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The glucocorticoid receptor in the distal nephron is not necessary for the development or maintenance of dexamethasone-induced hypertension

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Abstract

Glucocorticoids are used as a treatment for a variety of conditions and hypertension is a well-recognized side effect of their use. The mechanism of glucocorticoid-induced hypertension is incompletely understood and has traditionally been attributed to promiscuous activation of the mineralocorticoid receptor by cortisol. Multiple lines of evidence, however, point to the glucocorticoid receptor as an important mediator as well. We have developed a mouse model of glucocorticoid-induced hypertension, which is dependent on the glucocorticoid receptor. To determine the site(s) of glucocorticoid receptor action relevant to the development of hypertension, we studied glucocorticoid-induced hypertension in a mouse with a tissue-specific knockout of the glucocorticoid receptor in the distal nephron. Although knockout mice had similar body weight, nephron number and renal histology compared to littermate controls, their baseline blood pressure was mildly elevated. Nevertheless, distal nephron glucocorticoid receptor knockout mice and controls had a similar hypertensive response to dexamethasone. Urinary excretion of electrolytes, both before and after administration of glucocorticoid was also indistinguishable between the two groups. We conclude that the glucocorticoid receptor in the distal nephron is *not* necessary for the development or maintenance of dexamethasone-induced hypertension in our model.

Keywords

mouse model; steroids; kidney; hypertension

INTRODUCTION

Glucocorticoids (GCs), whether endogenous or exogenous, induce hypertension (HTN) [1]. The lack of understanding of the underlying mechanisms of GC-induced HTN leads to empiric, and often suboptimal, treatment of this condition. While traditionally GCs have commonly been believed to increase blood pressure (BP) via promiscuous activation of the

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mineralocorticoid receptor (MR) in the kidney, available data argue that this is not the only, or perhaps even the primary, mechanism underlying this form of HTN.

We demonstrated that administration of dexamethasone in drinking water induces a rapid and sustained elevation of BP in wild-type mice. Furthermore, we have shown that this response is mediated via the glucocorticoid receptor (GR) and is substantially independent of MR [2]. To identify sites of action of GR activity relevant to BP regulation, we have developed mice in which we systematically removed GR from candidate tissues. In this study we have focused on GR in the distal nephron.

A variety of studies have suggested an important role of GR in the distal nephron in regulating sodium balance. Dexamethasone increases mRNA abundance of a number of genes central to renal sodium reabsorption, including *sgk-1*, α ENaC and *GILZ* [3]. RU28362, a pure GC agonist devoid of mineralocorticoid activity, induces mineralocorticoid-like effects in cultured collecting duct cells; this activity is blocked by glucocorticoid, but not mineralocorticoid, antagonists [4]. Most intriguingly, glucocorticoids have an antinatriuretic effect *in vivo* in MR KO mice via an induction of amiloride-sensitive current while inducing an increase in systemic BP [5;6]. This finding strongly suggests that GCs possess an anti-natriuretic activity in the kidney via GR *in vivo*.

Although the sum of these observations seem to point to an anti-natriuretic role of GCs in the distal nephron, studies performed in live animals have consistently demonstrated that GCs induce natriuresis in conjunction with a prominent kaliuresis [3;7;8;9;10]. Consequently, the contribution of GCs to renal sodium handling has remained difficult to comprehend. A likely confounder in these studies is that the acute increases in blood pressure induced by GC effects on vascular tissue [11] may well complicate these data.

METHODS

Generation of Ksp Cre GR knock-out mice

Wild type (WT) BL6 mice with floxed GR alleles, designated GR^{loxP/loxP} were a generous gift from the laboratory of Dr. Gunther Schutz [12], and were mated with WT males (+/+) possessing Ksp-cadherin (hereafter denoted as Ksp Cre). By selective breeding, Ksp Cre + GR^{loxP/loxP} homozygotes and Cre- GR^{loxP/loxP} littermate controls were generated. Mouse DNA was isolated from tails by standard methods and analyzed by PCR. The primers for the floxed GR allele were: 5'GGCATGCACATTACTGGCCTTCT 3' and 5' CCTTCTCATTTCCATGTCAGCATGT 3'. Primers for detection of Cre were: 5' CCGGGCTGCCACGACCAA 3' and 5' GGCGCGGCAACACCATTTTT 3'. Experiments were performed according to a protocol approved by the Institutional Animal Care and Use Committee at the Yale University School of Medicine, and were consistent with the NIH Guidelines for the Care of Laboratory Animals.

