal nephron 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 in the regulation of ENaC activity by dietary salt intake. Simultaneous inhibition of MR and Ang II type 1 receptors 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 2 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 aldosterone-sensitive distal nephron. In contrast, in the setting of Ang II–dependent hypertension, ENaC activity is upregulated 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. (Hypertension. 2013;62:1111-1122.) • Online Data Supplement

Key Words: collecting duct • hypertension • nephrons

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.\(^1\) Dysfunction of sodium transport at this site has been tightly linked to disturbances in circulating volume and blood pressure abnormalities.\(^{1,2}\) Activity of the epithelial Na\(^+\) channel (ENaC) underlies electrogenic Na\(^+\) reabsorption at ASDN, which includes the connecting tubule (CNT) and the cortical collecting duct (CCD).\(^{3,4}\) 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, arise from gain-of-function and loss-of-function mutations in ENaC, respectively.\(^{5,7,11}\)

ENaC activity in the ASDN is inversely related to dietary sodium intake.\(^{12,14}\) Activation of the renin–angiotensin–aldosterone system (RAAS) in response to sodium restriction increases ENaC activity and expression via aldosterone–mineralocorticoid receptor (MR) pathway.\(^{3,5,8,15-17}\) 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,\(^{18,19}\) points to a role for other signaling components of renin–angiotensin–aldosterone system, most likely angiotensin (Ang) II, in the regulation of ENaC in response to volume contraction.

Ang II is the principal effector of RAAS.\(^{20,21}\) Increases in circulating Ang II levels induce vasoconstriction, secretion of aldosterone, and elevations in systemic blood pressure.\(^{22}\) In the kidney, Ang II promotes antinatriuresis, in part, by stimulating luminal Na\(^+\) entry and tubular Na\(^+\) reabsorption in the distal nephron.\(^{23-25}\) Virtually all Ang II actions to enhance renal sodium transport are mediated by Ang II type 1 receptors (AT1R). AT1R 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.\(^{26,27}\) We\(^{28}\) and others\(^{25,29}\) have documented that Ang II acutely increases ENaC activity and open

Received May 31, 2013; first decision June 28, 2013; revision accepted August 30, 2013.

From the Department of Integrative Biology and Pharmacology (M.M., O.Z., O.P.), Center for Laboratory Animal Medicine and Care (V.B.J.), and Institute of Molecular Medicine (P.A.D.), The University of Texas Health Science Center at Houston, TX; and Department of Physiology and Hypertension and Renal Center of Excellence, Tulane University School of Medicine, New Orleans, LA (M.C.P., L.G.N.).

The online-only Data Supplement is available with this article at http://hyper.ahajournals.org/lookup/suppl/doi:10.1161/HYPERTENSIONAHA.113.01797/-/DC1.

*These authors contributed equally to this work.

Correspondence to Oleh Pochynyuk, Department of Integrative Biology and Pharmacology, University of Texas Health Science Center at Houston, 6431 Fannin, Houston, TX 77030. E-mail Oleh.M.Pochynyuk@uth.tmc.edu

© 2013 American Heart Association, Inc.

