Effect of Mineralocorticoid Treatment in Mice with Collecting Duct-Specific Knockout of Endothelin-1.

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Renal Aldosterone-ET-1 Feedback

Abstract (250 limit) - 245

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ABSTRACT (250)

Aldosterone increases blood pressure (BP) by stimulating sodium (Na) reabsorption within the distal nephron and collecting duct (CD). Aldosterone also stimulates endothelin-1 (ET-1) production that acts within the CD to inhibit Na reabsorption via a negative feedback mechanism. We tested the hypothesis that this renal aldosterone-endothelin feedback system regulates electrolyte balance and BP by comparing the effect of a high-salt (NaCl) diet and mineralocorticoid stimulation in control and CD specific ET-1 knockout mice (CD ET-1 KO). Metabolic balance and radiotelemetric BP were measured before and after treatment with desoxycorticosterone pivalate (DOCP) in mice fed a high-salt diet with saline to drink. CD ET-1 KO mice consumed more high-salt diet and saline and had greater urine output than controls. CD ET-1 KO mice exhibited increased BP and greater fluid retention and body weight (BW) than controls on a high-salt diet. DOCP with high-salt feeding further increased BP in CD ET-1 KO mice and by the end of the study the CD ET-1 KO mice are substantially hypernatremic. Unlike controls, CD ET-1 KO mice failed to respond acutely to or escape from DOCP treatment. We conclude that local ET-1 production in the CD is required for the appropriate renal response to Na loading and lack of local ET-1 results in abnormal fluid and electrolyte handling when challenged with a high-salt diet and with DOCP treatment. Additionally, local ET-1 production is necessary, under these experimental conditions, for renal compensation to, and escape from, the chronic effects of mineralocorticoids.

KEYWORDS - Na reabsorption, desoxycorticosterone, blood pressure, aldosterone escape, renal, aldosterone, endothelin
The kidney is normally the primary organ that responds to changes in dietary sodium (Na) intake with commensurate changes in Na excretion to maintain normal Na balance. Derangements in the renal response to such changes can result in either Na depletion and hypotension, or excessive Na retention. Abnormal aldosterone activity for the degree of Na intake is an important element in the pathogenesis of many of these conditions. Aldosterone, produced by the adrenal cortex, is the most potent salt-(NaCl) retaining corticosteroid, and its action is opposed by multiple mechanisms, including endothelin-1 (ET-1) in the renal collecting duct (CD). Aldosterone increases systemic blood pressure (BP) in part by increasing Na absorption via the apical epithelial Na channel (ENaC) and basolateral Na,K-ATPase. Aldosterone action is opposed by humoral, autocrine, and paracrine systems that include the natriuretic peptides, atrial natriuretic peptide and brain natriuretic peptide, nitric oxide, prostaglandins, and ET-1 that increase renal Na and water excretion to lower BP.

We previously identified the ET-1 gene (Edn1) as an early aldosterone-responsive gene in a mouse inner medullary CD cell line. Further studies in our laboratory confirmed this action in vivo and demonstrated that aldosterone increases transcription of ET-1 via the mineralocorticoid receptor (MR) interacting directly with the Edn1 promoter at specific hormone response elements. We recently reported the first direct measurement of the quantitative effect of the acute application of ET-1 on Na reabsorption in the in vitro microperfused CD. In the presence of ET-1, ENaC-mediated Na reabsorption was
significantly inhibited which supports several other studies examining the effects of ET-1 in the CD. (3; 4; 40; 48) Collectively, these studies suggest that the stimulation of ET-1 production by aldosterone acts locally within the CD as a negative feedback mechanism to inhibit Na and water reabsorption.

CD-specific ET-1 KO (CD ET-1 KO) mice have salt-sensitive hypertension. (1; 24) Studies suggest that relative over-activity of the renin-angiotensin-aldosterone system is responsible for the pathogenicity of this mouse model. Despite being hypertensive on a high-salt diet, the CD ET-1 KO mice did not exhibit greater suppression of plasma renin activity and aldosterone levels than control mice. (1; 11) Ge et al. further demonstrated that either angiotensin II or MR antagonism normalized BP in the CD ET-1 KO mice under normal salt conditions and partly corrected the hypertension during high-salt intake. (11)

During experimentally induced primary hyperaldosteronism, a process known as “aldosterone escape”1 prevents chronic Na retention. (2; 23; 25; 27; 37; 41; 46; 47) Administration of exogenous aldosterone in the presence of adequate Na intake results in initial Na retention. However, this effect is transient and after a period of positive Na balance, renal excretion adjusts to match Na intake although blood pressure remains elevated. Various mechanisms have been proposed to mediate or contribute to aldosterone escape, but relatively few studies have quantified their contribution.