Immunofluorescence

Kidneys were removed, embedded in Tissue-Tek OCT compound and snap frozen prior to sectioning. Sectioned tissue was fixed by incubation in 100% EtOH followed by incubation in 100% acetone. Sections were rehydrated by incubation in PBS and permeabilized by incubation in 0.2% Triton-X. Blocking solution was applied for 30-60 minutes. Tissue sections were then washed 10X quickly in PBS. Primary antibody was applied and incubated 1-3 hours at room temperature. Sections were again washed 10X in PBS. Secondary antibody was applied and incubated for 30-60 minutes at room temperature. Sections were mounted with Vectashield and allowed to harden overnight at 4° C.

Histology

Kidneys were removed and immediately placed in 10% formalin for 4 hours at room temperature. They were then transferred to 70% EtOH and incubated at 4° C overnight. Kidneys were sectioned, mounted and stained with H &E. Two blinded observers viewed each slide.

Telemetric measurement of blood pressure and heart rate

Male Ksp Cre + GR^{loxP/loxP} mice and Cre- littermate controls were implanted with radiotelemetric blood pressure catheters as described [13], and allowed one week to recover from surgery. Adequate recovery was deemed to have occurred if mice demonstrated normal diurnal variation of blood pressure for three consecutive days. Mice were housed individually in a controlled environment with 12-hour light and dark cycles and had free access to water and standard mouse chow. After 3 days of a normal blood pressure pattern, mice were treated with dexamethasone (DEX) in their drinking water at a concentration of 15 mg/L. The estimated ingested dose of DEX for each mouse was approximately 1.5-2.5 mg/kg/day. In other experiments male Ksp Cre+ mice with an unfloxed GR allele and Cre- littermate controls were implanted with catheters and their baseline BP was measured for three days as described above.

Assessment of mouse circadian rhythm

Mice were housed in facilities with 12h light-dark cycles. Data were collected using the DataQuest System software time averaged systolic, diastolic and mean BPs for 4 hour intervals. All mice studied demonstrated a maximum change in BP from the time period of 8PM-midnight to 8AM-noon. This difference was calculated for the mean BP of each mouse at baseline and for each day of DEX treatment.

Aldosterone measurement

Baseline aldosterone measurements were obtained prior to treatment with DEX in control and Ksp Cre + GR^{loxP/loxP} groups. The mice were allowed 1 week to recover from the blood draw. Then, DEX at 15 mg/L was administered in the drinking water for 1 week. On the 7th day of treatment, blood was drawn again for aldosterone levels. Measurement of levels was by radioimmunoassay using the DSL-8600 Active Aldosterone Coated-Tube RIA Kit from Diagnostic Systems Laboratories.

Mouse urine collection

Eight to twelve week old male Ksp Cre + GR^{loxP/loxP} mice and Cre- littermate controls were housed individually in mouse-specific metabolic cages with free access to water and standard mouse chow. Mice were allowed to acclimatize for 24 hours. On day 2, a baseline 16-hour urine sample was collected. On day 3, another 16-hour urine sample was collected with mice simultaneously treated with DEX 15 mg/L in the drinking water. Urine electrolytes were determined by the Yale Mouse Phenotyping Core Facility, which uses the COBAS Mira assay (Roche). Each mouse's urine electrolytes were normalized to its own urine creatinine to control for differences in GFR.

Determination of nephron number

Glomeruli were isolated according to a previously published protocol [14], and Dynabeads were inactivated as per manufacturer's instructions. Male Ksp Cre + GR^{loxP/loxP} and Cre- littermate controls 8-10 weeks old were used in these experiments.

Statistical analysis

Data are expressed as means $\pm$ SEM. Statistical differences were determined by paired and unpaired t-tests or by Wilcoxon signed rank test. A p value of < 0.05 was considered significant.

RESULTS

Effective deletion of GR from the distal nephron

Generation of mice with GR KO in the distal nephron was achieved through the use of Ksp Cre, which has been estimated to provide approximately 80% excisional efficiency in the distal nephron and less than 5% estimated excisional efficiency in the proximal nephron [16]. Mean weights of male (31.4 ± 1.1 g, n=4) and female (22.4 ± 0.4 g, n=5) Ksp Cre + GR^{loxP/loxP} mice did not differ from those of control Cre⁻ GR^{loxP/loxP} male (30.9 ± 1.0 g, n=4) and female (22.3 ± 0.6 g, n=10) mice at 10-12 weeks of age (Table 1).