Hypertension is available at http://hyper.ahajournals.org

DOI: 10.1161/HYPERTENSIONAHA.113.01797
probability \( (P) \) in freshly isolated split-opened mouse and rat ASDN. The signaling pathway involves AT, R-dependent activation of NADPH oxidase and generation of reactive oxygen species, likely superoxide and peroxide, to stimulate ENaC and to diminish inhibitory actions of the arachidonic acid metabolites. Of note, acute stimulation of ENaC \( P \), by Ang II persists during satiruation and inhibition of MR cascade indicating aldosterone-independent nature of this regulation. 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. It remains unclear whether physiologically relevant changes in Ang II levels have their own nonredundant 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, intrarenal Ang II levels become much higher than those in plasma because of activation of the intrarenal and intratubular renin angiotensin system (RAS). Inappropriately stimulated intrarenal RAS leads to excessive sodium retention, in part, because of possible activation of ENaC in ASDN. 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, receptors (AT, ) exhibit a marked reduction in \( \alpha \)ENaC abundance in the kidney despite slightly elevated aldosterone levels. Furthermore, the effect of AT, R blockade on \( \alpha \)ENaC expression was not prevented by spironolactone, suggesting a direct role of AT, R in regulation of \( \alpha \)ENaC gene expression. Systemic infusion of Ang II increases \( \alpha \)ENaC protein abundance in rat kidney cortex. 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 hypertensive effects. In contrast, direct ENaC blockade with amiloride does attenuate blood pressure in Ang II–infused rats. This may indicate a dominant role of Ang II–driven aldosterone-independent ENaC activation in Ang II–infused models of hypertension.

In the current study, we use systemic pharmacological inhibition of MR and AT, 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 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 \( P \). In contrast, chronic Ang II infusion overstimulates 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. Animal use and welfare adhered to the National Institutes of Health 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 to 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\(^+\); TD.90228, regular \( 0.32\% \) Na\(^+\); TD.79112, and high \( 2\% \) Na\(^+\); TD.90234 sodium for 1 week. All diets were purchased from Harlan Teklad (Madison, WI). Spironolactone (30 mg/kgBW), mifepristone (30 mg/kgBW), and 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 for 3 consecutive days (2.4 mg/injection per animal) before the experimentation, similar to what we have done previously.

Male C57BL/6 mice (6 weeks; \( >25 \) 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 of 400 ng/kgBW per minute for 14 days following well-established protocols.

Mice were subjected to inhaled anesthesia with isoflurane, 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-day recovery phase, baseline systolic blood pressure levels were measured, and the mice were then subjected to a similar anesthesia for osmotic minipump subcutaneous implantation. Mice were infused with Ang II (400 ng/kgBW per minute) 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 ASDNs suitable for electrophysiology has been described previously. ENaC activity in principal cells was determined in cell-attached patches on the apical membrane made under voltage clamp conditions (\( V = −60\)mV) using standard procedures (see online-only Data Supplement for more details).

**Immunofluorescent Microscopy**

Kidneys were fixed in 10% formalin at 4°C overnight, processed for paraffin embedding, sectioning (3 \( \mu \)m), and sequentially immunostained using rabbit anti-\( \alpha \)ENaC and goat anti-AQP2 antibodies as described previously. The primary antibodies used were anti-\( \alpha \)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-minute incubations, respectively. For immunofluorescent detection, we used donkey antirabbit or donkey antigoat 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 and ×100 (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 for further use. Before experimentation, the kidneys were homogenized in 3 volumes of icecold hypotonic lysis buffer containing 50 mmol/L Tris, 1% Triton X-100, 5 mmol/L EDTA (pH=7.4) supplemented with 1 mmol/L PMSF and 2 mg/mL protease inhibitor cocktail (Complete mini; Roche Diagnostics, Germany). Homogenates were centrifuged at 1000g for 15 minutes at 4°C. The supernatants were split in 2 parts to assess total cellular proteins and plasma membrane proteins using plasma membrane protein extraction kit (Biovision Inc) following the manufacturer’s protocol. Protein concentration in the homogenates was determined with a Bradford assay. The samples (25 μg/lane) were separated on 9% polyacrylamide gels at 150 V for 1 hour 15 minutes and transferred to nitrocellulose membranes at 100 V for 1 hour 45 minutes. The membranes were blocked for 1 hour 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-αENaC primary rabbit antibodies (1:1000; Stressmarq Biosciences, Canada) followed by peroxidase-conjugated goat antirabbit secondary antibodies (1:20000; Bio-Rad) for 1 hour at RT. The membranes were reprobed with anti-α-tubulin (1:200; Abcam, United Kingdom) primary rabbit antibodies and peroxidase-conjugated goat antirabbit secondary antibodies (1:20000; Bio-Rad). When total cellular αENaC expression was assessed, αENaC band intensities were normalized to the intensities of the corresponding α-tubulin bands. For membrane-enriched fractions, band intensities were normalized to the intensity of the first αENaC band from the first control sample. All experiments were repeated ≥3 times.

