Down-regulation of the expression of endothelial NO synthase is likely to contribute to glucocorticoid-mediated hypertension

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Hypertension is a side effect of systemically administered glucocorticoids, but the underlying molecular mechanism remains poorly understood. Ingestion of dexamethasone by rats telemetrically instrumented increased blood pressure progressively over 7 days. Plasma concentrations of Na+ and K+ and urinary Na+ and K+ excretion remained constant, excluding a mineralocorticoid-mediated mechanism. Plasma NO2−/NO3− (the oxidation products of NO) decreased to 40%, and the expression of endothelial NO synthase (NOS III) was found down-regulated in the aorta and several other tissues of glucocorticoid-treated rats. The vasodilator response of resistance arterioles was tested by intravital microscopy in the mouse dorsal skinfold chamber model. Dexamethasone treatment significantly attenuated the relaxation to the endothelium-dependent vasodilator acetylcholine, but not to the endothelium-independent vasodilator S-nitroso-L-cysteine. Incubation of human umbilical vein endothelial cells, EA.hy 926 cells, or bovine aortic endothelial cells with several glucocorticoids reduced NOS III mRNA and protein expression to 60−70% of control, an effect that was prevented by the glucocorticoid receptor antagonist mifepristone. Glucocorticoids decreased NOS III mRNA stability and reduced the activity of the human NOS III promoter (3.5 kilobases) to ~70% by decreasing the binding activity of the essential transcription factor GATA. The expression-dependent down-regulation of endothelial NOS III may contribute to the hypertension caused by glucocorticoids.

dexamethasone | dihydrocortisol | RNase protection assay | Western blot | Reporter gene assay |

Endothelium-derived NO is a physiologically significant vasodilator. Pharmacological inhibition of the endothelial NO synthase (NOS III) increases blood pressure (1), and mice lacking the NOS III gene are hypertensive (2). Endothelial NO is also an inhibitor of platelet aggregation and adhesion (3, 4) and can prevent leukocyte adhesion to the endothelium by down-regulating the leukocyte adhesion glycoprotein complex CD11/CD18 (5). Finally, NO also has been shown to inhibit DNA synthesis, mitogenesis, and proliferation of vascular smooth muscle cells (6, 7). Therefore, endothelial NO is likely to represent a protective antiatherogenic principle (8), and up- or down-regulation of NOS III could have important consequences for vascular homeostasis (9).

Hypertension with diverse sequelae such as increased atherosclerosis, stroke, cerebral hemorrhage, and hypertensive cardiovascular disease is the major cardiovascular side effect of systemically administered glucocorticoids (10, 11). There is evidence for the presence of glucocorticoid receptors on endothelial cells (12) and vascular smooth muscle cells (13). Therefore, actions of glucocorticoids on the vasculature are conceivable. Indeed, effects of glucocorticoids on vascular resistance have been demonstrated and have been explained in part by an increased response of the vasculature to catecholamines and angiotensin II (10, 11).

We now demonstrate in vitro and ex vivo that glucocorticoids down-regulate endothelial NOS III expression by decreasing the stability of NOS III mRNA and reducing NOS III gene transcription. The resulting reduction in endothelial NO production could contribute to the increase in blood pressure seen with glucocorticoid therapy.

Methods

Reagents. Actinomycin D, dexamethasone, Nω-nitro-l-arginine methyl ester (L-NAME) and S-nitroso-N-acetyl-l-arginine (SNAP), goat anti-rabbit antibody conjugated to alkaline phosphatase, horse anti-mouse antibody conjugated to alkaline phosphatase, and mouse monoclonal antibody to β-tubulin were obtained from Sigma. DMEM and Ham’s F-12 nutrient mixture were from Life Technologies (Paisley, Scotland). Restriction enzymes, random hexamer primers, SureClone Ligation Kit, Taq DNA polymerase, Taq polymerase reaction buffer and 12 Sequencing Kit were from Amersham Pharmacia. Custom oligonucleotides were from MWG Biotech (Heidelberg, Germany). 3-Isobutyl-1-methylxanthine and superoxide dismutase were from Boehringer Ingelheim. pCR-Script and NucTrap probe purification columns were from Stratagene. DNase I, RNase A, RNase T1, proteinase K, T3/T7 RNA polymerase, and nitrate reductase were from Roche Diagnostics. Luciferase assay system with reporter lysis buffer was from Promega. [α-32P]UTP was from ICN. Mifepristone (RU 38486) was a generous gift of Roussel-UCLAF. Rabbit polyclonal antibody to NOS III was from Transduction Laboratories (Lexington, KY).