Here we have conducted complete metabolic balance experiments which to our knowledge are the first full quantitative assessment of the role of a single gene product to

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1 Aldosterone escape should not be confused with “aldosterone breakthrough” a response to therapy that may occur in patients treated with angiotensin converting enzyme (ACE) inhibitors or angiotensin receptor blockers (ARBs) to reduce plasma aldosterone levels. In this condition after initial reduction, plasma aldosterone increases to pretreatment levels. These patients tend to have a worse clinical prognosis than those that do not exhibit breakthrough (36)
aldosterone escape. If CD ET-1 KO mice have impaired feedback control of the renin-
angiotensin-aldosterone axis, treatment with mineralocorticoid should result in abnormal Na
conservation. In the present study, we addressed this question by treating CD ET-1 KO mice on
a high-salt diet with the long acting aldosterone analog, desoxycorticosterone pivalate (DOCP)
and measured electrolyte balance and BP response.

The current findings are consistent with a role for CD ET-1 as a component of a local
feedback system that regulates mineralocorticoid stimulated renal Na absorption. CD ET-1 KO
mice exhibit increased fluid and Na retention and BP when challenged with a high-salt diet. On
this diet, DOCP treatment resulted in acute Na retention in controls, but not in CD ET-1 KO
mice. This was followed by restoration of neutral Na balance in controls (escape), whereas CD
ET-1 KO mice exhibited progressive Na retention. These data demonstrate that the absence of
ET-1, specifically in the CD, impairs the renal response to a Na load and identifies ET-1 as a
necessary component of mineralocorticoid escape. We conclude that disruption of a renal
aldosterone-endothelin feedback mechanism prevents adequate renal compensation to the
effects of chronic mineralocorticoids on electrolyte balance.
**Materials and Methods**

*Animals.* All animal use was in compliance with the American Physiological Society’s Guiding Principles in the Care and Use of Laboratory Animals, and animal use protocols were approved by the North Florida/South Georgia Veterans Administration Institutional Animal Care and Use Committee. Mice homozygous for floxed ET-1 were imported from University of Utah Health Sciences Center, Salt Lake City, Utah and were the kind gift of Dr. Donald Kohan. The floxed ET-1 mice were bred with AQP2-Cre transgenic females (kind gift from Dr. David Weiner, University of Florida) also homozygous for floxed ET-1. Mice were genotyped as follows:

Genomic DNA was prepared according to standard methods from tail samples and amplified by PCR in a single reaction using the following oligonucleotides: ET-1 F (5’-agc-aa-gctg-tgc-tgc-cca-aag-a-3’); ET-1 R (5’-gac-tgc-cta-ttc-cta-ctc-gct-gatg-g-3’); AQP2 F (5′ cct ctg cag gaa ctg gtg ctt g-3’); CreTag R (5′-gcg aac atc ttc agg ttc tgc gg-3’). CD-specific ET-1 knockout (CD ET-1 KO) mice yield bands at 349 bp (floxed ET-1) and 671 bp (Cre transgene).

Age-matched C57BL/6J control and CD ET-1 KO adult male mice were implanted with PA-C10 radio transmitters (Data Sciences International (DSI) St. Paul, MN), were place in metabolic cages (Techniplast, Exton, PA) for 19 days and fed ad libitum a 2% NaCl (high-salt) gelled diet [45% TD99191 (0.2% Na), Teklad, Madison, WI, 54% water, 1% agar, 1.8% Na as NaCl] and given normal saline (0.9% NaCl) to drink without access to solute-free water. On day 8, mice were treated with desoxycorticosterone pivalate (DOCP, 0.07mg/g, IM). Each day, body weight, food intake, and saline intake were measured and urine and feces were collected for [Na] and [K] analysis using a digital flame analyzer (Cole-Parmer, Model 2655-00, Chicago, IL). BP and heart rate (HR) were continually recorded every ten minutes for 2 minutes. A moving
average was generated for each hour using DSI Dataquest A. R. T. Analysis Software Version 4.3.

On day 19, blood was collected via the abdominal aorta under isoflurane anesthesia. Plasma was analyzed for [K] as describe above. In a separate experiment, CD ET-1 KO mice were adapted to either a normal (0.2%) Na diet or a high-salt gelled diet and given the choice of a water bottle or a saline bottle for 8 days. The volume of water and saline consumed daily was recorded and averaged for days 6-8.

