Glucocorticoids Inhibit Tetrahydrobiopterin-Dependent Endothelial Function

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Tetrahydrobiopterin (BH_4) acts as an important co-factor for endothelial nitric oxide synthase (eNOS). Glucocorticoids have been shown to inhibit expression of the rate-limiting enzyme for tetrahydrobiopterin synthesis, GTP cyclohydrolase, in other cell types. We hypothesized that endothelium-dependent vasodilator responses would be blunted in rats made hypertensive with dexamethasone. Further, we hypothesized that treatment of rat vascular segments with dexamethasone would result in attenuation of endothelial function accompanied by decreased GTP cyclohydrolase expression. We report that endothelium-dependent relaxation responses to the calcium ionophore A23187 are reduced in aortic rings from dexamethasone-hypertensive rats compared with sham values. Dexamethasone incubation abolishes contraction to N^{ω}-nitro-L-arginine (L-NNA, 10^{-5} *M*) in endothelium-intact aortic rings, and inhibits expression of GTP cyclohydrolase. We conclude that inhibition of BH_4 synthesis by glucocorticoid regulation of GTP cyclohydrolase expression may contribute to reduced endothelium-dependent vasodilation characteristic of glucocorticoid-induced hypertension. [E.B.M. 2001, Vol 226:27–31]

Key words: tetrahydrobiopterin; endothelial nitric oxide synthase; glucocorticoids

Nitric oxide (NO) is a freely diffusable vasodilatory second messenger synthesized by nitric oxide synthase (NOS) in response to agonists such as acetylcholine, A23187, bradykinin, and histamine (1, 2). Optimal function of endothelial NOS requires the presence of several co-factors such as flavonoids and tetrahydrobiopterin (BH_4). BH_4 is synthesized by the rate-limiting action of GTP cyclohydrolase (3). Glucocorticoids have been shown to inhibit expression of GTP cyclohydrolase in other cell types (4). Clinical conditions characterized by elevated glucocorticoids (i.e., Cushing's syndrome) are associated with marked hypertension and impaired endothelium-dependent vasodilation (5, 6). Others have shown that pharmacological inhibition of GTP cyclohydrolase results in attenuation of endothelium-dependent vasodilation (7). The purpose of this study was to examine the effects of glucocorticoids on the BH_4-dependent component of vascular function. We hypothesized that endothelium-dependent vasodilator responses would be blunted in rats made hypertensive with dexamethasone. Further, we hypothesized that treatment of rat vascular segments with dexamethasone would result in attenuation of endothelium-dependent relaxation through decreased GTP cyclohydrolase expression.

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Materials and Methods

Animals and Tissue Preparation. Male Wistar rats, weighing 200–250 g, were used for this study. In the first series of experiments, rats were made hypertensive by treatment with dexamethasone. Six rats were anesthetized with sodium pentobarbital (50 mg/kg, ip), and dexamethasone (Innovative Research, 5 mg/kg as a pellet) was implanted subcutaneously. Sham-operated normotensive rats ($n = 6$) served as controls for this series of experiments (pellet without dexamethasone). Postoperatively, the rats received a standard diet and tap water *ad libitum*. Twenty days after implant, systolic blood pressures were measured in the conscious state using a tail cuff procedure (pneumatic transducer). In the second series of experiments, normotensive rats ($n = 10$) were used.

On the day of an experiment, rats were anesthetized with sodium pentobarbital (50 mg/kg), and the thoracic aorta was removed and placed in ice-cold physiological solution. The methods for isometric force recording in rat thoracic aortic rings were conducted as previously described (8). In all experiments, indomethacin (10^{-5} *M*) was added to the muscle bath to inhibit cyclooxygenase. In some experiments, the endothelium was removed from the aortic rings using a rubbing procedure (8). Verification of the rubbing procedure was assessed by the lack of relaxation to acetylcholine (10^{-6} *M*) following contraction induced by phenylephrine (EC_{30}; see below for method to determine this concentration).

In each aortic sample, a concentration-response curve to phenylephrine was constructed (cumulative addition; 10^{-9}–10^{-4} *M*). This served as a basis for selection of specific concentrations of phenylephrine to be used in subsequent protocols to assess endothelial function. This was necessary since the magnitude of relaxation and contractile responses to agents that alter endothelial function is influenced by the contractile state of the arterial segment.

In the first series of experiments on aortic rings from sham-normotensive and dexamethasone-hypertensive rats, a concentration of phenylephrine causing a half-maximal response (EC_{50}) was used to induce contraction. Once the contractile response had reached a plateau, relaxation responses to A23187 (10^{-10}–3×10^{-7} *M*) were evaluated. These drugs were rinsed from the muscle bath, and approximately 2 hr later, the aortic segments were again contracted with an EC_{50} of phenylephrine, and relaxation responses to nitroprusside (10^{-10}–3×10^{-7} *M*) were determined.

