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Octamer Binding Protein–1 Is Involved in Inhibition of Inducible Nitric Oxide Synthase Expression by Exogenous Nitric Oxide in Murine Liver Cells[1]

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Nitric oxide (NO) has diverse effects on immune responses and hepatic functions. In BNL CL.2 cells, the murine embryonic liver cells, inducible nitric oxide synthase (iNOS) mRNA expression appeared after 3 h of treatment with IFN-γ and LPS. Interestingly, mRNA and protein expression of iNOS was down-regulated by sodium nitroprusside (SNP) and diethylamine dinitric oxide in a time- and dose-dependent manner, but not by H_2O_2. TNF-α gene expression was also dramatically reduced by SNP, but IL-6 gene expression was inhibited much less. IFN-γ and LPS-induced chloramphenicol acetyltransferase activity of iNOS promoter constructs was inhibited by SNP. Electrophoretic mobility shift assay showed that SNP inhibited IFN-γ plus LPS-induced Oct-1 binding activity, and the inhibition was reversed by DTT. Mutation in the Oct-1 site completely abolished iNOS promoter activity. In addition, supershift assay and Southwestern analysis demonstrated that the Oct-1 binding activity was inhibited by SNP. Taken together, these results indicate that NO suppresses IFN-γ plus LPS-induced iNOS expression, and that Oct-1 is an important element in this process.

Key words: down regulation of iNOS, iNOS, NO, Oct-1.

There is growing recognition that nitric oxide (NO) acts as a major physiologic messenger in the nervous and cardiovascular systems. Furthermore, several studies have revealed that NO plays a protective role by preventing cell damage, organ ischemia, and infection (*1*, *2*). Besides mediating normal functions, NO has been implicated in pathophysiologic states as diverse as septic shock, hypertension, stroke, and neurodegenerative diseases. The biological roles of NO were first established in inflammatory responses and blood vessel reactivity (*3*, *4*). Also, there are several reports that NO, produced from NO-generating compounds including sodium nitroprusside (SNP), *S*-nitroso-*N*-acetylpenicillamine (SNAP), and diethylamine dinitric oxide (DEA/NO), has a variety of functions as a signal molecule, for example, in activation of G-proteins (*5*), regulation of NF-κB binding activity (*6*), activation of tyrosine phosphatase (*7*), and self-regulation of iNOS enzyme activity as a negative feedback modulator (*8*, *9*). Although NO has been shown to inhibit leukocyte adhesion and mitogenesis, some data suggest that NO may lead to lymphocyte activation, resulting in an increase in the secretion of TNF-α and other cytokines that enhance inflammatory processes such as fibrogenesis and scar formation (*10*).

Many studies have shown that the coculture of rat hepatocytes and Kupffer cells stimulated with lipopolysaccharide (LPS) produces large amounts of nitrite (NO_2^-) and nitrate (NO_3^-), the stable end products of the NO pathway (*11*). Furthermore, it has been demonstrated that hepatocytes also produce NO *in vivo* during chronic hepatic inflammation (*12*), and *in vitro* in response to the conditioned culture supernatant of Kupffer cells (*13*) or to a mixture of LPS and the cytokines such as TNF-α, IL-1β, and IFN-γ (*14*). Some reports showed that hepatic iNOS was expressed in endotoxemia and sepsis, suggesting that NO plays a critical role in the liver as a part of the host immune response (*15*, *16*). The significant induction of iNOS mRNA within only a few hours and the inhibitory effect of actinomycin D suggest that hepatocyte iNOS expression is primarily regulated at the transcriptional level, as seen in the case of macrophage iNOS. However, essentially nothing has been reported about the direct signal pathways and the effects of NO produced by neighboring cells on hepatic iNOS gene expression in the liver. The induction of iNOS expression in the liver is regulated by multiple signals, so further studies are needed to define the precise mechanism by which these signals activate the iNOS gene. Therefore, this study was undertaken to characterize the molecular regulation of iNOS expression by exogenous NO (SNP, SNAP, and DEA/NO) in murine normal embryonic liver cells.

In the present study, we observed that SNP inhibited

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Abbreviations: DEA/NO, diethylamine dinitric oxide; EMSA, electrophoretic mobility shift assay; NO, nitric oxide; iNOS, inducible nitric oxide synthase; SNAP, *S*-nitroso-*N*-acetly-DL-penicillamine; SNP, sodium nitroprusside; TNF-RE, tumor necrosis factor response element.

iNOS mRNA expression induced by IFN-γ plus LPS in a time- and dose-dependent manner, but not the expression of early response genes and constitutively expressed genes. This inhibitory effect of SNP was regulated at the transcriptional level and was controlled by inhibiting the binding activity of the Oct-1.

MATERIALS AND METHODS

Cells—BNL CL.2, a normal murine embryonic cell line (TIB 73), and Hepa-1c1c7, a murine hepatoma cell line (CRL-2026), were obtained from American Type Culture Collection, Rockville, MD and routinely grown in T/75 culture flasks in Dulbecco's Modified Eagle's Medium (DMEM; 4.5 g/liter glucose) supplemented with 10% fetal bovine serum (FBS). The cells were harvested from the culture flask by treatment with trypsin-EDTA and maintained by subculturing every 3–4 days at a density of 2×10^5 cells/ml. The cells were plated on a 96-well plate or 100-mm diameter Petri dish at a concentration of $0.5–1 \times 10^5$ cells/ml and cultured overnight for measuring the production of nitrite concentration or for preparing total RNA and nuclear extracts.

Reagents—DMEM and trypsin-EDTA were purchased from GIBCO Lab. (Grand Island, USA). FBS was obtained from Hyclone Laboratories (Logan, UT). Murine rIFN-γ and anti-mouse TNF-α were purchased from Genzyme (Cambridge, MA). TNF-α, IL-1β, and IL-6 were obtained from Genentech (South San Francisco, CA). TLC plates (Silica gel 60), LPS (*E. coli* 0128:B12), SNP, H_2O_2, pyrrolidine dithiocarbamate (PDTC), actinomycin D, sodium nitrate, sodium nitrite, *N*-(1-naphthyl)-ethylenediamine dihydrochloride, and sulfanilamide were purchased from Sigma Chemical (St. Louis. MO). SNAP and DEA/NO were obtained from Alexis Biochemicals (San Diego, CA). N^G-Monomethyl-L-arginine (N^GMMA) was obtained from Calbiochem (Lucerne, Switzerland). Dupont-NEN (Boston, MA) was the source of [α-^{32}P]dCTP and [α-^{32}P]UTP, and 1-deoxydichloroacetyl-1-[^{14}C]chloramphenicol was obtained from Amersham (Arlington Heights, IL).

Assay of Nitrite Concentration—Accumulated nitrite (NO_2^-) plus nitrate (NO_3^-) in the culture supernatant was measured by an automated colorimetric assay based on the Griess reaction (*11*). Briefly, equal volumes (50 μl) of sample and nitrite reductase were mixed and incubated for 90 min at 37°C. After reduction of nitrate to nitrite by incubating with nitrate reductase at 37°C for 1 h, 100-μl samples were treated with the Griess reagent (1% sulfanilamide, 0.1% napthyl-ethylenediamine dihydrochloride in 2.5% H_3PO_4) at room temperature for 10 min, and the nitrite concentration was determined by measuring the absorbance at 540 nm in a Tirertek Multiscan MCC340 (Flow Laboratories, North Ryde, Australia). The standard curve was constructed with known concentrations of sodium nitrite and nitrate.

Northern Blot Analysis—Cells were solubilized with RNAsol and the total cellular RNA was purified according to the manufacturer's recommended procedure. Samples of 10–20 μg of total RNA were size-fractionated in a 2% agarose-formaldehyde gel and transferred to a nylon membrane. The RNA blot was cross-linked by using UV cross-linker (UV Stratalinker, Stratagene, San Diego, CA) and baked at 80°C for 1 h in a vacuum dry-oven. After prehybridization at 42°C for 4 h in a buffer containing 50% formamide, 5× SSPE (20× SSPE, pH 7.4; 3 M NaCl, 0.2 M NaH_2PO_4, 0.02 M EDTA), 0.1% SDS, 0.5 mg/ml denatured salmon sperm DNA, and 2× Denhardt's solution (50× Denhardt's solution; 1% ficoll, 1% polyvinylpyrrolidone, 1% bovine serum albumin), hybridization was allowed to proceed at 42°C for 16–24 h in the same buffer containing $1–2 \times 10^6$ cpm/ml of [α-^{32}P]dCTP labeled probes. For the iNOS probe, PCR product (forward primer, 5′-CAGAAGCAGAATGTGACCATC-3′; reverse primer, 5′-CTTCTGGTCGATGTCATGAGC-3′) was prepared and sequenced. For the TNF-α probe, PCR product (forward primer, 5′-GATCATCTTCTCAAAATTCG-3′, reverse primer, 5′-TTGACGGCAGAGAGGAGGTT-3′) was prepared and sequenced. The blot was washed sequentially in 1× SSC/0.1% SDS and 0.2× SSC/0.1% SDS for 20 min at room temperature, then in 0.1× SSC/0.1% SDS for 20 min at 65°C. The membrane was exposed at −70°C to Kodak XAR-5 film using an intensifying screen. It was reused for detection of another message by deprobing with boiled strip buffer (0.1× SSC/0.5% SDS).