To determine whether we had effectively removed GR from the distal nephron, we stained sections from the kidneys of Cre⁺ mice with an antibody directed against GR, counterstaining with an antibody to aquaporin-2 (AQP-2) to identify collecting duct tissues (Figure 1). Control animals showed robust staining of GR in all kidney tissues. In contrast, while GR staining was preserved in most tissues of the kidney in Cre⁺ mice, there was an almost complete absence of GR/AQP-2 co-localization, indicating that we had achieved high levels of GR excision specifically in the distal nephron.

Higher baseline BP in Ksp Cre + GR^{loxP/loxP} mice compared to controls

To determine if there was a blood pressure phenotype in these mice, 8-12 week old male Ksp Cre⁺ GR^{loxP/loxP} mice and gender-matched littermate controls (n=4/group) were implanted with radiotelemetric catheters and allowed to recover for one week.

Initial BP measurements revealed a significant difference between the mean baseline BP of controls and Ksp Cre⁺ GR^{loxP/loxP} mice (103.8 ± 2.2 mm Hg vs. 111.4 ± 1.6 mm Hg, respectively, p = 0.032, Figure 2). We considered the possibility that the expression of the Cre transgene itself was causing elevated BP in Ksp Cre + GR^{loxP/loxP} mice. However, we found no difference in baseline BP between KspCre⁺ mice and Cre⁻ wild-type littermates (data not shown). As GCs have been implicated in renal development, inducing hypertension and reduced nephron number in rat pups born to mothers given dexamethasone during pregnancy [15;16], we wondered whether a developmental effect of GR deficiency in the distal nephron might have resulted in the observed BP discrepancy. We therefore quantified nephron number in 8-10 week old male Ksp Cre⁺ mice and controls (n=4/group). We found no significant difference between these two groups. Control mice had $16,680 \pm 464$ nephrons while Ksp Cre⁺ GR^{loxP/loxP} mice had $17,104 \pm 1055$ nephrons (p=0.68, Table 1). The nephron numbers we identified in these mice are consistent with other published reports of glomerular number in mice [14]. We also examined kidney histology in the controls and Ksp Cre⁺ GR^{loxP/loxP} mice, but found no obvious differences between the two groups (Figure 3). These data, coupled with the findings of normal renal histology (Figure 3), suggest that the absence of GR in the distal nephron does not cause a discernible effect on renal development.

We also assessed aldosterone levels to attempt to understand the difference in BP between these two groups of mice. However, there was no significant difference between baseline aldosterone levels between the 2 groups. At baseline, control mice (n=12) had a mean aldosterone level of 710.9 ± 73.8 pg/ml and Ksp Cre + GR^{loxP/loxP} mice (n=9) had a level of 573.4 ± 92.2 pg/ml (p=0.26) (Table 1). The trend towards lower aldosterone levels in the

Cre+ mice suggests that alterations in the renin-aldosterone system cannot explain the observed HTN.

Glucocorticoids have been implicated in the circadian control of blood pressure and so we assessed circadian blood pressure rhythms in these mice. We found that both Ksp Cre + GR^{loxP/loxP} mice and their controls have normal circadian blood pressure variation, although, as expected in the nocturnal mouse, the dip in BP occurs during the daytime (Table 2). At baseline both groups demonstrate a normal circadian variation of blood pressure with mean nighttime BP higher than mean daytime BP by 12.4 mm Hg in the KO animals and 10.9 mm Hg in the controls, indicating that distal nephron GR does not contribute to circadian BP variation.

Unchanged hypertensive response to DEX in Ksp Cre + GR^{loxP/loxP} mice

We next administered DEX 15 mg/L in the drinking water and monitored BP continuously for 1 week. Previous studies in our laboratory have shown that the hypertension produced in mice in this model is GR dependent, as the HTN observed is significantly attenuated by the GR antagonist RU486 but not by the MR antagonist spironolactone. We found no difference in the blood pressure response of Ksp Cre+ and Cre- mice to DEX administration. Consistent with previous observations in our lab [11], mean BP rose rapidly after initiation of DEX in control mice and remained elevated for the duration of GC therapy. We found a similar rise in BP in Ksp Cre+ littermates. There was no difference between the mean rise in BP in Cre+ or Cre- mice in the first day after DEX administration or in any day thereafter (Figure 2b). Importantly, this occurred despite the baseline elevation of blood pressure in Cre+ mice, indicating that the relative absence of GR in the distal nephron offers no protection from GC-induced HTN in this model.

