

GTP Cyclohydrolase 1 Downregulation Contributes to Glucocorticoid Hypertension in Rats

Brett M. Mitchell, Anne M. Dorrance, R. Clinton Webb

Abstract—NO, a potent vasodilator, has been implicated in the pathogenesis of glucocorticoid hypertension. NO synthase requires the cofactor tetrahydrobiopterin for the production of NO. Guanosine-triphosphate (GTP) cyclohydrolase 1 is the rate-limiting enzyme for the production of tetrahydrobiopterin, and in the presence of low levels of tetrahydrobiopterin, NO production is decreased. We have previously shown that tetrahydrobiopterin-dependent vasodilation is impaired in rats with glucocorticoid hypertension. However, the role GTP cyclohydrolase 1 plays in the pathogenesis of glucocorticoid hypertension has not been investigated. Therefore, we tested the hypothesis that downregulation of GTP cyclohydrolase 1 contributes to the development and maintenance of glucocorticoid hypertension in rats. Rats were implanted with dexamethasone ($0.79 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) or sham-operated, and systolic blood pressures were measured at baseline and after 12 hours, 4 days, or 15 days. Blood pressure increased significantly after dexamethasone treatment. Isometric force generation was measured in endothelium-intact aortic ring segments. Aortas from dexamethasone-treated rats exhibited a significant time-dependent decrease in maximal relaxation to acetylcholine compared with control rats. Incubation with sepiapterin (10^{-4} mol/L , 1 hour), which produces tetrahydrobiopterin via a salvage pathway, restored vasodilation to acetylcholine in aortas from 4- and 15-day dexamethasone-treated rats. GTP cyclohydrolase 1 mRNA expression levels also significantly decreased in a time-dependent manner. These results support the hypothesis that downregulation of GTP cyclohydrolase 1 contributes to increased blood pressure in glucocorticoid hypertensive rats. (*Hypertension*. 2003;41[part 2]:669-674.)

Key Words: glucocorticoids ■ endothelial nitric oxide synthase ■ endothelium ■ hypertension, experimental

Glucocorticoids are among the most widely prescribed drugs by physicians, and excess glucocorticoids can elevate blood pressure in humans (Cushing's syndrome) and animals.¹⁻³ Previous studies have shown that glucocorticoid hypertension is associated with an increased pressor response to angiotensin II and norepinephrine, and reduced production of vasodilators.^{3,4} In addition, endothelium-dependent vasodilation has been shown to be reduced in blood vessels from humans and animals with glucocorticoid hypertension.^{1,4,5} A reduction in the bioavailability of NO, a potent vasodilator, has been implicated in glucocorticoid-induced hypertension, and glucocorticoids affect many proteins involved in NO-mediated vasodilation.⁶

NO plays a key role in vascular tone, and decreases in NO production and bioavailability have been shown to reduce endothelium-dependent dilation and increase blood pressure. NO can be produced by the conversion of arginine and oxygen to citrulline via 3 isoforms of NO synthase (NOS). Endothelial NOS (eNOS) requires the cofactor tetrahydrobiopterin (BH4), which aids in the stabilization of the eNOS dimer and increases the affinity of eNOS for arginine.⁷ BH4 is produced via a de novo pathway from guanosine-triphosphate (GTP) by GTP cyclohydrolase I (GTPCH1), the

rate-limiting enzyme. BH4 can also be produced via a salvage pathway in which sepiapterin is converted to the intermediate dihydrobiopterin by sepiapterin reductase and then to BH4.⁸ GTPCH1 activity can be regulated by end-product inhibition via the GTP cyclohydrolase feedback regulatory protein (GFRP).⁹

In the presence of low levels of BH4, eNOS can become uncoupled and generate increased superoxide anions and decreased NO, both of which contribute to endothelial dysfunction and increased blood pressure.¹⁰⁻¹² Exogenous BH4 has been shown to restore endothelial function in humans with coronary artery disease¹³ and to suppress the development of elevated blood pressure when given to the spontaneously hypertensive rat, a genetic form of hypertension.¹⁴ Taken together, these observations suggest that BH4 levels are decreased and/or BH4 biosynthesis is altered in these disease conditions.

