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Nitric oxide suppresses inducible nitric oxide synthase expression by inhibiting post-translational modification of IκB

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Abbreviations: CM, cytokine mixture; eNOS, endothelial nitric oxide synthase; iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor-kappa B; NMA, N^G-monomethyl-L-arginine; nNOS, neuronal nitric oxide synthase; NO, nitric oxide; SNAP, S-nitroso-N-acetyl-D,L-penicillamine

Abstract

The expression of inducible nitric oxide synthase (iNOS) is a critical factor in both normal physiological functions and the pathogenesis of disease. This study was undertaken to determine the molecular mechanism by which nitric oxide (NO) exerts negative feedback regulation on iNOS gene expression. Isolated rat hepatocytes stimulated with cytokines exhibited a marked increase in NO production as well as iNOS mRNA and protein levels, which were significantly reduced by pretreatment of the NO donors S-nitroso-N-acetyl-D,L-penicillamine (SNAP) and V-PYRRO/NO. This effect of SNAP was inhibited when NO was scavenged using red blood cells. Pretreatment with oxidized SNAP, 8-Br-cGMP, NO_2^-, or NO_3^- did not suppress the cytokine-induced NO production. Moreover, LPS/IFN-γ-stimulated RAW264.7 cells, which produce endogenous NO, expressed lower levels of iNOS, IL-1β, IL-6 and TNF-α mRNAs, without changes in their mRNA half-lives, than those in the presence of the iNOS inhibitor N^G-monomethyl-L-arginine. The iNOS gene transcription rate exhibited an 18-fold increase after cytokine stimulation, which was significantly inhibited by SNAP pretreatment. SNAP also blocked cytokine-induced increase in NF-κB activation, iNOS promoter activity, nuclear translocation of cytosolic NF-κB p65 subunit, and IκBα degradation, which correlated with its inhibitory effect on phosphorylation and ubiquitination of IκB. These data indicate that NO down-regulates iNOS gene expression and NO production by inhibiting the post-translational processes of IκBα thereby preventing NF-κB activation. These results identify a novel negative feedback mechanism whereby NO down-regulates iNOS gene expression.

Keywords: feedback inhibition; hepatocytes; IκB; inducible nitric oxide synthase; NF-κB; nitric oxide

Introduction

Nitric oxide (NO) is an important regulatory molecule involved in many homeostatic functions including neurotransmission, blood pressure control, and antimicrobial defense mechanisms (Nathan, 1992). There are three known nitric oxide synthase (NOS) isoforms, each produced as a separate gene product. Both the endothelial (eNOS) and neuronal (nNOS) isoforms are constitutively expressed and are predominantly regulated post-translationally by calcium and calmodulin (Nathan, 1992). The low levels of NO produced by both eNOS and nNOS participate in cell signaling and regulate processes such as endothelium-dependent vascular relaxation and neurotransmission, respectively. iNOS, the third isoform, while constitutively expressed in some tissues (Asano *et al.*, 1994; Mannick *et al.*, 1994), is expressed only after cells are exposed to lipopolysaccharide (LPS) and/or cytokines (Geller *et al.*, 1995). iNOS expression results in sustained, high level of NO production which is responsible for the refractory hypotension associated

with hemorrhagic and septic shock (Petros *et al*., 1991) as well as direct cell cytotoxicity seen in chronic inflammatory conditions (Koprowski *et al*., 1993).

NO is a key central molecule in cellular biochemical processes, as it freely diffuses and traverses cell membranes to reach different targets, alters signaling networks by redox-sensitive modifications, and transcriptionally regulates multiple gene families (Kim *et al*., 1995; Kim *et al*., 1997a, b). Physiological levels of NO production following iNOS up-regulation are associated with increased wound healing and repair in tissue injury (Yamasaki *et al*., 1998). It is also known to activate multiple gene and cell signaling pathways through processes such as nitrosation and cGMP production (Kim *et al*., 1997; Stamler *et al*., 2001). Furthermore, numerous studies have shown that NO possesses anti-tumor effects and that forced expression of iNOS causes regression of tumors (Xie *et al*., 1995; Xu *et al.,* 2002). However, large quantities of NO production induce many detrimental events, such as neurologic disorder (Koprowski *et al*., 1993), septic shock (Xu *et al*., 2002), atherosclerosis (Chen *et al*., 2003), and apoptotic cell death (Kim *et al*., 1999).

The involvement of nuclear factor-kappa B (NF-κB) in the induction of the murine iNOS gene is consistent with the well described role of this transcription factor in regulating inflammation-associated genes. NF-κB has been shown to be required for iNOS induction in many cell types including hepatocytes (Geller *et al*., 1995), rodent macrophages (Xie *et al*., 1994), and vascular smooth muscle cells (Spink *et al*., 1995). This transcription factor has been also implicated in the induction of pro-inflammatory genes encoding TNF-α, IL-1β, IL-6, and COX-2 (Makarov, 2000). NF-κB is activated as a consequence of phosphorylation, ubiquitination, and subsequent proteolytic degradation of IκB protein through activation of IκB kinase (IKK) (de Martin *et al*., 1993). The liberated NF-κB translocates into nuclei and binds as a transcription factor to κB motifs in the promoter of target genes, leading to the induction of iNOS mRNA expression and NO production.

Studies have shown that iNOS expression is transcriptionally regulated (Lowenstein *et al*., 1993; Xie *et al*., 1993; de Vera *et al*., 1996) and that *in vivo* inhibition of NO formation leads to a significant increase in iNOS mRNA and protein levels (Luss *et al*., 1994). In addition, NO produced by a NO donor or NOS can inhibit NF-κB-dependent gene expression including iNOS (Taylor *et al*., 1997) and other genes (Khan *et al*., 1996) by the suppression of NF-κB-DNA complex formation (Marshall *et al*., 2001) or the up-regulation of IκBα mRNA and protein (Peng *et al*., 1995). However, the molecular mechanism by which NO can inhibit iNOS gene expression and NO production in association with phosphorylation, ubiquitination, and subsequent proteolytic degradation of IκB upstream of NF-κB-DNA binding has not been clearly elucidated. Therefore, experiments were undertaken to determine whether NO could directly regulate post-translational processes of IκB protein and subsequent iNOS gene expression in primary rat hepatocytes. Our data shows that NO suppresses iNOS gene expression through a mechanism which includes the inhibition of NF-κB activation *via* preservation of cytosolic IκBα.

Materials and Methods

Reagents

Murine recombinant TNF-α was purchased from Genzyme (Cambridge, MA), human recombinant IL-1β was generously provided by Craig Reynolds (National Cancer Institute), and murine recombinant IFNγ was obtained from Gibco BRL (Grand Island, NY). T4 polynucleotide kinase was purchased from United States Biochemical (Cleveland, OH). N-Acetyl-D,L-penacillamine was purchased from Sigma (St. Louis, MO). The NO donor S-nitroso-N-acetyl-D,L-penacillamine (SNAP) was generated by S-nitrosylation of N-acetyl-D,L-penicillamine *via* an acid catalyzed reaction as previously described (Ceneviva *et al*., 1998). N^G-monomethyl-L-arginine (NMA) was obtained from Alexis Corporation (San Diego, CA). Monoclonal iNOS antibody was purchased from Transduction Laboratories (Lexington, KY), and polyclonal antibodies for IκBα and NF-κB p65 subunit were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). [γ-^{32}P] ATP was obtained from NEN Life Science Products. Oxidized SNAP was generated by exposing SNAP to light and room temperature for 2 weeks. V-PYRRO/NO was provided by Larry Keefer (National Cancer Institute). All other reagents were purchased from Sigma (St. Louis, MO), unless otherwise indicated.

