Deficiency of Renal Cortical EGF Increases ENaC Activity and Contributes to Salt-Sensitive Hypertension


*Departments of Physiology and †Medicine, Medical College of Wisconsin, Milwaukee, Wisconsin

ABSTRACT

Various stimuli, including hormones and growth factors, modulate epithelial sodium channels (ENaCs), which fine-tune Na⁺ absorption in the kidney. Members of the EGF family are important for maintaining transepithelial Na⁺ transport, but whether EGF influences ENaC, perhaps mediating salt-sensitive hypertension, is not well understood. Here, the ENaC inhibitor benamil attenuated the development of hypertension in Dahl salt-sensitive rats. Feeding these salt-sensitive rats a high-salt diet led to lower levels of EGF in the kidney cortex and enhanced the expression and activity of ENaC compared with feeding a low-salt diet. To directly evaluate the role of EGF in the development of hypertension and its effect on ENaC activity, we infused EGF intravenously while continuously monitoring BP of the salt-sensitive rats. Infusion of EGF decreased ENaC activity, prevented the development of hypertension, and attenuated glomerular and renal tubular damage. Taken together, these findings indicate that cortical EGF levels decrease with a high-salt diet in salt-sensitive rats, promoting ENaC-mediated Na⁺ reabsorption in the collecting duct and the development of hypertension.

More than 76 million American adults have high BP¹ and the likelihood of developing hypertension significantly increases with age. Nearly 40% of African Americans aged >20 years exhibit hypertension and nearly 70% of these individuals have a form of hypertension that is highly sensitive to salt intake. A reduced ability to maintain sodium homeostasis and normal levels of arterial pressure is a hallmark of all forms of hypertension.² In the kidney, discretionary Na⁺ reabsorption in response to endocrine input to the aldosterone-sensitive distal nephron (ASDN) is a determinant of the pressure-natriuresis relationship, which is of fundamental importance in the long-term control of arterial pressure.²,³ Although sodium transport in ASDN accounts for a small proportion of renal sodium transport (<10%), ENaC activity is the rate-limiting step for this discretionary Na⁺ reabsorption.⁴

The Dahl salt-sensitive (SS) rat strain used in this study is a genetic animal model of hypertension and kidney disease that reveals disease traits similar to those observed in humans. This inbred strain exhibits a low-renin, sodium-sensitive form of hypertension that is associated with severe and progressive proteinuria, glomerulosclerosis, and renal interstitial fibrosis.⁵–⁷

The EGF and related hormones are multipotent agents⁸–¹¹ involved in regulation of various renal
functions and, particularly, ion channel activity. For instance, EGF stimulation rapidly induces TRPC511 and TRPM610 channel translocation to the plasma membrane. Members of the EGF family play an important role in the expansion of renal cysts12 and promote glomerular injury and renal failure in rapidly progressive crescentic glomerulonephritis.13 Moreover, Groenestege et al. described a mutation in the pro-EGF encoding gene, which is responsible for development of isolated autosomal recessive hypomagnesemia, associated with renal Mg2+ wasting.14

A role for EGF and its related growth factors in the regulation of ENaC-mediated sodium absorption has been proposed, although contradictory results have been observed with respect to ENaC activity and sodium transport.15–17 Although EGF was shown to stimulate ENaC-mediated renal salt absorption in some studies, others reported that EGF decreases sodium transport and ENaC activity.17 Our recent data show that EGF and its related growth factors (TGF-α, HB-EGF, and amphiregulin) have a biphasic effect on sodium absorption as represented by the experiments on cultured murine mpkCCDc14 principal cells.15 Basolateral application of the EGF family growth factors to polarized mpkCCDc14 principal cells grown on permeable supports acutely increases Na+ reabsorption, whereas chronic treatment of the monolayers with EGF and its related growth factors leads to significant inhibition of ENaC-mediated sodium absorption.15 Similar observations were made in Xenopus laevis A6 principal cells.16 There are a number of potential mechanisms mediating downstream signaling. Thus, it was proposed that EGF effects could be mediated by either the extracellular signal–regulated kinase 1/2 and mitogen-activated protein kinase pathway, Akt,16,18 or reactive oxygen species production.19