We have previously shown that rats with glucocorticoid hypertension (dexamethasone, $0.79 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ for 20 days) have reduced BH4-dependent vasodilation, and vessels incubated with dexamethasone have decreased GTPCH1 mRNA expression levels.⁵ With respect to NO, Wallerath et al¹⁵ found that eNOS mRNA expression was downregulated

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in aortas from rats treated with dexamethasone in the drinking water ($0.3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$). Expression of eNOS mRNA was decreased to 60% to 70% of control values within 3 days and remained at this level over 9 days. In addition, systolic blood pressure was increased to ≈ 140 mm Hg, and endothelium-dependent dilation to acetylcholine was decreased. Because superoxide anions can be produced by eNOS in the presence of low levels of BH₄, it is possible that the downregulation of GTPCH1 by glucocorticoids may precede eNOS downregulation, thus causing increased oxidative stress, which contributes to the decreased NO bioavailability and elevated blood pressure. Therefore, the purpose of the present study was to examine the time-course of the downregulation of GTPCH1 and its role in the pathogenesis of glucocorticoid hypertension in rats. Experiments were performed 12 hours, 4 days, or 15 days after beginning dexamethasone treatment to examine the role of GTPCH1 in the onset, development, and maintenance of hypertension, respectively. We hypothesized that a time-dependent downregulation of GTPCH1 would decrease endothelium-dependent dilation and contribute to the increased blood pressure in rats made hypertensive with synthetic glucocorticoids.

Methods

Animals and Blood Pressure Measurements

Male Sprague-Dawley rats (obtained from Harlan, Indianapolis, Ind; 300 to 324 g) were used, and all procedures were approved by the Medical College of Georgia's Animal Use for Research and Education Committee. All rats were maintained on a 12-hour/12-hour light/dark cycle and had access to water and standard rat chow ad libitum throughout the study. Systolic blood pressure was measured by tail-cuff procedure (pneumatic transducer). Blood pressure measurements were taken at the same time each day.

After baseline blood pressure measurements, rats were anesthetized with ketamine-xylazine cocktail (10 mg/kg IM) and were subcutaneously implanted with a pellet (Innovative Research) containing dexamethasone (5 mg pellet, $0.79 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) or were sham-operated (controls). Rats were killed 12 hours (12hr-DEX), 4 days (4d-DEX), or 15 days (15d-DEX) after dexamethasone implantation to examine the onset, develop, and maintenance of hypertension, respectively.

Organ Chamber Experiments

On the day of experiments, rats were anesthetized with sodium pentobarbital (50 mg/kg IP). The thoracic aorta was excised and immediately placed in cold physiological salt solution (PSS; composition in mmol/L: NaCl 130.0, KCl 4.7, KH₂PO₄ 1.18, MgSO₄·7H₂O 1.17, NaHCO₃ 14.9, dextrose 5.5, EDTA 0.26, CaCl₂ 1.6). The isolated endothelium-intact aortic segment was cleaned of connective tissue and cut into rings (3 to 4 mm). The aortic rings were then connected to an isometric force transducer in a 50-mL organ chamber filled with 37°C PSS and bubbled with 95% O₂/5% CO₂. Aortic rings from dexamethasone-treated and control rats were studied in parallel. All experiments were performed in the presence of indomethacin (10^{-5} mol/L) to inhibit cyclooxygenase. Vessels were set at a passive force of 3.5 to 4.0 g, and isometric force generation was recorded continuously. After a 60-minute equilibration period, all vessels were contracted with phenylephrine (PE, 10^{-7} mol/L) to test viability. Acetylcholine (ACH, 10^{-6} mol/L) was administered to test the functional integrity of endothelium as measured by relaxation. Concentration-response curves were obtained in a half-log, cumulative fashion. Responses to ACH and sodium nitroprusside (SNP) were generated after preconstriction to PE (10^{-7} mol/L). Incubation time was 60 minutes for indomethacin and sepiapterin, and 20 minutes for N^o-nitro-L-arginine (L-NNA).

Relaxation responses to ACH and SNP were expressed as percentage of relaxation from submaximal PE-induced constriction (10^{-7} mol/L). To determine EC₅₀ values for relaxation responses to SNP after NOS inhibition, data were expressed as a percentage of maximal relaxation. Regression analysis using 3 data points along the linear section of the concentration-response curve was used to generate an equation from which the EC₅₀ value was determined. These values were then averaged, and the geometric mean was compared between groups.