Cell culture

Rat hepatocytes were isolated from male, 200-250 g, Sprague-Dawley rats (Harlan Sprague-Dawley, Madison, WI) using a modified *in situ* collagenase (type IV, Sigma, St. Louis, MO) perfusion technique (Seglen, 1976). After separation from hepatic nonparenchymal cells, 5×10^6 hepatocytes were plated onto 100 mm gelatin-coated cell culture dishes (Corning Co., Corning, NY) and maintained at 37°C, 95% air, and 5% CO_2 in William's medium E (Gibco, Grand Island, NY) supplemented with insulin (10^{-6} M), HEPES (15 mM), L-glutamine, penicillin, streptomycin, and 5% low endotoxin calf serum (Hyclone Laboratories, Logan, Utah). After an 18 h incubation, cells were pretreated with SNAP for 8 h, followed by incubation with a

cytokine mixture (CM) of LPS (10 μg/ml), TNF-α (500 U/ml), IL-1β (200 U/ml), and IFNγ (100 U/ml). RAW267.4 cells were cultured in DMEM containing 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% FBS.

Nitrite plus nitrate assay

Culture supernatants were collected 24 h after cytokine treatment and assayed for nitrite plus nitrate (NOx^-), the stable end products of NO oxidation, using a nitric oxide quantitation kit (Alexis, Carlsbad, CA). Nitrite was determined in cell culture media of RAW264.7 cells by Griess reagents.

Northern blot analysis

Total RNA was extracted from hepatocytes by the RNAzol method (Kim *et al*., 1997). The RNA (20 μg) was electrophoresed on 1% agarose gel containing 1% formaldehyde, transferred onto a nylon membrane, and prehybridized with whale sperm DNA at 43°C overnight. Membranes were hybridized with ^{32}P-labeled cDNA probes (~2×10^6 cpm/ml) of iNOS, IL-1β, IL-6, and TNF-α, washed three times, and exposed to autoradiography film. To control mRNA loading, the membranes were stripped by boiling with 5 mM EDTA and 0.1% SDS and rehybridized with ^{32}P-labeled 18S ribosomal RNA probe using the protocol described above.

Preparation of cytosolic fraction and nuclear extract

Cells were lysed by three cycles of freezing and thawing in lysis buffer containing protease inhibitor mixture (0.1 mM phenylmethylsulfonyl fluoride, 5 μg/μl aprotinin, 5 μg/μl pepstatin A, and 1 μg/μl chymostatin). Crude cell lysate was obtained by centrifugation at 30,000 rpm for 30 min at 4°C. cells were washed and scraped into phosphate-buffered solution, and centrifuged at 4,500 rpm for 5 min in a microcentrifuge (Beckman). For preparation of cytosolic and nuclear fraction, the pelleted cells were suspended in buffer A (10 mM Tris, pH 7.5/1.5 mM $MgCl_2$/10 mM KCl/0.5% Nonidet P-40) at approximately 10 times the packed cell volume and lysed by gentle pipetting. Nuclei were recovered from cytosolic fraction by centrifugation at 7,000 rpm for 5 min. The nuclear proteins were extracted at 4°C by gentle resuspension of the nuclei (at approximately 2 times the packed nuclear volume) in Tris buffer (20 mM Tris pH 7.5, 10% glycerol, 1.5 mM $MgCl_2$, 420 mM NaCl, and 0.2 mM EDTA), followed by 30 min of platform rotation. The nuclear protein suspension was obtained by centrifugation at 12,000 *g* for 15 min.

Western blot analysis

Proteins (50 μg) were separated on SDS-PAGE and transferred to nitrocellulose membranes. Non-specific binding to the membrane was blocked by 5% nonfat dry milk in PBS containing 0.1 % Tween 20 (PBST) overnight at 4°C. Blots were washed in PBST and then incubated for 1 h with antibodies against mouse iNOS, NF-κB p65 subunit, and IκBα in PBST containing 1% nonfat milk. After washing three times with PBST, the membranes were hybridized with horseradish peroxidase-conjugated secondary antibodies for 1 h. Following five washes with PBST, membranes were incubated with chemiluminescent solution for 2 min, and the protein bands were visualized on X-ray film.

in vitro ubiqutination assay

Ubquitinqtion of IκBα was assayed as previously reported (Yaron *et al*., 1997). HA-tagged IκBα cDNAs was translationally labeled with [^{35}S]methionine *in vitro* using a transcription/translation-coupled transcription and translation system (Promega) and immunoprecipitated with HA-antibody conjugated with agarose. The translated protein was added directly to the reaction mixture containing 100 μg hypotonic HeLa cell lysate, 50 mM Tris (pH 7.6), 2 mM $MgCl_2$, 1 mM DTT, 20 nM okadaic acid, 1 mg/ml bovine ubiquitin, and proteosome inhibitor lactacystin (10 μM) at 30°C for 90 min. The reaction mixture was boiled in SDS-buffer and the sample was analyzed by SDS-PAGE (9%), followed by fluorography.

Assays for nuclear run-on and iNOS activity

Hepatocytes were harvested and nuclei were purified as previously reported (Lowenstein *et al*., 1993; Xie *et al*., 1993). Briefly, 2.1×10^7 nuclei were incubated at 30°C for 30 min in 300 μl of reaction mixture containing 5 mM Tris-HCl (pH 8.0), 2.5 mM $MgCl_2$, 150 mM KCl, 0.25 mM (each) GTP, ATP, and CTP, [α-^{32}P]UTP (250 μCi, specific activity 3,000 Ci/mmol), (NEN Life Science, Boston, MA), and 40 U RNAsin (Promega). Radiolabeled nuclear RNA was extracted from the reaction mixture by TriZol (Gibco BRL) according to the manufacturer's instructions. The labeled nuclear RNA was purified on a Sephadex G-50 column. Linearized plasmid constructs (10 μg) containing full length human iNOS, actin, and the empty vector pBluescript were denatured with 0.4 M NaOH at 60°C for 1 h and immobilized onto Genesceen-Plus membrane (NEN Life Science) *via* a slot-blot apparatus, immobilized and crosslinked (de Vera *et al*., 1996). Membrane was prehybridized at 43°C in a buffer containing 50% deionized formamide, 4× SSC, 2× Denhardt's solution, 50 mM PIPES (pH

7.0), 2.0 mM EDTA, 0.5% SDS, 200 μg/ml of yeast tRNA, 200 μg/ml denatured salmon sperm DNA, and 100 μg/ml Poly(A) for at least 4 h. Hybridization was carried out at 43°C for 48 h in the same buffer used for the prehybridization with equivalent counts per minute per milliliter of ^{32}P-labeled RNA added to each membrane. The membranes were washed in 2× SSC; this was followed by a 30-min digestion period with 10 μg/ml of RNase A and 10 U/ml RNase T1 (GIBCO BRL). Finally, the blots were washed twice at 53°C for 15 min with 0.1× SSC-0.5% SDS. The blots were exposed to X-ray film for autoradiography. iNOS activity was assayed as previously reported (Kim *et al*., 1993). In brief, enzyme reaction was initiated by mixing cell extract (30 μl) with a reaction mixture consisting of 1 mM NADPH, 20 μM FAD, 20 μM FMN, 0.5 mM BH_4, 4 mM L-arginine, and 5 mM glutathione, in a final volume of 200 μl in 40 mM Tris-HCl, pH 7.7. The reaction were carried out at 37°C and terminated after 30 min by the addition of 0.5 N NaOH and 400 μl of 10% $ZnSO_4$. After centrifugation, NO_2^- plus NO_3^- were measured by Griess reaction-based HPLC method.