ENaC dysfunction leads to disturbances in total body Na+ homeostasis associated with abnormal regulation of BP as observed in patients with Liddle’s syndrome and pseudohypoaldosteronism type 1.20 However, the exact role of ENaC in mechanisms mediating salt-sensitive hypertension remains unclear. It was proposed that the expression of ENaC is abnormally regulated by dietary sodium in SS rats, and this abnormal expression is one of the factors causing salt-sensitive hypertension.21–24 Here we confirm that ENaC expression is upregulated on a high-salt (HS) diet and provide direct evidence that ENaC activity is abnormally upregulated by dietary sodium in hypertensive SS rats, and this enhanced activity is one of the major factors causing salt-sensitive hypertension. In addition, our studies identify EGF as a key molecular substrate for a mechanism that diminishes development of salt-sensitive hypertension.

RESULTS

ENaC Plays a Role in Development of Salt-Sensitive Hypertension in SS Rats

In this study, we first addressed the question whether ENaC plays a role in the development of salt-sensitive hypertension of the SS inbred rat strain, which is a well established model for the study of this disease.6 To address this issue, we performed chronic BP monitoring in SS rats and investigated ENaC functionality in the connecting tubules (CNTs) and/or cortical collecting ducts (CCDs). Three weeks on a HS diet (4%) increased the mean arterial pressure (MAP) from 112.0 ± 3.9 mmHg on a low-salt diet (LS) (0.4%) to 173.3 ± 15.0 mmHg (Figure 1), as previously reported.7 Benzamil, an ENaC inhibitor,25 administered through the drinking water (15 mg/L), significantly reduced the level of salt-induced hypertension as previously observed with another ENaC inhibitor amiloride.24

Protein expression profiles of β- and γ-ENaC subunits were significantly increased in the kidney cortex of SS rats fed a HS diet compared with a LS diet (Figure 2, A and B). α-ENaC expression was not different in SS rats on a HS diet. Similar unchanged α-ENaC level and upregulation of β- and γ-ENaC subunit expression and abundance (along with cleavage and shift of γ-ENaC from 85 to 70 kD) was described in SS rats fed an 8% diet for 4 weeks.24 Interestingly, an increase in α-ENaC, but not β- and γ-ENaC, protein abundance was a result of aldosterone infusion in normal rats.26 Although plasma aldosterone concentration is typically suppressed in SS rats on a HS diet, the activity of the intrarenal renin-angiotensin-aldosterone system is increased27 and may contribute to the higher expression of β- and γ-ENaC subunits. To confirm our findings, a salt-resistant consomic rat strain containing a Brown Norway (BN) rat chromosome 13 substituted into SS rat genetic background (SS.13BN) was used as a negative control.28 Chromosome 13 contains several critical genes such as genes encoding renin and NADPH subunit p67phox and several studies utilizing this model illustrate its critical role in BP regulation.28–30 After 3 weeks on a HS diet, MAP was significantly lower in SS.13BN rats compared with SS rats, as
ENaC expression and activity are upregulated in SS rats compared with consomic SS.13BN rats fed the same HS diet or SS rats on a LS diet.

**β-ENaC Expression in Servo-Controlled Rats**

Although it is widely recognized that hypertension is a strong independent risk factor for renal failure, the effectiveness of BP control in the reduction of renal injury varies greatly between subpopulations of hypertensive patients.37 These observations have obscured the question of how much physical factors related to the elevation of renal perfusion pressure (RPP) actually contribute to renal injury in hypertension. This issue presents a great challenge, given the difficulty in sustaining a chronic increase of arterial pressure without concomitantly altering the systemic neurohormonal factors, such as circulating levels of angiotensin II, known to cause tissue injury independent of elevated arterial pressure.2,6 Servo-controlled measurements (Figure 5B) allowed us to determine the contribution of RPP into the development of renal injury in SS rats. For several weeks, the system maintained the RPP to the left kidney of the instrumented SS rat at the control level, whereas RPP to the right kidney increased in response to a HS diet. Both left and right kidneys were therefore exposed to an identical systemic neurohormonal and metabolic environment, but controlled levels of RPP within the left kidney protected it from the high pressure.38 Similarly to previous data,38 we observed significantly lower renal damage in the controlled organs as measured by glomerular injury scoring (Figure 5, D and E). As shown by immunohistochemistry, β-ENaC expression was increased in the uncontrolled kidneys compared with the controlled left kidneys (Figure 5, A and C).