Cloning of the iNOS Promoter—A genomic DNA library derived from the leukocytes of BALB/c mouse was purchased from Clontech Lab. (Palo Alto, CA, USA). It was screened by using [α-^{32}P]-labeled iNOS probe complementary to the 5′ segment of the iNOS cDNA extending from position 1 to 870. Plaque hybridization was carried out by the manufacturer's recommended procedure. Positive clones were confirmed by rescreening in the same manner to obtain isolated first clones. Cosmid DNA of the tertiary screened clones was isolated, digested with various restriction enzymes and then Southern hybridization was carried out by using the 5′ end fragment of the iNOS cDNA, positions 1 to 198. A DNA fragment that reacted positively with the probe was subcloned into pBluescript II KS(−) and then sequenced. The nucleotide sequence was analysed for transcription factor motifs using the transcription factor database and the SIGNALSCAN program.

CAT Assay—The 2-kb (kilobase) *Eco*RI restriction fragment upstream from the iNOS gene was digested with uniquely cleaving specific restriction enzymes and cloned into the CAT basic vector system (Promega, Madison,WI). All constructs were then sequenced to characterize them. In this experiment, the 1,542-bp *Sph*I fragment and the 420-bp *Xba*I fragment were used. Plasmid DNA was purified twice by equilibrium centrifugation in CsCl/ethidium bromide gradients. BNL CL.2 cells were plated at a density of 2×10^6 cells per 100-mm Petri dish 1 day before transfection. On day 2, fresh medium was added and cells were transfected by the calcium phosphate-DNA coprecipitation method with a total of 10 μg of piNOSCAT construct DNA and 5 μg of β-galactosidase expression vector (pCH110-β-gal, Pharmacia) as an internal control for transfection efficiency. After 18 h, cells were washed with HBSS, and fresh medium containing 10% FBS was added. Cells were then incubated for 24 h in the presence of appropriate cytokines or chemicals. The cell extracts were heat-inactivated at 65°C for 10 min and centrifuged, and the protein content of the supernatant was quantified by using Bio-Rad protein assay kit. CAT enzyme assays were performed in whole cell extracts (20 μg) at 37°C for 30 min, after normalization for β-galactosidase activity. The CAT activity was calculated as cpm of acetylated chloramphenicol/unit of β-galactosidase.

Electrophoretic Mobility Shift Assays (EMSA)—Nuclear

extracts were prepared by the modified method of Dignam *et al.* (17). BNL CL.2 cells were initially grown to semiconfluency (about 80%) before stimulation. The cells were washed with fresh complete medium and stimulated with various stimuli. After stimulation, the cells were washed with ice-cold PBS three times, harvested, and resuspended in hypotonic buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 1 mM PMSF, and 10 μg/ml leupeptin, aprotinin) for 10 min on ice. The cells were centrifuged for 5 min at 2,000 rpm, resuspended in buffer A containing 0.1% NP-40 and homogenized with 20 strokes of a B pestle Dounce homogenizer. Nuclei were separated from cytosol by centrifugation for 5 min at 2,000 rpm and resuspended in buffer C (20 mM HEPES, pH 7.9, 25% glycerol, 0.3 M NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF) for 30 min on ice. Nuclear extracts were obtained by centrifugation at 15,000 rpm for 30 min and the protein concentration was measured. The following synthetic oligonucleotides were used in this study: NF-κB: 5′-GGCAACTGGGGACTCTCCCTTT-3′ and 5′-GGCAAAGGGAGAGTCCCCAGTT-3′, Oct-1: 5′-GGGAACAGTTATGCAAAATAG-3′ and 5′-GAGCTATTTTGCATAACTGTT-3′, TNF-RE: 5′-CATCATGAATGAGCTAACTTGCA-3′ and 5′-GGTGTGCAAGTTAGCTCATTCAT-3′. For binding reactions, 5 μg of nuclear extracts were incubated in a total reaction volume of 9 μl containing 20 mM HEPES, pH 7.9, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, 5% glycerol, 200 μg/ml BSA, and 2 μg of poly(dI-dC)·(dI-dC) for 30 min at 4°C. The ^{32}P-labeled oligonucleotide (1 ng, 1 × 10^5 cpm) was then added to the reaction mixture and incubated for 30 min at 4°C. The reaction products were analyzed on a 4% polyacrylamide gel with 0.5× TBE buffer. The gel was dried and analyzed by autoradiography. For competition assays, excess oligonucleotide (100 molar excess) competitor was preincubated with nuclear extract for 30 min at 4°C. For supershift assays, antibodies were added and incubated for 60 min at 4°C before the standard binding reaction.

Southwestern and Immunoblot Analyses—Twenty micrograms of nuclear extracts from activated BNL CL.2 cells were separated by 10% SDS-PAGE and blotted on a nitrocellulose membrane. For Southwestern analysis, the nitrocellulose membrane was immediately neutralized with neutralizing buffer and hybridized with ^{32}P-radiolabeled 108-bp probe (−150 to −43) containing TNF-RE, NF-κB, and Oct-1 elements for 30 min at room temperature. The blot was washed and exposed to X-ray film. Immunoblotting was performed using anti–Oct-1 antibody as described elsewhere (*18*).

RESULTS

SNP Inhibited the Expression of iNOS mRNA Induced by IFN-r Plus LPS in BNL CL.2 Cells, Murine Embryonic Liver Cells—It has been reported that NO inhibits the iNOS enzyme activity (*19*) and the expression of several genes such as the Ia (*20*) and PKC genes (*21*). Another

