

Expressional Down-Regulation of Neuronal-Type Nitric Oxide Synthase I by Glucocorticoids in N1E-115 Neuroblastoma Cells

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ABSTRACT

Neuronal-type nitric oxide synthase (NOS I) is involved in ischemia-induced brain damage, and glucocorticoids have been reported to protect from brain damage. This prompted us to investigate if the activity or expression of NOS I was influenced by glucocorticoids. We used the murine neuroblastoma cell line N1E-115 as our experimental model. Short-term incubation (30 min) of the N1E-115 cells with dexamethasone (10 nM to 1 μ M) or hydrocortisone (100 nM to 10 μ M) did not change the enzymatic activity of NOS I. However, the glucocorticoids inhibited NOS I mRNA expression in a concentration-dependent fashion (down to $53.3 \pm 2.5\%$ of control). In time-course experiments with 100 nM dexamethasone, maximum down-regulation of NOS I mRNA was seen after 24 hr ($55.6 \pm 6.3\%$ of control). Similar effects were seen with 10 μ M hydrocortisone. The effect

of 100 nM dexamethasone was completely reversed by 1 μ M of the glucocorticoid receptor antagonist mifepristone. In experiments with actinomycin D (10 μ g/ml), the half-life of the NOS I mRNA was determined to be approximately 12 hr and remained unchanged after glucocorticoid incubation. Nuclear run-on analyses indicated that the decrease in NOS I mRNA was the result of a glucocorticoid-induced inhibition of NOS I gene transcription. In Western blots, the 160-kDa NOS I protein band was down-regulated to $68.5 \pm 8.4\%$ of control after an incubation of the N1E-115 cells with 100 nM dexamethasone for 26 hr. Similarly, NO production was down-regulated to $57.8 \pm 8.7\%$ of control. These data demonstrate that glucocorticoids reduce the expression of NOS I without changing its activity.

NO is synthesized by three isoforms of NO synthase (NOS; for review, see Förstermann *et al.*, 1995). The neuronal-type NOS (NOS I) and the endothelial-type NOS (NOS III) are regulated by Ca^{2+} and calmodulin, whereas the inducible-type NOS (NOS II) is largely or completely Ca^{2+} -independent. NOS I is found constitutively expressed in brain, peripheral neurons and other cell types (Förstermann *et al.*, 1995).

In the central nervous system, NO is a mediator of long-term potentiation of synaptic transmission (Yun *et al.*, 1996; for review, see Hölscher, 1997). Studies on mice with targeted disruptions of the NOS I- and the NOS III gene demonstrated that both isoforms are important for long-term potentiation (Son *et al.*, 1996). Beside these physiological and beneficial effects, NO produced by NOS I plays an important role in pathophysiology, such as glutamate-mediated neurotoxicity via NMDA receptors in focal ischemia (for review, see

Iadecola, 1997). After occlusion of the middle cerebral artery, NOS I mRNA was up-regulated and the number of NOS I-immunoreactive neurons in the ischemic area were increased (Zhang *et al.*, 1994). The detrimental role of NOS I in the development of ischemic injury is supported by studies on NOS I-knockout mice. Mice deficient in NOS I activity developed smaller infarcts after occlusion of the middle cerebral artery than normal mice (Huang *et al.*, 1994; Hara *et al.*, 1996). Similarly, the NOS inhibitor 7-nitroindazole, which exhibits a certain specificity for NOS I *in vivo*, reduced cerebral ischemic damage in rats caused by proximal middle cerebral artery occlusion (Yoshida *et al.*, 1994). Interestingly, the glucocorticoid dexamethasone also prevented brain damage when administered 24 hr before hypoxia-ischemia (Tuor *et al.*, 1993).

The present study was performed in search of a pharmacological tool controlling NOS I expression and/or activity. The murine neuroblastoma cell line N1E-115 was used as an *in vitro* cell model known to express NOS I (Förstermann *et al.*, 1990; Tracey *et al.*, 1993). This cell line represents an adrenergic clone that expresses tyrosine hydroxylase (Amano

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ABBREVIATIONS: NO, nitric oxide; NOS, nitric oxide synthase; NOS I, neuronal-type nitric oxide synthase; NOS II, inducible-type nitric oxide synthase; NOS III, endothelial-type nitric oxide synthase; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; PBS, phosphate-buffered saline; SSC, saline-sodium citrate buffer; TBS, Tris-buffered saline; nt, nucleotide; PIPES, piperazine-*N,N'*-bis(2-ethanesulfonic acid); SDS, sodium dodecyl sulfate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IBMX, 3-isobutyl-1-methylxanthine.

et al., 1972). The cell line retains several characteristics of neuronal cells such as neurite outgrowth during differentiation, electrical excitability (Kimhi *et al.*, 1976), and receptor-mediated NO production (Ishii *et al.*, 1989). It possesses several receptors for neurotransmitters. Using this cell line, we demonstrate that glucocorticoids down-regulate NOS I mRNA and protein, but have no effect on NOS I activity. The decreased NOS I expression results from a reduced NOS I transcription rate with unchanged NOS I mRNA stability.