Reverse Transcriptase-Polymerase Chain Reaction

Thoracic aorta was removed and cleaned free of adherent fat, connective tissue, and blood before being snap frozen. RNA was extracted by using TRIZOL reagent following the manufacturers protocol (GIBCO). The RNA was quantified by spectrophotometry, and 1 μg of RNA was used to produce cDNA. Contaminating cDNA was removed by using DNase enzyme before reverse transcription with AMV reverse transcriptase using oligo dT as a primer. Occasional RNA samples were subjected to the polymerase chain reaction (PCR) procedure without prior reverse transcription to control for the presence of contaminating genomic DNA in the sample. PCR amplifications were performed on a portion of the cDNA produced. Each PCR reaction contained 5 pmol/L of each oligonucleotide primer, 200 $\mu\text{mol/L}$ dNTP, and 0.2 U TAQ in the manufacturer's buffer. Optimum annealing temperature was assessed using a gradient block thermal cycler. Cycle number and template dilution factor were determined for each amplicon before experimentation to ensure linearity. The cDNA produced was resolved on a 2% agarose gel, and the amount of DNA present was identified by using ethidium bromide staining. The results were quantified by using KODAK 1D software and an EDAS 290 imaging system (Eastman Kodak). The specific oligonucleotide primers were designed by using an Internet-based primer design program, GeneFisher. Rat GTPCH1 (forward, 5'-ATTTGTGGGAAGGGTCCA-3'; reverse, 5'-CAGATAACGCTGGCCTCA-3') and eNOS (forward, 5'-GACATTGAG-AGCAAAGGGCTGC-3'; reverse, 5'-CGGCTTGTCACCT-CCTGG-3') primers were obtained from Biosource.

Reagents

The following compounds were purchased from Sigma Chemical Co.: ACH, indomethacin, L-NNA, PE, sepiapterin, and SNP. L-NNA and sepiapterin were dissolved in PSS, and a stock solution of indomethacin was dissolved in ethanol (<0.1% final concentration in organ chamber). All other drugs were dissolved in distilled water. All reagents were prepared fresh on the day of experiments.

Statistical Analyses

Results are presented as mean \pm SEM. ANOVA was used for multiple comparisons, followed by the Student-Newman-Keuls post-hoc test when necessary. The significance level was set at 0.05.

Results

Blood Pressure Measurements

Baseline systolic blood pressure did not differ between the dexamethasone-treated and control rats ($P > 0.05$) (Figure 1). After dexamethasone implantation, systolic blood pressure increased significantly from baseline (baseline, 125 ± 1 mm Hg; 12hr-DEX, 149 ± 3 mm Hg; 4d-DEX, 161 ± 3 mm Hg; 15d-DEX, 184 ± 4 mm Hg; all $P < 0.05$ versus previous time point) and was significantly higher than the control rat systolic pressures on each day ($P < 0.001$ for all).

Effect of Dexamethasone on Endothelium-Dependent Vasodilation

Maximal relaxation to ACH was significantly decreased in the 4d-DEX and 15d-DEX rats compared with the 12hr-DEX and control rats (relaxation from PE-induced contraction 10^{-7}

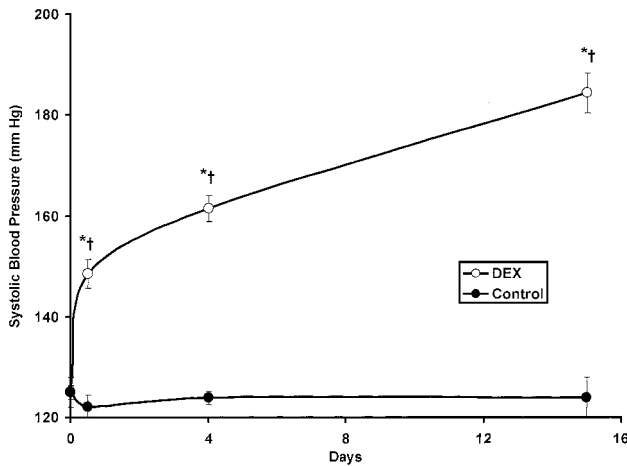


Figure 1. Dexamethasone (DEX, 0.79 mg · kg⁻¹ · d⁻¹) increases systolic blood pressures in male Sprague-Dawley rats. Results are expressed as mean ± SEM (n=5 per time point). *P<0.05 vs control; †P<0.05 vs previous time point (ANOVA with Newman-Keuls multiple comparison test).