In the second series of experiments, vessels were incubated in physiologic salt solution with either dexamethasone (1.3×10^{-6} *M*), dexamethasone plus sepiapterin, a BH_4 donor (10^{-4} *M*), or vehicle for 6 hr at 37°C. Half of each vessel was used for muscle bath experiments whereas the other half was used for extraction of mRNA. For these muscle bath experiments, a concentration of phenylephrine causing a 30% maximal response (EC_{30}) was used to contract each vessel segment to elicit equal magnitudes of contractile activation. During the plateau phase of the phenylephrine contraction, N^{ω}-nitro-L-arginine (L-NNA, 10^{-5} *M*) was added to the muscle bath, and the contractile response was recorded.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Amplification of GTP Cyclohydrolase cDNA. Aortic tissue segments were homogenized, and RNA was extracted using an RNeasy minispin column (Qiagen, Inc., Valencia CA). RNA concentration per sample was determined using UV spectroscopy to ensure equal template concentration for reverse transcription. RT-PCR reaction was performed using the methods of Brosius *et al.* (9). Rat GTP cyclohydrolase I primers, forward: 5′-CGAGATGGTGATTGTGA-3′ and reverse: 5′-TCCTGATGAGTGTGAGGA-3′ flanking a PCR product of 350 base pairs and a rat GAPDH primer set from the University of Michigan Biomedical Research Facility were used in conjunction with [^{32}P]-dCTP for radioactive labeling of PCR product. Optimum cycle number and template dilution factor were determined for each amplicon prior to experimentation. PCR products were identified on polyacrylamide gels, using a phosphorimager (Bio-Rad, Hercules, CA) and quantitated using Multi-Analyst software. Values were normalized and expressed as a percentage of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels.

Pharmacological Agents. The following compounds were purchased from Sigma Chemical Co. (St. Louis, MO): L-phenylephrine HCl, N^{ω}-nitro-L-arginine, dexamethasone, sepiapterin, acetylcholine, A23187, and indomethacin. Dexamethasone, A23187, and sepiapterin were solubilized in absolute ethanol. Ethanol concentrations in the tissue baths did not exceed 0.1%. At this concentration, ethanol did not alter functional measures of contractile activity. All other drugs were dissolved in distilled water.

Statistics. Results are presented as the mean ± SEM. For multiple comparisons, Student's *t* test was used with the Bonferroni correction with a *P*-value of less than 0.05 being considered significant.

Results

After 20 days of treatment, rats receiving the subcutaneous implant of dexamethasone had systolic blood pressures that were significantly higher than those in sham rats (168 ± 3 mmHg vs 123 ± 3 mmHg, respectively; $P < 0.05$).

Aortic segments from hypertensive rats were more sensitive to the contractile effects of phenylephrine as indicated by the leftward shift in the concentration-response curve and lower EC_{50} value (–log EC_{50} value = 7.8021 ± 0.0803; antilog = 1.6×10^{-8} *M* for hypertensive rats vs –log EC_{50} value = 7.1274 ± 0.0417; antilog = 7.5×10^{-8} *M* for normotensive rats; $P < 0.05$, $n = 6$ in each group). Maximal force development to phenylephrine in aortic rings from hypertensive rats (1585 ± 173 mg) was not significantly different from that in aortic rings from sham rats (1417 ± 190 mg).

Following contraction to an EC_{50} of phenylephrine, aortic rings were made to relax in response to the cumulative addition of A23187 to the muscle bath (Fig. 1A). Relaxation responses to this endothelium-dependent agonist were blunted in aortic rings from hypertensive rats compared with those in aortic rings from sham rats. Aortic rings from hypertensive rats were also less sensitive to the dilator actions of A23187 as indicated by the rightward shift in the concentration response curve and the higher EC_{50} value (–log EC_{50} value = 7.6763 ± 0.0400; antilog = 2.1×10^{-8} *M* for hypertensive rats vs –log EC_{50} value = 8.2461 ± 0.1038; antilog = 0.6×10^{-8} *M* for normotensive rats; $P < 0.05$, $n = 6$ in each group).

