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Knockout of the vascular endothelial glucocorticoid receptor abrogates dexamethasone-induced hypertension

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Abstract

Glucocorticoid-mediated hypertension is incompletely understood. Recent studies have suggested the primary mechanism of this form of hypertension may be through the effects of glucocorticoids on vascular tissues and not to excess sodium and water reabsorption as traditionally believed.

Objective—The goal of this study was to better understand the role of the vasculature in the generation and maintenance of glucocorticoid-mediated hypertension.

Methods—We created a mouse model with a tissue-specific knockout of the glucocorticoid receptor in the vascular endothelium.

Results—We show that these mice are relatively resistant to dexamethasone-induced hypertension. After one week of dexamethasone treatment, control animals have a mean blood pressure increase of 13.1 mm Hg while knockout animals have only a 2.7 mm Hg increase ($p<0.001$). Interestingly, the knockout mice have slightly elevated baseline BP compared to the controls (112.2 ± 2.5 mm Hg vs. 104.6 ± 1.2 mm Hg, $p = 0.04$), a finding which is not entirely explained by our data. Furthermore, we demonstrate that the knockout resistance arterioles have a decreased contractile response to dexamethasone with only 6.6% contraction in knockout vessels compared to 13.4% contraction in control vessels ($p=0.034$). Finally, we show that in contrast to control animals, the knockout animals are able to recover a significant portion of their normal circadian blood pressure rhythm suggesting that the vascular endothelial glucocorticoid receptor may function as a peripheral circadian clock.

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Conflicts of interest: none

Conclusions—Our study highlights the importance of the vascular endothelial GR in several fundamental physiologic processes, namely blood pressure homeostasis and circadian rhythm.

Keywords

mouse model; steroids; endothelium; circadian rhythm

INTRODUCTION

Multiple hypotheses have been proposed to explain how glucocorticoids (GCs) may influence blood pressure (BP) through extra-renal tissues, many of which have focused on vascular effects of GCs. There is evidence to confirm the presence of GR in the vascular endothelium [1–3], but little is known about its role here. Many studies have focused on the proposed effects of GCs on superoxide production and subsequent endothelial dysfunction, though these studies have all been performed in cell culture [4–6]. To date, there has not been a reliable way to assess the role of the glucocorticoids in the vascular endothelium *in vivo.*

Similarly, the presence of GR has also been confirmed in vascular smooth muscle cells [7, 8]. Multiple investigations, both *in vitro* and *in vivo*, have aimed to demonstrate potentiation of endogenous vasoconstrictors, notably norepinephrine and angiotensin II, as a plausible mechanism by which steroids induce hypertension [7, 9–11]. While this conclusion has indeed been reported in some studies, a significant subset of these experiments, performed in humans as well as pigs, rats and rabbits, has resulted in contradictory data [12–15], with greater vessel relaxation noted in steroid-treated cohorts.

In addition to the conflicting data that has emerged while trying to investigate the contribution of vascular smooth muscle cells and endothelial cells independently, there is ongoing debate as to how the interactions of these two tissues may be affecting vascular processes that ultimately result in hypertension. For example, in one study, rats treated with deoxycorticosterone acetate (DOCA), a mineralocorticoid, demonstrated reduced endothelium-dependent relaxation of vessels to acetylcholine which was hypothesized to be the primary mechanism underlying the resultant hypertension [16], while in another study rats receiving the same treatment exhibited increased mesenteric reactivity to norepinephrine which was attributed solely to the actions of vascular smooth muscle cells, with any contribution of the endothelium postulated to be purely compensatory [17]. Some groups have speculated that the contributions of the respective tissues are dictated by the blood vessel caliber with rat conductance vessels exposed to DOCA showing hypersensitivity to phenylephrine secondary to loss of endothelium-derived nitric oxide-dependent relaxation and resistance vessels exposed to the same treatment experiencing a reduced sensitivity to phenylephrine through an endothelium-derived non-nitric oxide dependent mechanism that is not present in normotensive animals [18] Thus, despite a huge body of literature and multiple experimental models in several animals species, vascular responses to GCs are still poorly understood.

In an attempt to more precisely address the role of the glucocorticoids in the development of hypertension we have created a mouse model enabling tissue-specific deletion of the glucocorticoid receptor. We haven shown previously that administration of dexamethasone (DEX) in the drinking water induces a rapid and sustained elevation of BP in wild-type mice that is substantially independent of the mineralocorticoid receptor [19]. We have also demonstrated that elimination of the glucocorticoid receptor in vascular smooth muscle attenuates, but does not prevent, the development of dexamethasone-induced hypertension *in vivo* [20], suggesting that the vascular smooth muscle glucocorticoid receptor may be

sufficient but is not necessary for the appearance of this form of hypertension. Thus, our current model integrates the information from our previous work by providing specificity for both the glucocorticoid receptor and the endothelium. In this study, we demonstrate a decisive role of vascular endothelial GR in the development of the acute and chronic phases of GC-mediated hypertension (HTN) in an *in vivo* model.

METHODS

Generation of vascular endothelial GR knock-out mice

WT BL6 mice with floxed GR alleles, designated $GR^{loxP/loxP}$ [20, 21], were mated with WT males (+/+) possessing Tie-1 Cre. By selective breeding, Tie-1 Cre+ $GR^{loxP/loxP}$ homozygotes and Tie-1 Cre− $GR^{loxP/loxP}$ controls were generated. Mouse DNA was isolated from mouse tail clipping by standard methods and analyzed by PCR to determine mouse genotype. The primers for the floxed GR allele were: 5′GGCATGCACATTACTGGCCTTCT 3′ and 5′ CCTTCTCATTCCATGTCAGCATGT 3′. Primers for detection of Cre were: 5′ CCGGGCTGCCACGACCAA 3′ and 5′ GGCGCGGCAACACCATTTTT 3′. All 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

Mouse tissues including aortas, kidneys, livers and brains were dissected out and fixed in 4% paraformaldehyde for 4 hours at room temperature followed by overnight incubation in PBS @ 4° C. Tissues were stained according to the protocol in reference 20.