iNOS promoter activity assay

A deletional construct containing 1.6 Kbp of the 5' flanking region of the murine iNOS gene ligated to upstream of the reporter gene luciferase (gift of Charles Lowenstein, Johns Hopkins University) was transiently transfected into hepatocytes using liposomes as described (Lowenstein *et al*., 1993). Serum-free transfections were carried out in 6-well plates using 1 μg of DNA and 10 μg of lipofectin for 6 h. After an overnight recovery, hepatocytes were pretreated with 750 μM SNAP for 8 h and then stimulated with CM for 12 h. Cells were lysed with lysis buffer containing 1% Triton X-100, 5 mM dithiothreitol, 50% glycerol, 10 mM EDTA, and 125 mM Tris-phosphate (pH 7.8). Luciferase activity was measured by luminometer.

Immunoprecipitation

Hepatocytes were pretreated with or without SNAP, followed by incubation with or without CM in the presence of inorganic ^{32}P and 40 μM lactacystin. Cells were harvested and washed with ice-cold PBS, and the pellets were resuspended in 80 μl of immunoprecipitation lysis buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, 5 mM EDTA, 1 mM DTT, 100 mM NaF, 2 mM sodium pyrophosphate, 2 mM sodium orthovanadate, and protease inhibitor mixture) and stored on ice for 20 min before centrifugation (14,000 *g*, 20 min, 4°C). IκBα was immunoprecipitated by incubation for 1 h at 4°C with polyclonal IκBα antibody bound to agarose. The immunoprecipitates were washed twice with immunoprecipitation buffer and suspended in SDS-PAGE sample buffer. Phosphorylated IκBα was examined by SDS-PAGE, followed by autoradiography.

Electrophoretic mobility shift assay

Hepatocytes were pretreated with different concentrations of SNAP for 8 h and stimulated with CM for 1 h. Preparation of nuclear extracts and NF-κB activation was assayed as described (de Vera *et al*., 1996). A double stranded NF-κB-specific oligonucleotide (5'-AGTTGAGGGGACTTTCCCAGGC-3') was labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase and purified on a Sephadex G-50 column. The nuclear extracts (10 μg of protein) were incubated with 40,000 cpm (0.5 ng) of ^{32}P-labeled oligonucleotide for 20 min at room temperature. Samples were separated on a 5% native polyacrylamide gel, which was dried and subjected to autoradiography.

Statistical analysis

Data are presented as the mean ± SD of at least three separate experiments. Comparisons between two groups were analyzed using Student's *t*-test. *P* values less than 0.05 were considered to be statistically significant.

Results

SNAP inhibits cytokine-induced NO production and iNOS expression in primary cultured rat hepatocytes. To investigate the effects of exogenous NO on CM-induced NO production and iNOS expression, we examined the effects of the NO donor SNAP on NO production as well as iNOS mRNA and protein expression in CM-stimulated hepatocytes. Hepatocytes were pretreated with or without 750 μM SNAP for different time periods, followed by incubation with or without CM. As we have previously shown (Kim *et al*., 1997), CM treatment markedly increased NO production in non-pretreated hepatocytes, as measured by the end-products nitrite and nitrate. However, this increase was decreased in a time-dependent manner, with maximum inhibition at 8 h incubation, by SNAP pretreatment (Figure 1A). When pretreated with different SNAP concentrations for 8 h, the CM-induced NO production was dose-dependently inhibited by SNAP whereas NO was not synthesized without CM treatment (Figure 1B). To further examine the effect of SNAP on iNOS gene expression, we measured the levels of iNOS mRNA and protein by Northern blot and Western blot analyses, respectively. Treatment with SNAP inhibited a CM-induced increase in iNOS mRNA level in a concentration- dependent

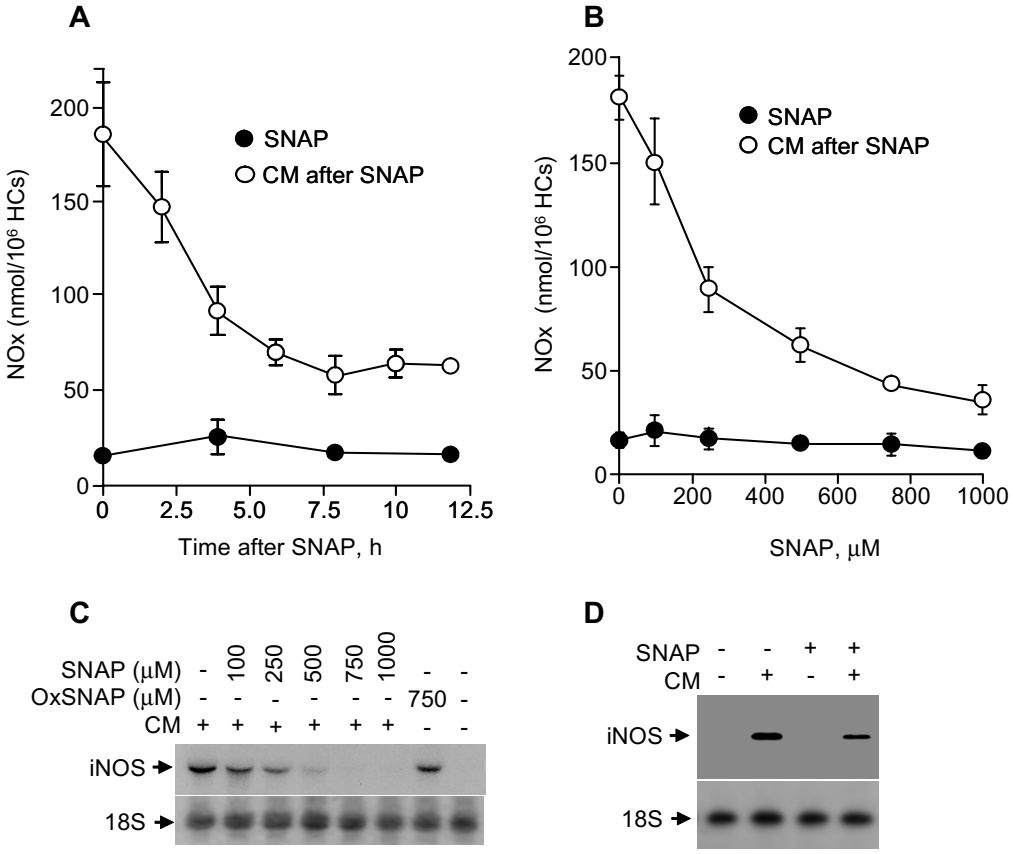

Figure 1. SNAP inhibits CM-induced NO production and iNOS gene expression in rat hepatocytes. Cultured rat hepatocytes pretreated with SNAP (0-1000 μM) or oxidized SNAP (750 μM) for different time periods or 8 h were washed twice with fresh medium, followed by incubation with CM (10 μg/ml LPS, 500 U/ml TNF-α, 200 U/ml IL-1β, and 100 U/ml IFNγ). (A) Culture supernatants were collected 24 h after CM exposure, and NOx (NO_2^- plus NO_3^-) production was measured by Griess reaction. (B) Hepatocytes were pretreated with different concentrations of SNAP for 8 h, followed by incubation with CM for 24 h. NOx was measured in the culture media. (C) At 8 h incubation of CM following SNAP pretreatment for 8 h, total RNAs were isolated, and NOS mRNA level was measured by Northern blot analysis. Equal loading of RNA in each lane was confirmed by 18S ribosomal RNA probe. (D) At 12 h incubation of CM following SNAP (500 μM) pretreatment for 8 h, cell extracts were isolated and iNOS protein level was measured by Western blot analysis. Data presented in (A) and (B) are the mean ± SD of three independent experiments.

