Mineralocorticoid and Glucocorticoid Receptors Stimulate Epithelial Sodium Channel Activity in a Mouse Model of Cushing Syndrome

Matthew A. Bailey, John J. Mullins, Christopher J. Kenyon

Abstract—Experiments in Cushing patients and healthy control subjects receiving adrenocorticotropic hormone (ACTH) indicate that transient renal sodium retention may contribute to the generation of hypertension. Here we have investigated the effect of chronic ACTH infusion on renal sodium handling in adult male C57BL/6J mice using selective antagonists to dissect mineralocorticoid and glucocorticoid receptor–mediated pathways. Mice were infused via osmotic minipump with ACTH (2.5 μg/d) or saline for 2 weeks before being anesthetized for renal function experiments. ACTH caused an increase in blood pressure and a reduction in fractional sodium excretion associated with enhanced activity of the epithelial sodium channel. Given separately, spironolactone and RU38486 blunted the pressor response to ACTH and the increased epithelial sodium channel activity; combined mineralocorticoid and glucocorticoid receptor blockade was required to resolve the response to ACTH excess. Dietary sodium depletion also prevented ACTH-induced hypertension. The effect of increased sodium reabsorption in the distal nephron is offset by downregulation of Na-K-Cl cotransport in the loop of Henle. Sodium excretion is normalized chronically, but blood pressure remains high; acute blockade of V1 receptors and \( \alpha \)-adrenoceptors in combination restored blood pressure to control values. In summary, ACTH excess promotes renal sodium reabsorption, contributing to the increased blood pressure; both glucocorticoid and mineralocorticoid receptor pathways are involved. These data are relevant to conditions associated with overactivity of the hypothalamic-pituitary-adrenal axis, such as obesity and chronic stress. (Hypertension. 2009;54:00-00.)

Key Words: renal clearance • amiloride • 11β-hydroxysteroid dehydrogenase • tubular sodium reabsorption • furosemide

The severity of hypertension in Cushing syndrome is an important predictor of morbidity and mortality, but the underlying cause remains uncertain. Renal sodium retention may contribute, with sodium balance being restored at the expense of elevated blood pressure. Impaired natriuretic capacity and hypokalemic alkalosis in Cushing syndrome may reflect mineralocorticoid excess, and aldosterone levels can indeed be high because of a direct action of adrenocorticotropic hormone (ACTH) on CYP11B2. However, the increase in aldosterone is transient. It is also possible that the mineralocorticoid receptor (MR) is activated by high levels of glucocorticoid. Nevertheless, glucocorticoid hypertension is not fully prevented by the MR antagonist spironolactone and glucocorticoid receptor (GR)–mediated pathways may also contribute to sodium retention.

Glucocorticoids stimulate renal sodium transport by increasing the activity of the basolateral membrane Na-K-ATPase. Direct activation of apical membrane transport proteins has also been demonstrated. Thus, glucocorticoids increase the following: (1) sodium-hydrogen exchange by Na\(^+\)/H\(^+\) exchanger 3 in the proximal tubule; (2) furosemide-sensitive cotransport by Na-K-Cl cotransporter (NKCC 2) in the thick limb of Henle; (3) thiazide-sensitive cotransport by Na-CI cotransporter (NCC) in the distal tubule, and (4) the epithelial sodium channel (ENaC) in the connecting tubule and collecting ducts. The net effect on sodium reabsorption is, however, difficult to predict, because glucocorticoids can also inhibit mineralocorticoid action and promote natriuresis.

The aim of the present study was to assess the impact of ACTH excess on renal sodium homeostasis. We found evidence for activation of ENaC through both the GR and MR; the net effect of increased sodium reabsorption was offset by increased glomerular filtration and downregulation of sodium transport in the thick limb of Henle.

Methods

All of the experiments were performed in accordance with United Kingdom Home Office regulations. Cohorts of male C57BL/6J mice were maintained on either a standard rodent diet (RM1 diet; 0.3% sodium, SDS Diets) or a low-sodium diet (0.03% sodium, SDS Diets) with free access to water. On day 0, osmotic minipumps...
(model 2002, ALZET) containing either ACTH (Synacthen, Ciba-Geigy; 2.5 μg/d) or vehicle (0.9% NaCl) were implanted SC.