mol/L: controls, 90 ± 2%; 12hr-DEX, 87 ± 5%; 4d-DEX, 67 ± 4%; 15d-DEX, 65 ± 3%; P<0.05 for 4d-DEX and 15d-DEX versus controls) (Figure 2). This effect was dependent on the length of dexamethasone treatment. NOS inhibition by using L-NNA (10⁻⁵ mol/L) significantly decreased maximal relaxation to ACH in all groups (Figure 2), except the 15d-DEX vessels, in which NOS inhibition slightly decreased maximal relaxation to ACH. Sepsipaterin (10⁻⁴ mol/L), a BH4 donor, restored maximal vasodilation responses to ACH in the 4d-DEX and 15d-DEX segments to that of controls (Figure 2). The dose-response curve and EC₅₀ value for aortas from all groups after sepsipaterin were similar to that of the control rats (data not shown).

Effect of Dexamethasone on Endothelium-Independent Vasodilation

Endothelium-independent dilation was examined by using SNP. Except for 1 data point, relaxations to SNP in aortic

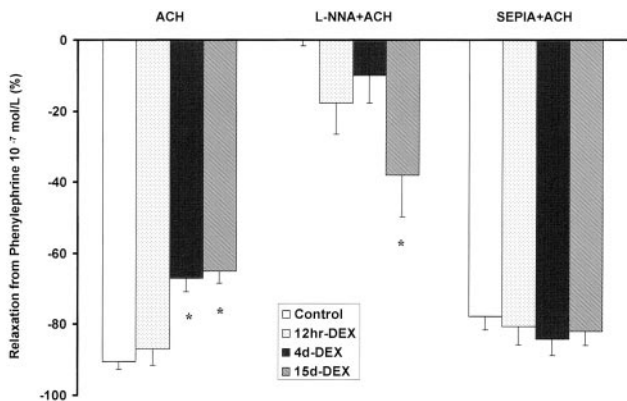


Figure 2. Maximal relaxation responses to ACH (10⁻⁵ mol/L) with and without L-NNA (10⁻⁵ mol/L) or sepsipaterin (SEPIA, 10⁻⁴ mol/L) in aortic rings after precontraction with PE (10⁻⁷ mol/L). Rats were treated with dexamethasone (DEX, 0.79 mg · kg⁻¹ · d⁻¹) for 12 hours, 4 days, or 15 days. Results are expressed as mean ± SEM (n=15 in control group and n=5 in each dexamethasone group). *P<0.05 vs control (ANOVA with Newman-Keuls multiple comparison test).

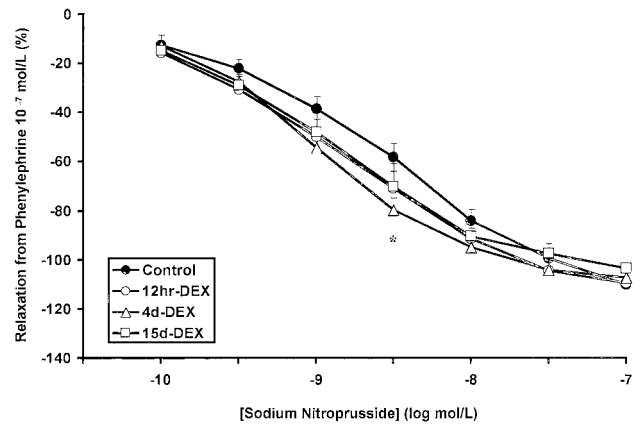


Figure 3. Endothelium-independent relaxations to sodium nitroprusside in aortic rings after precontraction with PE (10⁻⁷ mol/L). Rats were treated with dexamethasone (DEX, 0.79 mg · kg⁻¹ · d⁻¹) for 12 hours, 4 days, or 15 days. Results are expressed as mean ± SEM (n=15 in control group and n=5 in each dexamethasone group). *P<0.05 vs control (ANOVA with Newman-Keuls multiple comparison test).