manner. SNAP alone did not stimulate iNOS expression, and oxidized SNAP (not liberating NO) failed to inhibit CM-induced iNOS mRNA expression (Figure 1C), indicating that the inhibitory effect was in fact due to the NO release from SNAP. To determine the effect of NO on iNOS protein levels, Western blot analysis was performed with pretreatment with SNAP (500 μM). At 12 h stimulation with CM following SNAP pretreatment for 8 h, Western blot showed that CM-stimulated iNOS protein expression was also suppressed by pretreated SNAP (Figure 1D). SNAP generates NO, which is oxidized to NO_2^- and NO_3^- by reacting with molecular oxygen and produces cGMP from GTP by activating guanylate cyclase via interaction with its heme group. Therefore, we next examined the effects of these compounds and red blood cells (the biological scavenger of NO) on CM-induced hepatocyte NO production (Figure 2). SNAP pretreatment in the presence of red blood cells blocked the suppressive effect of SNAP on CM-induced NO production. Pretreatment with the membrane-permeable cGMP analog 8-Br-cGMP, NO_2^- or NO_3^- did not affect CM-induced hepatocyte NO production compared with control cells. Thus, NO generated by SNAP, but not its reaction products, suppresses NO production and iNOS gene expression in hepatocytes stimulated with CM.

Liver-specific NO donor inhibited hepatic iNOS gene expression

Biological NO is synthesized in the cytosol by the catalytic reaction of three isotypes of NOS and directly interacts with biological molecules to reveal its

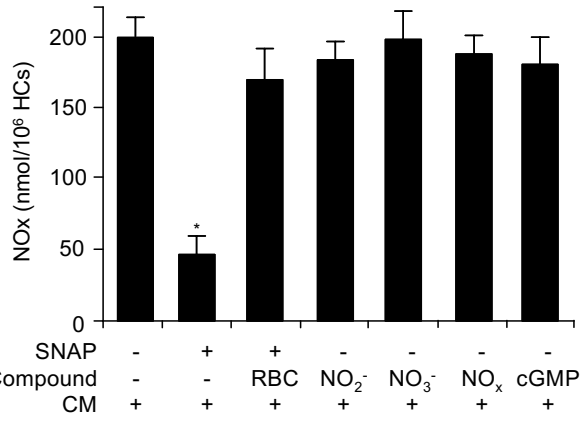

Figure 2. NO reaction products did not affect CM-induced NO production and red blood cells reversed the suppressive effects of SNAP. Hepatocytes were pretreated with NO_2^- (500 μM), NO_3^- (500 μM), NO_2^- (500 μM), NO_2^- + NO_3^- (250 μM + 250 μM) or 8Br-cGMP (500 μM) for 8 h, washed twice with fresh medium, and then stimulated with CM for 24 h. For studying the effect of red blood cells, hepatocytes were pretreated with SNAP (750 μM) in the presence of red blood cells (750 μM of hemoglobin) for 8 h, washed twice with fresh medium, and then stimulated with CM for 24 h. NOx was measured in the culture media by Griess reaction. Data presented are the mean ± SD of three independent experiments. *$P < 0.01$ vs. CM alone.

biological action (Nathan, 1992; Stamler *et al*., 2001). The general NO donor SNAP, however, produces NO in the culture media, diffuses into cells, and interacts with various intacellular molecules. To examine the effect of intracellularly generated NO on CM-induced iNOS expression, hepatocytes were pretreated with or without different concentrations of V-PYRRO/NO (a hepatocyte-specific NO donor that mimics endogenous NO sources) (Saavedra *et al*., 1997) for 8 h, followed by incubation with or without CM. V-PYRRO/NO pretreatment inhibited a CM-induced increase in iNOS enzyme activity (Figure 3A). We next examined the effect of the liver specific NO donor V-PYRRO/NO on iNOS mRNA and protein expression. Northern blot showed that an increased iNOS mRNA level induced by CM was inhibited in a dose-dependent manner by pretreatment with increasing concentrations of V-PYRRO/NO (Figure 3B). Western blot showed that CM-stimulated iNOS protein expression was also suppressed by treatment with V-PYRRO/NO (Figure 3D). Thus, these data indicate that NO produced within cells inhibited CM-induced NO-producing activity through the inhibition of iNOS mRNA and protein expression in primary cultured rat hepatocytes.

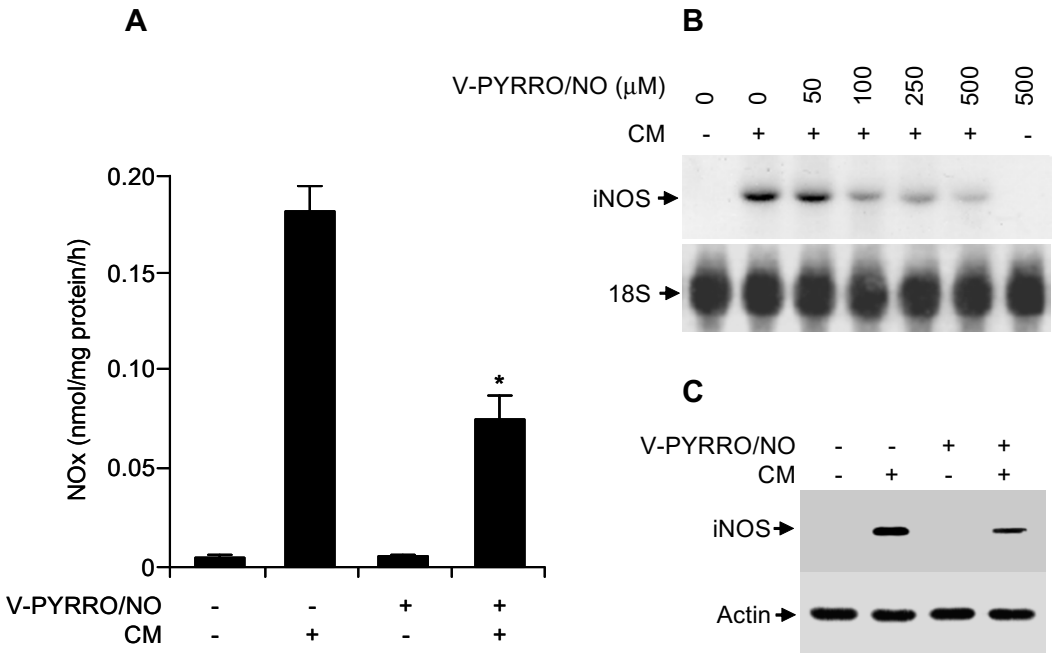

Figure 3. The liver specific NO donor V-PYRRO/NO inhibits iNOS mRNA and protein in hepatocytes stimulated with CM. Hepatocytes were pretreated with or without V-PYRRO/NO (0-500 μM) for 8 h and stimulated with CM. (A) iNOS activity was determined in the cytosolic extract as described in the section of materials and Methods. Data presented are the mean ± SD of three independent experiments. *$P < 0.01$ vs. CM alone. (B) After 8 h stimulation, total RNAs were isolated and iNOS mRNA levels were measured by Northern blot analysis. Equal loading of RNA in each lane was confirmed by 18S ribosomal RNA probe. (C) After 12 h stimulation, cytosolic extracts were prepared and iNOS protein level was measured by Western blot analysis.