Relaxation responses to nitroprusside were also evaluated in aortic rings from hypertensive and normotensive rats (Fig. 1B). The aortic rings were made to contract to an EC_{50} of phenylephrine and after the contraction had reached a plateau, nitroprusside was added cumulatively to the muscle bath. Relaxation responses to this endothelium-independent agonist were increased at mid-range concentraions (10^{-9}–10^{-8} *M*) in aortic rings from hypertensive rats compared with those in aortic rings from sham rats. Aortic rings from hypertensive rats were also more sensitive to the dilator actions of nitorprusside as indicated by the leftward shift in the concentration-response curve and the lower EC_{50} value (–log EC_{50} value = 8.7217 ± 0.0745; antilog = 1.9×10^{-9} *M* for hypertensive rats vs –log EC_{50} value = 8.3215 ± 0.0562; antilog = 4.8×10^{-9} *M* for normotensive rats; $P < 0.05$, $n = 6$ in each group).

To evaluate the effects of dexamethasone on endothe-

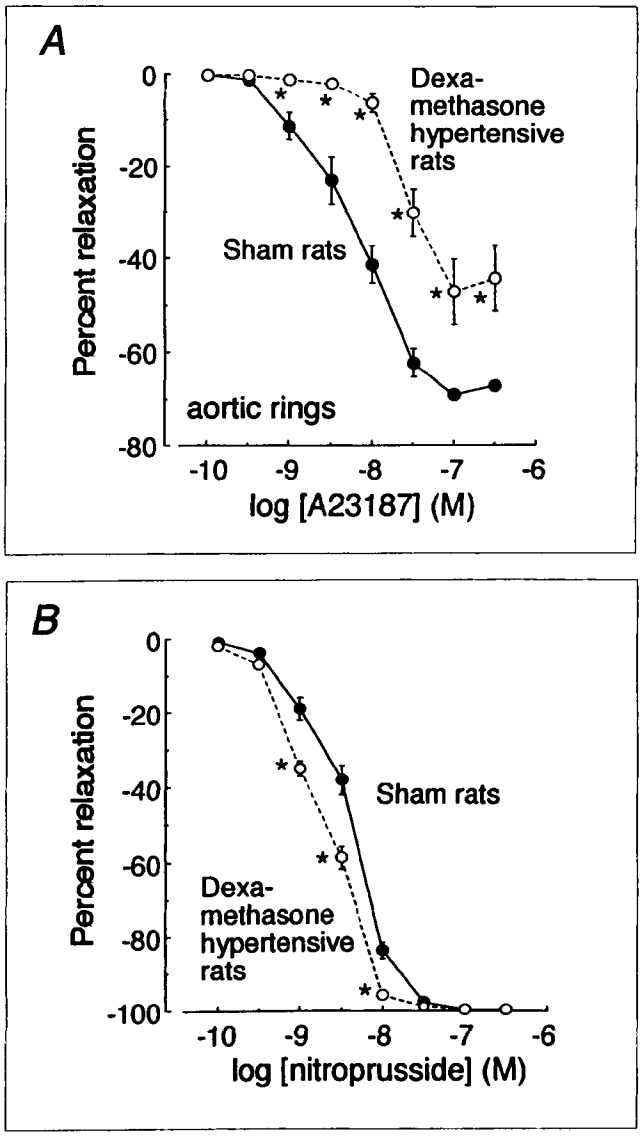

Figure 1. (A) Blunted endothelium-dependent relaxation to A23187 in aortic rings from dexamethasone-hypertensive rats. Aortic rings (endothelium intact) from sham and hypertensive rats were made to contract in response to phenyleprhine (EC_{50}). After the contraction had reached a plateau, A23187 was added to the muscle bath in a cumulative fashion. Aortic rings from both groups of rats relaxed in response to the calcium ionophore, and the magnitude of the relaxation was less in those from dexamethasone-hypertensive rats. (B) Relaxation responses to nitroprusside are increased in aortic rings from dexamethasone-hypertensive rats. Following contraction to phenyleprhine (EC_{50}) in aortic segments from sham and hypertensive rats, nitroprusside was added to the muscle bath in a cumulative fashion. Aortic rings from both groups of rats relaxed in response to nitroprusside, and the magnitude of the relaxation was greater at 10^{-9}–10^{-8} *M* in those from dexamethasone-hypertensive rats. Asterisks indicate a significant difference between sham and hypertensive rats with a *P*-value less than 0.05 ($n = 6$).