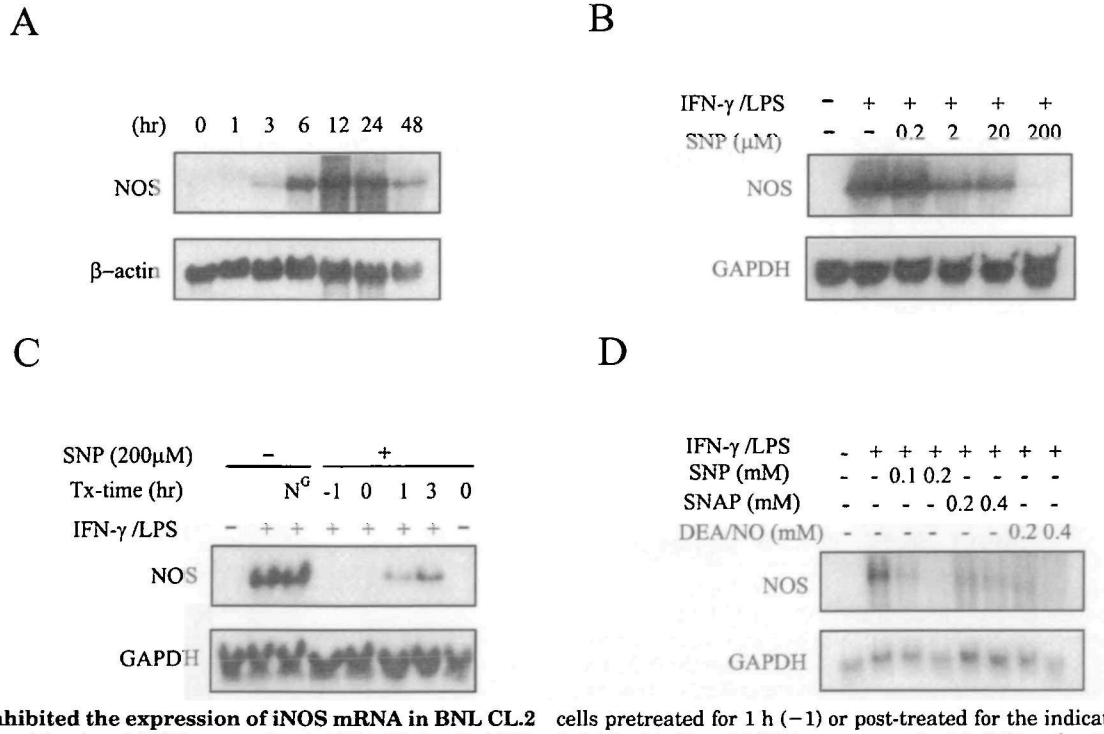

Fig. 1. NO inhibited the expression of iNOS mRNA in BNL CL.2 cells. (A) Time-kinetics of iNOS expression in BNL CL.2 cells. RNA was prepared from BNL CL.2 cells stimulated with IFN-γ (10 U/ml) plus LPS (1 μg/ml) for indicated times. The blot was hybridized with the cDNA probe for macrophage iNOS. (B) The effects of SNP on iNOS mRNA expression induced by IFN-γ plus LPS. BNL CL.2 cells pretreated with various concentrations of SNP for 30 min were treated with IFN-γ plus LPS, and further incubated for 6 h. (C) Time-dependent inhibition of iNOS mRNA expression by SNP. BNL CL.2 cells pretreated for 1 h (−1) or post-treated for the indicated time (0, 1, 3 h) with 200 μM SNP were treated with IFN-γ plus LPS for 6 h. N^G, 0.5 mM N^GMMA. The expression of iNOS mRNA was analyzed by Northern blotting. (D) The effects of several NO-generating compounds on iNOS mRNA expression. BNL CL.2 cells pretreated with various concentrations of SNP, SNAP, or DEA/NO for 30 min were treated with IFN-γ plus LPS for 6 h. The expression of iNOS mRNA was analyzed by Northern blotting.

report (*22*) demonstrated that NO increased the TNF-α secretion without increasing the TNF-α mRNA expression in human neutrophils. To investigate the molecular mechanisms of regulation of NO-mediated iNOS expression, we determined the effect of an exogenous NO source, sodium nitroprusside (SNP), on the mRNA expression and promoter activity of iNOS. SNP released NO dose-dependently (10.9 ± 0.6 and 22.0 ± 0.4 μmol/liter/min nitrite + nitrate at 10 and 100 μM SNP, respectively) and it did not show any cellular toxicity up to 200 μM SNP (data not shown). Expression of iNOS mRNA induced by IFN-γ plus LPS was first detected after 3 h of stimulation and reached its maximum after 12 h (Fig. 1A). As shown in Fig. 1B, iNOS mRNA expression was inhibited by SNP in a dose-dependent manner in BNL CL.2 cells and almost completely inhibited by 200 μM SNP. To characterize the inhibition, BNL CL.2 cells were treated with 200 μM SNP at various times and the steady-state level of iNOS mRNA induced by IFN-γ plus LPS was analyzed (Fig. 1C). When the cells were treated with SNP before or simultaneously with stimulation with IFN-γ plus LPS, the expression of the iNOS mRNA was completely blocked. In contrast, when the cells were treated with SNP 1 or 3 h after the stimulation, the amount of the iNOS mRNA appeared to increase gradually. These inhibitory patterns were similar to those of iNOS mRNA in RAW 264.7 cells (data not shown), and they implied that SNP might modulate iNOS expression at the transcriptional level (see below). When the cells were treated with N^G-monomethyl-L-arginine (N^GMMA), an iNOS enzyme inhibitor, the expression of the iNOS mRNA was not affected when compared to that of the untreated control (Fig. 1C, lane 3), suggesting that endogenous NO had no effect on the regulation of iNOS gene expression at least within 6 h after stimulation. Apparent endogenous NO production was observed from 14 h after IFN-γ plus LPS treatment (*23*). The treatment with SNP alone had no effect on iNOS expression (Fig. 1C, lane 8). Other NO-generating compounds, *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) and diethylamine dinitric oxide (DEA/NO), showed similar inhibitory effects on IFN-γ and LPS-induced iNOS expression (Fig. 1D), indicating that these inhibitory effects were due to the released NO rather than other by-products (*18*).

To determine whether the inhibitory effect on the expression of the iNOS gene is SNP-dependent, the effect of H_2O_2, a potent oxidant, on the expression of iNOS mRNA was tested. As shown in Fig. 2A, H_2O_2 did not block the expression of iNOS mRNA. To test whether SNP had an oxygen radical effect, the cells were treated with SNP and 5 μM pyrrolidine dithiocarbamate (PDTC), a well-known antioxidant. In this case, the inhibitory effect of SNP on iNOS expression was not restored by PDTC. In fact, PDTC is also known as an NF-κB inhibitor and exhibits a suppressive

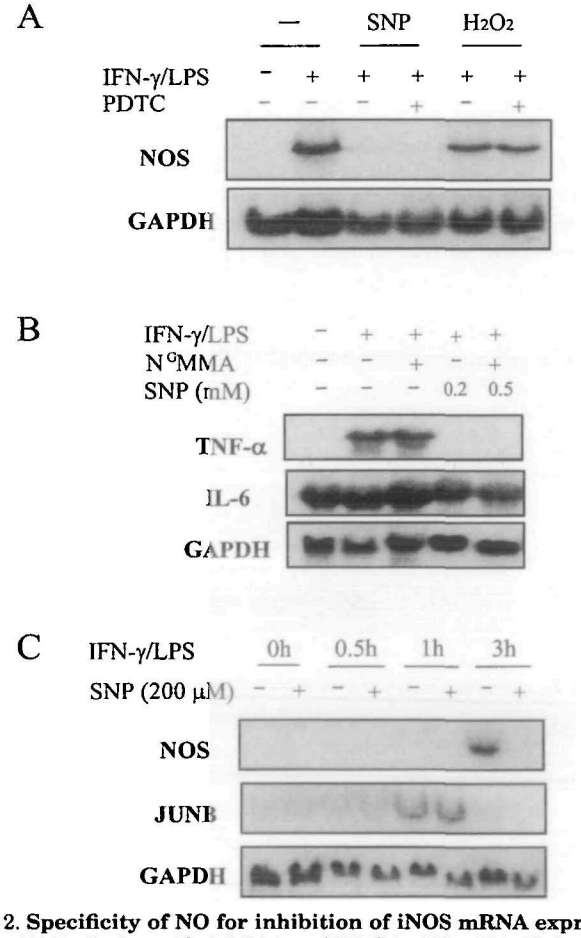

Fig. 2. **Specificity of NO for inhibition of iNOS mRNA expression.** (A) Radical specific inhibition of iNOS by NO. BNL CL.2 cells pretreated with or without 5 μM PDTC for 30 min were treated with 200 μM SNP or 0.5 mM H_2O_2 for another 30 min. Then the cells were stimulated with IFN-γ plus LPS for 6 h. Northern blot analysis was performed as described in "MATERIALS AND METHODS." (B) The effects of NO on cytokine gene expression. BNL CL.2 cells pretreated with N^GMMA or various concentrations of SNP for 30 min, and then stimulated with IFN-γ plus LPS for 6 h. The cells were harvested and Northern blot analysis was carried out as described in "MATERIALS AND METHODS." (C) The effects of NO on Jun-B expression. BNL CL.2 cells pretreated with 200 μM SNP for 30 min were treated with IFN-γ plus LPS for various times. The cells were harvested and Northern blotting was done using the specific probes.

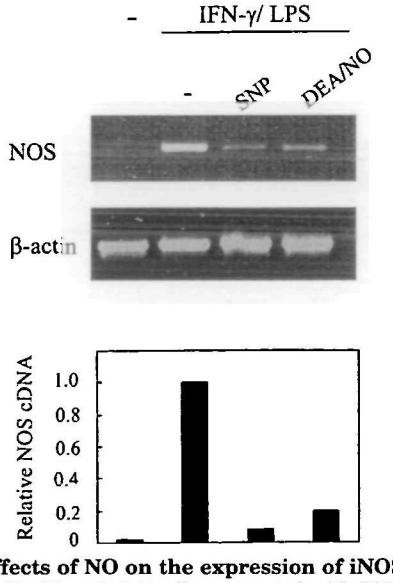

Fig. 3. **The effects of NO on the expression of iNOS mRNA in hepatoma cells.** Hepa-1c1c7 cells pretreated with 200 μM SNP or DEA/NO for 30 min were treated with IFN-γ plus LPS for 6 h. The expression of iNOS mRNA was analyzed by RT-PCR using the primers specific for iNOS or β-actin (*23*, top). Relative iNOS expression was quantitated by densitometry (bottom).

effect on NO synthesis in macrophages (*24*). However, our preliminary data showed that PDTC had little effect on iNOS mRNA expression in BNL CL.2 cells. These results indicated that the inhibitory effect of iNOS mRNA is SNP-dependent and the mechanism of action is different from an oxidant such as H_2O_2.

