Biochemical Pharmacology, Vol. 55, pp. 1873–1880, 1998.

ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(98)00078-1

Molecular Cloning and Analysis of the Rat Inducible Nitric Oxide Synthase Gene Promoter in Aortic Smooth Muscle Cells*

Hanfang Zhang,† Xunsheng Chen, Xingwu Teng, Connie Snead and John D. Catravas

Vascular Biology Center and Department of Pharmacology and Toxicology, Medical College of Georgia, Augusta, GA 30912-2500, U.S.A.

ABSTRACT. We have cloned five DNA fragments (−0.32, −0.48, −1.7, −3.2, and −5.1 kb) of the 5′-flanking region of the rat inducible nitric oxide synthase (iNOS) gene from rat genomic DNA. The functional importance of the 5′-flanking region was determined by transient expression of iNOS promoter-luciferase constructs in cultures of rat aortic smooth muscle cells. The −0.48 kb construct, containing one nuclear factor κB (NF-κB) binding site, expressed basal promoter activity but showed only a 1.5- and 1.7-fold increase in luciferase activity in response to lipopolysaccharide (LPS) or a cytokine mixture, respectively. However, the −3.2 kb construct (containing a second NF-κB binding site) showed full promoter activity with a 24-fold increase in response to LPS or cytokine mixture. The −5.1 kb construct showed no further increase in luciferase activity, suggesting that the 1.9 kb upstream of −3.2 kb may not be important in rat iNOS regulation. Rat iNOS promoter induction did not appear to be transcriptionally regulated by NO since NOS inhibitors did not affect induction. These data are in marked contrast to the mouse iNOS promoter in which a DNA sequence as short as a −85 bp, containing one NF-κB site, confers 10-fold inducibility by LPS. The present findings demonstrate that the rat iNOS gene is transcriptionally regulated by cytokines and LPS, but, unlike the mouse gene, the downstream NF-κB site does not appear to be a key region in responses to cytokines and LPS. These data suggest that the regulation of the rat gene may require the coexistence of at least two NF-κB sites or other elements upstream of −0.48 kb of the 5′-flanking region. BIOCHEM PHARMACOL **55**;11:1873–1880, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. iNOS promoter; NF-κB; cytokines; LPS; vascular smooth muscle cells

NO‡ plays important roles in the normal physiology and pathophysiology of many organ systems [1–3]. Of the three types of NOS catalyzing conversion of L-arginine to NO, two are constitutively expressed primarily in vascular endothelial cells (type III or ecNOS) and brain neuronal cells and skeletal muscle (type I or ncNOS), respectively. A third type, the inducible NOS (type II or iNOS), is expressed following stimulation by inflammatory mediators, such as cytokines or LPS. In VSMC, induction of iNOS and the consequent vast amounts of NO produced are believed to be responsible for the systemic hypotension seen in septic shock [4, 5], as well as for the side-effects of anti-tumor therapy with cytokines [6, 7].

The molecular mechanisms for the transcriptional regulation of the iNOS gene have been studied by cloning the mouse iNOS promoter and inducing expression of the promoter-reporter gene constructs in a cultured mouse macrophage cell line, RAW 264.7 [8–10]. One thousand bases out of the 1.5-kb mouse iNOS promoter confer full inducibility by the mixture of interferon-γ and LPS in this cell line [9, 10]. A key region in the promoter for LPS responsiveness is a downstream NF-κB site [11], whereas the synergistic effect of interferon-γ and LPS requires the presence of an additional 5′-region of the promoter [12].

Unlike the mouse iNOS gene promoter, a −1.09 kb of the 5′-flanking region of the human iNOS gene does not respond well to LPS [13]. Recently, promoter analysis of the human iNOS showed that the first −3.8 kb upstream of the gene resulted in only basal promoter activity and demonstrated at most a slight response to cytokines [14, 15]. A 3- to 5-fold induction was found in promoter segments containing up to −5.8 and −7.0 kb, and a 10-fold activation

* The nucleotide sequence data reported in this paper have been submitted to the GenBank with the accession number AF042085.

† Corresponding author: Dr. Hanfang Zhang, Vascular Biology Center, Medical College of Georgia, Augusta, GA 30912-2500. Tel. (706) 721-6338; FAX (706) 721-9799; E-mail: hzhang@mail.mcg.edu.

‡ *Abbreviations:* γ-IRE, interferon-γ response element; AP-1, activator protein 1; CM, cytokine mixture (500 U/mL IL-1β + 150 U/mL TNF-α); CRE, cyclicAMP response element; ecNOS, type III or endothelial nitric oxide synthase; GAS, γ-activated site; IL-1β, interleukin-1β; iNOS, type II or inducible nitric oxide synthase; L-NAME, N^{ω}-nitro-L-arginine-methyl ester; L-NMMA, N^{G}-monomethyl-L-arginine; LPS, lipopolysaccharide; ncNOS, type I or neuronal nitric oxide synthase; NF-κB, nuclear factor κB; NO, nitric oxide; NOS, nitric oxide synthase; RASMC, rat aortic smooth muscle cells; TNF-α, tumor necrosis factor α; and VSMC, vascular smooth muscle cells.

Received 22 September 1997; accepted 18 January 1998.

was observed in a construct containing −16 kb of the promoter although the full sequence and responsive elements of this promoter are unknown [14]. More recently, Eberhardt *et al.* [16] published a −1.7 kb segment of the rat iNOS promoter, and the promoter segment only showed a three-fold induction in response to cytokines, although the promoter contains two NF-κB sites similar to the location of the mouse iNOS promoter. These data suggest species-dependent variations in the molecular mechanisms of iNOS gene regulation.