**Urinary Sodium Excretion**

Urine was collected from bladders of euthanized animals and frozen at −20°C until analyzed. The time of urine sampling was the same (10:00 AM to 11:00 AM) 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).

**Data Analysis**

All summarized data are reported as mean±SEM. Data from different experimental sets were compared using a Student (2-tailed) t test or a 1-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% Na+). As illustrated in Figure 1A, we mechanically isolated bifurcations of the distal nephrons representing merging of 2 CNTs into a CCD. ENaC activity was surveyed using patch clamp electrophysiology in cell-attached configuration within split-opened areas located up to 100 μm above (CNT region) and 300 μm below (CCD region) the bifurcations. To reliably assess ENaC activity (NP), the mean NP was corrected to a frequency of observing patches with active channels (f=number of patches with channels/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 (Ppo) and the number of functional ENaC within a patch (M) 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 mg/kgBW for 7 days) in mice maintained on a regular salt intake. This treatment significantly diminished but did not abolish ENaC activity (NP) from 0.40±0.03 (n=223) to 0.21±0.05 (n=52). To ensure that the concentration of spironolactone is sufficient for complete inhibition of MR, we injected mice with deoxycorticosterone acetate for 3 consecutive days before the assessment. Saturation of mineralocorticoid status significantly increased ENaC activity to 0.61±0.08 (n=54) in ASDNs from control mice but had no effect on ENaC (0.23±0.04; n=58) in ASDNs from mice treated with spironolactone (Figure S1 in the online-only Data Supplement).

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 1 week (low, <0.01%; regular, 0.32%; and high, 2% Na+) in the absence and presence of systemic inhibition of GR with mifepristone (30 mg/kgBW, for 7 days). This concentration is similar to that used previously for GR inhibition. ENaC activity augmented during low Na+ diet and diminished under high Na+ diet (Figure 2A; see also Figure 3, top row for representative traces of ENaC activity).

![Figure 1](http://hyper.ahajournals.org/)
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 Table S1 for the absolute values).

Overall, we concluded that aldosterone uses 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 \( Na^+ \) diets for 1 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 but 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 and 4A). Detailed patch clamp analysis revealed that inhibition of MRs prevented changes in functional ENaC expression in response to dietary salt variations (Figure 4B; 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 Table S1). Thus, we concluded that aldosterone-independent stimulation of ENaC activity by dietary salt restriction involves augmentation of ENaC \( P_o \).

**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,\( R \) is capable of increasing ENaC \( P_o \) and this regulation persists even in the presence of saturated mineralocorticoid status. 28 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,\( R \) 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 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,\( R \) blockade, ENaC \( P_o \) was only slightly different in mice placed on 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 \( P_o \).

**Inhibition of Aldosterone–MR and Ang II–AT,\( R \) Cascades Additively Produces Natriuresis**

We next probed whether 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 with 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, although indicative, support the concept that aldosterone and Ang II signaling cascades stimulate ENaC

\[ \text{Figure 2. Glucocorticoid receptors do not contribute to regulation of epithelial Na}^+ \text{ channel (ENaC) by systemic salt intake. Summary } \]

\[ \text{graph of ENaC activity (NP}_{\text{ENaC}}; \text{A}; \text{functional ENaC expression (fN; B); and ENaC open probability (P}_{\text{o; C}) in mice kept on low (<0.01%),}\]

\[ \text{regular (0.32%), and high (2%) Na}^+ \text{ diets for 1 week, respectively, in the control (light grey bars) and after treatment with glucocorticoid receptor antagonist mifepristone (black bars).} \]
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+ transporting systems in the kidney, most notably the sodium chloride cotransporter (NCC).44,45