segments from all dexamethasone-treated rats were not statistically different compared with controls (Figure 3). However, NOS inhibition by L-NNA (10⁻⁵ mol/L) significantly increased the sensitivity to SNP in the vessels from all dexamethasone-treated rats compared with controls (Figure 4). The EC₅₀ values (Figure 4) for the 12hr-DEX (-8.8951 ± 0.0237; anti-log, 1.3 × 10⁻⁹), 4d-DEX (-8.8987 ± 0.0593; anti-log, 1.3 × 10⁻⁹), and 15d-DEX (-8.9463 ± 0.1611; anti-log, 1.1 × 10⁻⁹) rat aortas were significantly increased compared with the control vessels (-8.4838 ± 0.0744; anti-log, 3.3 × 10⁻⁹) (P<0.05 for all).

Effect of Dexamethasone on mRNA Expression

GTPCH1 mRNA expression was significantly reduced in the 4d-DEX and 15d-DEX vessels compared with controls and 12hr-DEX vessels (intensity in arbitrary units: controls, 122.2 ± 1.1; 12hr-DEX, 120.6 ± 3.6; 4d-DEX, 113.6 ± 2.5; 15d-DEX, 111.0 ± 1.7; P<0.05) (Figure 5). Similarly, eNOS mRNA expression was significantly decreased in the 4d-DEX and 15d-DEX vessels compared with controls and 12-hour-DEX vessels (intensity in arbitrary units: controls, 118.1 ± 1.8; 12hr-DEX, 111.8 ± 3.4; 4d-DEX, 103.4 ± 1.0; 15d-DEX, 98.4 ± 1.0; P<0.05, data not shown). There were no significant differences in GTPCH1 and eNOS mRNA levels between the 12hr-DEX vessels and controls (P>0.05).

Discussion

This study examined the role of GTPCH1 in the onset, development, and maintenance of glucocorticoid hypertension. We found that dexamethasone significantly elevated blood pressure and decreased endothelium-dependent dilation in a time-dependent manner. We also demonstrated that endothelium-dependent relaxation could be restored with sepsipaterin, a BH4 donor, in aortas from dexamethasone-treated rats. Finally, dexamethasone downregulated GTPCH1 and eNOS mRNA expression in a time-dependent fashion.

NO plays an important role in blood pressure regulation, and inhibition of NO production leads to elevated blood pressure, as demonstrated in rats given exogenous L-NNA

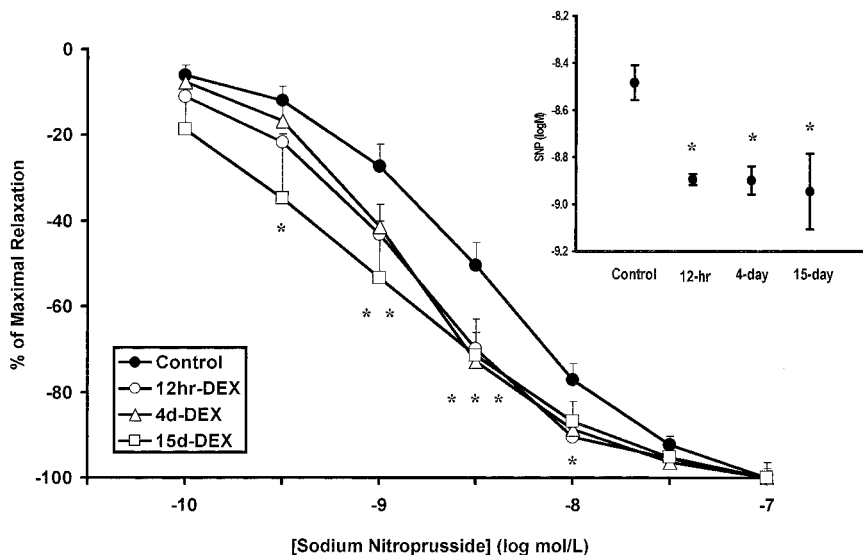


Figure 4. Endothelium-independent relaxations to sodium nitroprusside after NOS inhibition (L-NNA, 10^{-5} mol/L) and precontraction with PE (10^{-7} mol/L). Values are expressed as percentage of maximal relaxation. In the insert, the EC_{50} values for each group are reported. Results are expressed as mean \pm SEM (n=15 in control group and n=5 in each dexamethasone group). * P <0.05 vs control (ANOVA with Newman-Keuls multiple comparison test).