Next, the effects of SNP on the expression of other genes which are involved in inflammation were tested. As shown in Fig. 2B, TNF-α mRNA expression induced by IFN-γ plus LPS was completely inhibited by 200 μM SNP, in contrast to that in human neutrophils (*22*) and murine peritoneal macrophages (*24*). In our previous study (*23*), using BNL CL.2 cells, TNF-α and anti-TNF-α antibody did not modulate the induction of iNOS mRNA by IFN-γ plus LPS. Therefore, it is thought that the inhibition of TNF-α mRNA by SNP involves another physiological control during the activation of BNL CL.2 cells. The expression of IL-6 mRNA (Fig. 2B) and collagen type I and IV mRNA (data not shown), which are constitutively expressed in BNL CL.2 cells, was not altered by SNP. Also, SNP did not cause a decrease in the expression of housekeeping genes such as GAPDH or β-actin. In contrast, the expression of Bax mRNA, which is related to apoptosis, was slightly increased by SNP (data not shown). In addition, when the cells were treated with IFN-γ plus LPS, JunB, an early response gene, was induced 1 h after stimulation, but its expression was not inhibited by SNP at all (Fig. 2C). These data indicate that the effect of SNP on iNOS gene expression is gene-specific, rather than non-specific. In Fig. 3, SNP and DEA/NO also inhibited iNOS gene expression in Hepa-1c1c7 cells, well-known murine hepatoma cells, as observed in Figs. 1 and 2, suggesting that Hepa-1c1c7 cells and BNL CL.2 cells have a similar character in terms of NO-mediated iNOS gene regulation.

Inhibition of iNOS mRNA Induction by SNP Is Regulated at the Transcriptional Level—Next, the effects of NO on iNOS protein expression were tested. As shown in Fig. 4A, the expression of iNOS protein was inhibited by SNP, DEA/NO, and SNAP. To determine whether the inhibition of iNOS mRNA expression is regulated at the transcriptional level or at the post-transcriptional level, the effect of SNP on the stability of iNOS mRNA was tested. The BNL CL.2 cells were stimulated with IFN-γ plus LPS for 6 h to

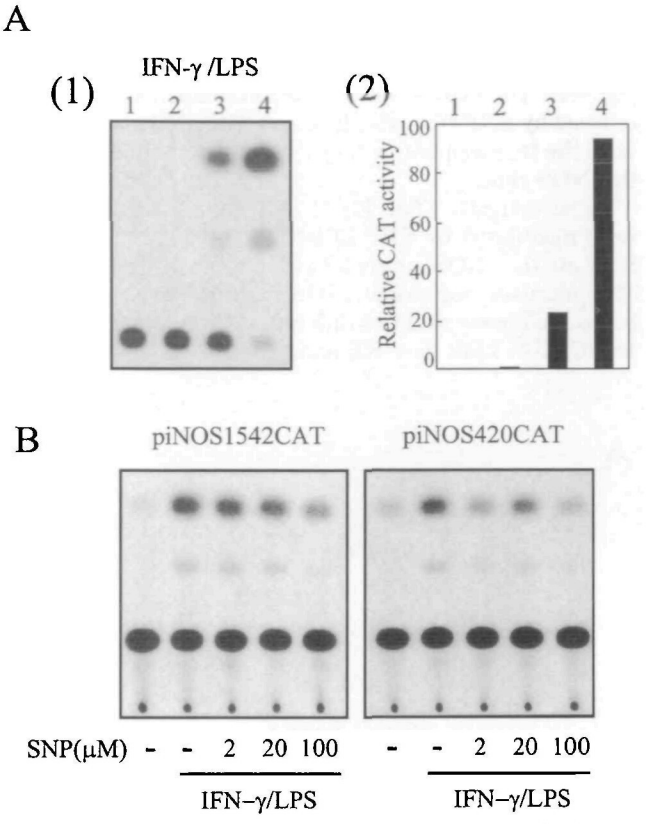

Fig. 5. **NO suppressed the promoter activity of the iNOS.** (A-1) The activation of iNOS promoter by IFN-γ plus LPS in BNL CL.2 cells. Twenty micrograms of each piNOSCAT construct was transfected in BNL CL.2 cells by the calcium phosphate–DNA coprecipitation method. Cells were then treated with IFN-γ plus LPS for 24 h. CAT activity was measured in whole cell extracts after normalizing the amount of extracts using β-galactosidase as an internal control. (A-2) Relative CAT activities were quantitated by densitometry. lane 1, no transfection; lane 2, pCAT, basic; lane 3, piNOS420CAT; lane 4, piNOS1542CAT (B) Dose-dependent effects of SNP on iNOS promoter activity. BNL CL.2 cells were transfected with piNOS-1542CAT or piNOS420CAT, then treated with various concentrations of SNP in the presence of IFN-γ plus LPS. The CAT activity was measured as described in "MATERIALS AND METHODS."

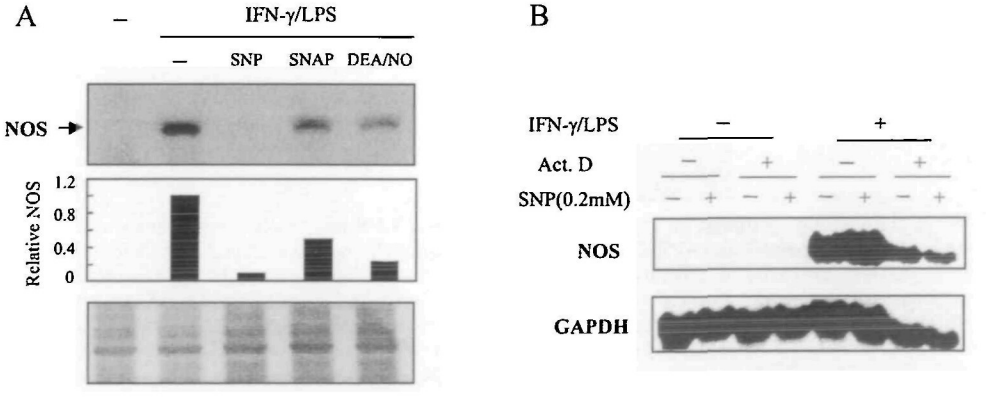

Fig. 4. **The effects of SNP on protein expression and mRNA stability of iNOS.** (A) BNL CL.2 cells were pretreated with 200 μM SNP, SNAP, or DEA/NO and stimulated with IFN-γ plus LPS for 48 h. Cell extracts were then immunoblotted with anti-iNOS antibody as described in "MATERIALS AND METHODS" (top). Relative iNOS protein expression was quantitated by densitometry (middle). The same cell extracts were also stained with Coomassie blue extracts (bottom). (B) BNL CL.2 cells were stimulated with IFN-γ plus LPS for 6 h, and then treated with 5 μg/ml of actinomycin D, which is inhibitor of transcription. The cells were further incubated for 6 h in the presence or absence of 200 μM SNP. Northern blot analysis was performed as described in "MATERIALS AND METHODS."

induce iNOS mRNA. The cells were then treated with actinomycin D to block the transcription of iNOS mRNA and further cultured for 4 h in the presence or absence of 200 μM SNP. The expression level of iNOS mRNA was slightly reduced by SNP (Fig. 4B). It seems that SNP mainly modulates the transcription rather than the post-transcription of the iNOS gene.

To investigate what kinds of transcriptional elements were modulated by SNP in BNL CL.2 cells, the effects of SNP on the iNOS promoter activity were first analyzed. The promoter regions of iNOS were cloned from murine leukocyte library and two different CAT constructs, piNOS-1572CAT (−1542 to + 82) and piNOS420CAT (−420 to + 82), were transfected into BNL CL.2 cells. CAT activity was measured 24 h after exposure to IFN-γ plus LPS. IFN-γ alone did not induce CAT activity at all, and LPS alone induced <10% of CAT activity obtained with the combination of IFN-γ plus LPS (data not shown). These results coincided with our previous finding (*23*) that IFN-γ or LPS alone did not induce NO synthesis, and iNOS mRNA expression was induced to <10% of that induced by IFN-γ plus LPS.