**Figure 3.** Coordinated regulation of epithelial Na\(^+\) channel (ENaC) activity by aldosterone and angiotensin (Ang) II signaling cascades. Representative continuous current traces from cell-attached patches monitoring ENaC activity in mice on low and high Na\(^+\) diets in the control (top), after treatment with mineralocorticoid receptor antagonist spironolactone (middle), and after combined treatment with spironolactone and Ang II type 1 receptor blocker losartan for 1 week (bottom). Patches were held at a test potential of \(V_h = V_p = -60\) mV. Inward Li\(^+\) currents are downward. Dashed lines indicate the respective current states marked as \(o\) and \(c\) denotes the closed state.

**Figure 4.** Inhibition of mineralocorticoid receptor (MR) and angiotensin II type 1 receptors (AT1R) abolishes stimulation of epithelial Na\(^+\) channel (ENaC) activity by dietary sodium restriction. Summary graphs of ENaC activity (\(fN_P\); 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\(^+\) diets for 1 week, respectively, in the control (light grey bars) and after treatment with MR antagonist spironolactone (dark grey bars). *Significant decrease vs respective control values; #significant increase vs spironolactone 2% Na\(^+\). Summary graphs of ENaC activity (\(fN_P\); D), functional ENaC expression (\(fN\); E), and ENaC open probability (\(P_o\); F) in mice kept on low (<0.01%), regular (0.32%), and high (2%) Na\(^+\) diets for 1 week, respectively, in the control (light grey bars) and after combined treatment with spironolactone and AT1R blocker losartan (shaded dark grey bars). *Significant decrease vs respective control values.
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 and diabetes mellitus. Studies in both rats and mice have demonstrated that chronic Ang II infusions lead to increased urinary levels of angiotensinogen and Ang II. These data suggest that augmented intratubular Ang II signaling enhances renal sodium reabsorption, in part, by overstimulating 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+) in response to chronic infusion of Ang II (400 ng/kgBW per minute 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 versus 115±2 mmHg; P=0.06). By day 5, systolic blood pressures were augmented in mice receiving Ang II (144±4 versus 117±1 mmHg; P<0.05) and continued to increase by day 9 (151±3 versus 118±2 mmHg; P<0.001) and day 13 (157±9 versus 120±1 mmHg; P<0.05) compared with the control group. The time course of developing hypertension is consistent with previous reports using a similar experimental strategy.

As visualized by the representative traces of ENaC activity (Figure 6B) and as summarized in Figure 7A, chronic Ang II

Figure 6. Chronic angiotensin (Ang) II infusion elevates systemic blood pressure and drastically increases functional epithelial Na+ channel (ENaC) activity. A, The time courses of changes in systolic blood pressure in response to chronic infusion of vehicle (circles) and 400 ng/kgBW per minute of Ang II (diamonds) as determined by telemetry. *Significant increase vs respective systolic blood pressure values in control vehicle-infused mice. B, Representative continuous current traces from cell-attached patches monitoring ENaC activity in mice after 2 weeks' infusion of vehicle (top), Ang II (middle), and Ang II in the presence of spironolactone (bottom). All other conditions are identical to that described in Figure 4.
infusion leads to a robust (≈6 times) elevation of ENaC activity compared with 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 and 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 $P_o$ (Figure 7D). The absolute values of ENaC activity in vehicle-infused and Ang II–infused animals are reported in Table S2.

To assess the relative contributions of aldosterone-dependent and aldosterone-independent mechanisms in activation of ENaC activity in Ang II–infused animals, mice were provided with spironolactone for the 14-day period of treatment. Inhibition of MR did attenuate the stimulatory effect of Ang II on ENaC (Figure 6B). However, ENaC activity remained >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). 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 MR fails to efficiently suppress ENaC activity, thus suggesting a critical contribution for Ang II–mediated mechanisms independent of aldosterone.