β-gal staining

Tie-1 Cre+ mice and their Cre− littermates were mated with mice homozygous for the ROSA26 gene such that β-galactosidase was expressed only upon Cre-mediated recombination as has been previously described [22].

Telemetric measurement of blood pressure and heart rate

Male Tie-1 Cre+ and Cre− littermate controls were implanted with radiotelemetric blood pressure catheters as described [23], 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. Measurement of the following 6 parameters occurred every one minute using the DataQuest System (Datasciences, St. Paul, MN): systolic BP, diastolic BP, mean BP, pulse pressure, heart rate and activity level. Measurements were averaged over 4-hour time periods using an Excel program developed specifically for this purpose. 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. Based upon an average water intake of 3–5 ml/day and an average weight of 30 g/mouse, the ingested dose of DEX for each mouse was approximately 1.5–2.5 mg/kg/day.

Mouse urine collection

Tie-1 Cre+ 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 acclimate to their new cages for 24 hours. On day 2, a 16-hour urine collection was started at 5 PM, shortly before the beginning of the dark cycle. A thin film (50 μl) of mineral oil was

used to coat the collection tube and prevent urine evaporation. On day 3, another 16-hour urine collection was started and mice were simultaneously treated with DEX 15 mg/L in the drinking water. Cages were cleaned thoroughly in between each urine collection. 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.

Measurement of plasma nitrate and nitrite

Mice were fasted overnight to minimize the effect of dietary nitrates. Measurement was as described previously [24].

Two-photon Laser Scanning Microscopy and 4D image analysis

Male mice, 8–12 weeks old, were anesthetized, shaved with a hair clipper and depilated. The resistance arterioles supplying the intestinal arcades were exposed by exteriorizing a loop of intestine through a ventral mid-line incision. The mesentery was kept moist with warm saline throughout the procedure and inhaled isoflurane anesthesia was used throughout the experiment. Prior to imaging each mouse was injected with 100 μl rhodamine-conjugated dextran via the retro-orbital plexus. Imaging was conducted before and after administration of dexamethasone, 2 mg/kg retro-orbitally. In other experiments phenylephrine 100 μg/kg was administered and the same imaging sequence was conducted. Each vessel acted as its own control. An Olympus BX61WI fluorescence microscope in combination with a 20×, 0.95NA Olympus objective and LaVision Biotec multiphoton microscopy system was used for imaging of mesenteric arteries. An auto-tunable Titanium-Sapphire two-photon laser (Chameleon Vision II, Coherent) pumped by a Verdi laser source was used for the excitation light source. Emitted light was collected with non-descanned detectors outfitted with the following bandpass filters: 435/90 nm, 525/50 nm and 615/100 nm. Stacks of 201 optical sections with 1 μm z-spacing were acquired before and 1hr and 15min after treatment with the laser set at a wavelength of 850 nm. Stacks of 11 to 13 optical sections with 5 μm z-spacing were acquired every 30s after treatment with the laser set at a wavelength of 850 nm. The field of view for each x–y plane was 500 × 500 mm, at a resolution of 0.977 mm per pixel. Volocity software was used to create volume renderings, sequences of time resolved images and obtain measurements of vessel diameters over time.

Reagents

Dexamethasone phosphate, donkey serum, PBS, paraformaldehyde and sodium nitroprusside were obtained from Sigma Chemicals (St. Louis, MO). Triton-X, acetone and alcohol were obtained from American Bioanalytical. The antibody to GR was obtained from Affinity Bioreagents (Golden, CO). Anti-CD31 antibody was courtesy of Dr. Joseph Madri (New Haven, CT). A stock solution of dexamethasone was dissolved in distilled water and prepared fresh prior to each set of experiments.

Statistical analysis

Data represent means ± SEM. Statistical differences were determined by 2-way and 2-way repeated measures ANOVA. Post-hoc comparisons were made by Bonferroni's test. A p value of < 0.05 was considered significant.

RESULTS

Effective deletion of GR from the vascular endothelium

We generated vascular endothelial GR knockout (KO) mice via the use of Tie-1 Cre which has been estimated to provide approximately 80 % excisional efficiency [25]. Mice

homozygous for the $GR^{loxP/loxP}$ allele were mated with mice homozygous for $GR^{loxP/loxP}$ allele and heterozygous for Tie-1 Cre. Cre+ mice and Cre− littermate controls were born in the expected 1:1 ratio. Mean weights of male (24.9 ± 1.4 g, n=5) and female (23.0 ± 1.1 g, n=9) Tie-1 Cre+ mice did not differ from those of Cre− male (26.0 ± 0.8 g, n=6) and female (23.4 ± 1.8 g, n=7) mice at 10–12 weeks of age.

We stained sections of mouse aorta, kidney, liver and brain from Tie-1 Cre+ mice and Cre− controls with an antibody to GR (Figure 1). Control animals demonstrated robust staining of the endothelium by GR while Tie-1 Cre+ mice showed minimal, if any, endothelial staining. To confirm that excision of GR staining was specific to the vascular endothelial layer, we also counterstained these tissues with an antibody to the endothelium-specific protein, CD31 [26, 27]. As shown in Figure 1, there is clear colocalization of GR with CD31 in the control animal, but this colocalization is almost entirely lost in the Tie-1 Cre+ mice. Importantly, expression of GR in non-endothelial structures was preserved in the liver and brain of Tie-1 Cre+ mice.