Experimental Procedures

Materials. Actinomycin D, bovine serum albumin fraction V, calcium ionophore A23187, cycloheximide, dexamethasone, goat anti-rabbit antibody conjugated to alkaline phosphatase, horse antimouse antibody conjugated to alkaline phosphatase, hydrocortisone, mouse monoclonal antibody to β -tubulin, Nonidet P-40, and polyvinylpyrrolidone were obtained from Sigma Chemical (St. Louis, MO). DMEM, Ham's F-12 nutrient mixture, and SuperScript reverse transcriptase were from Life Technologies (Paisley, UK). ATP, CTP, *EcoRV*, Ficoll 400, GTP, *NcoI*, random hexamer primer, SureClone Ligation Kit, *Taq* DNA polymerase, *Taq* polymerase reaction buffer, and 32 P Sequencing Kit were from Pharmacia (Uppsala, Sweden). IBMX and superoxide dismutase were from Boehringer Ingelheim Bioproducts (Heidelberg, Germany). pCR-Script was from Stratagene (La Jolla, CA). *BstEII*, DNase I, RNase A, RNase T1, proteinase K, and T3 RNA polymerase were from Boehringer Mannheim (Mannheim, Germany). [α - 32 P]UTP was from ICN (Costa Mesa, CA). Mifepristone (RU 38486) was a generous gift of Roussel-Uclaf (Romainville, France).

Cell culture, drug treatment, and RNA isolation. The murine neuroblastoma cell line N1E-115 (American Type Culture Collection, Rockville, MD) was cultured in DMEM with 10% fetal bovine serum and 2 mM L-glutamine. For NOS I mRNA analyses, cells were incubated for 6 to 48 hr with dexamethasone (0.1 nM to 1 μ M), with hydrocortisone (10 μ M) or the vehicle DMSO. DMSO (concentration \leq 0.01%) did not affect NOS I mRNA expression. Neither dexamethasone, hydrocortisone, nor DMSO (at the above concentration) had any effect on cell viability. In other experiments, N1E-115 cells were incubated for 24 hr with 100 nM dexamethasone in the presence of the glucocorticoid receptor antagonist mifepristone (0.1 to 3 μ M; added 1 hr before dexamethasone). For determination of the half-life of the NOS I mRNA, cells were incubated for 6 to 24 hr with actinomycin D (10 μ g/ml) alone (control) or in the presence of dexamethasone (100 nM). Total RNA was isolated from the N1E-115 cells by acid guanidinium thiocyanate/phenol/chloroform extraction (Chomczynski and Sacchi, 1987).

Cloning of a murine NOS I- and β -actin cDNA fragment. Total RNA was isolated from mouse cerebellum as described above. Two micrograms of this RNA were annealed with 40 ng of random hexamer primers and reverse transcribed with SuperScript reverse transcriptase following the manufacturer's instructions. Reverse transcription-generated cDNAs encoding for murine NOS I and β -actin were amplified using the polymerase chain reaction. Oligonucleotide primers for NOS I were: 5'-ACCATCTTCAGGCTTCAAGTAC-3' (sense) and 5'-TGGACTCAGATCTAAGGCGGTTG-3' (antisense), corresponding to positions 3363–3386 and 4313–4335 of the murine NOS I cDNA (Ogura *et al.*, 1993). Oligonucleotide primers for β -actin were: 5'-ACCAACTGGGACGACATGGAG-3' (sense) and 5'-AGGATCTTCATGAGGTAGTC-3' (antisense), corresponding to positions 151–171 and 481–500 of the murine β -actin cDNA (Alonso *et al.*, 1986). The amplified cDNA fragments (NOS I, 973 base pairs; β -actin, 350 base pairs) were cloned into the *EcoRV* site of vector pCR-Script with the SureClone Ligation Kit, generating the cDNA clones pCR-NOS I-mouse and pCR- β -actin-mouse. NOS I- and β -actin cDNA were sequenced using the dideoxy-mediated chain termination method (32 P Sequencing Kit).