Fecal Digestion. Fecal samples were dried overnight at 80°C and digested as follows: representative samples (~0.1 g) and a control blank were dissolved in 2 mL 1:1 nitric acid:deionized water heated to 95°C for 5 minutes. After cooling, 1 mL concentrated nitric acid was added and the samples were refluxed for 10-15 minutes at 95°C. After cooling, 1 mL deionized water and 1 mL 30% hydrogen peroxide were added. The samples were heated until clear and effervescence had subsided. After cooling, 1 mL 1:1 hydrochloric acid:deionized water and 2 mL deionized water were added and the samples were refluxed for 10 minutes at 95°C. The samples were quantitatively transferred to a 15 mL conical tube and diluted to 10 mL total volume in deionized water.

Statistical Analysis. The results are expressed as mean ± SEM. One-way or two-way repeated measures ANOVA and post-hoc Tukey t-test were used to determine the effect of treatment (days 0-8 high-salt; days 9-19 high-salt/DOCP unless described otherwise) and genotype using Sigma Plot for Windows Version 12. Paired and unpaired Student’s t-tests were performed.
using Microsoft Excel Version 2010 for comparison of groups and when interactions were present. Differences between groups were considered statistically significant at $P < 0.05$. 
Results

High Salt Diet Intake

The development of deoxycorticosterone acetate (DOCA) salt-induced hypertension in mice is influenced by the genetic background. The mouse strain used in the present study (C57BL/6) is more resistant to DOCA-induced increases in BP than other strains. Several studies reported robust DOCA-induced increases in BP in C57BL/6J mice when supplemented with NaCl in the drinking water. (31; 43) Availability of dietary Na is an important consideration during mineralocorticoid treatment. In order to achieve a maximum hypertensive response, we chose a high salt model that included 2% Na in a gelled diet and 0.9% NaCl in the drinking water (saline).

The CD ET-1 KO mice had a two-fold greater saline intake than the control mice (Figure 1A) before and after treatment with DOCP when normalized for body weight. In a separate group of CD ET-1 KO mice, a preference study was performed (Figure 1B). Given the option of water or saline, CD ET-1 KO mice drank small and equal volumes of water and saline each day on a normal Na diet (0.2%). However, the mice drank almost exclusively from the water bottle when adapted to the high-salt diet. During the first eight days of the high-salt diet, saline intake was significantly increased in CD ET-1 KO mice (Repeated Measures ANOVA, F=4.209, p<0.01) but not in controls. Both genotypes increased their saline intake after DOCP treatment (Control F=76.126 & CD ET-1 KO F=11.056; p<0.001). The CD ET-1 KO ate more of the high-salt gelled diet (2% Na) than control mice before and after DOCP treatment (Figure 1E).
Urinary Volume and Osmolality

Commensurate with greater saline intake, CD ET-1 KO mice had nearly a two-fold greater daily urinary volume than controls both before and after DOCP treatment (Figure 1D). DOCP treatment increased the urinary volume in both genotypes. CD ET-1 KO mice had significantly lower urine osmolality than control mice before and after DOCP treatment (Figure 1E), and DOCP treatment decreased urinary osmolality in both genotypes. Over the course of the first eight days of the high-salt diet, the daily urinary volume was significantly increased (Repeated Measures ANOVA F=4.646, p<0.001) and the urine osmolality significantly decreased (p<0.05, Day 1 vs. 4&5) within the CD ET-1 KO mice but not within the controls. Urine pH was not significantly different between genotypes either before or after DOCP treatment except for on day 1 (data not shown).

Fluid Balance and Body Weight

Fluid balance and body weight were measured in order to assess whether treatment with high salt and DOCP caused more fluid retention (positive fluid balance) and body weight gain in the CD ET-1 KO mice compared with controls. Fluid balance was calculated as the difference between fluid intake and the urinary output normalized for body weight (Figure 2A). After 3 days of acclimation to the high Na diet and metabolic cages, the CD ET-1 KO mice came into steady-state with constant fluid intake and urine output. Accordingly, analysis was performed on days 4-8 of the HS Diet. When fed the high-salt diet, CD ET-1 KO mice exhibited greater positive fluid balance (Figure 2A) and body weight gain than controls (Figure 2B). After DOCP treatment, control mice exhibited an increase in fluid balance and body weight whereas
CD ET-1 KO mice maintained a chronic state of positive fluid balance and increased body weight.

Radiotelemetry

We evaluated systolic BP and heart rate in response to the high salt diet before and after DOCP treatment. Daytime (06:00 – 17:00) and nighttime (18:00 – 05:00) systolic BP measurements are shown in Figure 3A, top and bottom respectively. When placed on the high-salt diet, systolic BP was increased in the CD ET-1 KO mice compared with pretreatment recordings on a normal-salt diet during both the day and night. In contrast, the high-salt diet did not increase BP in the control mice. DOCP treatment increased systolic BP in the CD ET-1 KO mice during both day and night. DOCP treatment increased BP in the control mice during the resting phase (daytime), but failed to reach statistical significance during the active phase (nighttime).