We verified the specificity of our Cre staining by crossing Tie-1 Cre+ and Cre− mice with a Rosa26 reporter mouse and staining tissues of interest with X-gal to verify β-galactosidase expression in the distribution of the endothelium in the Cre+ animals. β-galactosidase is not observed in the Cre− animals, while the Cre+ animals show intense β-galactosidase staining in an endothelial distribution in the aorta and carotid arteries as well as the saphenous arteries, which are resistance vessels. Circumferential staining is evident as well as *en face* staining in the larger vessels (Figure 2A). We also performed molecular analysis by qPCR of the smooth muscle and adventitial layers of mouse aortas from each genotype to further demonstrate the specificity of GR excision. Aortas for Cre− (n=3) and Tie-1 Cre+ (n=6) mice were dissected out and digested with a solution of collagenase and elastase to allow mechanical separation of the two layers. After RNA extraction and rt-PCR the resulting cDNA was used in qPCR to evaluate for the expression of GR, Cre recombinase and α-SMA mRNA in each layer (Figure 2B–E). There was no difference in the expression of GR in Cre+ animals in either the media or the adventitia compared to controls, though some variability was noted. The level of expression in the media was roughly 1–2× higher than that in the adventitia. The expression of Cre recombinase was undetectable in the media of both the Cre− and Cre+ aortas and in the adventitia of the Cre− aortas. There was very low level expression of Cre recombinase (>cycle 34) in the adventitia of some of the KO aortas which may represent expression in the endothelial vasa vasorum. The expression of α-SMA was similar in the medial and adventitial layers of both groups.

Higher baseline BP in Tie-1 Cre+ mice compared to controls

To determine if there was a blood pressure phenotype in these animals, 8–12 week old male Tie-1 Cre+ mice (n=7) and gender-matched littermate controls (n=5) were implanted with radiotelemetric catheters and allowed to recover for one week. Adequacy of recovery was verified by normal diurnal variation of BP for at least three days [28].

Baseline blood pressure in Tie-1 Cre+ mice was significantly higher than in Cre− control mice (112.2 ± 2.5 mm Hg vs. 104.6 ± 1.2 mm Hg respectively, $p = 0.04$). The significant difference in mean baseline BP between the two groups suggests that vascular endothelial GR may be involved in BP homeostasis. As Cre in our model is active during the embryonic period [25], we cannot rule out a developmental effect of GR on vascular or renal biology that serves to alter baseline BP. However, gross inspection and histologic analysis of kidneys from Cre− control and Tie-1 Cre+ did not show any differences between the two groups (Figure S1). To determine whether alterations in the renin-angiotensin-aldosterone system might be playing a role in the observed hypertension, we assessed aldosterone levels in these mice. However, they did not differ significantly between the groups. Tie-1 Cre+

mice had a mean aldosterone level of 436.1 ± 77.3 pg/ml while controls had a mean aldosterone level of 488.6 ± 100.3 pg/ml (p=0.68, n=10/group).

Glucocorticoids have been implicated in the circadian control of blood pressure. Therefore, we assessed circadian BP in these mice. We found that both Tie-1 Cre+ mice and their controls have normal circadian BP variation, though, as expected in the nocturnal mouse, the dip in BP occurs during the daytime. At baseline both groups demonstrate a normal circadian variation of blood pressure with mean nighttime BP higher than mean daytime BP by 17.6 mm Hg in the Cre+ animals and 18.2 mm Hg in the Cre− animals, indicating that vascular endothelial GR does not contribute to circadian BP variation at baseline.

Attenuated BP response to DEX in Tie-1 Cre+ GR knockout mice

To determine the effect of the Tie-1 Cre+ GR knockout in a model of GC-induced HTN, we administered DEX 15 mg/L in the drinking water and monitored BP continuously for 1 week. As we have noted previously [19], DEX induced a consistent and highly significant rise in BP within hours of the initiation of DEX therapy in Cre− mice. In contrast, the BP rise in Tie-1 Cre+ littermates was markedly attenuated (Figure 3). For example, on day 2 of DEX treatment, MAP increased by 9.7 ± 0.8 mm Hg in the control mice, but only 3.7 ± 1.1 mm Hg in the Cre+ animals (p = 0.004). Further analysis shows that, overall, the control mice become quite hypertensive after a week of DEX with a starting BP of 104.6 ± 1.2 mm Hg and an ending BP of 117.7 ± 2.1 mm Hg (p=0.0008). In contrast the starting and ending BPs in the Cre+ animals were not significantly different from each other (112.2 ± 2.5 mm Hg vs. 114.9 ± 2.8 mm Hg, respectively, p = 0.48). While the blood pressure of the Cre+ animals was lower than that of the Cre− animals after one week, the difference was not statistically significant (p=0.31).

In addition to their blunted hypertensive response to DEX, Tie-1 Cre+ mice also exhibited an alteration of the circadian blood pressure response to DEX when compared to Cre− controls (Table 1). Initially, both groups had a similar BP response to DEX; after only 1 day of treatment, circadian BP variation markedly decreased in each group with a nighttime/daytime difference of 2.64 ± 2.84 mm Hg vs. 2.70 ± 2.24 mm Hg in the Tie-1 Cre+ animals and Cre− controls, respectively (p = 0.99). In contrast to the Cre− controls, however, the Tie-1 Cre+ mice demonstrated partial recovery of their circadian rhythm on subsequent days. On days 2–7 of steroid treatment, Cre+ mice regained a highly significant portion of their normal circadian rhythm (p<0.001). Overall, Cre+ mice regained over 50% of their normal circadian variability whereas control mice could regain only about 20% (p<0.001). These data suggest that GCs may act, at least in part, through vascular endothelial GR to disrupt normal circadian blood pressure variation.