**EGF Deficiency in the Renal Cortex Contributes to Salt-Sensitive Hypertension**

As recently summarized, members of the EGF family of proteins, such as EGF, TGF-α, HB-EGF, and amphiregulin, chronically downregulate ENaC-mediated Na+ reabsorption in the kidney.17 Furthermore, renal cortical expression of the EGF receptor is increased in both prehypertensive and hypertensive SS rats compared with Sprague-Dawley rats.39 As measured in this study with ELISA assay, EGF concentration in the kidney cortex of SS rats fed a HS diet was significantly lower compared with SS rats on a LS diet or SS.13BN rats on both diets (Figure 6).

To fully evaluate the physiologic importance of EGF regulation of sodium transport in the CNTs/CCDs, we precisely
quantified the changes in ENaC as one of the potential EGF targets of this signaling system, by directly measuring channel activity. Freshly isolated tubules from SS rats fed a HS diet and chronically infused with EGF or vehicle were used to characterize the effects of EGF on ENaC and their relationship to the feedback regulation in response to salt. Three days of intravenous infusion of EGF (10 μg/h per kg) to SS rats led to the development of hypertension in SS rats. ΔMAP was 36.0 ± 11.8 mmHg and 0.3 ± 4.4 mmHg for vehicle and EGF, respectively. Importantly, chronic infusion of EGF decreased ENaC activity in isolated CNT/CCD compared with the control group (Figure 7). Thus, infusion of EGF precludes an increase of the BP in SS rats and ENaC might be the final target of this effect.

**DISCUSSION**

Our studies identify members of the EGF family as key molecular substrates for a new mechanism that diminishes development of salt-sensitive hypertension. The decline of EGF level in the kidney cortex results in inappropriate ENaC upregulation. Thus, this work provides significant insight into the molecular basis of salt-sensitive hypertension.

SS rats develop severe hypertension on a HS diet. One of the compelling reasons for studying the SS rat have been the remarkable similarities with phenotypic traits seen in salt-sensitive hypertension in African Americans, which include low renin, salt sensitivity, hyperinsulinemia, and early ESRD. However, despite extensive studies, exact mechanisms mediating salt-sensitive hypertension are not yet clear. Previous studies reported that the defects in salt and water handling in the kidney of prehypertensive and hypertensive SS rats are mediated in part by enhanced salt reabsorption in the medulla. However, changes in reabsorption in the distal nephron segments were also observed and, as summarized by Hawk and Schaefer, the role of ENaC-mediated Na⁺ reabsorption in the ASDN was unclear because there are no reports directly studying the...
The lack of studies in this area is not a consequence of unimportance of these critical sodium channels, but is due to the fact that few laboratories have the technical expertise to directly quantify changes in ENaC activity in native preparations. There is a panel of high-quality publications studying ENaC mRNA expression and the protein abundance in SS rats on various salt diets. The authors demonstrated that a HS diet drives abnormal regulation of ENaC and SGK1 expression in SS rats in contrast with salt-resistant animals.21,24,44 In addition to these previously published data, we have shown HS-induced ENaC activation in SS rats as directly measured by electrophysiology. Moreover, a unique chronic servo-control technique was used here to uncover the role of high BP in driving ENaC expression in SS rats. Here we provide direct evidence that ENaC plays a critical role in the development of salt-sensitive hypertension in SS rats.