On days 12 to 14, mice were anesthetized with Inactin (thiotub-abartial; 100 mg/kg IP), and renal function studies were performed as described; topical administration of a lidocaine solution was used during surgery. Mean arterial blood pressure (MABP) was recorded throughout (PowerLab, AD Instruments). Mice were infused throughout (0.2 mL/kg per 10 g IV) with a solution containing (in mM) 130 NaCl, 5 KCl, and 10 NaHCO₃. Fluorescein isothiocyanate-inulin (0.5%) was included for the measurement of glomerular filtration rate (GFR), and in some experiments 1.5% p-aminohippurate was included for the measurement of effective renal plasma flow. After a 60-minute equilibration period, 2 consecutive urine collections of 45 minutes each were made, bracketed by collections (20 μL) of arterial blood. After the first (control) urine sample, a bolus of either amiloride (2 mg/kg IV) or furosemide (2 mg/kg IV) was administered, and the second (control) urine sample, a bolus of either prazosin (0.1 mg/kg IV) or Manning compound adrenoreceptor or V₁-vasopressinergic receptor blockade were measured colorimetrically.¹⁸

Collection was then made. A 500-μL blood sample was then taken for electrolyte analysis.

In separate cohorts, the antihypertensive actions of either acute α₁ adrenoreceptor or V₁-vasopressinergic receptor blockade were measured. Blood pressure was monitored for 5 minutes at 2 Hz before a bolus of either prazosin (0.1 mg/kg IV) or Manning compound (1-mercaptop-cyclopentamethylene propionic acid 2-[0-[methyl] tyrosine] arginine vasopressin; Bachem; 10 μg/kg IV) was given. A subset of these mice received both compounds in a randomized order. Plasma volume was determined by dilution of Evans blue (1 μL/g of a 0.5% weight:volume solution IV); blood volume was calculated from plasma volume and hematocrit.

To identify the contribution of MR and/or GR to the ACTH excess phenotype, spironolactone and RU38468 were used either separately or in combination. The compounds were encapsulated in an elastomer pellet (Silastic, a gift from Dow Corning, Inc) in a 10:1 weight:weight ratio according to the manufacturer’s instructions. The pellets were cured and dried overnight at 37°C and implanted alongside the minipumps. The concentration of canrenone (the active metabolite of spironolactone) and/or RU38468⁹ in terminal plasma (days 13 to 14) was measured by mass spectrometry (Clinical Research Facility, University of Edinburgh). For canrenone this was 60.3±6.7 mmol/L; for RU38468 this was 109.7±9.2 mmol/L. For quantitative PCR, an additional cohort of ACTH-treated (n=12) or vehicle (n=12) mice was killed by cervical dislocation and the kidneys taken for RNA extraction using a TRIzol reagent (Invitrogen). The mRNAs of interest were quantified by validated TaqMan assays (Applied Biosystems) according to the manufacturer’s instructions using an ABI 7700 (Applied Biosystems). Data were normalized to 18S rRNA on a sample-by-sample basis.

### Statistical Analyses

All of the data are mean±SE. After tests for Gaussian distribution, comparisons were made using either unpaired t test or 1-way ANOVA with Holm-Sidak post hoc test, as appropriate. For correlation, the Pearson product-moment correlation coefficient was used. Analysis was performed using either Prism 4 (GraphPad Software, Inc) or SPSS 16 software (SPSS Inc).

### Results

ACTH treatment increased plasma corticosterone and aldosterone levels. ACTH-treated mice had a significantly higher hematocrit level than saline controls, which was attributed to hemoconcentration; depletion of plasma volume (~20%) was confirmed. ACTH-treated mice were hypokalemic and hypernatremic (Table 1).

MABP was significantly elevated in mice exposed to ACTH (Figure 1A), as was GFR (Figure 1B). Renal blood flow was reduced by ACTH excess (Figure 1C), suggesting that hyperfiltration is not secondary to hypertension. Increased GFR in the face of enhanced renal vascular resistance indicates a direct effect of ACTH excess on filtration fraction (Table 1).