Similar heart rate in Tie1 Cre+ and Cre− mice

At baseline, controls and Tie1 Cre+ mice had similar mean heart rates of 560 ± 15 beats per minute (bpm) and 574 ± 10 bpm respectively (p = 0.82). As we have observed previously [20], DEX administration induces roughly a 10–12% drop in heart rate over the first 24–48 hours of administration followed by a partial recovery of approximately 5% on day 3 of DEX administration [20]. This drop in heart rate was blunted in Tie-1 Cre+ mice, which experienced only a 7.5% drop in heart rate by day 2 (p=0.04, Figure S2). The attenuated heart rate decline in Tie-1 Cre+ mice may reflect the decreased hypertensive response, thus obviating the need for a relative reflex bradycardia.

Absent DEX-induced natriuresis in Tie-1 Cre+ mice

Despite, or perhaps owing to their acute hypertensive effects, GC's induce a rapid natriuresis in rodents [29–31]. Consistent with this observation, we found our Cre− mice (n=6)

markedly increased urine sodium excretion during the first 16 hours of treatment with DEX (Table 2). During this time period, sodium excretion increased from 0.42 ± 0.06 mmol/mg creatinine to 0.74 ± 0.06 mmol/mg creatinine (p = 0.004). In contrast, there was no significant change in urine sodium excretion in Tie-1 Cre + mice (n=7), going from 0.37 ± 0.07 mmol/mg creatinine before DEX to 0.33 ± 0.07 mmol/mg creatinine after DEX (p=0.75, Table 2). The finding of increased sodium excretion in the Cre− mice, but not in the Tie-1 Cre+ mice in this time period was mirrored by the changes in chloride excretion. Sixteen-hour chloride excretion rose from 0.75 ± 0.06 mmol/mg creatinine to 1.05 ± 0.09 mmol/mg creatinine in the Cre− animals (p = 0.017), but fell from 0.65 ± 0.16 mmol/mg creatinine to 0.51 ± 0.12 mmol/mg creatinine in the Tie1 Cre + mice (p = 0.55).

No difference in aortic morphology in Tie-1 Cre+ $GR^{loxP/loxP}$ mice

Given that Tie-1 Cre+ mice have higher baseline BP then controls, we hypothesized that they might develop chronic vascular changes such as LVH or medial hypertrophy over time. To investigate this possibility we evaluated heart weight, aortic histology, aortic medial thickness and aortic medial area in 10–11 month old male Tie-1 Cre+ and Cre− mice (Figure S3). Cre− animals (n=7) had a heart: body weight ratio of 0.54 ± 0.03% while Tie-1 Cre+ animals (n=6) had a ratio of 0.57 ± 0.02% (p=0.27). Aortic media thickness measured 25.5 ± 1.2 μm in Cre− mice and 27.9 ± 2.8 μm in Tie-1 Cre+ mice (p=0.45) and aortic media area was 26657 ± 1931 μm^2 in Cre− animals and 37435 ± 5502 μm^2 in Tie-1 Cre+ animals (p=0.11). We also assessed the aortas for fibrosis using Trichrome Masson staining. However, quantification of the mean intensities of each of the 3 color channels by histogram revealed no differences between the 2 groups (control 193.8 ± 3.2 units vs. Tie-1 Cre+ 194.5 ± 1.2 units, p=0.85).

Suppression of nitric oxide levels is similar in Tie-1 Cre+ and Cre− mice

It has been observed in both *in vitro* and *in vivo* systems that administration of glucocorticoids results acutely in overproduction of reactive oxygen species and decreased levels of nitric oxide [32, 33]. To investigate whether decreased nitric oxide levels were playing a role in the BP phenotype we observed in our model we assessed nitric oxide levels in Cre− and Cre+ mice at baseline, after 16 hours of dexamethasone treatment and after 1 week of dexamethasone treatment (Figure 4). At baseline we found no difference between the nitric oxide levels at steady-state with Cre− mice having a level of 708 ± 87 nM and Cre + mice having a level of 659 ± 108 nM (p=0.73). After 16 hours of DEX the nitric oxide level of the Cre− group declined to 399 ± 101 nM (p<0.01) and the level in the Cre+ group also declined similarly (397 ± 102 nM, p<0.01). After a full week of dexamethasone treatment, the levels in the Cre− mice sank further to 140 ± 44 nM (p<0.0001) and levels in the Cre+ mice also fell (144 ± 68 nM, p<0.0001). Measurement of nitric oxide levels in an eNOS KO mouse was included as a control and was 185 nM at baseline.