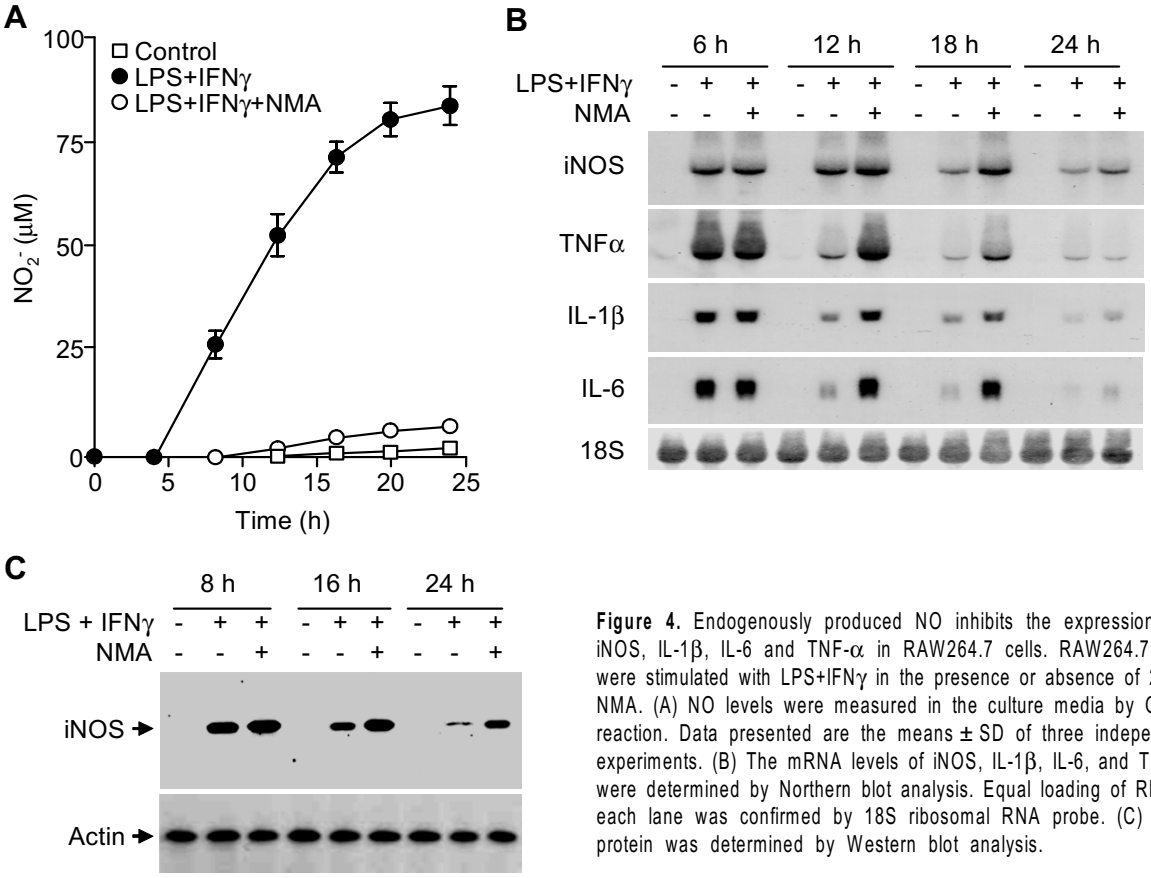

Figure 4. Endogenously produced NO inhibits the expression and iNOS, IL-1β, IL-6 and TNF-α in RAW264.7 cells. RAW264.7 cells were stimulated with LPS+IFNγ in the presence or absence of 2 mM NMA. (A) NO levels were measured in the culture media by Griess reaction. Data presented are the means ± SD of three independent experiments. (B) The mRNA levels of iNOS, IL-1β, IL-6, and TNF-α were determined by Northern blot analysis. Equal loading of RNA in each lane was confirmed by 18S ribosomal RNA probe. (C) iNOS protein was determined by Western blot analysis.

Table 1. The effect of NO on mRNA half-lives of iNOS, IL-1β, IL-6, and TNF-α in LPS+IFNγ-stimulated RAW264.7 cells. RAW264.7 cells were stimulated with LPS+IFNγ in the presence or absence of NMA for 8 h. Cells were washed with fresh media and incubated in fresh media containing actinomycin D (80 ng/ml) in the presence or absence of NMA. Total RNAs were isolated at different time points, and the levels of iNOS and 18S rRNA were determined by Northern blot analysis.

mRNA	Half-life, h	
	-NMA	+NMA
iNOS	2.4 ± 0.3	2.7 ± 0.4
IL-1β	3.2 ± 0.2	3.4 ± 0.3
IL-6	1.7 ± 0.2	2.0 ± 0.3
TNF-α	1.9 ± 0.2	2.3 ± 0.2

Biological NO inhibits iNOS and cytokine gene expression in RAW264.7 cells

To determine whether biologically produced NO would regulate the expression of iNOS and some other genes in different cell types, macrophage cell line RAW264.7 cells were exposed to LPS+IFNγ. in the presence or absence of the NOS inhibitor NMA. Treatment of RAW264.7 cells with LPS+IFNγ resulted in a strong increase in NO production, and this increase was reduced to the control level by the addition of NMA (Figure 4A). At 6 h stimulation with LPS+IFNγ, Northern blot showed a marked increase in iNOS mRNA expression compared with control, which was unchanged in the presence of NMA (Figure 4B). However, from 12 h when NO production was significantly increased (Figure 4A), the levels of iNOS mRNA induced by LPS+IFNγ were lower than those in the presence of NMA. Since it has been shown that LPS induces various pro-inflammatory cytokines in macrophages (Lee *et al.*, 2003), we determined whether the inflammatory genes were down-regulated by endogenous NO. The mRNA levels of IL-1β, IL-6 and TNF-α were significantly increased in cells stimulated with LPS+IFNγ, and these increases were further elevated in the presence of NMA (Figure 4B). In addition, NMA increased iNOS protein levels from 12 h of stimulation with LPS+IFNγ (Figure 4C), when NO production was significantly increased (Figure 4A). For each of these inflammatory mediators, mRNA stability was then assayed and determined to be unchanged in the presence or

absence of NMA (Table 1). These data indicate that NO production as well as expression of mRNAs of iNOS, IL-1β, IL-6, and TNF-α by NO are associated with transcriptional down-regulation of these genes.

NO suppresses iNOS gene transcription

The lake of changes in mRNA half-lives of iNOS, IL-1β, IL-6, and TNF-α strongly suggested that the increases in these genes' mRNA levels occurred at the level of transcription. To determine whether the suppressive effects of NO were occurring at the level of transcription, nuclear run-on assays were performed for iNOS in nuclei isolated from control and CM-stimulated hepatocytes. As shown in Figure 5, the level of iNOS transcript was undetectable in the nuclei of untreated cells. However, in nuclei isolated from CM-treated cells, transcription rate of iNOS exhibited an 18 fold increase compared with control, and the addition of SNAP resulted in about 70% decrease in the rate of iNOS transcription. These results indicate that NO inhibits iNOS expression by transcriptional down-regulation.