Daytime heart rate significantly increased in both genotypes when placed on the high-salt diet (Figure 3B). After DOCP treatment, heart rate significantly decreased in the control mice during both day and night.

Sodium Balance

Direct assessment of Na handling was performed measuring complete daily Na balance as the difference between total Na intake and excretion. We individually evaluated urinary and fecal excretion. In doing so, we were able to recognize distinct differences in Na handling in the CD ET-1 KO and control mice. Urinary Na excretion (Figure 4A) was significantly greater in CD ET-1 KO mice compared to controls before and after DOCP treatment. CD ET-1 KO mice
exhibited an increase in urinary Na excretion before and after DOCP treatment; whereas, the
control mice do not increase urinary Na excretion until after DOCP treatment. Control mice,
but not CD ET-1 KO, exhibited a significant decrease in urinary Na excretion on day 11, three
days after DOCP injection. Fecal Na excretion (Figure 4B) was about twenty-fold less than
urinary Na excretion. Fecal Na excretion was significantly higher in the CD ET-1 KO mice than
controls on the first two days of the HS diet and three days after DOCP injection on Day 11.

Daily Na balance was calculated as the difference between the total Na intake and
output (Figure 5A&B). Neither daily nor cumulative Na balance (Figure 5C&D) was different
between controls and the CD ET-1 KO mice before DOCP treatment. However, DOCP treatment
profundly increased daily and cumulative Na balance in the CD ET-1 KO mice resulting in a
significant difference between the genotypes. DOCP treatment did not increase the daily Na
balance in the control mice (which only exhibited a transient positive balance three days after
DOCP injection) but did increase their cumulative Na balance.

The control mice exhibited positive Na balance (Na retention) within 24 hours of
beginning the high-salt diet and rapidly returned to balance by day 2 (Figure 5B). Control mice
exhibit a sudden positive Na balance three to five days after the DOCP injection (most on day
11) and rapidly returned to balance. The CD ET-1 KO mice responded differently than control
mice to DOCP treatment. Of importance, they did not exhibit the abrupt positive Na balance
observed in the control mice after DOCP treatment. In response to DOCP, CD ET-1 KO mice
entered a prolonged positive balance that was significantly greater than controls.

Plasma Na and osmolality were measured on the final day of the experiment and
compared with mice fed a normal Na diet and are shown in Figures 5E&F. Plasma Na and
osmolality in the CD ET-1 KO mice were not different from controls when fed a normal Na diet. After chronic high salt diet and DOCP treatment, the CD ET-1 KO mice had a significant increase in plasma osmolality and plasma Na.

Potassium Balance

Aldosterone excess is commonly associated with hypokalemia although this hypokalemia does not reflect potassium deficiency but rather transcellular potassium redistribution.(15) We measured daily urinary and fecal K excretion and calculated daily and cumulative K balance throughout the duration of the high-salt/DOCP study. Urinary K excretion (Figure 6A) was not significantly different between control and CD ET-1 KO mice on the HS diet. Despite constant food intake, both genotypes exhibited diminishing urinary K excretion and increasing K balance (Figure 6B&C) after DOCP treatment. Furthermore, the CD ET-1 KO mice exhibited less urinary K excretion and greater K retention than the control mice after DOCP treatment. Plasma K, shown in Figure 6D, was not different between control and CD ET-1 KO mice on a normal-salt (0.2% Na) diet. With DOCP treatment and a high salt diet, plasma K significantly decreased in both genotypes. Urinary and fecal K excretions over the entire duration of the study are shown in Figure 7.


Discussion

Although aldosterone escape has been known for more than 50 years, its mechanism is not fully elucidated. Studies examining this mechanism have shown evidence that natriuretic peptides, prostaglandins, nitric oxide, specific transporters, and physical factors all participate in aldosterone escape. However, the quantitative importance of these mechanisms using the gold standard of complete metabolic balance, are quite limited. These studies represent one of the few experiments to quantify directly the importance of a mechanism to aldosterone escape. Our study implies that under the conditions of these experiments ET-1 expression by the CD is necessary for aldosterone escape. However, since many mechanisms may act in concert, the dependence of aldosterone escape on locally expressed ET-1 does not exclude the requirement of other factors or mechanisms. Purinergic signaling has been demonstrated to mediate aldosterone escape through down-regulation of ENaC. There is evidence that purinergic and the ET systems may work in combination to elicit a diuretic and natriuretic response. ET-1 stimulates nitric oxide (NO) production. NO inhibits ENaC, and the CD-specific KO of NO synthase 1 causes salt-sensitive hypertension. Aldosterone infusion in normal mice caused a marked increase in urinary microsomal prostaglandin E synthanse-1 (mPGES-1) and co-induction in proximal tubules, and the single-gene deletion of mPGES-1 caused impaired aldosterone escape by attenuating the down-regulation of many Na transporters including ENaC. The interaction between ET-1 and prostaglandins in the kidney requires further clarification.