Tie-1 Cre+ mice show decreased vessel reactivity in response to dexamethasone but not in response to phenylephrine

To further investigate the nature of the decreased hypertensive response of the Tie-1 Cre+ animals, we assessed the contractile response of resistance arterioles *in vivo* in response to DEX. Resistance arterioles from the intestinal mesentery from male Tie-1 Cre+ mice (n=6) and littermate Cre− controls (n=5), age 8–12 weeks, were imaged in real-time using intravital microscopy (Figure 5). In one group of experiments vessels were imaged before and 1 hour after intravenous administration of 2 mg/kg dexamethasone. This dose was similar to that used in the blood pressure measurements we performed. The diameter of control vessels contracted by 13.4 ± 2.6% 1 hour after dexamethasone while that of Tie-1 Cre+ vessels contracted by only 6.6 ± 0.5% (p=0.034, Figure 5) As a positive control, a separate cohort of Cre− (n=4) and Tie-1 Cre+ mice (n=4) were treated with phenylephrine

100 μg/kg, a dose that was found to result in hypertension *in vivo* [34]. In this case, the diameter of Cre− vessels contracted by 13.8 ± 2.8% 1 hour after phenylephrine administration while that of Tie-1 Cre+ vessels contracted similarly by 15.5 ± 2.6% (p=0.25). Prior to drug administration there were no differences in vessel diameter. This result suggests that decreased reactivity of Tie-1 Cre+ vessels to dexamethasone may be responsible for the blood pressure phenotype we observed, and furthermore, that this alteration in reactivity is likely specific to the GR ligand dexamethasone as we did not observe any difference with phenylephrine.

Vessel reactivity was also assessed by myograph studies in isolated saphenous artery rings from control and Tie-1 Cre+ pre-treated with 15 mg/L dexamethasone in their drinking water. However, in these *ex vivo* experiments no differences in vessel reactivity were seen between the two groups either at baseline or after dexamethasone pre-treatment in response to several vasoactive substances including phenylephrine (Figure 6).

DISCUSSION

Despite concerted effort, the molecular etiology of GC-induced hypertension remains unclear. To better characterize the *in vivo* role of GCs acting on the vascular endothelium, we have used advances in mouse genetics to create a mouse model in which GR has been specifically removed from the vascular endothelium. Our characterization of this model shows that growth, development and body weight are similar to controls. Interestingly while knockout mice have normal diurnal BP variation at baseline, they have higher baseline BP than Cre− control mice. These data suggest that GR in the vascular endothelium may play an important role in basal regulation of BP. While vascular anatomy and histology of the knockout mice are not grossly altered when compared to controls, we cannot rule out a significant developmental effect of the endothelial GR knockout.

The Tie-1 Cre+ mice exhibit neither an acute hypertensive response within the first 48 hours of DEX therapy nor a sustained hypertensive response to DEX after 1 week of treatment. In fact, their terminal BP is essentially unchanged from baseline. This observation reinforces the notion that the HTN in this model system is mediated by GR and not through MR. However, these findings must be viewed with caution, as the terminal BP of DEX-treated control mice did not differ significantly from that of the Tie-1 Cre+ mice. Definitive statements regarding the role of endothelial GR are difficult to make; nevertheless, our observations raise interesting questions about the molecular pathogenesis of GC-induced HTN.

Our knockout animals clearly demonstrated reduced vascular reactivity to dexamethasone at the level of the resistance vessels using an *in vivo* preparation, but not using an *ex vivo* preparation. There was no difference between control and knockout animals in response to phenylephrine in either the *in vivo* or *ex vivo* experiments. These results suggest that loss of GR in the endothelium has a specific effect on vascular reactivity to the GR ligand dexamethasone. Our inability to detect this effect in isolated vascular rings raises the possibility that this effect may be mediated by autocrine or paracrine input of surrounding tissues. In this regard, a variety of previous studies have suggested that glucocorticoids may act as mediators of local autocrine and paracrine effects [35–37], and, more recently, the concept of arterial hypertension as a result of paracrine dysfunction of local cell communities has emerged [38]. Elucidating the role of these autocrine and/or paracrine effects will require further study, but our findings do underscore the wide-ranging physiologic effects of glucocorticoids.

Our study is also intriguing with regard to its findings regarding diurnal variation of BP. Multiple lines of evidence exist which support an important role of GCs in circadian BP rhythms. Humans exhibit diurnal BP variation, with BP peaking soon after the morning cortisol surge, while BP peaks in mice and other nocturnal animals soon after the evening GC surge at the time of their awakening. Patients with disordered cortisol secretion, such as those with Cushing's syndrome, commonly lose their diurnal BP variation [39]. Studies have previously shown that the suprachiasmatic nucleus is the site of the central molecular clock in mammals [40]. More recent work has also shown that other tissues including the aorta, kidney and heart, contain peripheral clocks, which may affect cardiovascular function [41]. Although the signaling pathways that link the central and peripheral clocks are not well understood, it has been suggested that circulating hormones likely play a role [41]. Based upon the differences in circadian BP rhythm we observe in our model, we hypothesize that the vascular endothelium could be the site of one of these peripheral clocks with GCs, endogenous or exogenous, acting as a signal. Additional investigation will be necessary to determine the precise mechanism by which vascular endothelial GR may be involved in determining circadian rhythms.

In conclusion, our study demonstrates that vascular endothelial GR plays a critical role in mediating both the acute and chronic blood pressure response to steroids likely through effects on vascular reactivity and suggests that it may play a role in signaling mechanisms related to circadian BP variation. In addition our data suggest that GCs may have complex effects on vascular resistance and cardiovascular status, which are mediated through the endothelial GR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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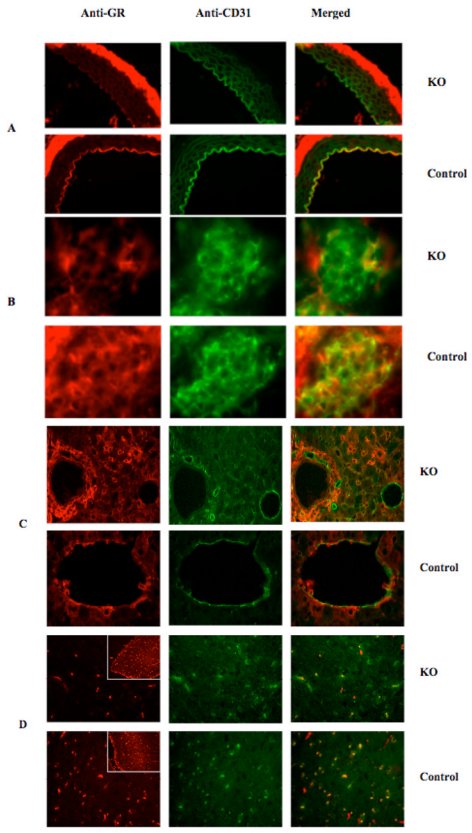

Figure 1.