When the region from −1542 bp to −421 bp of iNOS promoter was deleted, CAT activity decreased by a factor of about 10 in transfected BNL CL.2 cells stimulated with IFN-γ plus LPS (Fig. 5A). When the effects of SNP on the

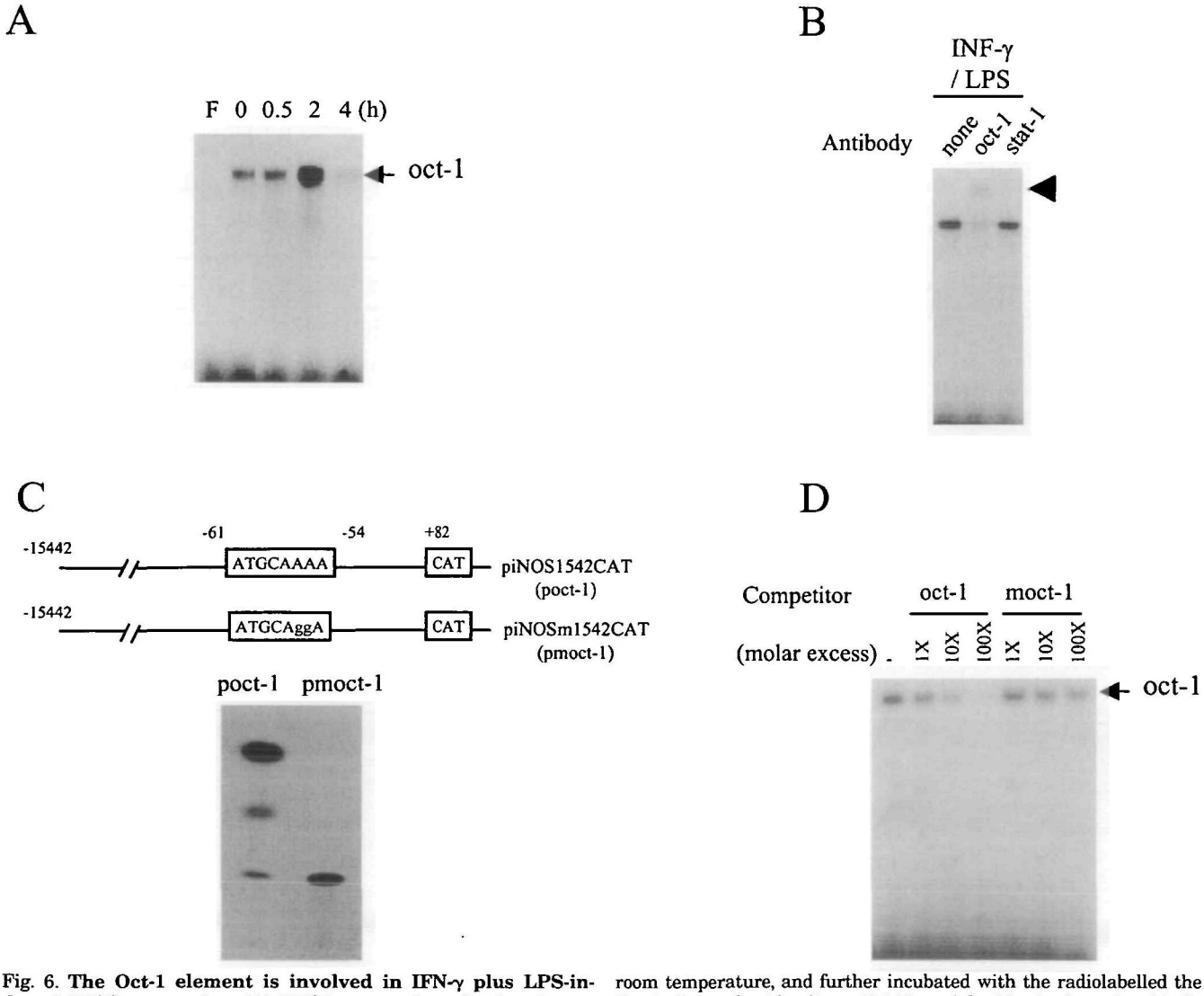

Fig. 6. **The Oct-1 element is involved in IFN-γ plus LPS-induced iNOS expression.** (A) EMSA was performed using the nuclear extracts prepared as indicated and the Oct-1 synthetic oligonucleotide. Time-kinetics for the Oct-1 binding activity induced by SNP and IFN-γ plus LPS (left). Competition assay was performed using the excess molar concentrations of Oct-1 or SRE, which does not exist within the iNOS promoter. Binding complexes were electrophoresed at 150 V for 2 h on a 4% polyacrylamide gel in 0.5× TBE buffer and analyzed by autoradiography. (B) Nuclear extracts prepared as indicated were incubated in the absence or presence of anti–Oct-1 antibody or anti-stat1 antibody (1 μg/reaction) for 20 min at room temperature, and further incubated with the radiolabelled the Oct-1 oligonucleotides (1 ng, 10,000 cpm) for 20 min. Arrowhead indicates the supershifted complex. (C) Twenty micrograms of each piNOS1542CAT construct or piNOSm1542CAT construct containing the mutated Oct-1 motif was transfected in BNL CL.2 cells by the calcium phosphate–DNA coprecipitation method. Cells were then treated with IFN-γ plus LPS for 24 h. CAT activity was measured in whole cell extracts as described above. (D) Competition assay was performed using the excess molar concentrations of wild-type Oct-1 or mutant Oct-1 (moct-1) oligonucleotides.

piNOS1542CAT and piNOS420CAT were tested, SNP inhibited the iNOS promoter activity in a dose-dependent manner in both constructs (Fig. 5B). These results suggest that NO inhibited the iNOS gene expression at the transcriptional level through the elements of the iNOS promoter.

Oct-1 Element Is Involved in the SNP-Mediated Inhibitory Effects on IFN-γ Plus LPS-Induced the iNOS Gene Expression—Sequence analysis of piNOS1542CAT and piNOS420CAT showed that the two constructs contain the common putative binding sites for the transcription factors Oct-1, NF-κB, and TNF-RE element. Oct-1 is known to be essential for iNOS promoter activity, and its activity and synthesis are regulated during M1 differentiation and activation (*25*). Recently, it was also reported that NO inhibited Oct-1 binding activity *in vitro* (*26*). To determine the precise elements related to the inhibitory effect of SNP, we performed the Oct-1 EMSA with extracts of nuclei isolated from BNL CL.2 cells. When the Oct-1 oligonucleotide was used as a probe, the binding activity of the Oct-1 element was maximal after 2 h of IFN-γ plus LPS treatment. Oct-1 element–specific binding was confirmed by competition assay using excess amount of oligonucleotide competitors as described in Experimental Procedures (Fig. 6A). The Oct-1 element (ATGCAAAA) in the iNOS promoter overlaps the GAS element (TTATGCAAA), and this region has been variously termed Oct-1 (27) and GAS (*28*). To identify the transcription factor activated by these stimuli, we performed supershift assay. Nuclear extracts from IFN-γ plus LPS-treated cells were treated with specific antibody against the transcription factors. Anti–Oct-1 antibody interfered with the formation of the Oct-1 binding complex (Fig. 6B), but antibody against stat1 did not. When the Oct-1 element was mutated by site-directed mutagenesis (ATGCAggA), iNOS promoter activity was completely abolished (Fig. 6C). Furthermore, the mutant Oct-1 oligonucleotide could not compete for Oct-1 binding activity induced by IFN-γ plus LPS treatment (Fig. 6D). These data indicate that Oct-1 binding is essential for iNOS promoter activity.