The present study demonstrates that the absence of ET-1 expression principally in cells of the CD impairs the renal response to dietary Na loading. The physiological effects of a high-
salt diet and DOCP treatment in CD ET-1 KO mice suggest a primary role for CD expressed ET-1 to modify the effects of aldosterone on CD Na reabsorption. Control mice responded to DOCP treatment predictably by acutely decreasing urinary Na excretion and entering a brief period of positive electrolyte balance (Figure 4A and Figure 5B). Control mice consequently developed modest positive fluid balance, body weight gain, and exhibited acute adaptation of Na balance within three to five days, similar to previous reports. (23; 41; 47) In contrast, the CD ET-1 KO mice exhibited no acute response to DOCP, but demonstrated progressive cumulative positive sodium balance. At the end of the study, the CD ET-1 KO mice were severely hypernatremic.

Increased fecal excretion of Na observed in CD ET-1 KO mice immediately following the start of the high-salt diet and DOCP treatment may reflect compensation to excessive renal Na retention (Figure 4B). However, in the absence of CD ET-1, other adaptive mechanism(s) fail to adequately compensate and restore neutral Na balance in response to chronic DOCP treatment. Previous studies have shown that CD ET-1 KO mice exhibit salt-sensitive hypertension.(1; 24; 26) The present data confirm this finding and support the hypothesis that CD ET-1 attenuates the renal action of aldosterone. Specifically, CD ET-1 KO mice have increased systolic BP during the day and night when placed on the high-salt diet; whereas, the control mice do not significantly increase BP on a high salt diet alone (Figure 3A).

The degree of hypertension observed in this study in mice on a C57BL/6J genetic background was not as pronounced as experiments conducted on a C57BL6/CBA background, but is consistent with the resistance of this strain to develop hypertension (1). This observation is also of interest because it is well know that there are major effects of the genetic background on the degree of hypertension exhibited by specific mice strains.(14; 14; 16) Indeed, genetic
differences between the Dahl salt-sensitive and salt-resistant strains of rat have been the
subject of extensive interest and research(5-7; 22; 29)
The protocol used in these studies has been used extensively as an accepted model of
desoxycorticosterone-salt hypertension to produce a model of hypertension similar to primary
hyperaldosteronism.(10; 28; 42; 44; 45) This protocol was specifically used in the present study
because the (C57BL/6J) mouse strain is resistant to DOCA-induced increases in BP. It has been
used to provide important information on the factors that contribute to or attenuate this form
of hypertension. Mice were monitored daily for adequate food and fluid intake and changes in
body weight and body condition scoring. However, prolonged isolation in metabolic cages can
be stressful to the mice and must be recognized. However, since both controls and CD-ET-1 KO
were subjected to identical conditions, the difference in the response of the CD ET-1 KO and the
controls demonstrates the importance of CD ET-1 expression in the response to
mineralocorticoids.

The changes in heart rate reflect the opposing effects of volume-independent (changes
in blood pressure via baroreceptors) and volume-dependent (changes in cardiac venous return
or hypovolemia). Although a reflex increase in blood pressure will activate baroreceptors and
decrease heart rate, isotonic sodium loading might be expected to increase in blood volume
which would increase venous return to the heart and consequently heart rate.

Despite slightly smaller average body weight, CD ET-1 KO mice consumed a substantially
larger volume of saline than controls. The propensity for the CD ET-1 KO mice to consume a
substantially greater volume of fluid during high-salt and DOCP treatment (Figure 1A) suggests
a central nervous system stimulation of thirst. Since these mice do not have access to water
without Na, apparently they cannot correct the hypernatremia and their condition is exacerbated with DOCP treatment. Indeed, by the end of the study the CD ET-1 mice are substantially hypernatremic. A formal comparison of the CD ET-1 KO mice preference for water versus saline revealed no selection of saline over water on a normal Na diet (Figure 1B).