Guytonian models of BP regulation predict that the kidney should respond to a hypertensive stimulus with a pressure natriuresis, a decrease in renal sodium reabsorption that serves to normalize BP. We hypothesized that if DEX acted on the distal nephron GR to inhibit this pressure natriuresis, that we would see a reduction of DEX-induced HTN after the initial hypertensive response in the Cre+ mice. However, though we continued the DEX for a period of one week, we found no diminution in the DEX-induced BP rise in either Cre+ or Cre- mice. The mean BP rise was 12.0 ± 1.7 mm Hg and 15.0 ± 4.3 mm Hg in Cre+ and Cre- mice, respectively ($p=0.54$) (Figure 2). This result suggests that GR in the distal nephron plays a limited role, if any, in the sustained hypertensive response to GCs.

Interestingly, aldosterone levels were similarly suppressed in both groups as well. After 1 week of treatment with DEX at 15 mg/L, the mean aldosterone level in control mice was markedly suppressed at 40.0 ± 3.3 pg/ml while the level in Ksp Cre + GR^{loxP/loxP} mice was similarly suppressed at 69.1 ± 16.4 pg/ml ($p=0.19$). This data indicates that the inhibition of pressure natriuresis is likely not mediated by the renin-angiotensin-aldosterone system.

Both groups demonstrated a similar circadian BP response to DEX administration. After only 1 day of treatment with DEX there was a dramatic blunting of the normal diurnal variation of BP in both groups such that the KO animals showed only a 2.8 mm Hg change between night and day, and the control animals showed only a 0.5 mm Hg difference during the same period. Both groups showed a similar degree of impaired diurnal variation of BP for the entire time DEX was administered (Table 2).

No alteration in urinary electrolyte excretion in Ksp Cre+ GR^{loxP/loxP} mice

Despite, or perhaps owing to, their acute hypertensive effects, GCs induce a rapid natriuresis and kaliuresis in rodents [3;8;10]. Consistent with prior observations, we found that our Cre- control mice markedly increased renal sodium and potassium excretion, as well as

chloride excretion, within 16 hours of DEX administration. We found that Ksp Cre+ GR^{loxP/loxP} mice behaved virtually identically, as would be expected from their BP response, with similar increases in urinary excretion of electrolytes. (Figure 4). These data suggest that distal nephron GR does not play a role in the acute natriuretic or kaliuretic response to DEX *in vivo*.

DISCUSSION

Historically, the role of glucocorticoids in the kidney is unclear. *In vitro* studies have implicated GCs in the regulation of ammoniogenesis, gluconeogenesis, GFR, Na-H exchange and Na-phosphate co-transport [1;8;18;19;20], all of which are proximal tubule processes. These observations are somewhat intuitive given the predominant localization of GR in the proximal nephron [21]. However, several *in vitro* and *in vivo* studies have demonstrated that GCs can exert mineralocorticoid-like effects, such as Na⁺ reabsorption and K⁺ secretion, in the distal nephron [4;22]. Furthermore, it was recently observed in a mouse model that ACTH dependent hypertension was dependent on both mineralocorticoid and glucocorticoid receptors acting to stimulate epithelial sodium channel activity [23].

Our characterization of a model with tissue-specific knockout of GR in the distal nephron shows that these mice develop normally, with similar body weight, nephron number and circadian blood pressure variation when compared to control mice. Interestingly, these mice have an elevation of mean BP at baseline.

We then tested these mice for DEX-induced HTN using a model that we have previously shown is dependent on GR and independent of MR [2]. Given the previously reported effects of GCs on distal nephron sodium handling mediated via GR, we had hypothesized that these mice lacking distal nephron GR would, in the presence of DEX, become acutely hypertensive due to vascular effects of GR [11], but would not sustain this HTN. However, we found that the hypertensive response to DEX is unchanged when compared to control mice. Furthermore, diurnal variation of BP and urinary electrolyte excretion, both before and after treatment with DEX were also indistinguishable between control and KO mice. These data suggest that distal nephron GR plays no significant role in the hypertensive response to GCs *in vivo* in this model.

The only phenotypic difference detected in the KO mouse is an approximately 7 mm Hg increase in baseline BP. The etiology of this BP difference is unclear at present. Shao *et al.* demonstrated that Ksp Cre expression was located exclusively in the tubular epithelial cells of the developing and mature kidney, and that the transgene was most highly expressed in the loop of Henle and the collecting duct [15]. Despite a known role of GCs in renal development, we found no alteration in nephron number in these mice nor were there any alterations in serum aldosterone levels. We also excluded the possibility that the BP alterations were due to the presence of the Cre transgene itself as there were no differences in BP between Cre+ and Cre- wild-type animals. Identification of the mechanisms underlying the baseline elevation of BP in these mice will require further study.