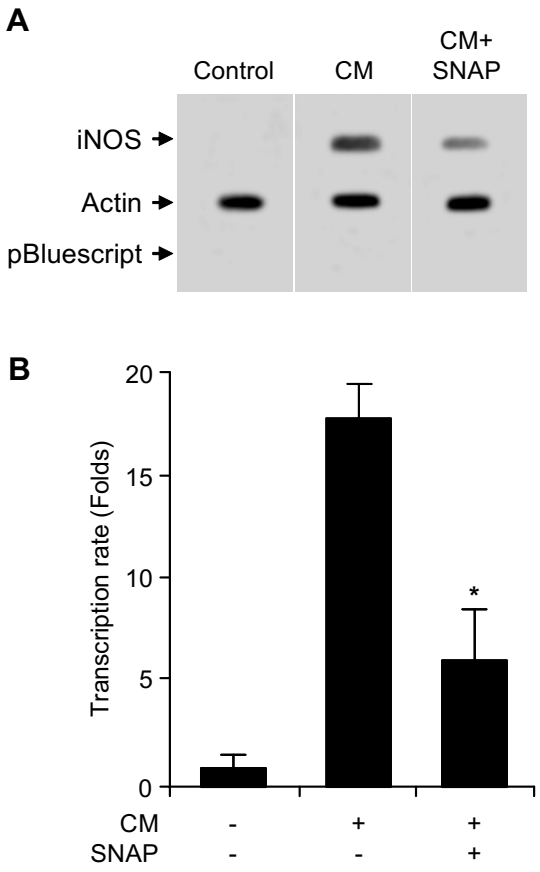

Figure 5. NO inhibits iNOS transcription rate. Hepatocytes were pretreated with or without 750 μM SNAP for 8 h and stimulated with CM for 1 h. Nuclei were isolated and incubated with [α-^{32}P]UTP to elongate the nascent RNAs. The elongated-nascent RNAs were hybridized to membranes containing mouse iNOS cDNA, β-actin, and empty vector pBluescript plasmids. Equivalent counts per minute per milliliter ^{32}P-labeled nuclear RNAs were used for hybridizations of each membrane. (A) An autoradiograph of elongated-nascent RNAs in isolated nuclei. (B) The normalized iNOS transcription rates as fold-increase over the value of untreated cells. Data presented are the means ± SD of three independent experiments. *$P < 0.05$ vs. CM alone.

NO inhibits NF-κB-DNA-binding activity and transcriptional activity of iNOS promoter.

The expression of iNOS and pro-inflammatory genes (IL-1β, IL-6, and TNF-α) requires the activation of the transcription factor NF-κB (Xie *et al*., 1994; de Vera *et al.,* 1996; Baeuerle *et al*., 1997). To investigate the effects of NO on the DNA-binding activity of NF-κB, electrophoretic mobility shift assays were performed using nuclear extracts from hepatocytes pretreated with SNAP for 8 h, followed by stimulation with CM. Nuclear extracts from control hepatocytes showed the undetectable level of NF-κB-DNA complex formation. CM stimulation resulted in a marked increase in NF-κB-DNA binding activity, and this binding activity was decreased in a concentration-dependent manner by pretreatment of SNAP (Figure 6A). The specificity of binding for NF-κB was demonstrated by cold competition with a 100-fold excess of cold probe. It has been shown that the iNOS promoter contains functional NF-κB binding sites (Xie *et al*., 1994; de Vera *et al*., 1996) and that NF-κB activation plays a key role in the transcriptional activation of the iNOS gene. Transient transfection was performed using a 1.6 Kbp murine iNOS-luciferase promoter construct to determine if NO blocks iNOS expression at the level of the promoter. Basal promoter activity was seen in transfected hepatocytes treated with media or SNAP alone. Conversely, CM- stimulated hepatocytes exhibited an 18-fold increase in promoter activity, which was then decreased by -50% under the condition of SNAP pretreatment (Figure 6B).

NO inhibits cytokine-induced IκBα phosphorylation and degradation.

Translocation of NF-κB to the nucleus is preceded by the sequential events of phosphorylation, ubiquitination, and proteolytic degradation of IκBα (Baeuerle *et al*., 1997). To determine the mechanism of NO-dependent inhibition of NF-κB activation, we first determined the cytosolic and nuclear NF-κB p65 subunit levels in hepatocytes treated with CM following SNAP pretreatment by Western blot analysis. CM treatment decreased the cytosolic p65 subunit level, resulting in an increase in the nuclear p65 level, while this subunit was mostly present in the cytosol from control

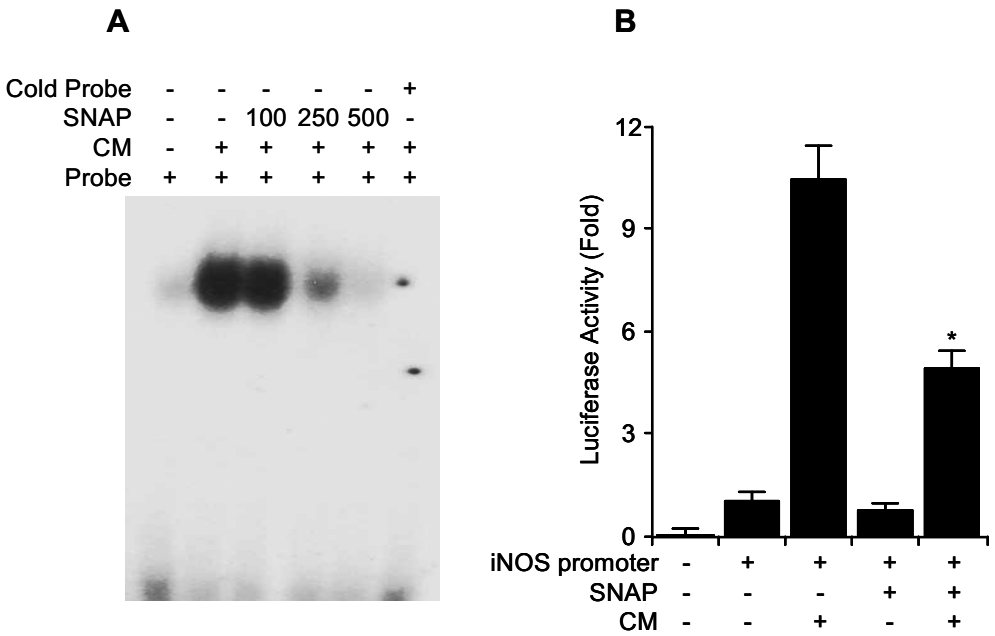

Figure 6. NO suppresses NF-κB activation and iNOS promoter actibity. Hepatocytes were stimulated with CM for 1 h following pretreatment with different concentrations of SNAP for 8 h. Cells were harvested, and nuclear extracts were prepared. Nuclear NF-κB activity was analyzed by electrophoretic mobility shift assay in the presence or absence of excess amount of cold probe. (B) Hepatocytes were transiently transfected by a lipofectamine method with a 1.6 Kbp murine iNOS promoter fragment linked upstream of luciferase. Cells were treated with CM following pretreatment with 750 μM SNAP for 8 h. After 12 h stimulation, luciferase activity in cell extracts was measured by luminometer. Data presented are the mean ± SD of three independent experiments. Data presented are the means ± SD of three independent experiments. $^{*}P < 0.02$ vs. CM.