Representative sections from Tie-1 Cre+mice and Cre− littermate controls. Sections of (A) mouse aorta (4 μm), (B) kidney/glomerulus, (C) liver and (D) brain are stained with both antibody to GR and antibody to CD31, which is endothelium specific. Tie-1 Cre+ mice demonstrate virtually no co-localization of GR in the vascular endothelium while controls show robust co-localization. Sections shown at 40× magnification except (D) inset which is 10×.

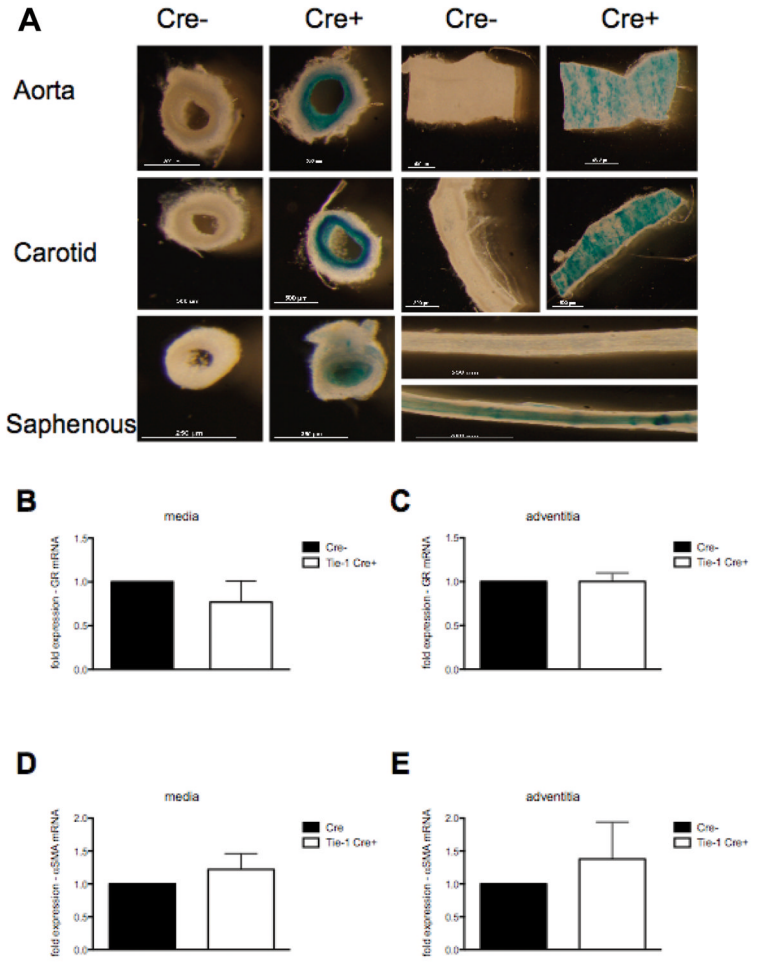

Figure 2.

(A) Beta-galactosidase staining is evident in the endothelial layer of Tie-1 Cre+ but not Cre− animals. Tie-1 Cre+ and Cre− animals were crossed with a Rosa26 reporter mouse and various tissues of the progeny were stained with X-gal. Cre+ animals show intense beta-galactosidase staining in the distribution of the endothelium as noted in both circumferential and en face preparations. All scale bars are 500 μm except circumferential saphenous artery, which is 250μm. (B–E) Molecular analysis of the medial and adventitial layers of control Cre− mouse aortas (n=3) and Tie-1 Cre+ mouse aortas (n=6). There were no significant differences noted in the expression of GR mRNA in the aortic media (B), or aortic adventitia (C), or in the α-SMA mRNA expression in the aortic media (D) or aortic adventitia (E).