Telemetric Measurement of Blood Pressure and Heart Rate in Rats. Male Wistar-Kyoto rats (from Charles River Breeding Laboratories, Sulzfeld, Germany) were housed singly under controlled environmental conditions (23 ± 1°C, light:dark, 12:12 h, light on at 7:00 a.m.). Food (regular rat chow) and water were always available. Food and water intake were measured continuously.

Abbreviations: L-NAME, Nω-nitro-l-arginine methyl ester; NOS III, endothelial-type nitric oxide synthase; SNAP, S-nitroso-N-acetyl-l-arginine; PLSD test, protected least-significant-difference test.

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with a PC-controlled monitoring system (Feeding/Drinking Monitor, Technical & Scientific Equipment, Bad Homburg, Germany). This device records hydrostatic pressure in the water bottle and the weight of the food container. Systolic and diastolic blood pressures were measured telemetrically in freely moving animals by using implanted transmitters as described (14). Monitoring of cardiovascular parameters started 1 week after implantation of the pressure transmitters. Measurements were taken every 5 min by using the DataQuest System (Datscience, St. Paul) and were averaged over 24 h by using DO-IT software (15). Based on the average water intake of the rats (~100 ml/kg of body weight/day; determined before the experiment), dexamethasone was added to the drinking water at a concentration of 3 mg/liter to reach a target dose of 0.3 mg/kg/day, and L-NAME was added at 100 mg/liter to reach a target dose of 10 mg/kg/day.

**Determination of NO2⁻/NO3⁻ in Rat Serum.** Serum NO2⁻/NO3⁻ was determined as NO2⁻ after enzymatic reduction with NO3⁻ reductase (16). NO3⁻ was determined by chemiluminescence after chemical reduction to NO by using a NOA 270B Nitric Oxide Analyzer (Sievers, Boulder, CO). Rats were fasted 24 h before blood and urine collection to minimize the influence of dietary NO2⁻/NO3⁻.

**Intravital Microscopy of Resistance Arterioles.** Titanium observation chambers were implanted into the dorsal skinfold of xylazine/ketamine-anesthetized C57/BL 6J mice (10 weeks old) to allow for intravital microscopy of a fine striated skin muscle. Five days after implantation, awake animals were placed under the microscope, the overshoot of the chamber was removed, and the tissue was superfused with Krebs solution (composition in mM: NaCl 120.0, KCl 4.7, KH2PO4 1.2, CaCl2·H2O 2.5, MgSO4·7H2O 1.2, NaHCO3 25.0, glucose 11.0; pH 7.4, 34°C) for an equilibration period of 30 min. The diameters of 1–2 resistance arterioles (diameter 30–60 μm) were measured in each chamber by using an ADOBE PHOTOSHOP (Adobe Systems, Mountain View, CA)-based image analysis at baseline and after continuous superfusion with acetycholine (10 μM) or SNAP (10 μM) according to previously published protocols (17). Experiments were performed in untreated control mice (n = 7) and mice receiving 0.3 mg/kg/day dexamethasone in their drinking water for 1 week before the experiment.

