Nitric oxide regulates nitric oxide synthase-2 gene expression by inhibiting NF- κ B binding to DNA

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Treatment of astroglial cells with interleukin 1 β and interferon γ transcriptionally activates the nitric oxide synthase (NOS)-2 gene. The duration of mRNA expression is brief because of transcript instability. In addition, NO donors reduce the expression of NOS-2 mRNA dramatically by reducing the rate of transcription. In this study we observed that the NO donor, spermine NONOate did not inhibit the activation and translocation of NF- κ B, a key transcription factor in the induction of

NOS-2, but inhibited formation of the NF- κ B–DNA complex. This effect was reversed by methaemoglobin (acting as an NO trap) and by the reducing agent dithiothreitol. Formation of the interferon-regulatory factor–DNA complex was unaffected by NO. These results suggest that NO can modulate its own production by interfering with NF- κ B interaction with the promoter region of the NOS gene, a negative feedback effect that may be important for limiting NO production *in vivo*.

INTRODUCTION

NO is produced by a variety of cells and participates in very diverse functions [1]. The activity of the inducible NO synthase (NOS-2) is independent of intracellular Ca²⁺ or exogenous calmodulin and can be induced in central nervous system astroglial cells *in vitro* by bacterial endotoxin or combinations of cytokines [2]. The cDNAs for the rodent and human astrocyte NOS-2 have been cloned and sequenced [3,4]. There is evidence for NOS-2 expression in astrocytes *in vivo*, associated with particular neuropathologies [5]. Since NOS-2 can produce a large amount of NO over a period of hours, its induction and expression must be finely regulated. Corticosteroids, anti-inflammatory cytokines [6] and a variety of neurotransmitters block transcriptional activation of the glial NOS-2 gene [7–9].

The expression of NOS-2 mRNA expression in astrocytes *in vitro* is brief [3,10] and it has been suggested that the transcript may be destabilized by a translation-dependent mechanism [11], as is the case for many short-lived cytokine and proto-oncogene transcripts [12]. In addition, the catalytic activity of NOS-2 is inhibited by NO itself [13,14]. We have shown previously that NO limits NOS-2 mRNA expression by blocking transcription [15,16], and others have reported that chronic NOS inhibition in a model of liver inflammation results in an increase in NOS-2 mRNA and protein [17]. Here, we provide evidence to suggest that NO inhibits transcription of the NOS-2 gene by interfering with binding of nuclear factor (NF)- κ B to its target DNA sequence.

MATERIAL AND METHODS

Materials

Unless otherwise specified, all reagents were obtained from Sigma or from sources previously cited. Antibodies specifically directed against p65 (RelA) and p50 subunits of NF- κ B were purchased from Rockland (Gilbertsville, PA, U.S.A.) and Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.).

Cell culture and induction of NOS-2

Astroglial cells (> 99% pure) were derived from neonatal rat cerebral cortex, and cultures passaged once as described previously [6]. To induce NOS-2, astrocytes were treated with a combination of recombinant human interleukin (IL) 1 β (500 pg/ml) and recombinant rat interferon (IFN) γ (50 units/ml) for the times indicated in the Figure legends.

Cell viability

This was determined in two ways. Cultures were treated with spermine NONOate (100 μ M, up to three times), and 6 h later the medium was assayed for lactate dehydrogenase as described previously [18]. In addition, a clonogenic survival assay [19] was performed. Neither suggested that such treatment with the NO donor altered viability of the cells.

Preparation of nuclear and cytosolic extracts

Nuclear and cytosolic extracts were isolated as described previously [7]. To prepare nuclear extracts for electrophoretic mobility-shift assays, dithiothreitol (DTT) was omitted from the extraction buffer, and the isolation procedure was conducted under nitrogen to protect NF- κ B from oxidation.

Western blotting

This was conducted as described previously [7]. Briefly, protein in cytosolic and nuclear extracts was estimated, and equal amounts (30 μ g) were resolved in a 7.5% polyacrylamide/SDS gel, transferred to nitrocellulose and probed with antibodies against the p65 and p50 subunits of NF- κ B.

Preparation of oligonucleotide probes

Each single strand for the NF- κ B, γ -activating sequence (GAS) and IFN-regulatory factor (IRF)-binding element (IRF-E) sites was synthesized (DNA Core, University of Iowa) on the basis of

Abbreviations used: DMPO, 5,5-dimethyl-1-pyrroline N-oxide; DTT, dithiothreitol; GAS, γ -activating sequence; Hb, methaemoglobin; IFN, interferon; IL, interleukin; IRF, interferon regulatory factor; NOS, nitric oxide synthase; NF- κ B, nuclear factor κ B.