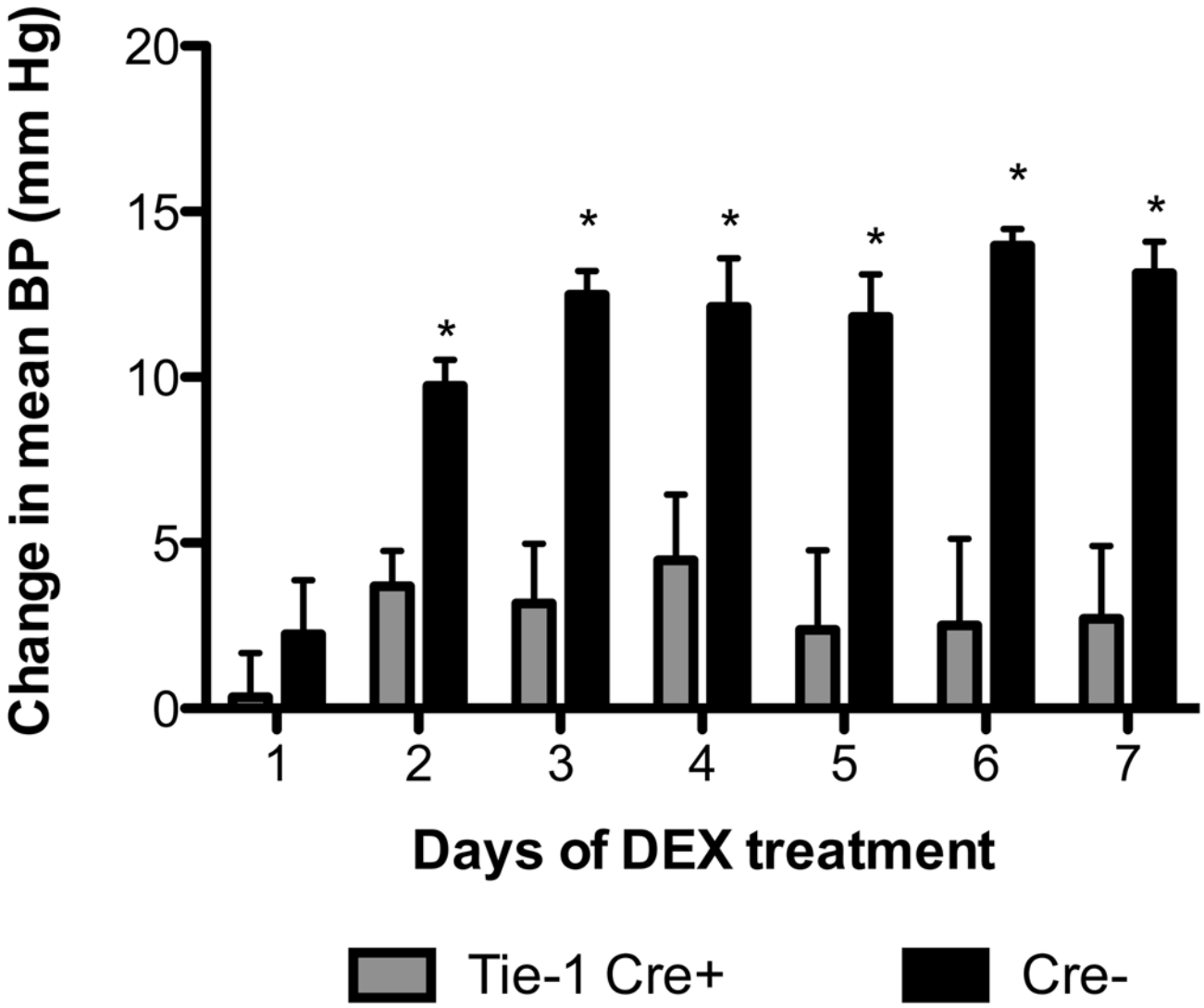

Figure 3.

Absence of DEX-induced HTN in Tie Cre+ mice. Animals were treated with DEX 15 mg/L in their drinking water. After 1 day of treatment with DEX, Tie-1 Cre+ mice has a mean BP increase of 0.34 ± 1.34 mm Hg while Cre− controls had a mean BP increase of 2.24 ± 1.64 mm Hg ($p=0.41$) After 1 week of treatment with DEX, Tie-1 Cre+ mice had a mean BP increase of 2.73 ± 2.20 mm Hg, while Cre− controls had a mean BP increase of 13.14 ± 0.95 mm Hg ($p=0.0008$). Controls had a statistically significant increase in mean BP for days 2–7 of DEX treatment. Values are means ± SE.

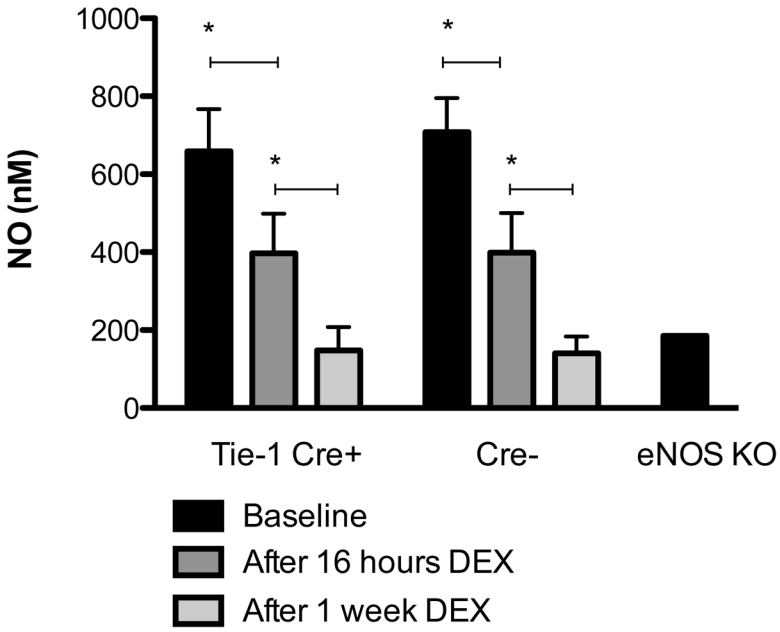

Figure 4.

Whole blood nitric oxide measurement. Baseline whole blood samples were taken from Tie-1 Cre+ mice and Cre− controls (n=5/group) after an overnight fast. Tie-1 Cre+ mice had a mean nitric oxide level of 659 ± 108 nM while Cre− controls had a level of 708 ± 87 nM (p=0.73). After 16 hours of DEX treatment nitric oxide levels were repeated and showed that mean Tie-1 Cre+ nitric oxide levels had decreased to 397 ± 102 nM while the Cre− mice showed a similar level of 399 ± 101 nM. After 1 week of DEX treatment, controls (n=7) showed a further suppression to 140 ± 44 nM and Tie-1 Cre+ animals (n=7) showed a similar suppression. Measurement of an eNOS KO mouse was included as a control. Values are means ± SEM.