**Cell Culture.** Human umbilical vein endothelial cells, the human umbilical vein endothelial cell-derived permanent cell line EA.hy 926 (18), and bovine aortic endothelial cells (19) were grown in DMEM with 10% fetal bovine serum, 2 mM l-glutamine, penicillin, and streptomycin. The three cell types were incubated for 18–36 h with dexamethasone (10 nM to 1 μM), dihydrocortisol (100 nM to 10 μM), or beclomethasone (10 nM to 1 μM), or were left untreated (control). Then, RNA or protein was extracted or the NOS activity of the intact cells determined (see below). In experiments with the glucocorticoid receptor antagonist mifepristone (1 to 10 μM), the antagonist was added 30 min before dexamethasone.

**RNase Protection Assay of NOS III mRNA.** Total RNA was isolated from the human or bovine endothelial cells and from several rat tissues by guanidium thiocyanate/phenol/chloroform extraction (20). Radiolabeled antisense RNA probes for human NOS III and human β-actin were prepared by in vitro transcription of the cDNA clones pCR-NOS III-Hu (21) and pCR-β-actin-Hu (22) as described (21, 23). Bovine and rat cDNAs were generated by reverse transcription of mRNA from bovine aortic endothelial cells and rat aorta, respectively. Bovine or rat NOS III cDNA fragments were amplified by PCR using the same oligonucleotide primers: 5’-GACATGAGAGCAGGAGGCTG-3’ (sense) and 5’-CGGCTTGTGCACCTCTGGG-3’ (antisense). Bovine β-actin and rat γ-actin cDNA fragments were amplified by PCR using the following oligonucleotide primers: 5’-ACCAACTGGGCAGCACCTGAG-3’ (sense) and 5’-CTGAGGATCTTCTATGAGGTAGTC-3’ (antisense) (24). The cDNA fragments (NOS III, 425 bp; γ-actin, 353 bp) were cloned into the EcoRV site of vector pCR-Script (generating the cDNA clones pCR-NOS III-rat and pCR-γ-actin-rat) and sequenced (TSequencing Kit). A portion (0.5 μg) of each DNA was in vitro transcribed by using T7/T3 RNA polymerase and [α-32P]UTP. The template DNA was degraded with DNase I, and the radiolabeled RNA was purified by using NucTrap probe purification columns. RNase protection assays were performed as described (21, 23) with a mixture of RNase A and RNase T1. Densitometric analyses of the RNase protection gels were performed by using a Phospho-Imager (Bio-Rad). The density of each NOS III band was normalized with the corresponding β-actin or γ-actin band.

**NOS III Protein Preparation and Western Blotting.** Protein isolation and Western blotting were done as described (21, 25). In brief, a CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) extract was prepared from the different types of endothelial cells and was separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (7.5% gels). The proteins were transferred to nitrocellulose membranes (Schleicher & Schüll) by electroblotting (Bio-Rad). Proteins were detected by using a polyclonal anti-NOS III antibody (1:500) and a monoclonal anti-β-tubulin antibody (1:500). After an incubation with the appropriate secondary antibodies conjugated to alkaline phosphatase as described (25), densitometric analyses were performed with a Video-Imager (Bio-Rad). NOS III protein bands were normalized by using the respective β-tubulin protein bands (21).

**Determination of NOS III Activity.** NOS III activity was bioassayed by using the stimulation of soluble guanylyl cyclase in RFL-6 rat lung fibroblasts as described (26). Dexamethasone-pretreated endothelial cells or control cells were incubated for 2 min at 37°C in 1 ml of Locke’s solution containing 0.3 mM 3-isobutyl-1-methylxanthine and 20 units/ml superoxide dismutase. Then, the conditioned media were transferred to the RFL-6 cells. After a 2-min incubation at 37°C on the RFL-6 cells, the reaction was stopped with 1 ml of ice-cold 50 mM sodium acetate buffer (pH 4.0) and by rapidly freezing the cells with liquid nitrogen. The cGMP content of the RFL-6 samples was determined by radioimmunoassay as described (26).

**Reporter Gene Assay Using the 5’-Flanking Region of the Human NOS III Gene Stably Transfected into EA.hy 926 Cells.** EA.hy 926 cells stably transfected with the plasmid pNOS III-Hu-3500-Luc-neo have been described (23). This plasmid contains 3.5 kilobases of the human NOS III promoter sequence (position −3,470 to +115) cloned before the luciferase reporter gene (23). Extracts of dexamethasone-treated cells and normal control cells were prepared by using the reporter lysis buffer, and luciferase activity was determined by using the luciferase assay system as described (21, 23).