EGF and its related growth factors act by binding to ErbB receptors at the plasma membrane, thus stimulating the intrinsic protein-tyrosine kinase activity of the receptor. Most ErbB-family ligands are expressed in the kidney.12 For example, HB-EGF is synthesized in the proximal tubule, EGF in thick ascending limb of Henle’s loop and distal convoluted tubule, and TGF-α in the distal convoluted tubule and collecting ducts.45,46 Here we identified that lessening of EGF level in the kidney cortex results in inappropriate ENaC upregulation. Therefore, our data directly demonstrate that enhancement of ENaC activity in SS rats fed a HS diet plays a major role in the development of salt-induced hypertension. Furthermore, these results suggest that alterations in EGF signaling are involved in the renal pathophysiology associated with cardiovascular diseases. It was shown that signaling cross-talk between Rac1 and the mineralocorticoid receptor modulates receptor activity and this identified Rac1 as a therapeutic target for CKD.47 The renal abnormalities in mice lacking Rho-GDP-dissociation inhibitor-α were associated with increased Rac1 and mineralocorticoid receptor (MR) signaling in the kidney.48 Our recent findings show that Rac1 increases ENaC activity.49,50 We hypothesize that the low EGF level in SS rats on a HS diet leads to high Rac1 and, consequently, increased ENaC activity that contributes to inappropriate salt retention and hypertension. This theory might explain the mechanism of beneficial effects of MR blockade on BP and renal and vascular damage.51,52

Whether the changes in EGF level are causative or reflective of renal pathophysiology requires further investigation. EGF receptor inhibition either with antibodies such as cetuximab or small molecule tyrosine kinase inhibitors is a well-established therapy in cancers.53–55 Thus, patients taking such drugs, especially ones with predisposition for salt-sensitive hypertension, may face an increased risk of renal injury. Collectively, our work identifies a crucial mechanism of
ENaC-mediated regulation of the sodium reabsorption in the CNTs/CCDs that is highly relevant in the development of salt-sensitive hypertension.

CONCISE METHODS

Experimental Animals
Experiments were performed on re-derived Rapp Dahl S rats (SS/JrHsdMcwi) that have been inbred for >50 generations at Medical College of Wisconsin. SS.13BN consomic rat model developed at the Medical College of Wisconsin served as the negative control. This inbred strain exhibits only 2% allelic differences compared with the SS strain, but salt-induced hypertension and renal injury are greatly attenuated in this strain (approximately 60%). Water was provided ad libitum and the salt content of each diet was 0.4% NaCl AIN-76 diet (Dyets, Bethlehem, PA) from weaning up to 8 weeks of age, when animals were transferred to experimental diets. At 8 weeks of age, the salt content of the chow was either maintained at 0.4% NaCl in the group fed a normal diet or increased to 4.0% or 8.0% NaCl, and the rats were maintained on these diets for another 1–3 weeks. Animal use and welfare procedures adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals following protocols reviewed and approved by the Medical College of Wisconsin Institutional Animal Care and Use Committee.

Surgical Procedure
The rats were deeply anesthetized with an intraperitoneal injection of ketamine/acepromazine (35/5 mg/kg) with supplemental anesthesia administered when needed. Using an aseptic technique, polyvinyl catheters were implanted in the femoral artery, tunneled subcutaneously, and exteriorized at the back of the neck in a lightweight tethering spring. Both antibiotic and analgesic agents were administered post-surgically, and the rats were allowed to fully awaken from anesthesia on a temperature-controlled pad. After recovery from anesthesia, all rats were placed into individual stainless steel cages that permitted daily measurement of arterial BP. The rats were allowed to recover for at least 3 days after surgery. After the recovery period, BP measurements were obtained constantly for the duration of the experiment. Plotted data represent mean measurements from 9:00 a.m. to 12:00 p.m. as previously described. We performed 10 μg/h per kilogram of EGF administration with continuous infusion at the rate of 6.9 μl/min through a venous catheter in PBS as a vehicle.