Although the inner medullary CD produces the greatest amount of ET-1, principal cells from all regions of the CD produce the autocrine.(24) The connecting segment (CNT) of the distal nephron has a large Na transport capacity and appears to be an important regulator of Na excretion.(33) Furthermore, an electroneutral thiazide-sensitive Na reabsorption mechanism identified as the NaCl cotransporter (NCC) in the distal convoluted tubule (DCT) is down-regulated during primary hyperaldosteronism.(9) Therefore, the effects of chronic mineralocorticoid treatment and possible inhibitory effects of ET-1 on Na transport mechanisms in the CNT and DCT may also contribute to the genotype effects.

A significant increase in urinary K excretion was not observed as a result of DOCP treatment in either genotype. In contrast, both genotypes progressively reduced urinary K excretion after DOCP treatment by about 20% in the controls and 50% in the CD ET-1 KO mice by the final days of the experiment (Figure 6A). Furthermore, the CD ET-1 KO mice retained more K than controls after DOCP treatment. Despite positive K balance, DOCP treatment induced significant hypokalemia in both genotypes (Figure 6D). In a recent review, Gumz et al. discuss intracellular redistribution of K by aldosterone.(15) Interestingly, chronic balance studies on the effect of mineralocorticoids in several species (dog, rabbit, pig, and mouse) have failed to find a significant effect of either desoxycorticosterone or aldosterone to produce a significant negative K balance.(8; 13; 18; 19; 34) One study has demonstrated that
desoxycorticosterone produced a positive K balance despite the development of hypokalemia.(12) Our laboratory has previously reported that mineralocorticoids increased HKα₂ H,K-ATPase activity and mice lacking this H-K pump exhibited negative K balance which supports a role for this K reabsorptive mechanism as one mechanism to maintain K balance with chronic mineralocorticoid treatment or excess. The current findings are consistent with these observations.(12)

In summary, the absence of ET-1 expression in cells of the CD alters the renal response to aldosterone. When fed a high-salt diet, the CD ET-1 KO mice have increased BP with fluid retention and body weight gain. DOCP treatment combined with the high-salt diet resulted in chronic electrolyte retention and substantial hypertension. Thus, disruption of a renal aldosterone-endothelin feedback mechanism prevented renal compensation to the effects of a mineralocorticoid-salt regiment. Such a locally acting system appears to be of primary importance in the strict regulation of Na and fluid balance.

The local autocrine-paracrine action of ET-1 to modulate Na transport in the CD involves multiple steps that are important for proper operation of this feedback control system as demonstrated by others(20; 21), and may also act in concert with humoral or physical factors.(25; 37; 41; 47) Further investigation is necessary to determine to what degree defects in other proteins or signaling mechanisms contribute to this impairment of fluid and electrolyte excretion and balance.
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Figure Legends

**Figure 1. Saline and Food Intake, Urine Volume and Osmolality.** Saline intake (Panel A), food intake (Panel C), and preference (water vs. saline, Panel B), are shown as mean ± SEM normalized for body weight. Urinary output (D) and osmolality (E) are shown as mean ± SEM. Control, n=9-10; CD ET-1 KO, n=6. HS, average days 6-8 of high-salt treatment; HS + DOCP, average days 17-19 of DOCP treatment

Panel A-D:
* p<0.05 vs. Control within same treatment, unpaired Student’s t-test.
† p<0.05 vs. HS treatment within same genotype, paired Student’s t-test.

Panel E:
*p<0.05 vs. Water same treatment, unpaired Student’s t-test

**Figure 2. Fluid Balance and Body Weight.** Fluid balance (Panel A) and percent change in body weight from day 3 (Panel B) are shown normalized for body weight. Data are shown as mean ± SEM. Control, n=9-10; CD ET-1 KO, n=6. HS, average days 4-8 of high-salt treatment; HS + DOCP, average days 17-19 of DOCP treatment

Panel A&B:
* p<0.05 vs. Control within same treatment, unpaired Student’s t-test.
† p<0.05 vs. HS treatment within same genotype, paired Student’s t-test.

**Figure 3. Systolic Blood Pressure and Heart Rate.** Systolic BP (Panel A) and heart rate (Panel B) are shown as mean ± SEM during the daytime (above) and nighttime (below). Control, n=7; CD ET-1 KO, n=4. NS, Normal salt; HS, average days 5-7 of high-salt treatment; HS + DOCP, average days 15-18 of DOCP treatment

Panel A&B:
* p<0.05 vs. NS within same genotype
**p=0.05 vs. NS within same genotype
†p<0.05 vs. HS within same genotype

**Figure 4. Sodium Excretion.** Urinary Na excretion (Panel A) and fecal Na excretion (Panel B) are shown as mean ± SEM. Solid lines are controls and dashed lines are CD ET-1 KO. Control, n=9-10; CD ET-1 KO, n=6. HS, high-salt