In absolute terms, sodium excretion was not different between the 2 groups of mice (Figure 1D), consistent with sodium balance. There was, however, a significant increase in filtered sodium load in ACTH-treated mice (40.5±2.8 versus 25.5±1.7 μmol/min; P<0.001), and, thus, fractional excretion was lower than in controls (Figure 1E). Despite the mild hypokalemia, potassium excretion remained robust in the ACTH-treated animals and was not different from controls (Figure 1F). There was no difference in the urinary sodium:potassium concentration ratio in the 2 groups of mice, and urine flow rate and fractional potassium excretion were similarly unaffected by ACTH excess (Table 1).

The reduction in fractional sodium excretion during ACTH treatment indicated increased tubular reabsorption, and we therefore measured the activity/expression of key sodium transport proteins. Enhanced ENaC activity was demonstrated; amiloride caused a significant natriuresis in both ACTH and saline-treated mice, but the effect was greater in the ACTH-treated mice (Figure 2A). An increase in ENaC-α mRNA was observed after ACTH treatment (Table 2), but this was to a lesser extent than the increase in functional channel activity. The possibility of enhanced ENaC trafficking to the apical membrane of the principal cell is suggested by the increased expression of serum glucocorticoid regulated kinase 1 (Table 2). The antikaliuretic effect of amiloride was confirmed.

### Table 1. Plasma Data, Urinary Sodium:Potassium Concentration Ratio, Fractional Potassium Excretion, and Urine Flow Rate in C57BL/6J Mice Treated With Either Vehicle (n=13) or ACTH (n=13) for 14 Days

<table>
<thead>
<tr>
<th>Variable</th>
<th>Vehicle</th>
<th>P</th>
<th>ACTH</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNa, mmol/L</td>
<td>147.2±0.8</td>
<td>&lt;0.01</td>
<td>154.1±1.6</td>
</tr>
<tr>
<td>PCr, mmol/L</td>
<td>4.47±0.17</td>
<td>&lt;0.01</td>
<td>3.65±0.19</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>41.8±0.6</td>
<td>&lt;0.01</td>
<td>44.6±0.7</td>
</tr>
<tr>
<td>P_aldo, pmol/L</td>
<td>851±86</td>
<td>&lt;0.01</td>
<td>1264±60</td>
</tr>
<tr>
<td>P_exo, mmol/L</td>
<td>133±29</td>
<td>&lt;0.01</td>
<td>745±124</td>
</tr>
<tr>
<td>Penv, mL</td>
<td>1.93±0.11</td>
<td>&lt;0.01</td>
<td>1.47±0.14</td>
</tr>
<tr>
<td>FEx, %</td>
<td>24.1±4.2</td>
<td>NS</td>
<td>16.6±2.0</td>
</tr>
<tr>
<td>Na/K</td>
<td>2.31±0.76</td>
<td>NS</td>
<td>2.00±0.34</td>
</tr>
<tr>
<td>V, μL/min</td>
<td>1.3±0.2</td>
<td>NS</td>
<td>1.6±0.3</td>
</tr>
<tr>
<td>RVR, mm Hg/mL·min⁻¹</td>
<td>45.4±2.7</td>
<td>&lt;0.01</td>
<td>76.1±4.3</td>
</tr>
<tr>
<td>FF, %</td>
<td>16.4±1.4</td>
<td>&lt;0.01</td>
<td>33.4±3.4</td>
</tr>
</tbody>
</table>

Renal vascular resistance and filtration fraction were obtained from separate cohorts of vehicle (n=6) and ACTH-treated (n=5) mice. Data are mean±SE; statistical comparisons were made using t test and P values (2 tailed) as given. Na/K indicates urinary sodium-potassium concentration ratio; FEx, fractional potassium excretion; V, urine flow rate; RVR, renal vascular resistance; FF, filtration fraction; NS, not significant; aldo, aldosterone; cort, corticosterone; vol, volume.

Although sodium excretion was not different between the 2 groups of mice (Figure 1D), consistent with sodium balance. There was, however, a significant increase in filtered sodium load in ACTH-treated mice (40.5±2.8 versus 25.5±1.7 μmol/min; P<0.001), and, thus, fractional excretion was lower than in controls (Figure 1E). Despite the mild hypokalemia, potassium excretion remained robust in the ACTH-treated animals and was not different from controls (Figure 1F). There was no difference in the urinary sodium:potassium concentration ratio in the 2 groups of mice, and urine flow rate and fractional potassium excretion were similarly unaffected by ACTH excess (Table 1).