**Regulation of Plasma Membrane αENaC 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 αENaC distribution in kidney sections at the cortex (Figure 8A and 8B) and inner medulla (Figure 8C and 8D) 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 αENaC fluorescent signal displays a predominant apical distribution in the collecting ducts from kidney

---

**Figure 7.** Mineralocorticoid receptor (MR) blockade fails to restore epithelial Na+ channel (ENaC) activity during chronic angiotensin (Ang) II infusion. A, Summary graph of ENaC activity ($f_{NP_o}$) in mice kept on regular salt intake after 2 weeks’ infusion of vehicle (control), Ang II, and Ang II in the presence of MR inhibitor spironolactone. Dashed line indicates the level of ENaC activity after spironolactone treatment in mice kept on regular salt intake (see also Figures 2 and 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 inhibitor spironolactone. Dashed line indicates the respective levels observed during regular salt intake plus spironolactone. *Significant increase vs control.
sections from Ang II–infused mice compared with control mice (Figure 8E and 8F). The αENaC immunoeexpression was present in the principal cells costained with the specific marker AQP-2 (Figure 8G–J).

Because immunofluorescent microscopy provides only qualitative evidence of the subcellular ENaC redistribution in Ang II–infused mice, we further monitored the fulllength 95-kDa αENaC in whole-kidney homogenates (total cell fraction) and plasma membrane–enriched fraction using a sucrose gradient–based membrane protein extraction kit (see Methods section). 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 α-tubulin staining. Importantly, we observed a shift of αENaC molecular weight, suggesting that plasma membrane αENaC is glycosylated. This is consistent with previously published results that cell surface αENaC in Madin-Darby Canine Kidney (MDCK) cells is almost exclusively glycosylated. The glycosylated αENaC in the total cell fraction was barely detectable (data not shown). This suggests that only a minor fraction of the total cell full-length αENaC protein pool resides on the plasma membrane. Chronic Ang II infusion had only a tendency to increase αENaC abundance in whole-kidney homogenates (Figure 9B and 9D), whereas plasma membrane αENaC levels were significantly higher in Ang II–treated mice (Figure 9C and 9E). Administration of MR inhibitor spironolactone decreased αENaC abundance in total cell (Figure 9B and 9D) and plasma membrane enriched–fraction (Figure 9C and 9E) of Ang II–infused animals below the values observed in control mice. We conclude that chronic Ang II infusion increases plasma membrane αENaC 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 and 7) is likely because of 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 2 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, and cannot be effectively suppressed by inhibition of aldosterone–MR axis.

In the current study, we found that inhibition of GR with mifepristone had a 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, although increasing αENaC abundance in rat kidneys in an aldosterone-independent manner, fails to activate Na+ currents measured in isolated split-open collecting ducts. We also demonstrate that maximal stimulation of mineralocorticoid signaling in deoxycorticosterone acetate–treated mice does not increase ENaC activity when MRs are inhibited with spironolactone (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, overstimulation 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 adrenocorticotropic hormone as a possible mechanism of hypertension.