When BNL CL.2 cells were treated with SNP, SNP inhibited IFN-γ plus LPS-induced Oct-1 binding (Fig. 7, A and B lane 4). Several reports (*29*, *30*) indicate that NO modulates enzyme activity through the *S*-nitrosylation of proteins. Recently, it was also reported that NO disrupts DNA binding of c-Myb (*31*) *via* redox-sensitive cysteine (C130) in a cell-free system. When nuclear extracts of BNL CL.2 cells were treated with 0.2 mM SNP, Oct-1 binding activity was completely inhibited (Fig. 7B lane 5). DTT is

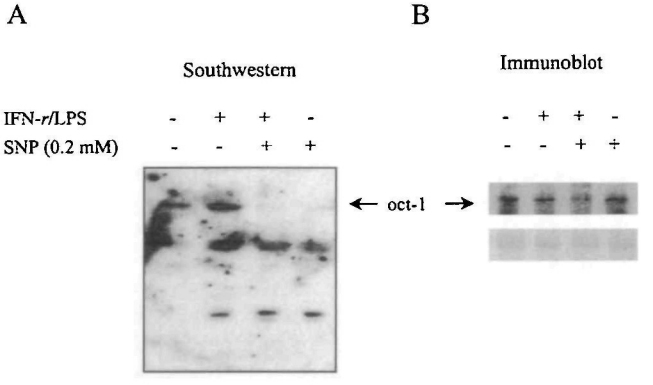

Fig. 8. **Identification of the Oct-1 protein by Southwestern and immunoblot analyses.** (A) Twenty micrograms of nuclear extracts were separated on a 10% SDS-PAGE and tested for binding activity to ^{32}P-radiolabeled 108 probe as described in "MATERIALS AND METHODS." (B) The same cell extracts were immunoblotted with anti-Oct-1 antibody (top). The gel was then stained with Coomassie blue (bottom).

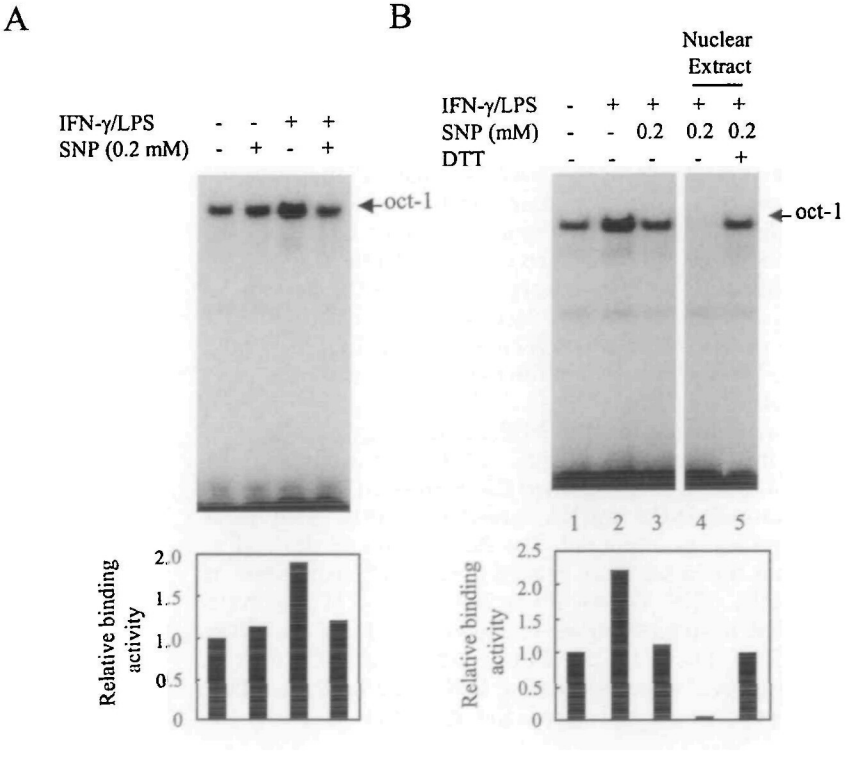

Fig. 7. **SNP inhibited IFN-γ plus LPS-induced Oct-1 binding activity.** (A) EMSA was performed by incubating 5 μg of nuclear extracts prepared from cells treated with 200 μM SNP or IFN-γ plus LPS for 2 h using the ^{32}P-labeled Oct-1 probe. Binding complexes were electrophoresed at 150 V for 2 h on a 4% polyacrylamide gel in 0.5× TBE buffer and analyzed by autoradiography. (B) EMSA was performed by incubating 5 μg of nuclear extracts prepared from cells treated with 200 μM SNP or IFN-γ plus LPS for 2 h using the ^{32}P-labeled Oct-1 probe (Lane 1–4). Nuclear extracts were directly treated with 200 μM SNP in the presence or absence of 1 mM DTT for 30 min before binding to the ^{32}P-labeled Oct-1 probe (lanes 5 and 6). Relative binding activities were quantitated by densitometry.

known to remove the thiol-bound NO groups from target proteins effectively (*31*, *32*). As shown in lane 6 of Fig. 7B, this inhibition was readily reversed by addition of excess DTT. When the effects of SNP on NF-κB activity were tested using the same extracts, SNP also inhibited NF-κB DNA binding activity (data not shown) as reported previously (*30*).

Finally, NO-mediated inhibition of Oct-1 binding was confirmed by Southwestern blotting and immunoblotting (Fig. 8). SNP inhibited Oct-1 binding to the radiolabeled 108-bp probe containing the Oct-1 site, but the binding activities of 65- and 40-kDa proteins were not affected by SNP. Immunoblotting of the same cell extracts showed that SNP did not reduce the synthesis of Oct-1. Taken together, these results indicate that SNP rapidly inhibited the Oct-1 binding activity induced by IFN-γ plus LPS, probably through protein modification such as *S*-nitrosylation of Oct-1.

DISCUSSION

The iNOS activity in murine macrophages has been reported to be maximally induced by IFN-γ and/or LPS, whereas both rat and human hepatocytes require the combination of four stimuli, LPS, TNF-α, IL-1, and IFN-γ, for the maximal induction. Meanwhile, others reported that the combination of IFN-γ plus LPS had a minimal effect on activating hepatocyte iNOS, but the combinations of IFN-γ plus TNF-α or TNF-α plus IL-1 were the most effective in activating hepatocyte iNOS (*33*). TNF-α has also been reported to stimulate NO formation directly in cultured hepatocytes isolated from rats exposed to LPS (10). Our previous study (*23*) showed that the combination of IFN-γ plus LPS was essential for iNOS mRNA expression and that TNF-α had no effect on the induction of iNOS mRNA in BNL CL.2 cells. The induction of iNOS mRNA within only 3 h suggested that the regulation of this type of iNOS mRNA expression might occur primarily at the transcriptional level, as seen in the case of macrophage iNOS (*33*, *34*). These studies suggest that the cytokine signals which induce iNOS gene expression differ quantitatively and qualitatively, depending on cell types. Hepatic iNOS is expressed in endotoxemia and in sepsis, suggesting that NO plays a critical role in the liver as a part of the host immune response. Also, it is clear that the induction of iNOS in the liver is regulated by multiple signals acting in synergy, and it is therefore necessary to define the precise mechanisms by which these signals activate the iNOS gene expression. Thus, the major object of this study was focused on the regulation of iNOS gene expression by IFN-γ plus LPS and the roles of NO in the murine embryonic liver cell line, BNL CL.2 cells.

NO synthesized by the iNOS activity regulates the expression of specific genes such as c-fos and c-Jun (*35*). In BNL CL.2 cells, SNP inhibited the expression of IFN-γ plus LPS-induced iNOS mRNA dose-dependently. SNP inhibited the expression of only the iNOS gene and the TNF-α gene, but the latter is not related to the iNOS expression in these cells. SNP did not affect the stability of the iNOS gene, but it suppressed its promoter activity of the iNOS gene. Even though the regulatory patterns of iNOS expression by several stimuli including IFN-γ and LPS are different depending on cell types, the inhibition of iNOS expression by NO seems to be common to liver cells (BNL CL.2) and hepatoma cells (Hepa-1c1c7) as well as macrophages (*18*) and microglial cells (*36*).

The inhibition of iNOS gene expression by its end product, NO, implies negative feedback regulation of iNOS synthesis. However, blocking iNOS activity with N^GMMA did not alter iNOS mRNA expression (Fig. 1C). This may be due to the lack of detectable NO production from BNL CL.2 cells during this time of incubation (6 h). NO production was detectable after 14 h stimulation with IFN-γ plus LPS in BNL CL.2 cells (*23*). Feedback inhibition is dependent on the concentration of the end product. In this case, endogenous NO production (less than 0.1 μmol/10^5 cells/min) of BNL CL.2 cells is much less than NO generation by NO donors (22.0 μmol/liter/min nitrite+nitrate at 100 μM SNP; 53.2 μmol/liter/min nitrite+nitrate at 100 μM DEA/NO). However, when an excess amount of NO (more than 500 μM) is generated during endotoxic shock and inflammation (*37*), the feedback inhibition of iNOS gene expression can be expected.

