Nitric Oxide Differentially Regulates Induction of Type II Nitric Oxide Synthase in Rat Vascular Smooth Muscle Cells Versus Macrophages

Hanfang Zhang, Connie Snead, John D. Catravas

Abstract—We studied effects of nitric oxide (NO) released by different NO donors on induction of inducible NO synthase (iNOS) in rat aortic smooth muscle cells (RASMC) and rat macrophage cell line NR8383. iNOS protein expression induced by a CM (interleukin-1β 250 U/mL, interferon-γ 150 U/mL, and tumor necrosis factor-α 150 U/mL) was not affected by the NO donor SNAP (0.2 to 1 mmol/L) in RASMC at 24 hours of incubation but was dose-dependently decreased by SNAP in macrophages (maximal 60% inhibition). A fully functional ~3.2-kb rat iNOS promoter was transfected into RASMC and macrophages. The CM-induced promoter activity in transfected macrophages was inhibited by SNAP (maximal 67% inhibition), but this inhibitory effect by SNAP was not observed in transfected RASMC. Electrophoretic mobility-shift assays demonstrated that nuclear factor-κB (NF-κB) binding patterns were different in 2 cell types and that the ratio of p50:p65 subunits was significantly lower in macrophages than in RASMC. Furthermore, NF-κB activity was not affected by SNAP in RASMC but was reduced by SNAP in macrophages. Another putative NO donor, NOR3 (1 mmol/L), completely inhibited iNOS induction by CM in RASMC, but this was accompanied by severe cytotoxicity, which resulted in cell death. Similar concentrations of SNAP did not exhibit cytotoxicity in RASMC, whereas macrophages demonstrated 88% viability compared with cells without SNAP. NO synthase inhibitor Nω-monomethyl-arginine significantly inhibited CM-induced nitrite production in both cell types and stimulated iNOS protein expression in macrophages but did not affect iNOS expression in RASMC. These data strongly suggest that NO may affect transcriptional regulation of iNOS differently in RASMC versus macrophages, possibly by means of regulation of NF-κB activation. (Arterioscler Thromb Vasc Biol. 2001;21:529-535.)

Key Words: gene induction ■ nitric oxide synthase ■ macrophage ■ muscle, smooth ■ nuclear factor-κB ■ nitric oxide donors

Generation of nitric oxide (NO) from its substrate L-arginine is catalyzed by NO synthase (NOS). Of the 3 isozymes of NOS, 2 are constitutively expressed primarily in vascular endothelial cells (eNOS or type III) and neuronal cells and skeletal muscle (nNOS or type I). A third type, inducible NOS (iNOS or type II), is induced after immunological or inflammatory stimuli with substances such as cytokines or Gram-negative bacteria. Induction of iNOS produces high output of NO and has been proposed to be a major factor involved in pathologic vasodilatation and tissue damage observed in patients with and in animal models of sepsis and septic shock and in side effects of antitumor therapy with cytokines. Understanding the molecular mechanisms of iNOS induction may provide the fundamental basis for developing reagents to control gene activation in pathophysiological conditions.

NO plays important roles in cellular signaling. Because NO is a gas and free radical, it signals by chemical reaction with its protein targets, which results in covalent modification and stable alteration in protein structure and function. NO may interact with the heme prosthetic group of NOSs to inhibit NOS catalytic activity at the protein level. Increasing evidence suggests that NO may be an important regulator of iNOS induction at the transcriptional level. In a model of hepatic inflammation, chronic NOS inhibition leads to a 2-fold to 3-fold increase in iNOS mRNA and protein level. In cultured human ramified microglial cells, rat hepatocytes, and CNS glial cells, cytokine-stimulated iNOS induction is inhibited by NO derived from NO donors. Induction of iNOS is amplified in the presence of NOS inhibitor or NO-trapping agents. Furthermore, researchers have reported that the NOS inhibitor Nω-monomethyl-L-arginine (L-NMMA) enhances mouse iNOS promoter activation in the mouse macrophage cell line RAW 264.7, activated by interferon-γ and lipopolysaccharide (LPS), and that nuclear factor-κB (NF-κB) activation is reduced by NO in rat hepatocytes, human endothelial cells, and ramified microglial cells exposed to NO donors. Park et al have demonstrated that the NO donor spermine NONOate dose not affect activation and translocation of NF-κB in rat astroglial cells.