Using a physiologically relevant object, that is, 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 (Figure 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 αENaC protein abundance in response to dietary NaCl restriction. However, ENaC redistribution to the apical plasma membrane was not prevented by inhibition of MR. 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 (ie, \( P_o \)) by dietary sodium is not affected by MR inhibition. In contrast, combined pharmacological blockade of AT\(_1\)R and MR nearly abolished changes in ENaC \( P_o \) when dietary Na\(^+\) was manipulated (Figures 3 and 4). This argues that Ang II cascade has nonredundant stimulatory actions on ENaC activity via control of channel gating. Consistently, we\(^{28}\) and others\(^{29}\) 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.\(^{33}\) Systemic Ang II infusion also increases αENaC protein abundance in rat renal cortex.\(^{36}\) Paradoxically, inhibition of MR with spironolactone\(^{33}\) and eplerenone\(^{32}\) 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.\(^{37}\) 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 on spironolactone treatment (Figure 7). The stimulatory effect of Ang II infusion on ENaC \( P_o \) in the presence of MR inhibition was anticipated\(^{28,29}\) 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, R– mice have decreased αENaC abundance in the presence of elevated circulating aldosterone levels.35 AT, R blockade also results in a fall in αENaC mRNA abundance, and downregulation of αENaC expression was not blocked by spironolactone.36 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 or trafficking. Although there was only a tendency of increasing αENaC levels in whole-kidney homogenates of Ang II–infused mice, the stimulatory effect of Ang II on αENaC was significant for the plasma membrane fraction (Figure 9). Consistently, our immunofluorescent studies demonstrate accumulation of αENaC predominantly in the apical region in principal cells of Ang II–infused mice (Figure 8). Strikingly, spironolactone treatment decreased αENaC 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 because of 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 and 7). Accumulating experimental evidence suggests a critical role of intrarenal RAS in development of excessive sodium retention and Ang II–dependent hypertension.21,52 Intrarenal synthesis of Ang II appears to be sufficient to affect sodium handling in the distal nephron, because luminal perfusion of CCDs with Ang I stimulates sufficient to affect sodium handling in the distal nephron, because luminal perfusion of CCDs with Ang I stimulates sodium entry in an ACE-dependent manner.51 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.54,55 This feed-forward mechanism is thought to underlie inappropriate accumulation of sodium reabsorption in the distal nephron.38,56 Indeed, it was recently reported that systemic infusion of Ang II results in increased urinary angiotensinogen levels, indicating intratubular RAS activation.57 In contrast, dietary sodium restriction does not lead to augmentation of urinary angiotensinogen and Ang II excretion rates in rats and, thus, to appreciable intratubular RAS activation.58 Because 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 probably controls ENaC 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 because of increased protein levels but rather stimulation of silent channels residing on the apical plasma membrane.

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+ 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, angiotensinogen, 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.

Sources of Funding

Research reported in this publication was supported by the National Institutes of Health (NIH) NIDDK DK095029 (to O.P.), NIH NIGMS P30GM103337 (to L.G.N.), S&R Foundation Ryuji Ueno award (to O.P.), and American Heart Association Grant-in-Aid 13GRNT16220002 (to O.P.)

Disclosures

None.

References


**Novelty and Significance**

**What Is New?**

- Coordinated actions of aldosterone and angiotensin (Ang) II cascades underlie respective increases in the number of functionally active epithelial Na⁺ channels (ENaC) and channel open probability when dietary sodium intake is reduced.
- During the pathology of Ang II–dependent hypertension, ENaC is overactive and no longer effectively regulated by the aldosterone–mineralocorticoid receptor (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 nonredundant role in adaptation of ENaC-mediated sodium reabsorption in the aldosterone-sensitive distal nephron to variations in dietary salt intake.

Chronic Ang II infusion overstimulates 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.
Chronic Angiotensin II Infusion Drives Extensive Aldosterone-Independent Epithelial Na⁺ Channel Activation

Mykola Mamenko, Oleg Zaika, Minolfa C. Prieto, V. Behrana Jensen, Peter A. Doris, L. Gabriel Navar and Oleh Pochynyuk

Hypertension. 2013;62:1111-1122; originally published online September 23, 2013; doi: 10.1161/HYPERTENSIONAHA.113.01797

The online version of this article, along with updated information and services, is located on the World Wide Web at:

http://hyper.ahajournals.org/content/62/6/1111

Data Supplement (unedited) at:

http://hyper.ahajournals.org/content/suppl/2013/09/23/HYPERTENSIONAHA.113.01797.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/
ONLINE SUPPLEMENT

Chronic Angiotensin II infusion drives extensive aldosterone-independent ENaC activation

Mykola Mamenko†, Oleg Zaika†, Minolfa C. Prieto, V. Behrana Jensen, Peter A. Doris, L. Gabriel Navar and Oleh Pochynyuk