**Electrophoretic Mobility Shift Assays.** Binding activities of transcription factors Sp1 and GATA in nuclear extracts from dexamethasone-treated or untreated cells were determined by electrophoretic mobility shift assays as described (21). Ten micrograms of nuclear protein were incubated with 17.5 fmol 32P-labeled double-stranded oligonucleotide containing a consensus Sp1-binding motif (5’-ATTTCGATCGGGCGCGCGACGC-3’, Promega), the Sp1 element (positions −109 to −91) of the human NOS III promoter (5’-GGTAGGGGGCGGGCG-
GAGG-3′) (27), a consensus GATA-binding motif (5′-CCTGATAAAGGATAACTCT-3′, Santa Cruz Biotechnology), or the GATA site (positions −239 to −218) of the human NOS III promoter (5′-GCTCCACTTACGCCTGATIGA-3′) (27). Specificity of binding was determined by adding excess (1.75 pmol) unlabeled oligonucleotide. Gels were autoradiographed on x-ray film.

**Statistics.** Data represent means ± SEM. Statistical differences were determined by Wilcoxon signed rank test or by factorial analysis of variance followed by Fisher’s protected least-significant-difference (PLSD) test for comparison of multiple means.

**Results**

**Ingestion of Dexamethasone Increased Blood Pressure and Decreased Serum NO2−/NO3− in Rats.** Untreated rats were normotensive with 24-h average blood pressures of 121.3 ± 1.1/88.1 ± 0.9 mmHg (systolic/diastolic). When the dexamethasone treatment was started, the water intake of the rats decreased initially; the target dose of 0.3 mg/kg/day was reached after ~5 days (Fig. 1). Dexamethasone treatment resulted in an increase in blood pressure that reached a plateau after 1 week of treatment (Fig. 1). When dexamethasone treatment was discontinued [by tapering off the daily dose over 3 days (Fig. 1)], blood pressure returned to normal levels (Fig. 1). When dexamethasone treatment was discontinued [by tapering off the daily dose over 3 days (Fig. 1)], blood pressure returned to normal levels (Fig. 1) and after (C) dexamethasone treatment. Values and bars represent means ± SEM obtained from five rats.

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Dexamethasone Treatment Decreased NOS III mRNA Levels in the Aorta and Other Tissues. When rats were treated with dexamethasone (0.3 mg/kg/day) as described above and killed on days 3, 6, and 9 of treatment, NOS III mRNA levels in the aorta were found to be reduced by up to 40% (Fig. 2). The dexamethasone treatment also reduced NOS III mRNA levels in the liver and the kidney (Fig. 2) but not in the heart (n = 5, data not shown).

Dexamethasone Treatment Selectively Attenuated Endothelium-Dependent Vasodilatation in Resistance Arterioles. Dexamethasone treatment (~0.3 mg/kg/day) of C57/BL 6J mice significantly attenuated the vasorelaxation in response to the endothelium-dependent vasodilator acetylcholine (10 μM; Fig. 3). The same dexamethasone treatment had no significant effect on the vasorelaxation of resistance arterioles to superfusion with 10 μM SNAP (Fig. 3).

Glucocorticoids Decreased NOS III mRNA Levels in Three Types of Endothelial Cells. When EA.hy 926 cells, human umbilical vein endothelial cells, or bovine aortic endothelial cells were treated with dexamethasone (10 nM to 1 μM for 18 h), NOS III mRNA decreased to 60–70% of control (Fig. 4). Also, beclomethasone
Asterisks indicate significant differences from control animals (**). Intravital microscopy in awake C57BL/6J mice. Shown is the vasorelaxation of resistance arteries as percent of baseline microvessel diameter. The mean baseline diameters were 37.9 ± 6.5 μm in control animals (Co, white bars) and 45.0 ± 9.8 μm in dexamethasone-treated animals (Dex, gray bars). Depicted in the figure are changes in the responses to acetylcholine (Ach, 10 μM) and SNAP (10 μM). Data represent means ± SEM of n = 7 animals per group. Asterisks indicate significant differences from control animals (***, P < 0.001, Wilcoxon rank sum test) n.s., not significant.