Preparation of antisense RNA probes. To generate radiolabeled antisense RNA probes for RNase protection analyses, the cDNA clones pCR-NOS I-mouse and pCR- β -actin-mouse were linearized with *NcoI* and *BstEII*, respectively, extracted with phenol/chloroform and concentrated by ethanol precipitation. This DNA (0.5 μ g) was *in vitro* transcribed for 60 min at 37°, using T3 RNA polymerase and [α - 32 P]UTP. Then, the template DNA was degraded with DNase I (RNase-free; 10 units/ μ l) for 45 min at 37°, and the labeled RNA was precipitated with ethanol.

RNase protection analyses. RNase protection analyses were performed with the above [α - 32 P]UTP-labeled probes as described (Sambrook *et al.*, 1989). Briefly, after a denaturation step for 10 min at 85°, 20 μ g of total RNA (isolated from N1E-115 cells as described above) were hybridized for 14 hr at 51° with a 200,000-cpm labeled NOS I cRNA probe (252 nt) and a 30,000-cpm labeled β -actin cRNA probe (222 nt) in hybridization buffer (40 mM PIPES, pH 6.7, 400 mM NaCl, 1 mM EDTA, 50% formamide). Then, the hybridization mixture was incubated for 30 min at 30° with 300 μ l digestion buffer (10 mM Tris-HCl, pH 7.4, 300 mM NaCl, 5 mM EDTA) containing 3.5 μ g RNase A and 25 units of RNase T1. The reaction was stopped by adding 70 μ l of a buffer (10 mM Tris-HCl, pH 7.8, 5 mM EDTA, 2.85% SDS) containing 70 μ g of proteinase K. After incubation for 15 min at 37° and phenol/chloroform extraction, the samples were concentrated by ethanol precipitation and analyzed by electrophoresis using 6% polyacrylamide/8 M urea gels. Gels were dried and exposed to X-ray films for two days. Densitometric analyses were performed using the Phospho-Imager system (BioRad, Munich, Germany). The protected RNA fragments for NOS I and β -actin were 180 nt and 108 nt, respectively.

Nuclear run-on transcription analysis. Nuclear run-on analyses were performed essentially as described previously (Kleinert *et al.*, 1998). Confluent N1E-115 cells were maintained in culture for 24 hr with or without dexamethasone (100 nM). Cells were scraped from the tissue culture plates in Versen buffer (8.1 mM NaH₂PO₄, 136.8 mM NaCl, 1.4 mM KH₂PO₄, 2.6 mM KCl, 1 mM EDTA, 1.1 mM glucose, pH 7.4), and centrifuged at 500 \times g for 10 min at 4°. The cellular pellet was then washed twice with ice-cold PBS, resuspended in a lysis buffer containing 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, and 0.5% Nonidet P-40, incubated on ice for 5 min and centrifuged at 500 \times g for 5 min. The pellet was washed two more times with the lysis buffer and the final nuclear pellet was resuspended in 100 μ l of a buffer containing 50 mM Tris-HCl, pH 8.3, 5 mM MgCl₂, 0.1 mM EDTA, and 40% (v/v) glycerol. *In vitro* transcription of nuclear pellet (100 μ l) was carried out at 30° for 45 min in a buffer containing 5 mM Tris-HCl, pH 8.0, 2.5 mM MgCl₂, 150 mM KCl, 0.25 mM each of ATP, CTP, GTP, and 80 μ Ci of [α - 32 P]UTP (800 Ci per mmol). The transcription reaction was terminated by the addition of 400 units of DNase I and a further 15 min-incubation at 30°. Proteins in the sample were digested at 37° for 30 min with 80 μ g proteinase K in 1% (final concentration) SDS. After a phenol/chloroform extraction, the radiolabeled RNA transcripts were collected by ethanol precipitation. Equal amounts (5 μ g) of purified, linearized, and denatured *XcmI*-based vectors (Borovkov and Rivkin, 1997) without insert, or containing murine NOS I (1.1 kilobase pairs) or murine glyceraldehyde-3-phosphate dehydrogenase (0.9 kilobase pairs) cDNA fragments generated by RT-PCR, were dotted onto nylon membranes. After cross-linking, the membranes were prehybridized at 65° for 4 hr in 6 \times SSC (0.9 M NaCl, 0.09 M sodium citrate, pH 7.0), 5 \times Denhardt's reagent (0.1 g of Ficoll 400, 0.1 g of polyvinylpyrrolidone, 0.1 g of bovine serum albumin fraction V in 100 ml of H₂O), 0.1% SDS, and 100 μ g/ml denatured salmon sperm DNA. Hybridization of the radiolabeled transcripts to strips of nylon membrane was carried out at 65° for 48 hr in the same buffer. After hybridization, the strips were washed twice for 30 min with 2 \times SSC and 0.1% SDS at room temperature and twice with 0.5 \times SSC and 0.1% SDS at 65° before autoradiography. Densitometric analyses were performed with a Phospho-Imager (BioRad).