lial NOS activity, we incubated vessel segments with 1.3 μ*M* dexamethasone and examined the contractile response to the NOS inhibitor, L-NNA. This concentration of dexamethasone was used because previous studies have shown that this concentration inhibits BH_4 synthesis (4), and that this concentration is achieved in the plasma of patients with Cushing's syndrome (6). Aortic rings that were treated with dexamethasone were more sensitive to the contractile effects of phenylephrine than control aortic rings as indicated by the leftward shift in the concentration response curve and lower EC_{50} value (–log EC_{50} value = 7.6976 ± 0.0328; antilog = 2.0×10^{-8} *M* for dexamethasone-treated rings vs –log EC_{50} value = 7.2390 ± 0.0510; antilog = 5.8×10^{-8} *M* for control aortic rings; $P < 0.05$, $n = 10$ in each group). Maximal force development to phenylephrine in aortic rings treated with dexamethasone (1640 ± 273 mg) was not significantly different from that in untreated aortic rings (1451 ± 191 mg). An EC_{30} of phenylephrine was used in subsequent experiments to evaluate functional activity of nitric oxide synthase (see below). The EC_{30} for phenylephrine in dexamethasone-treated rings was lower (–log EC_{30} value = 7.9675 ± 0.0406; antilog = 1.1×10^{-8} *M*) than in untreated aortic rings (–log EC_{30} value = 7.5712 ± 0.0503; antilog = 2.7×10^{-8} *M*; $P < 0.05$, $n = 10$).

Figures 2A & 2B show that in untreated aortic segments constricted with a concentration of phenylephrine (3×10^{-8} *M*) that elicited a submaximal response, L-NNA (10^{-5} *M*) caused a significant contraction. In aortic segments without endothelium, L-NNA did not cause a significant contraction (38 ± 18 mg, $n = 6$). Aortic segments without endothelium (–log EC_{30} value = 7.9749 ± 0.0585; antilog = 1.1×10^{-8} *M*) were more sensitive to the contractile effects of phenylephrine than intact segments (–log EC_{30} value = 7.5494 ± 0.0607; antilog = 2.8×10^{-8} *M*; $n = 6$, $P < 0.05$). Segments incubated with dexamethasone did not contract when treated with L-NNA, an effect that was reversible with sepiapterin (10^{-4} *M*), a BH_4 donor (Fig. 2B).

Figure 2C illustrates the effect of dexamethasone incubation on GTP cyclohydrolase mRNA levels in rat thoracic aorta. Incubation of aortic segments with dexamethasone resulted in a 50% decrease in GTP cyclohydrolase mRNA. These experiments indicate that dexamethasone decreases BH_4 levels.

Discussion

Two hypotheses were tested in this study. First, it was hypothesized that endothelium-dependent vasodilator responses would be blunted in rats made hypertensive with dexamethasone. Further, we hypothesized that treatment of rat vascular segments with dexamethasone would result in attenuation of endothelium-dependent relaxation through decreased GTP cyclohydrolase expression. Cushing's syndrome patients have elevated plasma glucocorticoids and are hypertensive (6). These patients demonstrate reduced vasodilator responses to endothelium-dependent agonists and when challenged with intravenous arginine, are unable to excrete NO-related metabolites (6). Further, treatment of rats with dexamethasone results in hypertension and impaired vasodilation to calcium ionophore, A23187 (Fig. 1)