(10 nM to 1 μM) and dihydrocortisol (0.1 to 100 μM) reduced NOS III mRNA expression in EA.hy 926 cells to a similar extent (n = 3 each, data not shown). The inhibition of NOS III mRNA expression by dexamethasone was prevented by the glucocorticoid receptor antagonist mifepristone (Fig. 4a). The antagonist alone (1 to 10 μM) had no effect on NOS III mRNA expression (Fig. 4a).

**Dexamethasone Treatment Reduced NOS III Protein and NO Production in Endothelial Cells.** Incubation of EA.hy 926 cells with dexamethasone (10, 100, and 1,000 nM) for 36 h reduced NOS III protein expression to 79.8 ± 4.5, 74.2 ± 3.1, and 68.5 ± 4.1% of control (n = 4; Fig. 5a). Also, NO production by EA.hy 926 cells was reduced after treatment of the cells with dexamethasone for 36 h (Fig. 5b) or with 10 μM dihydrocortisol for 36 h (reduction to 72.6 ± 4.3% of control, n = 3, data not shown).

**Dexamethasone Treatment Destabilized the NOS III mRNA and Inhibited the Activity of the NOS III Promoter.** When transcription was blocked with 10 μg/ml actinomycin D added to the incubation medium of EA.hy 926 cells and RNA was prepared from these cells 6 to 48 h later, NOS III mRNA levels were found to decline over time (Fig. 6a). The approximate half-life of the mRNA was 48 h. Treatment of EA.hy 926 cells with dexamethasone (100 nM, 18 h) reduced the stability of the NOS III mRNA (Fig. 6a). EA.hy 926 cells stably transfected with the plasmid pNOS III-Hu-3500-Luc-neo showed significant NOS III promoter activity. When the transfected cells were incubated for 18 h with 10–1,000 nM dexamethasone, promoter activity decreased to ~70% of control (Fig. 6b).

**Effect of Dexamethasone on the Binding of Nuclear Extracts to Transcription Factor-Binding DNA Motifs.** The known sequence of the human NOS III promoter (GenBank accession no. AF032908) contains no bona fide glucocorticoid response element. Therefore, we tested the effect of dexamethasone on the binding activity of other transcription factors known to be important for the activity of the human NOS III promoter, namely Sp1 and GATA (27–29). As shown in Fig. 7, dexamethasone treatment of EA.hy 926 cells reduced the binding activities of nuclear extracts to an oligonucleotide containing the GATA site of the human NOS III promoter (positions −239 to −218) in a concentration-dependent fashion (n = 4). The same results were obtained with an oligonucleotide containing a consensus GATA binding motif (n = 5, data not shown). On the other hand, nuclear extracts from dexamethasone-treated endothelial cells showed unchanged binding activity to oligonucleotides containing the Sp1 element of the human NOS III promoter (positions...
with dexamethasone on the NO production of EA.hy 926 cells stimulated for 36 h on NOS III protein expression and NOS activity. A Western blot analysis using a polyclonal anti-NOS III- and a monoclonal anti-β-tubulin-antibody (for normalization). Combined cytosolic and solubilized particulate protein fractions were prepared from untreated EA.hy 926 cells [control (Co)] and EA.hy 926 cells incubated with dexamethasone (10−1,000 nmol) for 36 h. M, molecular weight marker. The blot shown is representative of four independent experiments with similar results. b shows the effect of a 36-h incubation with dexamethasone on the NO production of EA.hy 926 cells stimulated for 2 min with Ca²⁺ ionophore A23187 (10 μM). NO production was measured by the activation of soluble guanylyl cyclase in RFL-6 reporter cells. cGMP content of the RFL-6 cells after a 2-min incubation was determined by radioimmunoassay. Basal cGMP content of the RFL-6 cells (2.12 ± 0.4 pmol/10⁶ cells) was subtracted from all samples. Data represent means ± SEM of three independent experiments. Asterisks indicate significant differences from untreated cells (***, P < 0.001 by ANOVA and Fisher’s PLSD test).