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However, in electrophoretic mobility-shift assays, this NO donor inhibits binding reaction of NF-κB to its consensus DNA sequence. This suggests that NO may decrease binding of NF-κB to DNA. Existence of a negative feedback mechanism of NO on iNOS induction in those cells may serve as a fine regulation to control output of NO.

Little is known about regulation of iNOS induction by NO in vascular smooth muscle cells (SMC). Treatments with the NO donor NOR3 decreased cytokine-induced iNOS expression in cultured rat aortic SMC (RASMC), and the mechanism of inhibition may involve decreased NF-κB activation by interfering with phosphorylation and degradation of IkB.16 However, when the fully functional rat iNOS promoter is transfected into RASMC, cytokine- or LPS-induced rat iNOS promoter activity is not changed in presence of NOS inhibitors, although nitrite production is significantly decreased.17 Vascular SMC represent the predominant cell type that expresses iNOS in rat septic models,18 and activation of NF-κB in vascular SMC and macrophages and to determine mechanisms that may be involved in NO-mediated regulation of iNOS gene induction.

Methods

Cell Culture

RASMC were harvested from Wistar rats (Harlan) by enzymatic dissociation by use of standard methods.22 Cells at passage 3 to 5 were used in the studies, and results of the study were from 9 separate harvests. Each harvest contained cells pooled from 4 rats. Rat alveolar macrophage cell line NR8383 was purchased from ATCC. The cell line, derived from normal rat alveolar macrophages, exhibits characteristics of macrophage and has been used extensively to study macrophage functions.23 All cultures were grown in a humidified incubator at 37°C under 5% CO2 in air.

Transient Transfection of DNA into RASMC and Macrophages and Luciferase Activity Assay

Transfection of RASMC with lipofectamine or lipofectamine-plus was used in RASMC according to published procedures.26 Macrophage NR8383 cells were transfected by electroporation. To control for efficiency of transfections, plasmid DNA that contained a cytomegalovirus promoter–driven β-galactosidase gene was co-transfected. Transfected cells were incubated with CM, tumor necrosis factor-α (150 U/mL, R&D Systems), interferon-γ (150 U/mL, R&D Systems), and interleukin-1β (250 U/mL, Boehringer) in the presence or absence of SNAP (Calbiochem) for 6 hours for RASMC and 12 hours for macrophage NR8383 cells. These time points were chosen because preliminary experiments showed that maximal luciferase activity was achieved at 6 hours for RASMC and 12 hours for NR8383 cells in response to CM. Concentrations of cytokines are similar to those used by other investigators in cell cultures.27 After being washed 3 times in PBS, cells were lysed with 0.35 mL of 1× cell-culture lysis reagent. Luciferase activity in 20 μL of cell lysate was measured with luciferase assay substrate (Promega) in a TD 20/20 luminometer (Turner Designs).

Western Blotting Analysis of iNOS Protein

At the end of experimental treatments, cells (in 6-well plate) were washed 3 times with ice-cold PBS. Then, 0.35 mL ice-cold RIPA lysis buffer (in mmol/L: 20 Tris-HCl [pH 7.4], 2.5 EDTA, 10 NaP04, 50 NaF, and 1 PMSF and 1% Triton; 10% glycerol, and 0.1% SDS) was added. Lysates that contained equal amount of protein (5 to 10 μg) were subsequently loaded on 7.5% SDS-polyacrylamide gels, and resolved proteins were electrophoretically transferred to nitrocellulose membrane. iNOS protein was specifically detected by rabbit polyclonal anti-mouse iNOS antibody with 1:5000 dilution (Transduction Laboratories). The second antibody was a peroxidase-conjugated donkey anti-rabbit IgG. Membrane was developed with an enhanced chemiluminescence detection system (Amersham) and exposed on film.

Nuclear Extracts

Confluent RASMC and macrophage NR8383 cells were treated with CM in presence or absence of SNAP for 120 minutes. Nuclear proteins were isolated with the modified protocol of Dignam et al29 at 4°C. Cells were washed with ice-cold PBS, collected by gentle scraping off the plates using a cell lifter, and then subjected to centrifugation. Cells were resuspended in 5 vol of hypotonic buffer (protease inhibitor cocktail and [in mmol/L] 10 HEPES-KOH [pH 7.9], 10 KCl, 1.5 MgCl2, and 0.5 DTT) for 15 minutes on ice and homogenized by being passed 10 times through a 27-gauge needle in the presence of 0.5% NP-40. After centrifugation at 13 400g at 4°C for 2 minutes and washing (once) with hypotonic buffer, pellets were resuspended in salt buffer (protease inhibitor cocktail, 25% glycerol, and [in mmol/L] 20 HEPES-KOH [pH 7.9], 400 KCl, 1.5 MgCl2, 0.2 EDTA, and 0.5 DTT). The resuspended nuclei solution was stirred with a rotator for 30 minutes at 4°C, and supernatants were collected by centrifugation at 20 000g at 4°C for 30 minutes.