1Department of Integrative Biology and Pharmacology; The University of Texas Health Science Center at Houston, USA; 2Center for Laboratory Animal Medicine and Care, The University of Texas Health Science Center at Houston, USA; 3Institute of Molecular Medicine, The University of Texas Health Science Center at Houston, USA; 4Department of Physiology and Hypertension and Renal Center of Excellence, Tulane University School of Medicine, New Orleans, LA, USA

To whom correspondence should be addressed:
Oleh Pochynyuk, Department of Integrative Biology and Pharmacology, University of Texas Health Science Center at Houston, 6431 Fannin, Houston TX, 77030, USA; Ph. (713) 500-7466; Fx. (713) 500-7455; Oleh.M.Pochynyuk@uth.tmc.edu.

† Both authors contributed equally to this work
METHODS

Tissue isolation. Briefly, mice were sacrificed by CO₂ administration followed by cervical dislocation and the kidneys were removed immediately. Kidneys were cut into thin slices (< 1mm) with slices placed into ice-cold physiologic saline solution buffered with HEPES (pH 7.4). The ASDN was identified as merging of CNT into CCD (see Figure 1A) and was mechanically isolated from cortical sections of kidney slices by micro-dissection using watchmaker forceps under a stereomicroscope. Isolated ASDN was attached to a 5 x 5 mm cover glass coated with poly-L-lysine. A cover-glass containing ASDN was placed in a perfusion chamber mounted on an inverted Nikon Eclipse Ti microscope and perfused with room temperature HEPES buffered (pH 7.4) saline solution. ASDNs were split-opened with two sharpened micropipettes, controlled with different micromanipulators, to gain access to the apical membrane. The tubules were used within 1-2 hr of isolation.

Electrophysiology. ENaC activity in principal cells was determined in cell-attached patches on the apical membrane made under voltage-clamp conditions (-V_p = -60 mV) using standard procedures 1-3. Current recordings were made in a permanently perfused bath (1.5 mL/min). Recording pipettes had resistances of 8-10 megaohms. Typical bath and pipette solutions were (in mmol/L): 150 NaCl, 5 mM KCl, 1 CaCl₂, 2 MgCl₂, 5 glucose and 10 HEPES (pH 7.4); and 140 LiCl, 2 MgCl₂ and 10 HEPES (pH 7.4), respectively. For each experimental condition, ASDNs from at least three different mice were assayed. Gap-free single channel current data from gigahm seals were acquired (and subsequently analyzed) with an Axopatch 200B (Axon Instr.) patch clamp amplifier interfaced via a Digidata 1440 (Axon Instr.) to a PC running the pClamp 10.2 suite of software (Axon Instr.). Currents were low-pass filtered at 100 Hz with an eight-pole Bessel filter (Warner Instr.). Events were inspected visually prior to acceptance. ENaC activity was analyzed over a span of 60-120 sec for each experimental condition. Using previously described analysis 1, we can reliably (P<0.05) estimate the maximal number of functional ENaC in a patch using this time span. Channel activity in individual patches, defined as \( N P_o \), was calculated using the following equation: \( N P_o = (t_1 + 2t_2 + \ldots + nt_n) \), where \( N \) and \( P_o \) are the number of ENaC in a patch and the mean open probability of these channels, respectively, and \( t_n \) is the fractional open time spent at each of the observed current levels. \( P_o \) was calculated by dividing \( N P_o \) by the number of active channels within a patch as defined by all-point amplitude histograms. To estimate total ENaC activity (\( fN P_o \)) under a particular physiological condition, we normalized \( N P_o \) to the frequency of observing patches with at least one active channel (\( f = \) number of patches with active channels / total number of patches). To assess functional ENaC expression for each experimental condition, the mean number of active channels within a patch was corrected to \( f \). For representation, current traces were corrected for a slow baseline drifts as necessary.
REFERENCES