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promoter region analysis of the mouse NOS-2 gene, and annealed by boiling for 10 min at 95 °C and by cooling at room temperature for 3 h. The oligonucleotide double strand was filled by the Klenow fragment of DNA polymerase I with [α - 32 P]dCTP and the three other non-radiolabelled dNTPs. The mutant NF- κ B oligomer was obtained from Santa Cruz Biotechnology; the mutation (ninth base, top mutant sequence) is italicized.

5'-CAGTGGGACTCTCCCT-3'	NF- κ B specific oligomer
3'-ACCCCTGAGAGGGAGCA-5'	
5'-AGTTGAGGCGACTTTCAGGC-3'	NF- κ B mutant oligomer
3'-TCAACTCCGCTGAAAGGGTCCG-5'	
5'-CACTGTCAATATTTAC-3'	IRF-E oligomer
3'-GACAGTTATAGTAAAGTATTA-5'	
5'-ATCCTAGAAGTTACTCTGTAGAC-3'	GAS oligomer
3'-ATCTTGAATGAGACATCTGGTCCG-5'	

Electrophoretic mobility-shift assay

To visualize DNA binding of transcription factors, nuclear extracts (10 μ g of protein) were incubated with 1 μ g of poly-(dI-dC)·poly(dI-dC) in 10 mM Tris/HCl, pH 7.5, containing 0.1 mM EDTA and 5 mM MgCl₂ for 15 min at room temperature. Where used, DTT, 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) or NO donors were added at this point. The 32 P-labelled oligonucleotide probe [(1–2) $\times 10^5$ c.p.m.] was then added and incubated for 30 min at room temperature. In competition assays, excess oligonucleotide competitor was preincubated with nuclear extracts for 15 min at room temperature. In supershift assays, antibodies were added 30 min before 32 P-labelled oligonucleotide. At the end of the incubation, 10 $\times$ DNA loading buffer was added and the sample was electrophoresed in a native 4.5 % polyacrylamide gel (acrylamide/bisacrylamide, 19:1, v/v) in buffer (pH 8.5) containing 90 mM Tris, 90 mM boric acid and 2 mM EDTA at a constant voltage (10 V/cm). Gels were dried and exposed to X-ray film with intensifying screens overnight at –70 °C.

RESULTS

Reactive oxygen intermediates are potential activators of NF- κ B [20], and we have observed that the spin-trapping agent DMPO and pyrrolidine dithiocarbamate, a metal chelator and free radical scavenger, significantly block NOS-2 mRNA induction in astrocytes [16]. Thus we suspected that the NO from spermine NONOate ($t_{1/2}$ = 40 min) might interact with an oxygen free radical essential for NF- κ B activation, and so we examined translocation of the p65 and p50 subunits of NF- κ B into astrocyte nuclei by immunoblotting with specific antibodies (Figure 1). Spermine NONOate alone appeared to increase the translocation of p65 into nuclei (Figure 1A) but did not increase p50 (Figure 1B), implying that p65 and p50 activation and/or translocation may be differentially regulated. We detected no obvious deficits in the translocation of p65 or p50 in astrocytes activated by cytokines in the presence of NONOate (Figures 1A and 1B), and there were no variations in the cytosolic levels of p65 and p50 between treatment groups (results not shown). Therefore it appears that the effect of NO on NOS-2 gene expression is

Figure 1 Translocation of NF- κ B subunits revealed by immunoblotting

Nuclear extracts were prepared as described in the Materials and methods section. Astrocytes were treated with 100 μ M spermine NONOate (NONO) 1 h before cytokine (IL-1 β /IFN- γ) treatment and harvested 1 h after cytokine treatment. The nuclear extracts (N; 20 μ g of protein) prepared in the absence or presence of cytokines (IL-1 β /IFN- γ) and/or 100 μ M spermine NONOate (NONO) were resolved by SDS/PAGE (7%) and immunoblotted using antibodies against the p65 (A) and p50 (B) subunits of NF- κ B. Extracts of cytoplasm (C) represent positive controls. A representative of two experiments is shown.

downstream from NF- κ B activation and translocation to the nucleus.