Discussion

Because NOS III is a vasoprotective enzyme (8), changes in its expression and/or activity may have important consequences for vascular homeostasis. The NO produced by NOS III inhibitors and mice lacking the NOS III gene (2). Cushing’s syndrome is associated with hypertension, and, also, synthetic glucocorticoids—when administered for prolonged periods of time—are known to increase blood pressure (11). Although cortisol-induced hypertension is characterized by Na⁺ retention and volume expansion, studies with synthetic glucocorticoids or Na⁺ restriction suggest that the hypertension is, to a substantial degree, independent of Na⁺ and volume (11). Also, in the current study, the blood pressure-elevating effects of dexamethasone are unlikely to be attributable to marked Na⁺ and water retention because no change in plasma or urine Na⁺ or K⁺ concentrations were noted. Evidence for separate mechanisms of glucocorticoid- and mineralocorticoid-induced hypertension comes from experiments with Wistar-Furth rats. This strain does not develop hypertension when treated with salt and mineralocorticoids. Wistar-Kyoto rats developed hypertension when treated with either deoxycorticosterone acetate/NaCl or dexamethasone whereas Wistar-Furth rats developed hypertension only after dexamethasone treatment (30).

Increase in cardiac output is not essential for the glucocorticoid-induced rise in blood pressure, but the precise role of increases in peripheral resistance as a primary mechanism has not been determined. Increased pressor responsiveness, particularly to catecholamines and angiotensin II, is a prominent feature, but its mechanism is poorly understood (10, 11). In vitro studies have shown that the number of angiotensin II type 1 receptors of vascular smooth muscle cells is increased by glucocorticoids (11). Also, a reduced activity of depressor systems, such as the kallikrein-kinin system, vasodilator prostaglandins and NO, has been discussed (11).

In the current study, we have demonstrated that glucocorticoids lead to a substantial down-regulation of endothelial NOS mRNA and protein. This has been shown in vitro in three types of cultured endothelial cells from two species (Figs. 4 and 5) and in the aorta and some other rat tissues ex vivo (Fig. 2). The down-regulation was prominent in the liver, which is a highly vascularized organ that contains a large number of endothelial cells. The finding that NOS III mRNA expression was not reduced in the heart may be explained by the fact that, in this organ, most of the NOS III is present in cardiomyocytes (31), where its expression may be regulated differently from endothelial cells. The decreased protein expression of NOS III resulted in a reduced NO production, as demonstrated by a smaller stimulation of soluble guanylyl cyclase in RFL-6 cells in vitro (Fig. 5b) and lower serum levels of NO₂⁻/NO₃⁻ in rats in vivo. This was paralleled by an increase in blood pressure. A similar increase in blood pressure was achieved with a moderate...
dose of the NOS inhibitor L-NAME, which produced a similar reduction in plasma NO$_3^-$/NO$_2^-$ concentrations. The pathophysiologic relevance of the dexamethasone effect also was demonstrated in vivo by intravital microscopy, in which resistance arterioles of dexamethasone-treated mice exhibited significantly attenuated vasorelaxation in response to the endothelium-dependent vasodilator acetylcholine but not to the endothelium-independent vasodilator SNAP (Fig. 3).

The molecular mechanism of NOS III down-regulation by glucocorticoids seems to involve both mRNA destabilization (Fig. 6) and reduced transcription of the NOS III gene (Fig. 7). In these experiments, dexamethasone reduced only the GATA binding site to position 230 of the human NOS III promoter by about 70% of control (27). This may contribute to the blood pressure increases seen during systemic administration of glucocorticoids.

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