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evident in both groups of mice but was lower in the ACTH group (Figure 2B). Amiloride increased the urine flow rate in both groups of mice, with the diuresis being greater in the ACTH group (Figure 2C). The \( \alpha \) and \( \beta \) subunits of the Na,K ATPase or the expression of NCC was not changed by ACTH treatment. For the former, it is possible that regional differences in mRNA expression are masked by the use of whole kidney homogenates. Expression of NKCC2 was signifi-

Figure 1. (A) MABP; (B) GFR; (C) renal blood flow; (D) absolute sodium excretion; (E) fractional sodium excretion; and (F) absolute potassium excretion in male C57BL/6J mice after 14 days treatment with ACTH (n=13) or saline vehicle (n=13). Data are mean±SE. Comparisons were made using an unpaired t test. *P<0.05; **P<0.01; ***P<0.001.

Figure 2. The net effect of amiloride (amiloride) on the excretion of (A) sodium, (B) potassium, and (C) urine in ACTH (n=8) and saline-treated (n=7) mice. The effect of furosemide on (D) sodium excretion, (E) urine flow rate, and (F) potassium excretion in ACTH (n=6) and saline-treated mice (n=6). Data are mean±SE. Comparisons were made using an unpaired t test. *P<0.05, **P<0.01.
Significantly downregulated (Table 2), and the natriuretic response to furosemide was therefore measured: ACTH caused a significant natriuresis in control mice but not in the ACTH group (Figure 2D), consistent with the downregulation of NKCC2. In contrast, the furosemide-induced diuresis was larger in the ACTH group than in the control group (Figure 2E). Despite the increased urine flow, furosemide did not promote kaliuresis in the ACTH-treated group (Figure 2F).

To investigate the contribution of increased renal sodium reabsorption to hypertension, mice were maintained on a low-sodium diet before and during the 14-day experimental protocol. In ACTH-treated mice fed a low-sodium diet, MABP (99.2±2.3 mm Hg; n=8) was not different from low-sodium control mice MABP (96.8±1.8 mm Hg; n=7). Volume depletion and hyperfiltration were similarly prevented by dietary sodium restriction (data not shown), hypokalemia persisted (3.63±0.27 versus 4.41±0.14 mmol/L; P=0.05). Spironolactone and RU38486 were used to resolve the effects of ACTH excess into MR- and GR-mediated components, respectively. Combined receptor blockade in sodium-replete control mice had no significant effect on basal MABP (Figure 3A) and tended to increase sodium excretion (Figure 3C), although this was not statistically significant. The natriuretic effect of amiloride was abolished (Figure 3D). Similar effects on sodium handling were observed in ACTH-treated mice; the rise in blood pressure was prevented, and, in this case, the natriuresis was statistically significant. Administration of either spironolactone or RU38486 alone partially rescued the pressor effect of ACTH (by ≈10 mm Hg in each case; P<0.05) and also blunted the increase in amiloride-sensitive sodium excretion. Notably, under single receptor blockade, fractional sodium excretion and amiloride-sensitive sodium reabsorption were not statistically different from either the vehicle and the ACTH groups. Across all of the sodium-replete experimental groups, however, there was a highly significant correlation between amiloride-sensitive sodium reabsorption and blood pressure (Pearson r=0.59; P<0.001).

There were no derangements in plasma sodium or potassium concentrations in any of the groups receiving either spironolactone or RU38486. The ACTH-induced volume depletion was, however, prevented by RU38486 but not by spironolactone. ACTH-induced hyperfiltration, considered to be a GR-mediated response, could only be prevented by combined GR and MR blockade (Figure 3B).

There was a highly significant positive correlation between MABP and both plasma sodium (r=0.66; P<0.001) and hematocrit (r=0.49; P<0.01) across all of the sodium-replete experimental groups. Because hypervolemia and volume depletion can stimulate the release of vasopressin and activity of the sympathetic nervous system, the effect on MABP of acute antagonism of either the V1 or α1 adrenoceptor was measured. Both V1 blockade (Figure 4A) and α1 adrenoceptor blockade (Figure 4B) caused a significant reduction in MABP in saline-treated control mice, but the antihypertensive effect was greater in mice receiving ACTH. A subset of control and ACTH-treated mice received both antagonists in a randomized order, and a sample recording, taken from an ACTH-treated mouse, is shown (Figure 4C). Combined blockade of V1 and α1 receptors restored MABP to control levels (Figure 4D).