To study downstream interactions between NF- κ B and its target DNA sequence, electrophoretic mobility-shift assays were conducted. Formation of the NF- κ B–DNA complex was abolished by an excess of non-radiolabelled wild-type NF- κ B-specific oligomer, but was not affected by a mutant NF- κ B oligomer (Figure 2A). The NF- κ B–DNA complex was supershifted almost completely with an antibody against p50, but there was little shift with an antibody against p65. Although we cannot rule out the possibility of p65 participation, the data suggest that p50 homodimers are important NF- κ B complexes for the regulation of rat astroglial NOS-2 gene expression. This result is similar to that of a study using mouse macrophages [21], but different from another study which shows that the major NF- κ B complex in rat astrocytes activated by tumour necrosis factor α is the p65 homodimer [22]. Thus the constituents of the NF- κ B complex activated may be different from cell to cell and vary with the nature of the stimulus.

We next investigated whether spermine NONOate interfered with the binding of NF- κ B to its target sequence. As seen in Figure 2(B), no NF- κ B–DNA complex was formed by nuclear extracts from cytokine-activated cells subsequently treated with NONOate. However, binding is readily apparent in the presence of a reducing agent, DTT (Figure 2B), suggesting that this loss of complex-formation is redox-regulated. The spin trap, DMPO, did not affect NF- κ B–DNA complex-formation when added directly to nuclei. Exposure to a spermine NONOate solution that had liberated all its NO (maintained at room temperature for 2 h) produced no effect.

Diethylamine NONOate, another NO-releasing agent but with a different side chain and which releases NO more rapidly ($t_{1/2}$ = 2 min), showed the same effect as spermine NONOate on the NF- κ B–DNA complex (Figure 3). This inhibitory effect was partially reversed in a dose-dependent manner by the subsequent addition of methaemoglobin (Hb), acting as an NO-trapping agent. Alone, Hb did not interfere with NF- κ B/DNA complex-

Figure 2 Electrophoretic mobility-shift assay of NF-κB present in nuclear extracts

Astrocytes were incubated in the presence (I) or absence (UI) of cytokines (IL-1 β /IFN- γ) for 1 h, and nuclear extracts prepared. In (A), shift assays were performed in the presence or absence of wild-type NF-κB-specific oligomer (wtNF-κB, 50-fold), mutant NF-κB oligomer (mNF-κB, 50-fold) and antibodies against p65 and/or p50 subunits. A representative of two experiments is shown. In (B), shift assays were performed in the presence or absence of 1 mM DTT, 100 μ M spermine NONOate (NONO) or 25 mM DMPO. A representative of two experiments is shown.

	without DTT									
Cytokines	-	+	+	+	+	+	+	+	+	+
D-NONO	-	-	+	+	+	+	+	+	+	-
Hb (μ M)	0	0	0	0.1	0.5	2	4	10	10	10

Figure 3 Reversal by Hb of the effect of NO on NF-κB-DNA complex-formation

Nuclear extracts (10 μ g of protein) prepared from astrocytes activated for 1 h in the presence or absence of cytokines (IL-1 β /IFN- γ) were subjected to shift assay in the presence or absence of diethylamine NONOate (D-NONO, 50 μ M) and/or various concentrations of Hb added 15 min after D-NONO.

formation even at the highest concentration (10 μ M). The inhibitory effect of diethylamine NONOate on NF-κB binding was also reversed when the reaction time was extended from 15 to 90 min, presumably because of the short half-life of this

	without DTT		
Cytokines	-	+	+
D-NONO	-	-	+

Figure 4 Effect of NO on binding of IRF-like protein to DNA

Nuclear extracts (10 μ g of protein) prepared from astrocytes activated for 2.5 h in the presence or absence of cytokines (IL-1 β /IFN- γ) were subjected to shift assay in the presence and absence of diethylamine NONOate (D-NONO, 50 μ M). A representative of two experiments is shown.

NONOate and the rapid oxidation of the NO liberated (results not shown).

To determine whether NO interferes with the binding of other transcription factors, shift assays were conducted with IRF-E in the presence and absence of diethylamine NONOate. Diethylamine NONOate did not affect IRF-DNA complex-formation (Figure 4). Formation of the IRF-DNA complex was maximal at 2.5 h after cytokine activation, and cycloheximide, a translation inhibitor, significantly decreased formation of the complex. Similarly, we found that binding of yet another transcription factor [probably STAT (signal transducer and activator of

transcription) 1] to a GAS site was unaffected by diethylamine NONOate (results not shown).