Panel A
* p<0.05 significant effect of genotype, repeated measures ANOVA
† p<0.05 significant effect of treatment within CD ET-1 KO, repeated measures ANOVA
‡ p<0.05 significant effect of genotype and treatment, repeated measures ANOVA
§ P<0.05 significant effect of treatment within Control, repeated measures ANOVA post hoc Day 11 vs. 8, 12-19

Panel B:
* p<0.05 significant effect of genotype, repeated measures ANOVA post hoc Days 1,2 & 11.
† p<0.05 significant effect of treatment within CD ET-1 KO, repeated measures ANOVA post hoc Day 2 vs. 8, 10, 13-19; Day 11 vs. 10,13-14, &17.
Figure 5. Sodium Balance, Plasma Sodium and Osmolality. Daily Na balance is shown in Panel A. Cumulative Na balance is shown in Panel C. HS, average days 6-8 of high-salt treatment; HS + DOCP, average days 17-19 of DOCP treatment. Daily and cumulative Na balance are shown for days 1-19 in Panels B and D, respectively. Solid lines are controls and dashed lines are CD ET-1 KO. Control, n=9; CD ET-1 KO, n=6. Plasma Na and Osmolality are shown in Panel E &F, respectively. Control, n=4-11; CD ET-1 KO, n=3-7. NS, normal-salt; HS, high-salt. All data are shown as mean + SEM.

Panel A&C:
* p<0.05 vs. Control within same treatment, unpaired Student’s t-test.
† p<0.05 vs. HS treatment within same genotype, paired Student’s t-test.

Panel B:
* p<0.05 within Control Day 1 vs. 2,3,6,7, Tukey Test
† p<0.05 within Control Day 11 vs 9-12&14-19, Tukey Test
‡ p<0.05 significant effect of genotype, repeated measures ANOVA.

Panel D:
* p<0.05 significant effect of treatment within both genotypes, repeated measures ANOVA
† p<0.05 significant effect of treatment within both genotypes, repeated measures ANOVA
‡ p<0.05 significant effect of genotype, repeated measures ANOVA, post-hoc Days 17-19

Panel E&F:
* p<0.05 vs. Control within same treatment, unpaired Student’s t-test.
† p<0.05 vs. NS treatment within same genotype, unpaired Student’s t-test.

Figure 6. Potassium Excretion and Balance and Plasma Potassium. Urinary K excretion is shown in Panel A. Daily and cumulative K balance is shown in Panel B and C, respectively. Control, n=9; CD ET-1 KO, n=6. HS, average days 6-8 of high-salt treatment; HS + DOCP, average days 17-19 of DOCP treatment. Plasma K is shown in Panel D. NS, normal Na. Control: n=8 NS, 4 HS; CD ET-1 KO: n=8 NS, 6 HS. All data are shown as mean ± SEM.

Panel A-C
* P<0.05 vs. HS diet within same genotype, paired Student’s t-test.
† p<0.05 vs. Control same treatment, unpaired Student’s t-test.

Panel D:
* p<0.05 vs. NS diet within same genotype, paired Student’s t-test.

Figure 7. Urinary and Fecal K Excretion. Urinary and Fecal K excretion are shown in Panel A and B, respectively. Solid lines are controls and dashed lines are CD ET-1 KO. Control, n=9; CD ET-1 KO, n=6. HS, high-salt

Panel A:
* p<0.05 significant effect of genotype, repeated measures ANOVA
†p<0.05 significant effect of treatment in both genotypes, repeated measures ANOVA

Panel B:
* p<0.05 significant effect of treatment within both genotypes, repeated measures ANOVA
**Figures**

**Figure 1. Saline and Food Intake, Urine Volume and Osmolality.** Saline intake (Panel A), food intake (Panel C), and preference (water vs. saline, Panel B), are shown as mean ± SEM normalized for body weight. Urinary output (D) and osmolality (E) are shown as mean ± SEM. Control, n=9-10; CD ET-1 KO, n=6. HS, average days 6-8 of high-salt treatment; HS + DOCP, average days 17-19 of DOCP treatment.

Panel A-D:
* p<0.05 vs. Control within same treatment, unpaired Student’s t-test.
† p<0.05 vs. HS treatment within same genotype, paired Student’s t-test.

Panel E:
*p<0.05 vs. Water same treatment, unpaired Student’s t-test.
Figure 2. Fluid Balance and Body Weight. Fluid balance (Panel A) and percent change in body weight from day 3 (Panel B) are shown normalized for body weight. Data are shown as mean ± SEM. Control, n=9-10; CD ET-1 KO, n=6. HS, average days 4-8 of high-salt treatment; HS + DOCP, average days 17-19 of DOCP treatment.