Discussion

ACTH infusion induces a robust pressor response in humans and experimental animals, associated with reduced sodium excretion.4,5 In our study, rigorous dietary sodium restriction prevented this response to ACTH excess, suggesting that enhanced ENaC-mediated sodium reabsorption is an important hypertensive event. Indeed, there was a highly significant correlation between the amiloride-sensitive sodium reabsorption and blood pressure. The functional ENaC channel complex is a heterodimer of α, β, and γ subunits; only αENaC was stimulated by ACTH excess in the present study and not to the same extent as the increase in physiological channel activity. Stimulation by corticosteroids of αENaC has been shown previously,20 with expression in the inner medullary collecting duct being the rate-limiting step for channel formation.20 It is possible that an increase in αENaC expression in this segment could have a disproportionate effect on renal sodium transport. In addition, we have evidence to suggest that trafficking of the channel complex to the apical membrane of the principal cell is stimulated by ACTH; expression of serum glucocorticoid regulated kinase 121 was greater in kidneys from ACTH-treated mice than from control mice.

By the end of the experiment, absolute sodium excretion was normal despite persistently high levels of ENaC. This is compatible with long-term sodium balance at the expense of chronically elevated blood pressure. Our data indicate that both hemodynamic and tubular mechanisms may compensate for a reduced natriuretic capacity. First, ACTH causes glomerular hyperfiltration and increases the filtered sodium load. Hyperfiltration occurs despite increased renal vascular resistance, and it is therefore unlikely to be a secondary response to hypertension. Notably, hyperfiltration is considered a GR-mediated response,22 and selective GR blockade tended to lower GFR. However, spironolactone was also required to fully prevent hyperfiltration. Aldosterone modulates angiotensin II action in the renal microvasculature23 and can directly increase glomerular pressure by inducing pathological glomerular remodeling.24

<table>
<thead>
<tr>
<th>Name</th>
<th>Gene ID</th>
<th>Vehicle</th>
<th>ACTH</th>
<th>P</th>
</tr>
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<tr>
<td>α-ENaC</td>
<td>Scnn1a</td>
<td>0.46±0.05</td>
<td>&lt;0.05</td>
<td>0.65±0.09</td>
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<td>β-ENaC</td>
<td>Scnn1b</td>
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<td>NS</td>
<td>0.56±0.07</td>
</tr>
<tr>
<td>γ-ENaC</td>
<td>Scnn1g</td>
<td>0.59±0.07</td>
<td>NS</td>
<td>0.66±0.07</td>
</tr>
<tr>
<td>NCC</td>
<td>Slc12a3</td>
<td>0.78±0.09</td>
<td>NS</td>
<td>0.66±0.07</td>
</tr>
<tr>
<td>NKCC2</td>
<td>Slc12a1</td>
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<td>&lt;0.001</td>
<td>0.70±0.07</td>
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<td>Atplα1</td>
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<td>β Na,K-ATPase</td>
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<td>11βHSD2</td>
<td>Hsd11β2</td>
<td>0.74±0.10</td>
<td>NS</td>
<td>0.81±0.12</td>
</tr>
</tbody>
</table>

Data are normalized to the expression of 18S RNA, which was not different between groups. Data are mean±SE. Statistical comparisons were made using the t test and P values (2 tailed) as given. NS indicates not significant; sgk1, serum glucocorticoid regulated kinase 1.
Second, expression and activity of NKCC2 were down-regulated. This was unexpected, because corticosteroids stimulate sodium reabsorption in the thick limb of Henle. Reduced NKCC2 activity, possibly as a result of mild potassium depletion, has ramifications for the transport of other electrolytes: the reduced electrochemical driving force for paracellular reabsorption through the cation shunt may cause the hypercalciuria induced by ACTH excess.

The expression of NCC was not affected by ACTH treatment despite regulation by glucocorticoids. We were also unable to demonstrate altered expression of either subunit of the Na,K-ATPase, although the α subunit has been linked to ACTH-induced hypertension. Nevertheless, activity of NCC and Na,K-ATPase can be increased without requiring gene transcription, and we cannot discount a role for these transporters in the response to ACTH excess.