Western blotting. For Western blotting, N1E-115 cells were incubated for 26 hr in DMEM with or without 100 nM dexamethasone. Combined soluble and CHAPS-solubilized particulate protein fractions from N1E-115 cells (100 μ g each) were separated on 7.5% SDS-polyacrylamide gels (Laemmli, 1970) and electroblotted to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Blots were blocked for 60 min at room temperature in TBS (10 mM Tris-HCl, pH 7.4, 154 mM NaCl) containing 5% (w/v) nonfat dry milk, 0.05% (w/v) Tween 20 and 10% (v/v) goat serum. They were then cut in half at about 65 kDa. The upper part was incubated overnight at 4° with a rabbit polyclonal antibody to NOS I (1:2,000; Schmidt *et al.*, 1992) in PBS containing 1% (w/v) bovine serum albumin and 0.1% (w/v) Tween 20; the lower part was incubated for standardization with a mouse monoclonal antibody to β -tubulin (1:750) in the same incubation medium. After three washes with TBS containing 5% (w/v) nonfat dry milk and 0.05% (w/v) Tween 20, the blots were incubated for 60 min at room temperature with the appropriate alkaline phosphatase-conjugated secondary antibodies (a goat anti-rabbit antibody and a horse anti-mouse antibody). After three washes with TBS, bands were visualized with 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium chloride.

Determination of NO production. NO production by N1E-115 cells was bioassayed using RFL-6 rat lung fibroblasts (Förstermann *et al.*, 1990). RFL-6 cells were cultured to confluence on 6-well plates in Ham's F12 nutrient mixture (supplemented with 15% fetal bovine serum and 1 mM L-glutamine). They were washed twice with PBS and incubated for 30 min at 37° in Locke's solution (154.0 mM NaCl, 5.6 mM KCl, 2.0 mM CaCl_2 , 1.0 mM MgCl_2 , 3.6 mM NaHCO_3 , 5.6 mM glucose, 10.0 mM HEPES, pH 7.4) containing 0.6 mM IBMX. Confluent N1E-115 cells were cultured in 6-well plates in DMEM (with 10% fetal bovine serum and 2 mM L-glutamine). Dexamethasone (10 nM to 1 μ M), hydrocortisone (100 nM to 10 μ M) or vehicle (\leq 0.01% DMSO) were added to the cells for the last 30 min before the experiments (effect on NOS activity). To determine its effect on NOS expression, 100 nM dexamethasone or vehicle (0.001% DMSO) was added during the last 26 hr before the experiments. After aspiration of the culture medium, cells were washed twice with PBS and incubated for 30 min at 37° in Locke's solution containing 20 units/ml superoxide dismutase. For determination of basal NO release, N1E-115 cells were incubated for 2 min at 37° in 1 ml Locke's solution containing 0.3 mM IBMX and 20 units/ml superoxide dismutase; other cells were stimulated with 10 μ M A23187 for 2 min. Then, the conditioned media were transferred to the RFL-6 cells. After a 2-min incubation at 37° on the RFL-6 cells, the reaction was stopped by aspiration of the solution, adding 1 ml of ice-cold 50 mM sodium acetate buffer, pH 4.0, and rapidly freezing the cells with liquid nitrogen. The cGMP content of the RFL-6 samples was determined by radioimmunoassay as described (Förstermann *et al.*, 1990).

Data analysis. Data represent mean \pm standard error of the indicated number of independent experiments. Statistical differences were determined by factorial analysis of variance followed by Fisher's protected least-significant-difference test for comparison of multiple means.

Results

Lack of an acute effect of glucocorticoids on NO production in N1E-115 cells. A 30-min incubation of N1E-115 cells with dexamethasone (10 nM to 1 μ M; four experiments) or hydrocortisone (100 nM to 10 μ M; four experiments) did not affect basal (data not shown; four experiments) or calcium ionophore (A23187)-stimulated NO production of the N1E-115 cells, which suggests that NOS activity was not affected (Table 1).