Several different NO-generating compounds such as SNAP, DEA/NO, and NO gas have been used to study the biological roles of NO *in vitro*. SNP has been used in many *in vitro* and *in vivo* studies, but spontaneous decomposition of SNP releases not only NO but also Fe^{2+} and cyanide, each of which may react with thiol groups of cellular proteins including transcription factors involving NOS expression. The direct effects of cyanide and other compounds were not tested in this study, but their toxic effects can be ruled out because cells remained viable during the experiments and the expression of IL-6, JunB, and SNP did not reduce expression in other housekeeping genes. In addition, DEA/NO, which has relatively inert by-products (*18*), had the same effects on iNOS mRNA expression.

In macrophages, two separate areas in the iNOS promoter containing almost identical *cis* elements are required for maximal activated expression (*29*, *30*). Since the upstream region alone is not related to the regulation of induction, it may act primarily as an enhancer.

In BNL CL.2 cells, these two regions were also required for the maximal induction, and the CAT activity of two constructs containing these regions was reduced by SNP in a dose-dependent manner (Fig. 4). These results suggested that the iNOS promoter, especially the downstream part, could be responsible for inhibition by SNP in addition to the activation by IFN-γ plus LPS-induced gene expression. It has been reported that transcription factors such as AP-1, Oct-1, and Oct-2 were found in the macrophage iNOS promoter, and LPS activated NF-IL6, NF-κB, AP-1, and ISRE binding proteins. IFN-γ activated interferon consensus sequence binding protein (ICSBP), interferon regulatory factor-1/interferon-stimulated gene factor 2 (IRF-1/ISGF-2), interferon-stimulated gene factor 3 (ISGF-3), and γ-interferon activating factor (GAF) (*38*, *39*). The promoter region of piNOS420CAT (downstream region) contains various *cis*-acting elements, including a particularly striking array of octamers, NF-κB, Oct-1, and TNF-RE. When the Oct-1 element was mutated by site-directed mutagenesis, CAT activity of mutant piNOS1542CAT (pmoct-1) was not detected at all in IFN-γ plus LPS- induced BNL CL.2 (Fig. 5), untreated BNL CL.2 cells, and macrophages (data not shown). These results including supershift and site-directed mutagenesis studies strongly suggest that the Oct-1 ele-

ment is essential for basal iNOS gene expression. However, Oct-1 binding activity was moderately induced by IFN-γ plus LPS treatment (Fig. 6), and the possibility cannot be ruled out that the Oct-1 may have some minor roles in iNOS inducibilty.

Oct-1, a member of the POU family, is involved in the ubiquitous expression of genes including immunoglobulin genes, snRNA genes, and histone H2B. It has two highly conserved domains, a POU homeodomain and a POU-specific domain, which are required for DNA binding and for the interaction with other transactivators such as VP16 and OBF-1 (*40*). The DNA binding activity of Oct-1 is known to be regulated by protein modification such as phosphorylation (*41*). Our results and others (*31*) suggested that the Oct-1 activity is also regulated by NO probably by *S*-nitrosylation. Actually, there are two cysteine amino acids which are located in the highly conserved domains of the POU homeodomain and POU-specific domain (*40*), and further analysis is required to elucidate the protein modification of Oct-1 by NO.

NF-κB is known to regulate the iNOS gene expression by LPS in murine macrophages (*42*), and it was also reported that NO inhibited NF-κB DNA binding (*30*, *36*, *43*). In BNL CL.2 cells, NO inhibited NF-κB binding activity when NF-κB element was used, but competition assay using the probe containing NF-κB, TNF-RE, and Oct-1 elements showed that NF-κB did not compete for the binding of the protein-DNA complex, although Oct-1 did (data not shown). One possible explanation for this could be that the interaction of NF-κB to the NF-κB element requires interaction with Oct-1, and Oct-1 is absolutely required for the iNOS promoter activity, as suggested by Sawada *et al.* (*25*). Our mutagenesis study of the Oct-1 element (Fig. 5) further supports this hypothesis. Thus, inhibition of Oct-1 binding by NO to the basal level may impair the whole transcriptional machinery for induction of iNOS gene expression.

The dose-response relationship between NO and iNOS mRNA expression in macrophages has been reported to be biphasic (*18*). In this report, low concentrations (1–12 μM) of NO showed stimulatory effects and high concentrations (>200 μM) of NO suppressed the iNOS gene expression in ANA-1 macrophages. In our study, a low concentration of NO (0.2–2 μM) showed an inhibitory effect on iNOS mRNA expression. As also discussed previously, NO derived from SNP did not influence the expression of many genes induced by IFN-γ plus LPS, except for TNF-α in BNL CL.2 cells. However, in ANA-1 macrophages, NO did not influence the IFN-γ plus LPS-induced TNF-α mRNA expression. NO increased TNF-α production in LPS-treated human neutrophils without increasing LPS-induced TNF-α mRNA expression (*44*). It remains to be determined whether TNF-α plays a role in mediating the effects of NO on iNOS expression in different cells.

Collectively, NO played a critical role by limiting the induction of iNOS expression, suggesting that overproduction of NO in the liver may cause physiological imbalance by shutting off iNOS expression. In this process, the binding activity of Oct-1, a critical transcriptional factor operating in inducible expression probably interacting with other factors, is regulated by NO.

REFERENCES

1. Schmidt, H.H.W. and Walter, U. (1994) NO at work. *Cell* **78**, 919–925
2. Geller, D.A., Nussler, A.K., DiSilvio, M., Lowenstein, C.J., Sharpiro, R.A., Wang, S.C., Simmons, R.L., and Billar, T.R. (1993) Cytokines, endotoxin, and glucocorticoids regulate the expression of inducible nitric oxide synthase in hepatocytes. *Proc. Natl. Acad. Sci. USA* **90**, 522–526
3. Garthwaite, J., Charles, S.L., and Chess-Williams, R. (1998) Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in brain. *Nature* **336**, 385–388
4. Bredt, D.S. and Snyder, S.H. (1989) Nitric oxide mediates glutamate-linked enhancement cGMP levels in the cerebellum. *Proc. Natl. Acad. Sci. USA* **86**, 9030–9033
5. Lander, H.M., Sehajpal, P., Levine, D.M., and Novogrodsky, A. (1993) Activation of human peripheral blood mononuclear cells by nitric oxide-generating compounds. *J. Immunol.* **150**, 1509–1516
6. Vincenti, M.P., Burrell, T.A., and Taffet, S.M. (1992) Regulation of NF-κB activity in murine macrophages: Effect of bacterial lipopolysaccharide and phorbol ester. *J. Cell. Physiol.* **150**, 204–213
7. Pan, M.G., Florio, T., and Stork, P.J.S. (1992) G protein activation of a hormone-stimulated phosphatase in human tumor cells. *Science* **256**, 1215–1217
8. Rogers, N.E. and Ignarro, L.J. (1992) Constitutive nitric oxide synthase from cerebellum is reversibly inhibited by nitric oxide formed from L-arginine. *Biochem. Biophys. Res. Commun.* **189**, 242–249
9. Vodovotz, Y., Kwon, N.S., Pospischil, M., Manning, J., Paik, J., and Nathan, C. (1994) Inactivation of nitric oxide synthase after prolonged incubation of mouse macrophages with IFN-r and bacterial lipopolysaccharide. *J. Immunol.* **152**, 4110–4118
10. Van Dervort, A.L., Yan, L., Madara, P., Cobb, J.P., Wesley, R.A., Corriveau, C.C., Tropea, M.M., and Danner, R.L. (1994) Nitric oxide regulates endotoxin-induced TNF-α production by human neutrophils. *J. Immunol.* **152**, 4102–4109
11. Ding, A.H., Nathan, C., and Stuer, D.J. (1988) Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. *J. Immunol.* **141**, 2407–2412
12. Luss, H., DiSilvio, M., Litton, A.L., Veda, L.M.Y., Nussler, A.K., and Billiar, T.R. (1994) Inhibition of nitric oxide synthesis enhances the expression of inducible nitric oxide synthase mRNA and protein in a model of chronic liver inflammation. *Biochem. Biophys. Res. Commun.* **204**, 635–640
13. Curran, R.D., Billiar, T.R., Stuer, D.J., Hofmann, K., and Simmons, R.L. (1989) Hepatocytes produce nitrogen oxides from L-arginine in response to inflammatory products of Kupffer cells. *J. Exp. Med.* **170**, 1769–1774
14. Curran, R.D., Billiar, T.R., Stuer, D.J., Ochos, J.B., Harbredcht, B.J., Flint, S.G., and Simmons, R.L. (1990) Multiple cytokines are required to induce hepatocyte nitric oxide production and inhibit total protein synthesis. *Ann. Surg.* **212**, 462–471
15. Billiar, T.R., Curran, R.D., Harbrecht, B.G., Stuehr, D.J., Demetris, A.J., and Simmons, R.L. (1990) Modulation of nitrogen oxide synthesis in vivo: N^G-monomethyl-L-arginine inhibits endotoxin-induced nitrite/nitrate biosynthesis while promoting hepatic damage. *J. Leukoc. Biol.* **48**, 565–571
16. MacMicking, J.D., Nathan, C., Hom, G., Chartrain, N., Fletcher, D.S., Trumbauer, M., Stevens, K., Xie, Q.-W., Sokoi, K., Hutchinson, N., Chen, H., and Mudgett, J.S. (1995) Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. *Cell* **81**, 641–650
17. Dignam, J.D., Levovitz, R.M., and Roeder, R.G. (1983) Accurate transcription initiation by RNA polymerase II in a stable extract from isolated mammalian nuclei. *Nucleic Acids Res.* **11**, 1475–1489