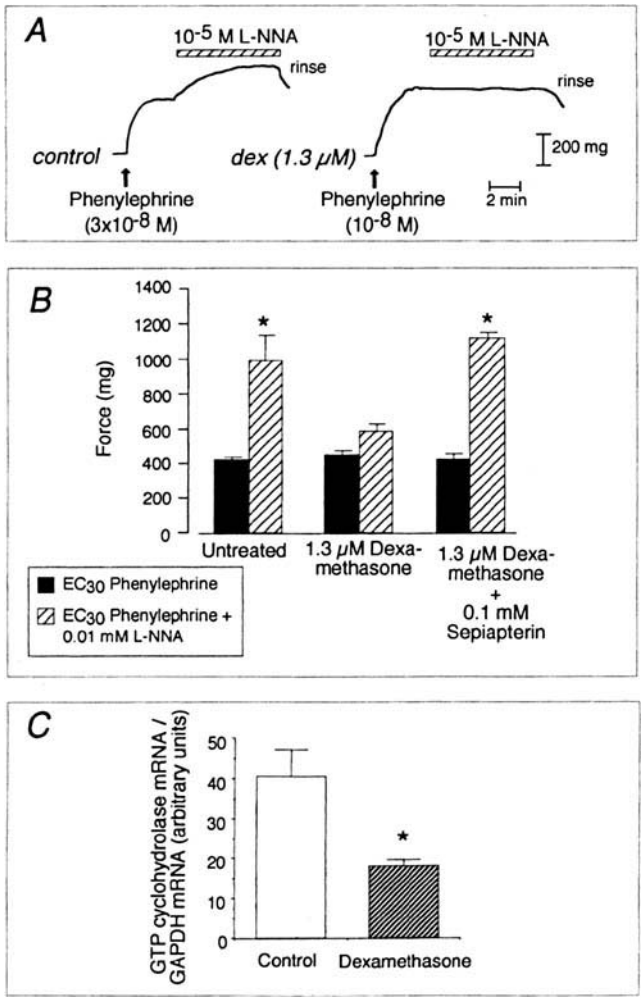

Figure 2. (A) Dexamethasone incubation blocks potentiation of phenylephrine contraction by L-NNA in rat thoracic aortic rings. Tracing illustrating potentiation of contraction by L-NNA and blockade of this effect by dexamethasone incubation. (B) L-NNA (10^{-5} *M*, hatched bars) potentiates contraction to phenylephrine (solid bars), an effect that is blocked by dexamethasone (1.3×10^{-6} *M*). Sepiapterin incubation (10^{-4} *M*) reverses the effect of dexamethasone. (C) Dexamethasone incubation decreases expression of GTP cyclohydrolase mRNA in rat thoracic aortic rings. GTP cyclohydrolase mRNA levels were reduced 50% in vessels incubated with dexamethasone. Asterisk indicates significance with a *P*-value less than 0.05 ($n = 10$).

(10). However, a causal mechanism for these observations has not been investigated. Here we described decreased expression of the rate-limiting enzyme for BH_4 synthesis in rat aortic segments incubated with dexamethasone. In segments from the same vessels, we described a blockade of the contractile response to L-NNA with dexamethasone incubation and reversal of this effect by sepiapterin, a BH_4 donor.

The presence of BH_4 is required by all isoforms of NOS, evidenced by *in vitro* studies that document up to a 30-fold increase in NOS activity in the presence of BH_4 compared with BH_4-free preparations (11). The importance of BH_4-mediated eNOS activity was reported in cultured endothelial cells by Schmidt *et al.* (12). Inhibition of GTP cyclohydrolase with 2,4-diamino-6-hydroxypyrimidine (DAHP) resulted in decreased NO formation in response to calcium ionophore (12). At the tissue level, Consentino *et al.* (7) showed that inhibition of GTP cyclohydrolase with DAHP resulted in impaired endothelium-dependent vasodilation in canine coronary arteries and in cerebral arteries (13). These observations indicated that alteration of BH_4 levels markedly influences eNOS activity and endothelial regulation of vascular tone.

The effect of glucocorticoids on eNOS expression has only recently been examined. Wallerath *et al.* (14) demonstrated that dexamethasone treatment of cultured endothelial cells results in decreased expression of eNOS mRNA and protein and reduced formation of NO in endothelial cells (14). Indeed, using the aortic tissue segments described in this study, we have confirmed the observation of Wallerath *et al.* that dexamethasone reduces eNOS mRNA expression (data not shown). Although this effect of glucocorticoids on eNOS expression and activity could represent a possible mechanism for the development of glucocorticoid-induced hypertension, the effect of altered expression of GTP cyclohydrolase on endothelium-dependent vascular function has only recently been investigated. Katusic *et al.* (15) demonstrated that cytokines increase expression of GTP cyclohydrolase in cultured human umbilical vein endothelial cells. It was postulated that regulation of GTP cyclohydrolase expression may influence production of NO and therefore may affect vascular contractility (15).

In contrast to the blunted dilator responses to A23187 in aortic rings from dexamethasone hypertensive rats, we observed that dilator responses to the endothelium-independent agonist, nitroprusside, were increased in hypertension compared with normotensive values. This may be due to an upregulation of the signaling pathway for nitric oxide in smooth muscle cells. This would be predicted to occur as a compensation due to a low eNOS activity produced by decreased expression of GTP cyclohydrolase. Regardless of the precise mechanism, it is apparent that the reduced dilator activity of A23187 in hypertensive rats is not the result of cellular events downstream of nitric oxide.

Here, we reported that incubation of rat thoracic aortic segments results in decreased expression of GTP cyclohydrolase mRNA and decreased eNOS activity, as assessed by potentiation of contraction by L-NNA. Endothelium-dependent relaxation responses to A23187 in aortic rings from dexamethasone-hypertensive rats were blunted compared with those in aortic rings from normotensive rats. We speculate that glucocorticoid-induced hypertension may occur through a similar downregulation of GTP cyclohydrolase expression resulting in decreased synthesis of BH_4. This is supported by the effects of dexamethasone, a nonmetabolized, synthetic steroid with selective glucocorticoid activity on GTP cyclohydrolase expression. We conclude that decreased expression of GTP cyclohydrolase results in attenuation of eNOS-dependent regulation of vascular contractility. This may represent one mechanism for development of glucocorticoid-induced hypertension.

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