Down-regulation of NOS I mRNA by glucocorticoids in N1E-115 cells. Incubation of N1E-115 cells with 100 nM dexamethasone for 6 to 48 hr resulted in a reduction of NOS I

mRNA expression (Fig. 1). Densitometric analyses of five independent experiments showed a down-regulation to $74.8 \pm 2.7\%$ of control at 6 hr, $62.2 \pm 4.5\%$ at 12 hr, $55.6 \pm 6.3\%$ at 24 hr and $61.0 \pm 5.5\%$ at 48 hr (Fig. 1; five experiments; $p < 0.001$ versus untreated control). Similarly, hydrocortisone (10 μ M) produced a time-dependent decrease of NOS I mRNA levels. The maximum effect was seen after 48 hr (inhibition to $60.0 \pm 1.5\%$ of control; four experiments; $p < 0.001$). Incubation of N1E-115 cells with increasing concentrations of dexamethasone ranging from 0.1 nM to 1 μ M for 6 or 24 hr also demonstrated a concentration-dependent reduction of NOS I mRNA expression (Fig. 2). The inhibition of NOS I mRNA expression by 100 nM dexamethasone was completely reversed by the glucocorticoid receptor antagonist mifepristone (Fig. 3). The antagonist alone had no effect on NOS I mRNA expression (three experiments; data not shown).

To investigate if dexamethasone leads to a destabilization of the NOS I mRNA, experiments were performed with 10 μ g/ml actinomycin D (Fig. 4). In these experiments, the half-life of the NOS I mRNA was about 12 hr and remained unchanged in the presence of 100 nM dexamethasone (Fig. 4).

Nuclear run-on analyses performed with nuclei from control and dexamethasone-treated N1E-115 cells demonstrated that dexamethasone inhibited NOS I gene transcription to $77.9 \pm 6.8\%$ of control (three experiments; Fig. 5).

Incubation of N1E-115 cells with the protein synthesis inhibitor cycloheximide (10 μ g/ml) for 6 hr did not block the down-regulation of NOS I mRNA expression in response to 100 nM dexamethasone ($79.4 \pm 8.3\%$ of control in the presence of cycloheximide versus $70.9 \pm 5.8\%$ in the absence of the inhibitor; three experiments).

Down-regulation of NOS I protein by dexamethasone in N1E-115 cells. Western blots using a specific polyclonal antibody to NOS I demonstrated the 160-kDa protein in the combined soluble and CHAPS-solubilized particulate fractions of N1E-115 cells (Fig. 6). Incubation of the cells with 100 nM dexamethasone for 26 hr reduced NOS I protein expression to $68.5 \pm 8.4\%$ of control (as quantified with a monoclonal antibody to β -tubulin; three experiments; $p < 0.01$; Fig. 6).

Down-regulation of NO production by dexamethasone in N1E-115 cells. A 2-min incubation of the RFL-6

TABLE 1

Lack of an acute effect of glucocorticoids on A23187-stimulated NO production in N1E-115 cells.

Cells were incubated for 30 min in the absence or presence of the indicated concentrations of dexamethasone or hydrocortisone. A23187 (10 μ M)-stimulated NO production was measured as cGMP increase in RFL-6 reporter cells produced by the conditioned media of N1E-115 cells. The basal cGMP content of the RFL-6 cells (2.32 ± 0.07 pmol/ 10^6 cells) was subtracted from all values. The cGMP production stimulated by nonglucocorticoid-treated N1E-115 cells (12.23 ± 0.75 pmol/ 10^6 cells) was set at 100%. Data represent mean \pm standard error of four experiments.

Glucocorticoid	A23187-stimulated NO production
μ M	% of untreated control
Dexamethasone	
0.01	108.82 \pm 15.38
0.1	91.44 \pm 9.92
1	101.22 \pm 16.66
Hydrocortisone	
0.1	113.64 \pm 16.51
1	99.86 \pm 6.96
10	91.99 \pm 28.68

fibroblasts with the conditioned medium of N1E-115 cells stimulated with 10 μ M of the calcium ionophore A23187 resulted in an about 11-fold increase in the cGMP content (Fig. 7). This increase was markedly reduced to $57.8 \pm 8.7\%$ of control values when N1E-115 cells were pretreated for 26

hr with 100 nM dexamethasone (Fig. 7). Basal NO production was also reduced in dexamethasone-treated cells compared with control cells (0.81 ± 0.12 pmol cGMP/ 10^6 cells versus 1.17 ± 0.20 pmol cGMP/ 10^6 cells; Fig. 7).