Histologic and Immunohistochemical Analyses
Kidneys were harvested for histologic and immunohistochemical analysis using previously described methods. The rats were euthanized with sodium pentobarbital (50 mg/kg, intraperitoneally); the kidneys were then removed and placed into zinc formalin. The kidney sections were cut at 4-μm slices, dried, and deparaffinized for subsequent streptavidin-biotin immunohistochemistry. After deparaffinization, the slides were treated with a citrate buffer (pH 6) for a total of 35 minutes. The slides were blocked with a peroxidase block (DAKO, Carpinteria, CA), avidin block (Vector Labs, Burlingame, CA), biotin block (Vector Labs), and serum-free protein block (DAKO). For kidney damage analysis, the tissue was stained with Gomori’s One-Step Trichrome. Individual glomeruli (average of 80 per kidney) were evaluated using the method of Raji et al. In brief, each visible glomerulus from a representative kidney slice was scored from 0 (no injury) to 4 (nonfunctional, sclerotic) and an average score was calculated for each kidney. Protein cast analysis was performed using a color thresholding method using Metamorph (Molecular Devices, Sunnyvale, CA) software. Results reported as the percentage of total cast area. For immunohistochemistry, tissue sections were wounded for 30 minutes with anti-β-ENaC antibodies (1:100 dilution, SPC-404; StressMarq Biosciences Inc.
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infused with vehicle or EGF (2 μg/h) infusion via i/v micropipettes controlled by micromanipulators. Slices were split open to gain access to the apical membrane with sharpened microdissection using forceps under a stereomicroscope. CCDs were isolated, split-open rat CCDs. CCDs were isolated from SS rats as described previously.58,59 The SS or SS.13BN rat kidneys were flushed with PBS in an anesthetized animal, excised, and cut in 1- to 2-mm slices under the binocular with ×6 magnification. The approximate apical kidney sections corresponding to the cortex were carved (approximately 1 g) and then diced into small pieces with a razor blade. Samples were pulse sonicated in gentle lysis buffer with protease inhibitor cocktail (Roche Instrument Company, Novato, CA). The resistance of the pipette in the corresponding bath medium was 7–12 MΩ. Typical bath solution was 1:100 NaCl, 1 CaCl2, 2 MgCl2, and 10 HEPES (pH 7.4). Pipette solution for cell-attached configuration was as follows (in mM): 140 LiCl, 2 MgCl2, and 10 HEPES (pH 7.4). Gap-free single-channel current data from GΩ seals in principal cells were acquired and subsequently analyzed. Patches were selected for low baseline noise levels with no drift of the baseline current (in general, low noise was associated with high-seal resistances). To assess the steady-state probability of channel opening, patches were clamped to a potential of −60 mV and channel activity was determined during an at least 1-minute recording period. The first 10 seconds of recording after applying a voltage step were omitted from the steady-state analysis. The channel events were analyzed by Clampfit 10.2 software (single-channel search in analyze function) in conjunction with Origin 7.0 (OriginLab, Northampton, MA). A 50% threshold cross-method was utilized to determine valid channel openings. All events were carefully checked visually before being accepted. NPo, the product of the number of channels, was used to measure the channel activity within a patch.

**Western Blot Analyses**

Kidney total cortical lysate was prepared as follows.58 The SS or SS.13BN rat kidneys were flushed with PBS in an anesthetized animal, excised, and cut in 1- to 2-mm slices under the binocular with ×6 magnification. The approximate apical kidney sections corresponding to the cortex were carved (approximately 1 g) and then diced into small pieces with a razor blade. Samples were pulse sonicated in gentle lysis buffer with protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) for 10 seconds and spin cleared at 10,000×g for 10 minutes. The resulting supernatant was subjected to PAGE and subsequently transferred onto nitrocellulose membrane (Millipore, Bedford, MA) for probing with antibodies and subsequently visualized by enhanced chemiluminescence (Amersham Biosciences Inc, Piscataway, NJ). Antibodies for α-, β-, and γ-ENaC (cat# SPC-403D, SPC-404D, and SPC-405D, respectively) were from Stress-Marq Biosciences Inc.26

**Isolation of CCDs**

Patch clamp electrophysiology was used to assess ENaC activity in isolated, split-open rat CCDs. CCDs were isolated from SS rats as previously described.35,49,50 Kidneys were cut into thin slices (<1 mm) and then placed into ice-cold physiologic salt solution (pH 7.4). Collecting ducts were mechanically isolated from slices by microdissection using forceps under a stereomicroscope. CCDs were split open to gain access to the apical membrane with sharpened micropipettes controlled by micromanipulators.