Electrophoretic Mobility-Shift Assay

The NF-κB oligonucleotide was derived from rat iNOS promoter (−972 to −949) that contained the upstream NF-κB binding site (underlined): 5′-TGCAAGGGGAGTTTTCCTCT-3′ and 5′-GGAGAGGGAAATCCCCCTGG-3′. Each oligomer was filled with [α-32P]dCTP and the other nonradioabeled dNTPs by the Klenow fragment of DNA polymerase 1. Nuclear protein (2.5 to 5 μg) was incubated with 340 000 cpm of [32P]labeled oligonucleotide at 30°C for 30 minutes in the gel-shift binding buffer (in mmol/L: 12 HEPES, 4 Tris-HCl, 60 KCl, 1 EDTA, and 1 DTT; 10% glycerol; 2 μg of poly(dI-dC); and 2.5 μg of BSA) in a final volume of 25 μL. Subsequently, free and the oligonucleotide-bound proteins were separated by electrophoresis on a native 5.5% polyacrylamide gel in 0.5×Tris borate–EDTA buffer. After electrophoresis, the gel was dried and exposed to Hyperfilm MP. The intensity of the bands was analyzed with a PhosphorImager (Molecular Dynamics). Competition experiments were conducted by adding excess unlabeled NF-κB oligonucleotide in the binding reaction mixture.

Statistical Analysis

Values are reported as mean±SE. Significant differences among means were estimated by the Student t test or ANOVA. Statistical significance was established at P<0.05.

Results

Differential Effects of NO on Cytokine-Induced iNOS Protein Induction in Macrophages and RASMC

RASMC and alveolar macrophages (NR8383 cells) were incubated with CM in presence of different concentrations of SNAP for 6 and 24 hours. iNOS protein level was determined at 6 and 24 hours. iNOS protein level was determined by Western blotting. Haddad et al30 reported that 100 μmol/L of SNAP releases an average of 1.2 μmol/L of NO in a PBS (pH 7.4) solution for 2 hours at 37°C. To test the NO release profile of SNAP in our system, fresh SNAP (500 and 1000 μmol/L) in DMEM/F12 medium with 10% FBS were incu-
bated at 37°C for 24 hours. At different time points, an aliquot of the medium was collected and nitrite assays (by the Griess reagent method) were performed immediately after collection. At the 0-hour time point, very little nitrite could be detected. Then, at 6 hours, 50% of total nitrite was released, and nitrite release continued for 24 hours. Because NO is known to degrade to nitrite in seconds, the experiment suggests that SNAP, under these experimental conditions, may release significant NO and serve as an effective NO donor for 24 hours. Macrophage NR8383 cells expressed a small amount of iNOS protein at rest, and iNOS protein was strongly induced by CM. Induction was dose-dependently decreased in the presence of the NO donor at both 6 (Figure 1A) and 24 (Figure 1B and 1C) hours of incubation. However, in RASMC, strong induction of iNOS protein by CM was not inhibited by presence of NO donor at either time point (Figure 1). Instead, a significantly increased induction of iNOS was observed in RASMC at 6 hours (Figure 1A).

Differential Effects of NO on iNOS Promoter Activity in Macrophages and RASMC

To determine whether inhibition of iNOS protein by NO in macrophages might involve altered transcriptional regulation, iNOS promoter activity was studied in the presence of CM and SNAP. We cloned a 3.2-kb DNA fragment upstream of the rat iNOS gene and linked it to a luciferase gene-containing vector, PGL-3 basic. The 3.2-kb promoter is fully inducible in response to CM. Small basal luciferase activity was observed in both macrophage NR8383 cells and RASMC. When both types of cells were exposed to CM, robust increase in luciferase activity was observed (Figure 2). Increased luciferase activity in macrophage NR8383 cells was dose-dependently decreased by SNAP (Figure 2). However, promoter activity was significantly enhanced by SNAP in RASMC (Figure 2). These data further indicate that NO differentially regulates iNOS induction in macrophages and RASMC, and the effect of NO on the regulation of iNOS gene may occur at the transcriptional level in macrophage NR8383 cells.