Discussion

NOS I was originally considered to be a constitutively expressed enzyme (Förstermann *et al.*, 1995). In recent years, however, evidence has accumulated that NOS I can be subject to expressional regulation by physiological and pathophysiological stimuli (for review, see Förstermann *et al.*, 1998). The present study demonstrates in N1E-115 cells that glucocorticoids have no acute effect on NOS I activity, but down-regulate the expression of NOS I mRNA and protein when present for prolonged periods of time. Our data show that glucocorticoids inhibit transcription of the NOS I gene. The half-life of NOS I mRNA remained unchanged after dexamethasone. Interestingly, the half-life of NOS I was found to be about 12 hr (i.e. much shorter than the

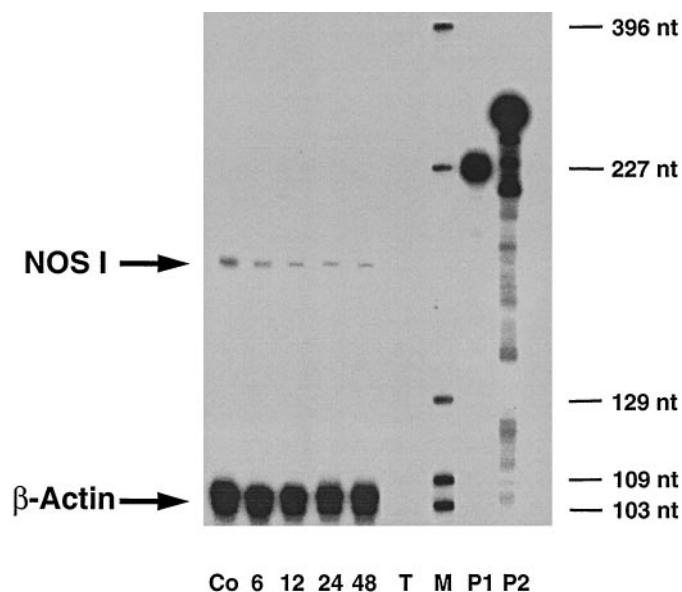


Fig. 1. RNase protection analysis of RNAs from N1E-115 cells using antisense RNA probes for murine NOS I and β -actin (for standardization). Cells were either left untreated (Co) or incubated for 6, 12, 24 or 48 hr with 100 nM dexamethasone. T, tRNA; M, molecular size marker; P1, undigested NOS I probe; P2, undigested β -actin probe. The gel is representative of five experiments with similar results.

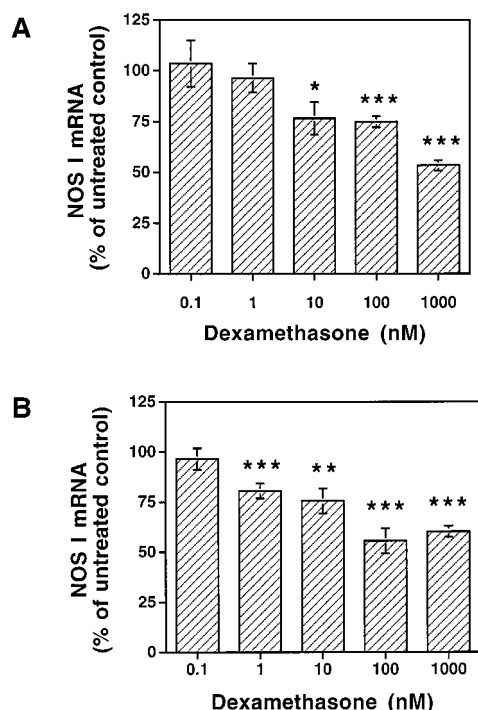


Fig. 2. Effect of dexamethasone on NOS I mRNA expression in N1E-115 cells. Confluent cells were incubated for 6 hr (A) or 24 hr (B) with increasing concentrations of dexamethasone. Total RNA was extracted and RNase protection analyses performed as described in Fig. 1. Columns, densitometric analyses of three to eight experiments (mean \pm standard error). ***, $p < 0.001$; **, $p < 0.01$; and *, $p < 0.05$ versus untreated control cells (100%).

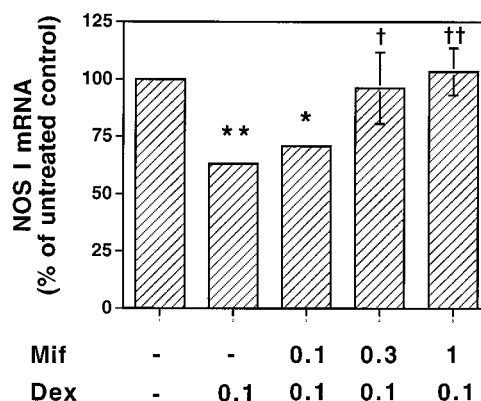


Fig. 3. Reversal of the dexamethasone (Dex)-induced down-regulation of NOS I mRNA by the glucocorticoid receptor antagonist mifepristone (Mif). N1E-115 cells were incubated for 24 hr with 100 nM dexamethasone alone or in the presence of increasing concentrations of mifepristone (0.1 to 3 μ M; added 1 hr before dexamethasone). Total RNA was extracted and RNase protection analyses performed as described in Fig. 1. Columns, densitometric analyses of three to five experiments (mean \pm standard error). **, $p < 0.01$ and *, $p < 0.05$ versus untreated control cells; †, $p < 0.01$; ††, $p < 0.05$ versus 100 nM dexamethasone.