In summary, we conclude that distal nephron GR is not necessary for the maintenance of GC-induced HTN in our model. However, we are unable to exclude the possibility that, given the lack of total Cre-mediated excision of GR in the distal nephron, only a small proportion of functioning GR need be present to manifest the DEX-mediated HTN in our model. This scenario seems unlikely though, given that, in our model and others, this form of HTN does not seem to depend on sodium retention. Similarly, we are unable to exclude proximal tubule effects of GCs as our model does not appear to materially alter GR expression in the proximal tubule. Indeed, studies of NHE3 *in vitro* have demonstrated that

treatment of OKP cells with DEX results in an increase of NHE3 at the apical membrane, though total protein abundance remains unchanged [24]. Assessing a possible role for proximal tubule GR in GC-induced HTN will require additional investigation.

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Figure 1.

Successful knockout of GR in the distal nephron. Kidney sections of Ksp Cre⁺ and Cre⁻ GR^{loxP/loxP} control kidney were double stained with antibodies to aquaporin-2 and GR. The control kidney shows clear co-localization of GR in the distal nephron, while the KO mouse shows minimal co-localization in both low power and higher power images.

Figure 2.

Sustained HTN in Ksp Cre + GR^{loxP/loxP} mice treated with DEX. Controls and Ksp Cre + GR^{loxP/loxP} mice (n=4/group) were treated with DEX 15 mg/L in the drinking water for 1 week and BP was monitored continuously by catheter. The 24 hour mean arterial pressure is presented. **A.** Baseline BP is elevated in Ksp Cre + GR^{loxP/loxP} mice compared to controls (p=0.032). **B.** There is no significant difference between the mean DEX-induced rise in BP in the 2 groups (p=0.54). *** p < 0.05.

Figure 3. Renal histology is similar in Ksp Cre + GR^{loxP/loxP} mice and controls. Shown are H & E stains of kidney cortex from male mice. **A.** Control 10x, **B.** Ksp Cre+ 10x, **C.** Control 40x, **D.** Ksp Cre+ 40x

Figure 4.

No alteration in DEX-induced urinary electrolyte excretion in Ksp Cre + GR^{loxP/loxP} mice. Urine from male Ksp Cre + GR^{loxP/loxP} (n=6) and control (n=7) mice was collected prior to DEX and for the first 16 hours following DEX administration. *** p < 0.05

Table 1

Baseline characteristics of Ksp Cre+ GR^{loxP/loxP} mice (n=4) and littermate controls (n=4). Values are means $\pm$ SE.

	Control	Ksp Cre ⁺	P value
<u>Body weight:</u>			
Male	30.2 $\pm$ 1.0 g (n=4)	31.4 $\pm$ 1.1 g (n=4)	0.74
Female	22.3 $\pm$ 0.6 g (n=10)	22.4 $\pm$ 0.4 g (n=5)	0.90
Baseline BP:	103.8 $\pm$ 2.2 mm Hg (n=4)	111.4 $\pm$ 1.6 mm Hg (n=4)	0.032
Nephron number	16,680 $\pm$ 464 (n=4)	17,104 $\pm$ 1055 (n=4)	0.67
Aldosterone level	710.9 $\pm$ 73.8 pg/ml (n=12)	573.4 $\pm$ 92.2 pg/ml (n=9)	0.26

Table 2

No effect of GR on circadian BP variation. Average difference in mean BP in mm Hg between 8PM-midnight and 8AM-noon for Ksp Cre+GR^{loxP/loxP} mice (n=4) and Cre-controls (n=4) at baseline and for each day of DEX treatment. Values are means $\pm$ SE.

	Ksp Cre⁺	Control	P value
Baseline	12.40 $\pm$ 1.89	10.91 $\pm$ 1.11	0.52
Day 1	2.78 $\pm$ 1.88	0.50 $\pm$ 2.63	0.51
Day 2	6.12 $\pm$ 1.63	3.63 $\pm$ 2.42	0.43
Day 3	10.61 $\pm$ 3.20	4.24 $\pm$ 1.66	0.13
Day 4	10.68 $\pm$ 2.75	6.94 $\pm$ 2.00	0.31
Day 5	12.74 $\pm$ 5.31	6.49 $\pm$ 0.48	0.37
Day 6	7.11 $\pm$ 3.42	6.76 $\pm$ 2.44	0.94
Day 7	6.47 $\pm$ 3.80	7.40 $\pm$ 2.82	0.86