DISCUSSION

Owing to its potential cytotoxicity, the unregulated production of NO may be detrimental to tissues. We showed previously [15,16] that treatment of astrocytes with exogenous NO markedly attenuated the transcriptional activation of NOS-2 in various cell types. Although the promoter region of the glial NOS-2 gene has not yet been sequenced, we predict from the mechanism of its activation that this will display response elements cognate to the mouse macrophage [23] and human NOS-2 genes [24], namely, sequence elements such as AP-1, NF- κ B, NF-IL₆, TNF-RE, IRF-E, ISRE and GAS. Of these transcription factors, NF- κ B [21,25] and IRF-1 [26,27] seem to be the key ones for NOS-2 transcriptional activation.

Recently, two other reports have described a similar effect of NO on NOS-2 gene expression. Although the mechanism was not addressed, in a study using a mouse macrophage cell line [28] a biphasic effect of an NO donor was observed. The authors suggest that a low concentration of NO augments NOS-2 mRNA expression but that a high concentration suppresses it. In the other study using human microglia [29] it was suggested that inhibition of NOS-2 gene expression by NO can be attributed to a decrease in NF- κ B activation. However, the effect of NO on activation and translocation of NF- κ B was not addressed directly. The results from our present study suggest that, rather than inhibiting translocation of NF- κ B into the nucleus, NO suppresses NOS-2 gene expression by interfering with the binding of NF- κ B to its target DNA sequence.

In addition to NF- κ B, many bioregulatory targets for nitrogen oxides have been reported [30], including ion channels, receptors, enzymes, signal-transducing proteins and transcription factors. Most of them have thiol or metal groups which may be regulated by the redox state or by nitrogen oxides. Cysteine-62 of the p50 subunit of NF- κ B seems to be directly involved in the NF- κ B-DNA interaction [31], and NO might alter critical thiol groups, resulting in disruption of DNA-binding ability. This is suggested by our observation that the reducing agent DTT reversed the effect of NONOate in a dose-dependent manner. In addition, we found that the effect of NO was time-dependent and also partially reversed by an NO-trapping agent (Hb). All of this evidence suggests that NF- κ B-DNA complex-formation may be influenced by the concentration of NO as well as the redox environment in the nucleus.

We have found that NO donors can interfere with NF- κ B binding to its target DNA sequence *in vitro*. The situation *in vivo* may be much more complex because high concentrations of glutathione and redox-active proteins such as thioredoxin, which may prevent thiol group alteration, are present in cells. However, the redox state of cells can be affected by a change in the ratio of the oxidized to reduced state of these reducing agents and a certain amount of NO may shift this state to one encouraging thiol group alteration. A recent study showed that NO caused a depletion of intracellular GSH via S-nitrosylation [32]. This may result in a cellular environment favourable to alteration by NO of critical thiol groups important for NF- κ B binding. In addition, we have to consider that the redox environment of compartments within a cell may be regulated in different way(s) so as to promote activation of transcription factors and their binding to target DNA sequences [33].

In conclusion, we suggest that the mechanism by which NO reduces NOS-2 mRNA expression in cytokine-activated astrocytes is downstream of NF- κ B translocation, via interference

with the binding of NF- κ B to its promoter response element. This modulatory effect may be significant *in vivo*, as the presence of NO is thought to provoke neuropathological damage if produced continuously. However, NO might limit its own production in cells already activated, and also prevent transcriptional activation of NOS-2 in adjacent cells. On the basis of these observations, we might predict that NO will regulate the expression of other genes under redox control and such evidence exists. Another transcription factor with critical cysteine residues, AP-1, is regulated by NO [34,35]. It has been shown that NO interacts with the redox-sensitive SoxR protein which triggers expression of the *soxRS* regulon in *Escherichia coli* [36]. The expression of monocyte chemoattractant protein-1 in human umbilical vein endothelial cells [37], early growth response-1 in rat lung macrophages [38] and protein kinase C δ [39] and IL-6 [40] in peritoneal macrophages are all down-regulated by NO. In addition, the replication of certain viruses in macrophages is inhibited by NO [41]. By way of contrast, NO up-regulates the expression of IL-8 in a human melanoma cell line [42], tumour necrosis factor in blood mononuclear cells [43], *c-fos* and *junB* in PC12 cells [44] and IL-1 α in peritoneal macrophages [40].

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