cells (Figure 7A). These results indicate that SNAP pretreatment inhibited CM-induced translocation of the cytosolic p65 subunit to nuclei. Since nuclear translocation of NF-κB is directly linked to IκB degradation (Baeuerle *et al*., 1997), we next performed Western blot analysis to determine the effects of NO on IκBα degradation. CM treatment significantly decreased IκBα protein level within 30 min compared with control, and pretreatment with SNAP before CM stimulation preserved IκBα protein levels (Figure 7B). We further examined whether NO would regulate IκBα ubiquitination in the cytosols from hepatocytes incubated with CM following pretreatment with SNAP for 8 h. The basal level of IκBα ubiquitination was detectable in the cytosol from untreated cells. However, IκBα ubiquitination was significantly increased in the cytosol from CM-stimulated cells, and this increase was inhibited in a concentration-dependent manner by SNAP pretreatment (Figure 7C). To investigate the effects of NO on IκBα phosphorylation, phosphorylated IκBα was analyzed on the SDS-PAGE after immunoprecipitating IκBα protein in the cytosol from hepatocytes pretreated with SNAP for 8 h, followed by incubation with CM in the presence inorganic ^{32}P. CM treatment significantly increased IκBα phosphorylation compared with the control, and this phosphorylation was inhibited by SNAP pretreatment (Figure 7D). These results indicate that NO blocks NF-κB activation by inhibiting IκBα degradation through the suppression of its phosphorylation and ubiquitination.

Discussion

Since the expression of iNOS contributes to the systemic manifestations of a number of acute and chronic diseases, it is important to elucidate the molecular mechanisms that regulate iNOS expression. We previously showed that when hepatocytes were recovered in fresh medium for 24 h after pretreatment with SNAP for 8 h, stimulation with cytokines increased iNOS-mediated NO production compared with untreated control by increasing tetrahydrobiopterin production without changing iNOS gene expression (Park *et al*., 2002). However, it has been reported that cotreatment with the NO-donor SNAP and cytokines (including LPS) suppresses iNOS expression and NO production in hepatocytes (Taylor *et al*., 1997), macrophages (Sheffler *et al*., 1995; Kim *et al*.,1998) and epithelial cells (Cavicchi *et al*., 2000). These results indicate that there present both negative and

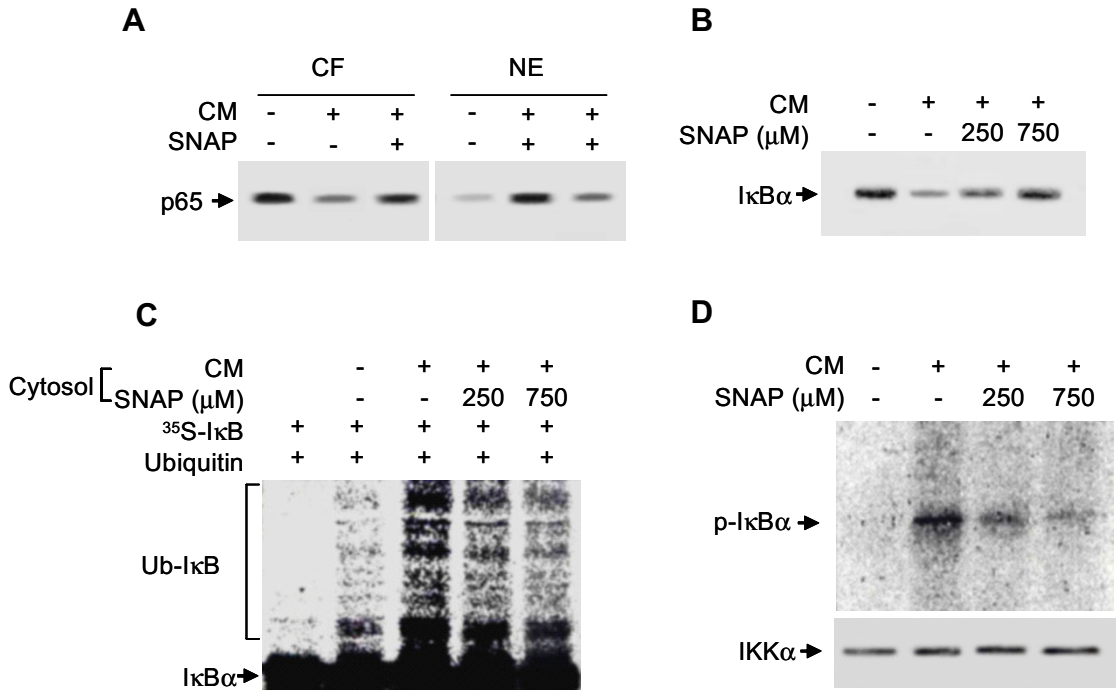

Figure 7. NO inhibits nuclear translocation of NF-κB p65 subunit as well as degradation, phosphorylation, and ubiquitination of IκBα. Hepatocytes were pretreated with 750 μM SNAP for 8 h and stimulated with CM. (A) After 1 h stimulation, cells were harvested, washed with ice-cold PBS, and resuspended in nuclear extraction buffer. Cytosolic fractions (CF) and nuclear extracts (NE) were prepared as described in the Method Section. Both samples (50 μg protein) were separated on SDS-PAGE, and the NF-κB p65 subunit was visualized by Western blot analysis. (B) After 20 min stimulation, whole cell lysates from hepatoctes were prepared in SDS-PAGE. IκBα protein was visualized by Western blot analysis. (C) Hepatocyte extracts were prepared from hepatocytes stimulated with CM for 30 min following pretreatment with or without 750 μM SNAP. IκBα was labeled with [^{35}S]methionine by *in vitro* transcription/translation method. Labeled IκBα was incubated with cell lysate in the presence of ubiquitin, proteosome inhibitor, and phosphatase inhibitor for 40 min at 37°C. Reaction was stopped by mixing with 2× sample loading buffer, and samples were separated on SDS-PAGE and visualized by autoradiography. (D) After 30 min stimulation with CM in the presence of proteosome inhibitor and inorganic ^{32}P, IκBα was immunoprecipitated with an anti-IκBα antibody. The pellets were separated on SDS-PAGE, and the phosphorylated IκBα protein was visualized by autoradiogaraphy. IκBα protein level was determined by Western blot analysis.

positive feedback regulatory mechanisms for NO production during NO generation and after recovery of a certain time period following exposure to NO, respectively. In this study, we investigated to identify a molecular feedback mechanism whereby NO down-regulates iNOS expression and NO production. Pre-treatment with SNAP suppressed NO production in rat hepatocytes stimulated with CM. Northern and Western blot analyses of CM-stimulated hepatocytes showed that NO production by SNAP and V-PYRRO/NO inhibited iNOS mRNA and protein levels in a concentration-dependent manner. Furthermore, RAW264.7 cells stimulated with LPS+IFNγ increased NO production, and the inhibition of iNOS activity by NMA further elevated the expression of iNOS, IL-1β, IL-6, and TNF-α genes. However, NO did not affect the mRNA stability of these genes. Nuclear run on assays confirmed that NO inhibited transcription rate of iNOS gene. SNAP also inhibited NF-κB activation and iNOS promoter activity. Moreover, SNAP blocked CM-induced nuclear translocation of the cytosolic NF-κB p65 subunit and degradation, phosphorylation, and ubiquitination of IκBα. The data in this study indicate that NO-mediated decreases in iNOS gene expression and NO production are due to a block in phosphorylation and ubiquitination of IκBα, which results in NF-κB remaining in its inactivated state.