Differential Effects of NO on Nuclear NF-κB Binding Activity in Macrophages and RASMC

Induction of iNOS gene by cytokines requires the activation of nuclear NF-κB activity. To determine whether NO donor may differentially affect the NF-κB activation in macrophage NR8383 cells and RASMC, we studied nuclear NF-κB binding activities in response to CM in the presence of SNAP in macrophage NR8383 cells and RASMC by EMSA. Both cell types showed 2 constitutively expressed bands (Figure 3A). On stimulation with CM, complex I was most prominent in macrophage NR8383 cells, whereas complexes II, I, and III were similarly induced in RASMC (Figure 3A). This enhanced nuclear binding activity was abolished by excess cold oligonucleotide in the binding reaction. Similarly, when...
RASMC were pretreated and subsequently incubated in the presence of the NF-κB inhibitor pyrrolidine dithiocarbamate (60 μmol/L) with CM, NF-κB activity was eliminated (Figure 3A). In macrophages, complex I was significantly inhibited in the presence of SNAP, but not by oxidized SNAP at the same concentration. The inhibitory effect of SNAP in macrophage NR8383 cells was not observed in RASMC.

Characteristics of the induced NF-κB complexes were investigated in supershift experiments with antibodies against p50 and p65 subunits. Figure 4A shows that complex I in both cell types mainly consists of the p50 and p65 subunits. Complexes II and III in RASMC include p50 and other subunits. Noticeably, the amount of p50 was much less than that of p65 in macrophage NR8383 cells compared with RASMC. Hence, the ratio of p50:p65 in macrophage NR8383 cells was only about half of that in RASMC (Figure 4A and 4B). Although the NO donor decreased activity of nuclear NF-κB, it did not change the characteristics of NF-κB compositions in macrophage NR8383 cells.

SNAP Does Not Influence the Effect of CM on IκBα

We further examined the dynamic change of IκBα in RASMC and NR8383 cells in response to CM in the presence or absence of SNAP (1 mmol/L). Western blotting experiments showed that, in response to CM, protein levels of IκBα decreased dramatically after 30 minutes exposure and returned to control levels by 120 minutes of exposure to CM. This effect was not significantly affected by SNAP (n=3 per cell type).
NO Donor NOR3 Decreases iNOS Protein Induction But Is Toxic in RASMC

Recently, Katsuyama et al.\(^{16}\) reported that the NO donor NOR3 inhibits cytokine-induced iNOS expression in cultured RASMC. Induction of iNOS protein by CM was completely inhibited in the presence of NOR3 (Calbiochem), a finding that is consistent with the report of Katsuyama et al.\(^{16}\) However, this inhibitory effect was accompanied by a highly toxic effect of NOR3 in RASMC. Shortly after exposure to NOR3, RASMC started to shrink. Most cells died by 24 hours, but no morphological changes were observed in the presence of SNAP. Taken together, these data suggest that NOR3 is highly toxic in RASMC and that the apparent inhibition of iNOS induction by NOR3 may be due to cell death.

Cytotoxicity of NO Donor SNAP in Macrophage NR8383 Cells and RASMC

The cytotoxic effect of NO donor SNAP was further studied in macrophage NR8383 cells and RASMC utilizing the MTT method. SNAP (1 mmol/L) did not exhibit any toxicity in RASMC, whereas macrophage NR8383 cells demonstrated 88% cell survival compared with cells without SNAP at 24 hours of incubation. These data indicate that inhibitions of iNOS protein, promotor activity, and nuclear NF-κB binding activity by SNAP in macrophage NR8383 cells are not related to cell toxicity.

Effect of Blockade of Endogenous NO Production by an NOS Inhibitor on iNOS Induction in Macrophage NR8383 Cells and RASMC

The high output of endogenous NO may serve as a regulator of iNOS induction. Therefore, preventing NO production by coexposure of cells to CM and an NOS inhibitor could reveal the role of NO on iNOS induction by CM. The NOS inhibitor L-NMMA effectively inhibited nitrite production, a stable product of NO, induced by CM in both macrophages and RASMC. Furthermore, L-NMMA increased iNOS protein expression in macrophage NR8383 cells but not in RASMC (Figure 5). These data further demonstrate that NO, either from exogenous sources released from a NO donor or by endogenous production, may differentially regulate iNOS gene induction in macrophage NR8383 cells and RASMC.