and in eNOS knockout mice.^{16,17} Excess glucocorticoids are known to increase blood pressure in humans and animals, and systolic blood pressure rises rapidly on administration of high doses of synthetic glucocorticoids. In the present study, we found systolic blood pressures significantly increased after 12 hours in rats implanted with a dexamethasone pellet (Figure 1). Systolic pressures continued to rise significantly and were further elevated 4 and 15 days after dexamethasone implantation.

Previous studies have shown that endothelium-dependent relaxation to ACH is decreased in humans and rats with glucocorticoid hypertension.^{1,4,5} Our data support these findings, and we further elucidated that this decrease in ACH-induced vasodilation was time-dependent (Figure 2). Although the 12hr-DEX vessel relaxation responses were not significantly different compared with that of controls, the 4d-DEX and 15d-DEX had significantly reduced vasodilation. These data suggest that a further reduction of vasodilation, most likely from the decreased bioavailability of NO, contributes to the development and maintenance of elevated

systolic blood pressure in glucocorticoid hypertensive rats, but not the onset. The increased blood pressure seen after 12 hours of dexamethasone without a subsequent reduction in vasodilation may be owing to a potentiation of vasoconstriction by angiotensin II and norepinephrine, previously shown to occur after glucocorticoid administration.^{3,4} The thoracic aorta, of which changes in tone do not influence systemic resistance, was used in the current study as a model for vascular tissue to examine the mechanism of circulating glucocorticoids and the effects on vascular reactivity and gene expression. NOS inhibition by using L-NNA (10^{-5} mol/L) abolished ACH-induced vasodilation in all groups except the 15d-DEX vessels, which showed a slightly reduced vasodilation to ACH (Figure 2). This would imply that decreased NO bioavailability and NO-mediated vasodilation by glucocorticoids may upregulate other mediators of vasodilation such as endothelium-derived hyperpolarizing factor (EDHF). Evidence to support this comes from a previous study that showed increased EDHF-mediated relaxation and gene expression in porcine coronary arteries after 24-hour incubation with cortisol.¹⁸ Importantly, we can eliminate cyclooxygenase-derived products as the cause of this finding because indomethacin (10^{-5} mol/L) was used in all experiments. In addition, increased sensitivity to NO by downstream mediators of vasodilation may also explain this finding.

Inhibition of GTPCH1, the rate-limiting enzyme in the production of BH₄, has been shown to decrease NO production and endothelium-dependent relaxation.^{19,20} In contrast, restoration of BH₄ levels in humans and animals increases endothelium-dependent relaxation in a number of disease conditions.^{21–23} Results from our study support these findings, as we showed that sepiapterin restored ACH-induced vasodilation in glucocorticoid hypertensive rats (Figure 2). Interestingly, we observed a small, but not significant, decrease in vasodilation in the control vessels after sepiapterin incubation for 60 minutes. BH₄ has been reported to be susceptible to auto-oxidation, which may increase oxidant stress and decrease NO bioavailability, thus leading to reduced vasodila-

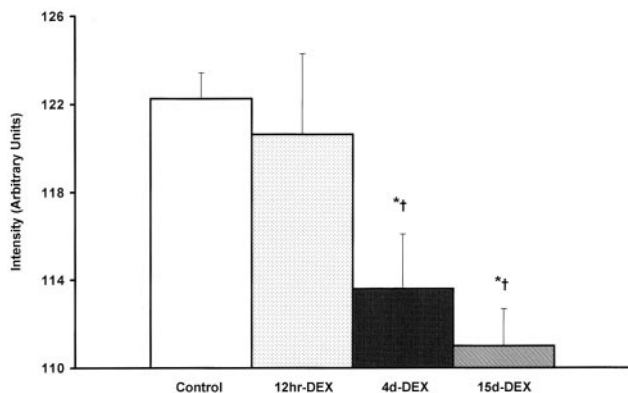


Figure 5. GTPCH1 mRNA expression in aortas from rats treated with dexamethasone (DEX, $0.79 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$). Results are expressed as mean \pm SEM (n=15 in control group and n=5 in each dexamethasone group). * P <0.05 vs control; † P <0.05 vs 12hr-DEX (ANOVA with Newman-Keuls multiple comparison test).

tion. Katusic²⁴ has suggested this mechanism as a caution in the use of exogenous BH4 in humans with normal endothelial function.