### Table S1
Complementary stimulation of ENaC by aldosterone and Ang II signaling cascades

<table>
<thead>
<tr>
<th>Experimental condition</th>
<th>ENaC activity, fNp₀</th>
<th>ENaC open probability, p₀</th>
<th>Functional ENaC expression, fN</th>
</tr>
</thead>
<tbody>
<tr>
<td>low sodium diet control</td>
<td>0.75±0.11</td>
<td>0.51±0.04</td>
<td>1.47±0.18</td>
</tr>
<tr>
<td>regular diet control</td>
<td>0.37±0.03</td>
<td>0.37±0.02</td>
<td>0.90±0.06</td>
</tr>
<tr>
<td>high sodium diet control</td>
<td>0.16±0.04</td>
<td>0.27±0.03</td>
<td>0.62±0.10</td>
</tr>
<tr>
<td>low sodium diet + mifepristone</td>
<td>0.83±0.08</td>
<td>0.50±0.02</td>
<td>1.68±0.16</td>
</tr>
<tr>
<td>regular diet + mifepristone</td>
<td>0.38±0.03</td>
<td>0.39±0.03</td>
<td>0.98±0.09*</td>
</tr>
<tr>
<td>high sodium diet + mifepristone</td>
<td>0.12±0.02</td>
<td>0.25±0.02</td>
<td>0.45±0.09*</td>
</tr>
<tr>
<td>low sodium diet + spironolactone</td>
<td>0.31±0.06*</td>
<td>0.52±0.07*</td>
<td>0.55±0.10*</td>
</tr>
<tr>
<td>regular diet + spironolactone</td>
<td>0.21±0.05*</td>
<td>0.36±0.05</td>
<td>0.46±0.05</td>
</tr>
<tr>
<td>high sodium diet + spironolactone</td>
<td>0.13±0.03</td>
<td>0.26±0.05</td>
<td>0.37±0.04</td>
</tr>
<tr>
<td>low sodium diet + spironolactone and losartan</td>
<td>0.21±0.04*</td>
<td>0.28±0.04*</td>
<td>0.51±0.06*</td>
</tr>
<tr>
<td>regular diet + spironolactone and losartan</td>
<td>0.15±0.04*</td>
<td>0.24±0.05*</td>
<td>0.45±0.06*</td>
</tr>
<tr>
<td>high sodium diet + spironolactone and losartan</td>
<td>0.10±0.03</td>
<td>0.22±0.04</td>
<td>0.34±0.07*</td>
</tr>
</tbody>
</table>

* P<0.05 versus control values on respective sodium diet. # P<0.05 versus high sodium diet + spironolactone
Table S2
Inhibition of MR receptors does not prevent activation of ENaC by systemic infusion of Ang II

<table>
<thead>
<tr>
<th>Experimental condition</th>
<th>ENaC activity, $fN_{P_0}$</th>
<th>ENaC open probability, $P_o$</th>
<th>Functional ENaC expression, $fN$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.31±0.06</td>
<td>0.32±0.03</td>
<td>0.87±0.12</td>
</tr>
<tr>
<td>Ang II infused</td>
<td>1.71±0.19#</td>
<td>0.55±0.03#</td>
<td>3.10±0.30#</td>
</tr>
<tr>
<td>Ang II infused + spironolactone</td>
<td>0.91±0.09#</td>
<td>0.52±0.03#</td>
<td>1.82±0.18#</td>
</tr>
</tbody>
</table>

#P<0.05 versus respective vehicle infused control values.
Figure S1. *Inhibition of mineralocorticoid receptors abolishes aldosterone signal to ENaC.* Summary graph of ENaC activity in mice kept on a regular salt diet (0.32%) and injected with deoxycorticosterone acetate (DOCA) for 3 consecutive days in the control (left panel) and after inhibition of MR receptors with spironolactone (right panel). * - significant change versus basal 0.32% Na+. # - significant decrease versus 0.32% Na+ DOCA.