**Electrophysiology**

Single-channel data were acquired and subsequently analyzed with Axopatch 200B or 700B amplifiers (Molecular Devices) interfaced via a Digidata 1440A to a PC running the pClamp 10.2 suite of software (Molecular Devices). When signals were acquired with an Axopatch 200B amplifier, currents were filtered with an 8-pole, low-pass Bessel filter LPF-8 (Warner Instruments, Hamden, CT) at 0.2 kHz. The pipette was pulled with a horizontal puller (Sutter P-97; Sutter Victoria, BC, Canada). Secondary detection was performed with Goat anti-Rabbit biotinylated IgG (Biocare, Concord, CA) followed by streptavidin horseradish peroxidase (Biocare) and visualized with dianinobenzidine (DAKO).

**ELISA Assay and Biochemical Analyses of Electrolytes**

Cortical EGF level was measured by a 96-well plate method with an ELISA kit (#CSB-E08029r; Cusabio Co. Ltd, Wuhan, Hubei, China). Total tissue lysate collected from the rat kidney cortex as described for Western blotting, was applied on the plate along with the standard and results were measured colorimetrically. Urine and blood samples were collected during infusion experiments and Na+, K+, and creatinine levels were analyzed as previously described.60,61

Figure 8. Chronic intravenous infusion of EGF prevents development of hypertension and kidney damage in SS rats fed a HS diet. Protocol (A) and effect of intravenous EGF infusion on the MAP (B) in the SS rats fed a HS diet as described in the corresponding protocol (n=3–4). (C) Light microscopy of Trichrome-stained sections of whole kidney (×1) and renal cortex (×60) of SS rats fed a HS diet for 10 days and intravenously infused with vehicle or EGF (2 μg/h). The averaged percentage of protein casts area in the renal outer medulla (D) and the glomerular injury score (E) are shown. *P<0.05, **P<0.01, and ***P<0.001 versus SS rats treated with vehicle.


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Figure 9. Effect of EGF infusion on urine and serum Na⁺ and K⁺ levels in SS rats fed a HS diet. (A) Na⁺ urinary concentrations normalized to creatinine in the vehicle- and EGF-treated groups before (day 0) and after (day 4, day 10) the HS diet started. (B and C) Na⁺ and K⁺ levels in serum remain unchanged during the experiment in both groups. n=4 in each group. §P<0.05 compared with day 0; *P<0.05 compared with saline, day 10.

Servo-Control of RPP
This set of servo-controlled experiments closely followed previously described studies. 38,42 In short, rats were instrumented with indwelling arterial (right carotid artery and left femoral artery) and venous (left femoral vein) catheters. An inflatable silastic vascular occluder was implanted around the aorta between the right and left renal artery branches and exteriorized with flexible Tygon tubing at the back of the neck. A servo-controlled turntable (Rodent Workstation with Raturn System; Bioanalytical Systems, West Lafayette, IN) was used that enabled free movement of the rat throughout the study. Rats were conditioned to the turntable for 3–4 days before undergoing any surgical procedures. The pressure signal from the femoral arterial catheter, reflecting RPP of the left kidney, was the input to the servo-control unit that initiated the inflation of the occluder cuff.

Statistical Analyses
All summarized data are reported as mean±SEM. Data were compared using either the (two-tailed) t test or a one-way ANOVA (post hoc comparison with Bonferroni multiple correction). Differences were considered statistically significant at P<0.05.

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DISCLOSURES
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