Panel A&B:
* p<0.05 vs. Control within same treatment, unpaired Student’s t-test.
† p<0.05 vs. HS treatment within same genotype, paired Student’s t-test.
Figure 3. Systolic Blood Pressure and Heart Rate. Systolic BP (Panel A) and heart rate (Panel B) are shown as mean ± SEM during the daytime (above) and nighttime (below). Control, n=7; CD ET-1 KO, n=4. NS, Normal salt; HS, average days 5-7 of high-salt treatment; HS + DOCP, average days 15-18 of DOCP treatment

Panel A&B:
*p<0.05 vs. NS within same genotype
**p=0.05 vs. NS within same genotype
†p<0.05 vs. HS within same genotype
**Figure 4. Sodium Excretion.** Urinary Na excretion (Panel A) and fecal Na excretion (Panel B) are shown as mean ± SEM. Solid lines are controls and dashed lines are CD ET-1 KO. Control, n=9-10; CD ET-1 KO, n=6. HS, high-salt

Panel A
* p<0.05 significant effect of genotype, repeated measures ANOVA
† p<0.05 significant effect of treatment within CD ET-1 KO, repeated measures ANOVA
‡ p<0.05 significant effect of genotype and treatment, repeated measures ANOVA
§ P<0.05 significant effect of treatment within Control, repeated measures ANOVA post hoc Day 11 vs. 8, 12-19

Panel B:
* p<0.05 significant effect of genotype, repeated measures ANOVA post hoc Days 1,2 & 11.
† p<0.05 significant effect of treatment within CD ET-1 KO, repeated measures ANOVA post hoc Day 2 vs. 8, 10, 13-19; Day 11 vs. 10,13-14, &17.
**Figure 5. Sodium Balance, Plasma Sodium and Osmolality.** Daily Na balance is shown in Panel A. Cumulative Na balance is shown in Panel C. HS, average days 6-8 of high-salt treatment; HS + DOCP, average days 17-19 of DOCP treatment. Daily and cumulative Na balance are shown for days 1-19 in Panels B and D, respectively. Solid lines are controls and dashed lines are CD ET-1 KO. Control, n=9; CD ET-1 KO, n=6. Plasma Na and Osmolality are shown in Panel E & F, respectively. Control, n=4-11; CD ET-1 KO, n=3-7. NS, normal-salt; HS, high-salt. All data are shown as mean ± SEM.

Panel A&C:
- * p<0.05 vs. Control within same treatment, unpaired Student’s t-test.
- † p<0.05 vs. HS treatment within same genotype, paired Student’s t-test.

Panel B:
- * p<0.05 within Control Day 1 vs. 2,3,6,7, Tukey Test
- † p<0.05 within Control Day 11 vs 9-12&14-19, Tukey Test
- ‡ p<0.05 significant effect of genotype, repeated measures ANOVA.

Panel D:
- *p<0.05 significant effect of treatment within both genotypes, repeated measures ANOVA
- † p<0.05 significant effect of treatment within both genotypes, repeated measures ANOVA
- ‡ p<0.05 significant effect of genotype, repeated measures ANOVA, post-hoc Days 17-19

Panel E&F:
- * p<0.05 vs. Control within same treatment, unpaired Student’s t-test.
- † p<0.05 vs. NS treatment within same genotype, unpaired Student’s t-test.
Figure 6. Potassium Excretion and Balance and Plasma Potassium. Urinary K excretion is shown in Panel A. Daily and cumulative K balance is shown in Panel B and C, respectively. Control, n=9; CD ET-1 KO, n=6. HS, average days 6-8 of high-salt treatment; HS + DOCP, average days 17-19 of DOCP treatment. Plasma K is shown in Panel D. NS, normal Na. Control: n=8 NS, 4 HS; CD ET-1 KO: n=8 NS, 6 HS. All data are shown as mean ± SEM.

Panel A-C
*P<0.05 vs. HS diet within same genotype, paired Student’s t-test.
† p<0.05 vs. Control same treatment, unpaired Student’s t-test.

Panel D:
*P<0.05 vs. NS diet within same genotype, paired Student’s t-test.
Figure 7. Urinary and Fecal K Excretion. Urinary and Fecal K excretion are shown in Panel A and B, respectively. Solid lines are controls and dashed lines are CD ET-1 KO. Control, n=9; CD ET-1 KO, n=6. HS, high-salt

Panel A:
* p<0.05 significant effect of genotype, repeated measures ANOVA
†p<0.05 significant effect of treatment in both genotypes, repeated measures ANOVA