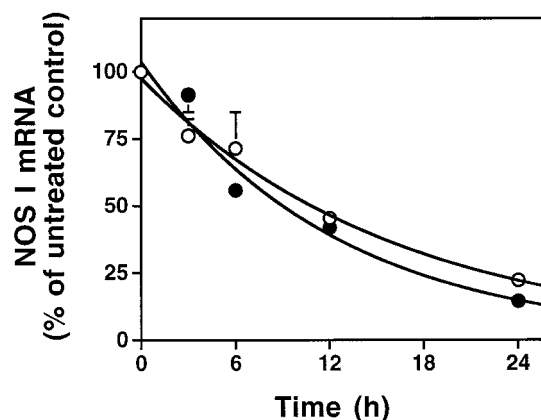


Fig. 4. Effect of dexamethasone on the half-life of the NOS I mRNA. N1E-115 cells were incubated for 24 hr with 10 μ g/ml actinomycin D in the presence (●) or absence (○) of 100 nM dexamethasone. Total RNA was extracted and RNase protection analyses performed as described in Fig. 1. Symbols, densitometric analyses of three to four experiments (mean \pm standard error; within symbol if not visible).

44–48 hr described for NOS III) (Yoshizumi *et al.*, 1993; Liao *et al.*, 1995; Li *et al.*, 1998). As cycloheximide had no effect on NOS I mRNA expression, the putative transcriptional down-regulation by dexamethasone does not seem to involve protein *de novo* synthesis. The effect of dexamethasone is likely to be a specific receptor-mediated process because it was inhibited by mifepristone. It occurred at concentrations equal to or below therapeutic plasma concentrations. Depending on the dosage and application method these can range from 10 nM to 1 μ M (English *et al.*, 1975; Brady *et al.*, 1987).

In *in vivo* models, steroid hormones have been shown previously to alter NOS I expression. Estradiol and pregnancy increased NOS I in rat hypothalamus and skeletal muscle (Weiner *et al.*, 1994; Ceccatelli *et al.*, 1996; Xu *et al.*, 1996). Corticosterone treatment (40 mg/kg) of rats for 20 days resulted in a decrease of NOS I RNA (and an increase of heme oxygenase 2 RNA) in the hippocampus (Weber *et al.*, 1994).

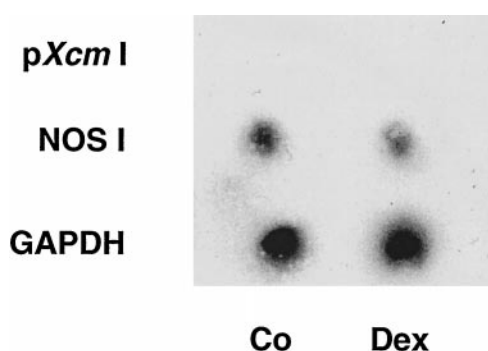


Fig. 5. Nuclear run-on analysis of nuclei from N1E-115 cells. Autoradiograph of a filter with linearized plasmids containing either a murine NOS I cDNA fragment (*central lane*), a murine glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) cDNA fragment (*lower lane*) or the plasmid DNA alone (*pXcm I*; *upper lane*). To this filter, radiolabeled RNA was hybridized that was obtained by *in vitro* transcription with nuclei from N1E-115 cells incubated for 24 hr in the absence (*Co*) or presence of 100 nM dexamethasone (*Dex*). The filter is representative of three experiments with similar results.