Although ACTH was not kaliuretic in C57BL/6J mice, potassium excretion remained robust in the face of mild potassium depletion. The potassium conservation deficit probably reflects reduced reabsorption in the thick limb of Henle and persistent secretion in the distal nephron. The potassium-sparing properties of amiloride observed in ACTH-treated mice are consistent with channel-mediated potassium secretion in the distal nephron. ROMK and BK (maxi-K) channels mediate potassium secretion in the mouse. However, BK channels are likely to be inactive during ACTH excess; furosemide is not kaliuretic despite a significant increase in the urine flow rate. That furosemide induces a robust diuresis in the absence of a natriuretic effect is somewhat perplexing, although similar findings have been reported in the ROMK null mouse. The diuresis might reflect reduced water reabsorption in the proximal tubule because of inhibition of carbonic anhydrase by furosemide.

The renal and hypertensive actions of ACTH excess can be attributed to combined activation of MR and GR, and there is no evidence for alternative pathways for stimulation of ENaC. In addition, MR and GR have reciprocal functionality, because single receptor blockade only blunted the enhanced ENaC activity: the hypertension was similarly reduced by single receptor blockade. With stimulation of aldosterone being transient, activation of MR by glucocorticoids, normally pre-

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**Figure 3.** (A) MABP; (B) fractional excretion of sodium (FENa); (C) the net effect of amiloride on sodium excretion (∆amilorideNa); and (D) GFR in C57BL/6J mice treated for 14 days with saline vehicle (Vehicle; n = 13), saline vehicle plus RU38486 and spironolactone (Vehicle+RU486+Spiro; n = 7), ACTH-treated mice plus RU38486 and spironolactone (ACTH+RU486+Spiro; n = 7), ACTH plus either RU38486 (ACTH+RU486; n = 7) or spironolactone (ACTH+Spiro; n = 8), and mice that received ACTH alone (ACTH; n = 11). Data are mean ± SE. Analysis was via a 1-way ANOVA with Holm-Sidak post hoc test for directed comparisons. *P < 0.05, **P < 0.01 vs vehicle group; other comparisons as stated.
vented by 11β-hydroxysteroid dehydrogenase type 2 (11βHSD2), must be considered. The ratio of urinary free cortisol:cortisone, an index of 11βHSD2 function, is increased in patients with Cushing syndrome, suggesting inhibition of 11βHSD2 activity by ACTH. However, renal hsd11b2 mRNA expression was not altered by ACTH excess, nor does ACTH inhibit enzymatic function. It is possible, therefore, that the protective barrier provided by 11βHSD2 is breached, allowing corticosterone to activate MR.

The notion that GR activation directly regulates ENaC is controversial. The channel can be activated by either receptors in a mouse cell line, and there is evidence for functional crossover in MR knockout mice and transgenic mice overexpressing GR in the collecting duct. In contrast, however, α-ENaC expression was not stimulated by a GR-specific dosage of dexamethasone, and collecting duct–specific deletion of MR prevents expression of ENaC.

In the present study, GR-dependent contraction of plasma volume predominated. Volume depletion, hyperosmolality, and hypernatremia stimulate vasopressin release, causing sustained activation of the sympathetic nervous system. Our data suggest that both systems sustain elevated blood pressure during ACTH excess. In the rat, neither chemical sympathectomy nor chronic V1 receptor antagonism fully prevent the pressor response to ACTH. ACTH-induced hypertension is also observed in Brattleboro rats, indicating that vasopressin is not essential. Other studies, however, find V1 antagonism an effective measure to counter enhanced vascular reactivity in glucocorticoid-mediated hypertension. In the present investigation, the acute effect of receptor antagonism in the maintenance phase of hypertension was measured, and there is clearly a need for chronic studies.

**Perspectives**

ACTH-dependent hypertension is sodium dependent and is associated with MR- and GR-mediated activation of ENaC. Renal sodium retention may ultimately sustain hypertension via stimulation of vasoactive factors, and this may occur independent of any blood pressure change. This study has relevance to hypertensive disorders associated with overactivity of the hypothalamic-pituitary adrenal axis: enhanced ENaC expression is found in a rat model of chronic stress, and a mouse obesity model displays impaired sodium excretion.

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**Disclosures**

None.

**References**


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