18. Sheffler, L.A., Wink, D.A., Melillo, G., and Cox, G.W. (1995) Exogenous nitric oxide regulates IFN-γ plus lipopolysaccharide-induced nitric oxide synthase expression in mouse macrophages. *J. Immunol.* **155**, 886–894
19. Assreuy, J.F., Cunha, F.Q., Liew, F.Y., and Moncada, S. (1993) Feedback inhibition of nitric oxide synthase activity by nitric oxide. *Br. J. Pharmacol.* **108**, 833–837
20. Sicher, S.C., Vazquez, M.A., and Lu, C.Y. (1994) Inhibition of macrophage Ia expression by nitric oxide. *J. Immunol.* **153**, 1293–1300
21. Jun, C.D., Choi, B.M., Lee, S.Y., Kang, S.S., Kim, H.M., and Chung, H.T. (1994) Nitric oxide inhibits the expression of protein kinase C delta gene in the murine peritoneal macrophages. *Biochem. Biophys. Res. Commun.* **204**, 105–111
22. Van Dervort, A.L., Yan, L., Madara, P.J., Cobb, J.P., Wesley, R.A., Corriveau, C.C., Tropea, M.M., and Danner, R.L. (1994) Nitric oxide regulates endotoxin-induced TNF-α production by human neutrophils. *J. Immunol.* **152**, 4102–4109
23. Lee, B.S., Kang, H.S., Pyun, K.H., and Choi, I. (1997) Roles of tyrosine kinases in the regulation of nitic oxide synthesis in murine liver cells: Modulation of NF-κB activity by tyrosine kinases. *Hepatology* **25**, 913–919
24. Mulsch, A., Schray-Utz, B., Mordvintcex, P.I., Hauschildt, S., and Busse, R. (1993) Diethyldithiocarbamate inhibits induction of macrophage NO synthase. *FEBS Lett.* **321**, 215–218
25. Sawada, T., Falk, L.A., Rao, P., Murphy, W.J., and Pluznik, D.H. (1997) IL-6 induction of protein-DNA complexes via a novel regulatory region of the inducible nitric oxide synthase gene promoter. *J. Immunol.* **158**, 5267–5276
26. Liu, X.K., Abernethy, D.R., and Andrawis, N.S. (1998) Nitric oxide inhibits Oct-1 DNA binding activity in cultured vascular smooth muscle cells. *Life Sci.* **62**, 739–749
27. Xie, Q.W., Whisnant, R., and Nathan, C. (1993) Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon γ and bacterial lipopolysaccharide. *J. Exp. Med.* **177**, 1779–1784
28. Lowenstein, C.J., Alley, E.W., Raval, P., Snowman, A.M., Snyder, S.H., Russell, S.W., and Murphy, W.J. (1993) Macrophage nitric oxide synthase gene: Two upstream regions mediate induction by interferon γ and lipopolysaccharide. *Proc. Natl. Acad. Sci. USA* **90**, 9730–9734
29. More, S., Stamler, J.S., and Brune, B. (1996) Posttranslational modification of glyceraldehyde-3-phosphate dehydrogenase by S-nitrosylation and subsequent NADH attachment. *J. Biol. Chem.* **271**, 4209–4214
30. Mattews, J.R., Botting, C.H., Panico, M., Morris, H.R., and Hay, R.T. (1996) Inhibition of NF-κB DNA binding by nitric oxide. *Nucleic Acids Res.* **24**, 2236–2242
31. Brendeford, E.M., Anderson, K.B., and Gabrielsen, O.S. (1998) Nitric oxide disrupts specific DNA binding of the transcription factor c-Myb in vitro. *FEBS Lett.* **425**, 52–56
32. Kim, Y.M., Talanian, R.V., and Billiar, T.R. (1997) Nitric oxide inhibits apoptosis by preventing increases in caspase-3-like activity via two distinct mechanisms. *J. Biol. Chem.* **272**, 31138–32248
33. Geller, D.A., Lowenstein, C.J., Shapiro, R.A., Nussler, A.K., DiSilvio, M., Wang, S.C., Nakayama, D.K., Wang, S.C., Nakayama, D.K., Simmons, R.L., Snyder, S.H., and Billiar, T.R. (1993) Molecular cloning and expression of inducible nitric oxide synthase from human hepatocytes. *Proc. Natl. Acad. Sci. USA* **90**, 3491–3495
34. Liu, S., Adcock, I.M., Old, R.W., Barnes, P.J., and Evans, T.W. (1993) Lipopolysaccharide treatment in vivo induces widespread tissue expression of inducible nitric oxide synthase mRNA. *Biochem. Biophys. Res. Commun.* **196**, 1208–1213
35. Peunova, N. and Enikolopov, G. (1993) Amplification of calcium-induced gene transcription by nitric oxide in neuronal cells. *Nature* **364**, 450–453
36. Colasanti, M., Persichini, T., Menegazz, M., Mariotto, S., Giordano, E., Caldarera, C.M., Sogos, V., Lauro, G.M., and Suzuki, H. (1995) Induction of nitric oxide synthesis mRNA expression: suppression by exogenous nitric oxide. *J. Biol. Chem.* **270**, 26731–26733
37. MacMicking, J.D., Nathan, C., Hom, G., Chartain, N., Fletcher, D.S., Trumbauer, M., Stevens, K., Xie, Q.W., Sokol, K., and Hutchinson, N. (1995) Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. *Cell* **81**, 641–650
38. Politis, A.D., Ozato, K., Coligan, J.E., and Vogel, S.N. (1994) Regulation of IFN-gamma-induced nuclear expression of IFN consensus sequence binding protein in murine peritoneal macrophages. *J. Immunol.* **152**, 2270–2278
39. Mamane, Y., Heylbroeck, C., Genin, P., Algarte, M., Servant, M.J., LePage, C., DeLuca, C., Kwon, H., Lin, R., and Hiscott, J. (1999) Interferon regulatory factors: the next generation. *Gene* **237**, 1–14
40. Suzuki, N., Peter, W., Ciesiolka, T., Gruss, P., and Scholer, H.S. (1993) Mouse Oct-1 contains a composite homeodomain of human Oct-1 and Oct-2. *Nucleic Acids Res.* **21**, 245–252
41. Segil, N., Roberts, S.B., and Heintz, N. (1991) Mitotic phosphorylation of the Oct-1 homeodomain and regulation of Oct-1 DNA binding activity. *Science* **254**, 1814–1816
42. Xie, Q.W., Kashiwabara, Y., and Nathan, C. (1994) Role of transcription factor NF-κB/Rel in induction of nitric oxide synthase. *J. Biol. Chem.* **269**, 4705–4708
43. Park, S.K., Lin, H.L., and Murphy, S. (1997) Nitric oxide regulates nitric oxide synthase-2 gene expression by inhibiting NF-κB binding to DNA. *Biochem. J.* **322**, 609–613
44. Pittner, R. and Spitzer, J.A. (1992) Endotoxin and TNF-α directly stimulate nitric oxide formation in cultured rat hepatocytes from chronically endotoxemic rats. *Biochem. Biophys. Res. Commun.* **185**, 430–435