Discussion

In the present study, we have demonstrated that NO from the NO donor SNAP differentially regulates induction of iNOS gene activation in vascular SMC and macrophages. NO decreased iNOS protein expression and iNOS promotor activity in macrophages. The inhibitory effect of NO in macrophages may involve decreased activation of nuclear NF-κB binding activity. Furthermore, inhibition of endogenous NO production by NOS inhibitor significantly increased iNOS protein expression in macrophages. These data support the observation that NO may transcriptionally inhibit iNOS induction in cultured hepatocytes and CNS glial cells.\(^{10-12}\) Recently, Katsuyama et al.\(^{16}\) reported that NO derived from NOR3 also inhibits NF-κB activation and iNOS induction in RASMC at passage 15-20. We performed similar experiments with SNAP in passage 20 RASMC and did not observe any inhibition of iNOS induction. When NOR3 was used as the NO donor, complete inhibition on iNOS protein was observed, but this was accompanied by a highly toxic effect of NOR3. The present study with RASMC was conducted at passage 3-5 cells from 9 separate harvests and showed that the inhibitory effect of NO on iNOS gene induction is absent in RASMC but present in macrophages.

Several reports suggest cell type–dependent iNOS gene induction. In macrophage cells, the downstream NF-κB site at position –76 to –85 bp of the mouse iNOS promoter functions as a core promoter, but the upstream NF-κB site of the iNOS promoter plays an important role in eliciting responses to cytokines in the A7r5 rat smooth muscle cell line.\(^{27}\) Similarly, when the iNOS promoter was transfected into cultured RASMC, a key region was located at –234 bp from the 5'-region.\(^{28}\) Data have also demonstrated that LPS activation of the human iNOS promoter exhibits cell-type specificity. When the 1.1-kb human iNOS promoter was...
transfected into macrophages and SMC, the induction in response to LPS was observed only in macrophages, not in vascular SMC, including the A7r5 cell line, freshly cultured RASMC, and human saphenous-vein SMC.19 The Jak/Stat pathway is reported to mediate full induction of iNOS in macrophage RAW 264.7 cells by LPS and interferon-γ.20 However, inhibition of the pathway enhances iNOS induction in RASMC by LPS and interferon-γ.21 Few studies have compared directly the 2 cell types from the same species. In the present study, the 2 cell types, RASMC and macrophages, are from the same species, rat. Furthermore, rat iNOS promoter is used in both cell types to eliminate possible species differences in expression of promoter activity. Inhibition of iNOS induction by a CM was observed only in macrophages and not in RASMC. These data strongly indicate a cell type–dependent variation in the molecular regulation of iNOS gene and that the feedback inhibition effect of NO on iNOS may be macrophage specific.

Mechanisms that underlie the differential effect of NO on regulation of iNOS induction in RASMC and macrophages remain unknown. NF-κB activation is a necessary factor in iNOS induction.27,32 NF-κB complex I (Figure 3) was remarkably induced in RASMC and macrophages and consisted of heterodimers of the p65 and p50 subunits (Figure 3 and 4). However, consistently, NF-κB complex II and III were mainly induced in RASMC, and these bands appear to contain p50 and other subunits of NF-κB (Figure 3). In supershift studies with antibodies against p65 and p50, ratio of subunits p50:p65 was much lower in macrophages than in RASMC (Figure 4). The induction patterns and compositional differences in NF-κB subunits are speculated to be the basis for the differential regulation of iNOS induction by NO in RASMC and macrophages.

NO reacts with reactive oxygen species, such as superoxide (O2·-) to generate molecules such as peroxynitrite, which are much more active than either O2· or NO alone; they modify and change the functions of several proteins.34 S-nitrosylation of a cysteine residue (C62) of p50 of NF-κB is an example of oxidative modification in gene regulation.35 Estimates of O2· production in neutrophils are 3-fold of those in vascular cells.36 The expected high amount of O2· in macrophages stimulated by cytokines would interact with NO to form highly reactive species that react with cysteine or tyrosine on NF-κB proteins and, hence, decrease the binding activities of NF-κB. Generation of O2· would be less in vascular SMC and, subsequently, NF-κB may not be adversely modified, even in the presence of NO. This cell type–specific oxidative status may explain why NO differential regulates NF-κB activity in macrophages versus vascular SMC.

In summary, we have presented evidence that NO differentially regulates iNOS expression in rat vascular SMC and macrophages. Feedback inhibition by NO in macrophages may provide a mechanism for controlling tightly amounts of NO produced and prevent the host from the toxic effects of NO. The lack of such feedback mechanism in RASMC may explain the high output of NO in septic shock that leads to hyporeactive vessel walls.

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References


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