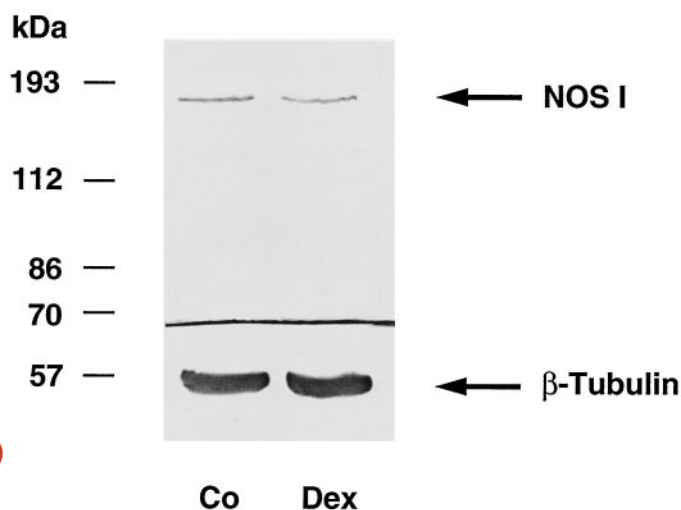


Fig. 6. Western blot analysis of the combined soluble and CHAPS-solubilized particulate fractions of N1E-115 cells. Cells were incubated for 26 hr in the absence (*Co*) or presence of 100 nM dexamethasone (*Dex*). Protein samples were separated on 7.5% polyacrylamide gels and electroblotted to nitrocellulose membranes. The upper part of the blot was incubated with a polyclonal antibody to NOS I; the lower part was incubated with a monoclonal antibody to β -tubulin (for standardization). The gel is representative of three experiments with similar results.

The increased NOS I mRNA levels in the rat hippocampus after lithium and tacrine administration could also be normalized by corticosterone (Bagetta *et al.*, 1993).

Corticosterone or dexamethasone has been shown to protect against glutamate-induced neurotoxicity in *in vivo* and *in vitro* studies (Zoli *et al.*, 1991; Ogata *et al.*, 1993; Page and Morton, 1995). Corticosteroids have also been shown to protect against cellular injury, such as that which occurs after ischemia and reperfusion (Hall, 1993). In a neonatal rat model of hypoxia-ischemia, pretreatment with dexamethasone for ≥ 3 hr prevented neuronal injury without affecting cerebral blood flow (Barks *et al.*, 1991; Tuor *et al.*, 1993). This protective effect was not seen when dexamethasone was given immediately before an hypoxic-ischemic episode (Barks *et al.*, 1991).

Neuronal damage in response to ischemia or mediated by glutamate-receptors is likely to involve the action of NOS (for review, see Yun *et al.*, 1996). In ischemic brain injury, all three isoforms of NOS are being up-regulated. Shortly after induction of ischemia, NOS I- and NOS III expression has been shown to increase, whereas NOS II induction occurred with a delay of 6 to 12 hr after occlusion of the middle cerebral artery (for review, see Iadecola, 1997). Studies with relative selective NOS inhibitors or with knockout mice lacking specific NOS isoforms imply that both NOS I (Hara *et al.*, 1996; Huang *et al.*, 1994) and NOS II (Iadecola *et al.*, 1997) contribute to the ischemic brain damage, whereas NOS III is likely to have protective effects (Huang *et al.*, 1994).

Glucocorticoids are well established inhibitors of NOS II induction (Di Rosa *et al.*, 1990; O'Connor and Moncada, 1991; Geller *et al.*, 1993; Kleinert *et al.*, 1996). The current study demonstrates that NOS I expression can also be controlled by these steroids. The inhibition of expression of NOS I and NOS II in neuronal cells may contribute to the neuroprotective effects seen with glucocorticoids.

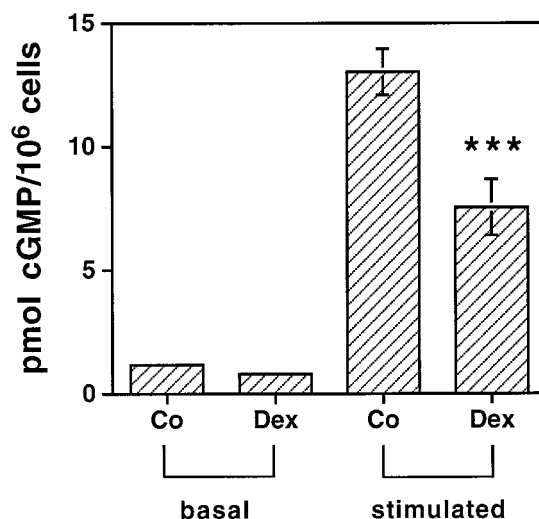


Fig. 7. Long-term effect of dexamethasone on NO production in N1E-115 cells. Cells were incubated for 26 hr in the absence (*Co*) or presence of 100 nM dexamethasone (*Dex*). Basal NO production, or NO production stimulated with 10 μ M A23187 were measured by the activation of soluble guanylyl cyclase in RFL-6 reporter cells. cGMP content of the RFL-6 cells after a 2-min incubation was determined by radioimmunoassay. The basal cGMP content of the RFL-6 cells (1.76 ± 0.01 pmol/ 10^6 cells) was subtracted from all samples. Data represent mean \pm standard error (four or five experiments). ***, $p < 0.001$, significant difference from stimulated control.

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