Several reports indicate that NO inhibits iNOS enzyme activity. Griscavage *et al* (Griscavage *et al*., 1993), as well, as Assreuy *et al* (Assreuy *et al*., 1993) demonstrated that NO functions as a negative feedback modulator by interacting with the enzyme bound heme of iNOS in rat alveolar macrophages and in a murine macrophage cell line, respectively. Their data demonstrated an NO-mediated 40-60% suppression of iNOS enzyme activity. Another possible negative feedback inhibitory mechanism of NO can be associated with limitation of intracellular assembly of active dimeric iNOS by preventing heme insertion and decreasing heme availability (Kim *et al*., 1995; Albakri

et al., 1996). We here examined if NO inhibited iNOS activity in CM-stimulated hepatocytes. Hepatocytes were stimulated with CM for 8 h, washed with fresh media, and incubated with or without 750 μM SNAP. After 8 h, cells were washed twice and incubated with fresh media for another 12 h. Hepatocytes stimulated with CM only produced 84 ± 6 μM of nitrite, and cells treated with SNAP following CM stimulation synthesized 79 ± 5 μM of nitrite (data not shown). This result indicates that NO did not directly inhibit iNOS activity in hepatocytes. Our data also showed that SNAP strongly inhibited the levels of iNOS mRNA and protein in CM-stimulated hepatocytes. These data demonstrate that NO feedback inhibition of enzyme activity plays a minor role in iNOS regulation and that inhibition of transcriptional gene expression is the major pathway of iNOS regulation. This indicates that the major mechanism of regulation of iNOS-dependent NO production is *via* transcriptional control and that NO inhibits iNOS enzyme activity *via* a minor mechanism of post-translational modification in rat hepatocytes.

Although NO can upregulate NF-κB-dependent cyclooxygenase-2 expression (Chun *et al*., 2004), other studies have demonstrated that exogenous or endogenous NO suppresses iNOS expression and NO production, probably by direct inhibition of DNA-binding activity of NF-κB (Matthews *et al*., 1992; Marshall *et al*., 2001), or up-regulation of IκB protein level (Peng *et al*., 1995). Chemical NO donors, sodium nitroprusside and spermine NONOate, suppressed iNOS mRNA expression and NO production in human microglial cells (Colasanti *et al*., 1995) and rat astroglial cells (Peng *et al*., 1995) stimulated with cytokines. In these studies, pre-treatment with NO donor inhibited cytokine-induced iNOS expression by interfering with the binding activity of NF-κB to its promoter response element, and this inhibition was reversed by preincubation of nuclear extract with dithiothreitol (Park *et al*., 1997). These data suggest that, rather than inhibiting IκBα degradation and translocation of NF-κB into the nucleus, NO suppresses iNOS gene expression by directly interfering with the binding of NF-κB to its target DNA sequence. NO is a well-known redox regulator by nitrosylating transition metal (Kim *et al*., 2000) and thiol group (Kim *et al*., 1997). NF-κB p50 subunit has cysteine residue at 62, which seems to be directly involved in the NF-κB-DNA interaction (Matthews *et al*., 1992), and NO might alter this critical thiol group, resulting in disruption of DNA-binding ability, which was recovered by dithiothreitol. However, our data showed that NO blocked the translocation of cytosolic NF-κB p65 subunit to the nucleus following CM treatment. Since cytosolic NF-κB translocates into the nucleus and then binds to DNA, the inhibitory effect of NO on nuclear translocation of NF-κB is a more important determinant for NF-κB-dependent gene expression than S-nitrosylation of Cys62 of the NF-κB p50 subunit. Another possibility for NO-mediated suppression of iNOS gene expression may be associated with the increase in up-regulation of IκBα mRNA and protein levels without changing the protein levels of the NF-κB subunits, p65 and p50 (Peng *et al*., 1995). Contrary to these results, we could not observe any significant changes in IκBα mRNA and protein levels in SNAP-treated hepatocytes by Northern blot and Western blot analyses (data not shown). This is not evident in hepatocytes and an alternative mechanism appears to be employed. The data in this study demonstrates that this down-regulation of iNOS gene expression is due to a decrease in NF-κB-DNA binding activity which is secondary to a block in IκBα phosphorylation and degradation.

The transcription factor NF-κB is a key factor for regulating the expression of iNOS and pro-inflammatory genes including IL-1β, IL-6, and TNF-α, which contain NF-κB-binding motifs within their respective promoters (Li *et al*., 2002). Our data showed that NO generated by SNAP down-regulated transcription rate of iNOS gene and iNOS promoter activity in hepatocytes. Furthermore, LPS+IFNγ-stimulated RAW264.7 cells, which produced large quantities of endogenous NO, suppressed the expression of IL-1β, IL-6, and TNF-α genes, compared with cells co-treated with the NOS inhibitor NMA. This regulation was directly associated with decreases in iNOS promoter activity, NF-κB-DNA complex formation, and nuclear translocation of cytosolic NF-κB. Because the activation of NF-κB is influenced by the phosphorylation, ubiquitination, and proteolytic degradation of IκB (Baeuerle *et al*., 1997; Piaggio *et al*., 2001; Kiemer *et al*., 2003), it suggests that NO may regulate a signaling cascade associated with NF-κB activation. The data in this study demonstrated that NO significantly inhibited phosphorylation, ubiquitination, and proteolytic degradation of IκB. Phosphorylation of Ser at 32 and 36 of IκB is a pre-requisite step for its ubiquitination and degradation. This phosphorylation occurred by increasing IKK activity in cells stimulated with immune stimulants including LPS and cytokines. We previously reported that NO induced an increase in both protein tyrosine and protein serine/threonine phosphatase activity and that phosphatase inhibitor, calyculin A or cantharidin, reversed the inhibitory effect of NO on ubiquitin-proteasome pathway (Kibbe *et al*., 2000). We here showed the similar results that NO inhibited *in vivo* IκBα phosphorylation and *in vitro* IκBα ubiquitinqtion. Therefore, our data suggest that the inhibitory effect of NO on CM-mediated NF-κB activation is through the prevention of IκBα protein degradation by the ubiquitin-proteasome pathway in association with

increased IKK-dependent Ser phosphorylation of IκBα. The regulation of iNOS expression is complex and tightly regulated and appears to occur at multiple levels, from the signal transduction pathway for the initiation of gene transcription to post-translational protein modifications. The work in this study further characterizes the molecular mechanism of feedback inhibition of iNOS gene expression by its reaction product NO. We speculate that as levels of NO increase to a critical level, NO-mediated feedback regulation begins to inhibit overproduction of NO by blocking IκBα degradation, thereby preventing the activation of NF-κB. This results in a down-regulation of iNOS transcription and serves to decrease the amount of subsequent NO to be produced. Hepatocytes can induce the synthesis large quantities of NO, and negative feedback regulation serves to attenuate this physiological response to possibly prevent further tissue injury.

In summary, our data identifies a novel negative feedback loop whereby NO down-regulates iNOS gene expression in a number of cell types and across species by NF-κB activation, through the suppression of IκBα phosphoryation and degradation. Because NO has such profound effects, its overproduction may signal to decrease its further production to prevent inappropriate transcription and further damage to the host.

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