To examine the site of glucocorticoid-induced decreases in vasodilation, endothelium-independent experiments were performed. By using SNP, we found no differences in the concentration-response curves in the aortas from dexamethasone-treated rats compared with controls except for 1 unrelated data point (Figure 3). However, we did demonstrate an increased sensitivity to SNP after NOS inhibition (Figure 4). By depleting endogenous NO in dexamethasone-treated and control vessels, we saw a significant decrease in SNP EC₅₀ values for aortas from dexamethasone-treated rats. These findings suggest that in the presence of low NO bioavailability, downstream mediators of NO-induced vasodilation, such as guanylate cyclase and cyclic guanosine monophosphate, may become upregulated. Although endothelium-dependent relaxations in the 12hr-DEX vessels were not different compared with that of controls, we did observe a significant shift of the concentration-response curve to SNP after NOS inhibition. This may be explained by a rapid downregulation of eNOS after glucocorticoid treatment, despite sufficient levels of NO to mediate vasodilation. In addition, endothelium-dependent relaxations in the 12hr-DEX vessels may be mediated via hydrogen peroxide, a known vasodilator.

Glucocorticoids have been shown to alter various proteins in the NOS-mediated production of NO.⁶ To examine the genomic effect of glucocorticoids on GTPCH1 and eNOS, we performed mRNA expression studies. We have previously shown that GTPCH1 mRNA levels were significantly decreased (50% of controls) in aortic segments incubated with dexamethasone (1.3×10^{-6} mol/L) after 6 hours.⁵ However, it is not known at what period in the development of hypertension that the enzyme responsible for BH4 biosynthesis decreases. In the current study, we hypothesized there would be little to no change in GTPCH1 and eNOS mRNA expression in the vessels from 12hr-DEX-treated rats, but there would be a significant decrease of both enzymes in the 4d-DEX and 15d-DEX compared with controls. Indeed, we found a significant downregulation of GTPCH1 and eNOS in the latter 2 time-points, 4d-DEX and 15d-DEX (Figure 5). Because BH4 biosynthesis is tightly coupled with eNOS production, this supports the finding by Wallerath et al,¹⁵ who found a 40% decrease in eNOS mRNA in aortas from rats after 3 days of dexamethasone in the drinking water ($0.3 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$). They found that although the eNOS promoter lacks a glucocorticoid response element, glucocorticoids can destabilize eNOS mRNA via prevention of the transcription factor GATA and reduced transcription of the eNOS gene. These data support previous findings that glucocorticoids can negatively affect gene expression of eNOS and GTPCH1, thus leading to reduced vasodilation and increased blood pressure. It is currently unknown if GTPCH1 contains a glucocorticoid response element. Of interest, there was a very strong correlation between GTPCH1 mRNA levels and blood pressure in the dexamethasone-treated rats ($r = -0.94$).

In conclusion, our evidence supports the hypothesis that downregulation of GTPCH1 expression decreased production of BH4 and NO and contributed to hypertension in rats

treated with synthetic glucocorticoids. GTPCH1 appears to be downregulated early in the development of hypertension, and a sustained decrease of GTPCH1 expression by glucocorticoids may contribute to the decreased NO bioavailability and increased blood pressure in rats.

Perspectives

Based on the results of this study, prevention of GTPCH1 downregulation in humans and animals with excess glucocorticoids may maintain endothelial function and suppress the development of hypertension. Future research to examine the molecular mechanism of GTPCH1 downregulation by glucocorticoids may provide a target that would eliminate potential negative side effects of excess glucocorticoids such as depression and hypertension.

Acknowledgments

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