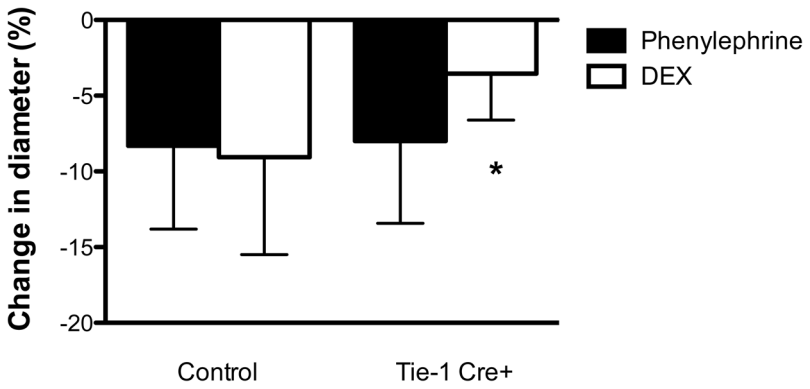

Figure 5.

Tie-1 Cre+ mice have decreased reactivity to dexamethasone compared to controls. Tie-1 Cre+ animals (n=6) and Cre− controls (n=5) were treated with 2 mg/kg dexamethasone IV. Resistance arterioles from the mesentery were imaged in real-time. After 1 hour the diameter of Tie-1 Cre+ vessels decreased by only 6.6 ± 0.5% while the diameter of Cre− control vessels decreased by 13.4 ± 2.6% (p=0.019). Reactivity to phenylephrine, 100 μg/kg, was assessed as a positive control. Values are means ± SE.

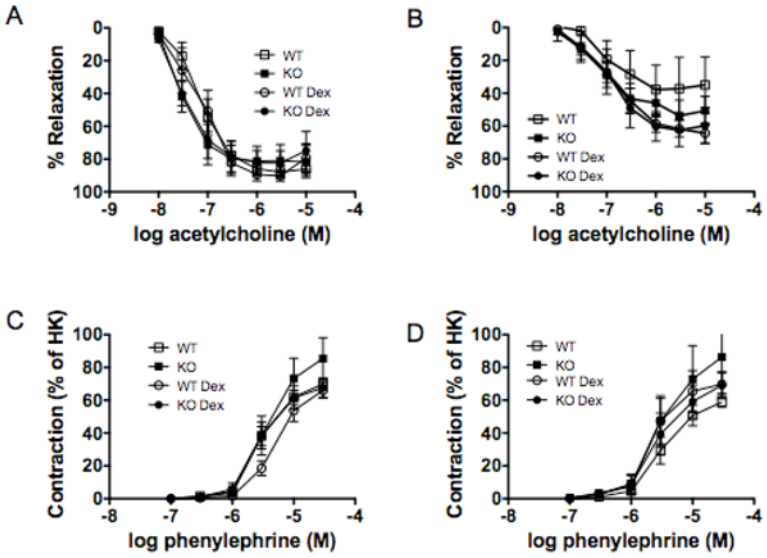

Figure 6.

No differences in ex vivo vessel reactivity. Control and Tie-1 Cre+ saphenous arteries (n=4/group) both at baseline and after pre-treatment of mice with 15 mg/L dexamethasone were mounted in a myograph. Dose response curves were constructed for (A) acetylcholine, (B) acetylcholine with L-NAME, (C) phenylephrine and (D) phenylephrine with L-NAME. No significant differences were observed between the two groups.

Table 1

Loss of GR in the vascular endothelium partially restores normal circadian blood pressure variation. Average difference in mean BP in mm Hg between 8 PM-midnight and 8 AM-noon for Tie-1 Cre+ (n=7) and control (n=5) mice at baseline and for each day of DEX treatment. Values are means ± SE.

	Tie-1 Cre+	Cre− controls	P value
Baseline	17.61 ± 1.49	18.17 ± 2.46	0.84
Day 1	2.64 ± 2.84	2.70 ± 2.24	0.99
Day 2	6.75 ± 2.01	3.84 ± 0.82	0.32
Day 3	10.94 ± 1.13	2.65 ± 2.86	**0.01**
Day 4	12.28 ± 1.96	7.57 ± 1.5	0.14
Day 5	10.37 ± 1.55	2.89 ± 3.44	**0.04**
Day 6	8.01 ± 1.2	2.81 ± 3.27	0.10
Day 7	8.20 ± 1.9	2.33 ± 3.21	0.12

Table 2

Urinary electrolyte excretion and urinary volume at baseline and acutely following DEX in Tie-1 Cre+ mice (n=7) and Cre− controls (n=6). Urine electrolytes are expressed as mg/mmol creatinine. Values are means ± SE.

	Baseline	After DEX	P value
Control Na^+	0.42 ± 0.06	0.74 ± 0.06	**0.004**
Control Cl^-	0.75 ± 0.06	1.05 ± 0.09	**0.017**
Control K^+	0.71 ± 0.07	0.90 ± 0.12	0.21
Urine Volume	681 μl	606 μl	0.78
Tie-1 Cre+ Na^+	0.37 ± 0.07	0.33 ± 0.07	0.75
Tie-1 Cre+ Cl^-	0.65 ± 0.16	0.51 ± 0.12	0.55
Tie-1 Cre+ K^+	0.77 ± 0.15	0.64 ± 0.12	0.54
Urine Volume	503 μl	660 μl	0.47