Although many different cell types can be induced to produce NO by LPS and proinflammatory cytokines [17], most studies of iNOS gene regulation have focused thus far on macrophages. Unlike macrophages, the upstream NF-κB site of the mouse iNOS promoter plays an important role in eliciting a response to cytokines in the A7r5 rat smooth muscle cell line [18]. Similarly, when the mouse macrophage iNOS promoter was transfected into cultured RASMC, it was shown that a key region in mediating the effect of IL-1β was located at −234 bp from the 5′-region of the gene, whereas a significant portion of iNOS induction was independent of the NF-κB site [19]. These data strongly indicate that, in addition to species-dependent variation, there is a cell type-dependent variation in the molecular regulation of iNOS induction.

Exposure of rats to LPS has served as a popular and useful model of septic shock. Because the high output of NO by rat VSMC plays an important part in the pathophysiology of septic shock, in the present study rat iNOS gene regulation in aortic VSMC has been investigated. We have cloned five DNA fragments of −0.32, −0.48, −1.7, −3.2, and −5.1 kb from the 5′-flanking region of the rat iNOS gene and studied the regulation of the promoter in cultures of rat aortic VSMC. We found that the −484-bp segment of the 5′-flanking region of the gene does not respond to LPS and/or cytokines. However, a 24-fold induction was observed in the −3.2-kb construct in response to LPS or cytokines, whereas the −5.1-kb construct showed a similar response, suggesting that the 1.9 kb upstream of the −3.2 kb may not be important in rat iNOS regulation. It was also observed that iNOS promoter induction is not affected by NO, since NOS inhibitors had no effect on the gene induction even though NO production was abolished completely. These data are in marked contrast with findings from the mouse iNOS promoter in either VSMC or macrophages and demonstrate that the rat iNOS is transcriptionally regulated by LPS and cytokines, but in a manner different from that in mouse.

MATERIALS AND METHODS

Reagents

Human recombinant IL-1β was obtained from Boehringer. TNF-α was purchased from R & D Systems. LPS, L-NMMA and L-NAME were purchased from Sigma. Tissue culture media and Lipofectamine were obtained from Life Technologies.

Cell Culture

RASMC were harvested from Wistar rats (Harlan) by enzymatic dissociation, using standard methods [20]. The cells were identified as smooth muscle by indirect immunofluorescent staining for α-actin, using mouse anti-α-actin antibody and anti-mouse IgG FITC conjugate. The positive stain was routinely 100%. RASMC were grown in 100-mm plates in 50% F12 nutrient medium and 50% Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, glutamine, penicillin (100,000 units/L), and streptomycin (100,000 × μg/L) and were subcultured (1:4) into 100-mm tissue culture plates. Cells at passage 2 or 3 were used in the studies. All cultures were grown in a humidified incubator at 37° under 5% CO_2 in air.

Molecular Cloning and Sequencing of the Rat iNOS Promoter

DNA fragments of the 5′-flanking region of rat iNOS promoter were amplified with the PromoterFinder DNA Walking Kit (Clontech) [21]. Briefly, five pools of DNA fragments were prepared by digestion of rat genomic DNA with five restriction enzymes, *Pvu*II, *Ssp*I, *Dra*I, *Sca*I, and *Eco*RV. Following the digestion, each pool of DNA fragments was ligated to an adapter. Two gene specific oligonucleotide primers (totally spanning 45 bp) were derived from the known sequence of the exon 1 of RASMC iNOS cDNA [22]. The sequences of the primers were 5′-GAG GAACCTCAGAAGCGTCTCTAAAAG-3′ (the primary gene specific primer, representing positions 80 to 106 of rat iNOS cDNA) and 5′-CTCTAAAAGTCCAGTC CCCTCACCAAG-3′ (the secondary nested primer, positions 62 to 88). The amplification was performed using the Advantage™ Genomic PCR Kit (Clontech), and the cycle parameters were as follows: 7 cycles of denaturation at 94° for 30 sec and annealing/extension at 72° for 4 min except for an initial denaturation of 1 min, and then 32 cycles of denaturation at 94° for 30 sec and annealing/extension at 67° for 4 min followed by 4 min at 67°. A 1.7 kb promoter fragment was also obtained by amplification with a 5′ primer (5′-GATGAAGCCTGAAGACGCGTCGAC TTTGATATACTGAAATTC-3′) at the −1.7 kb site with −3.2 kb as DNA template. The DNA fragments with identical 3′ ends were cloned into a luciferase gene-containing vector, PGL3-Basic (Promega), which does not contain a promoter and enhancer. The DNA fragments were sequenced on both strands in an ABI automated sequencing system, model 377 in the Molecular Biology Core Facility of the Medical College of Georgia.

Isolation and Purification of Plasmid DNA

Large-scale preparations of DNA of promoter-reporter constructs were obtained by modified alkaline lysis as described [23, 24]. The JM109 bacterial pellet was collected by a brief

centrifugation of 150–250 mL of Luria-Bertani medium overnight culture. The cell lysate was prepared by using equal volumes of Solution I (50 mM of glucose, 25 mM of Tris · Cl, pH 8.0, and 10 mM of EDTA), Solution II (0.2 N of NaOH and 1% SDS), and Solution III (3 M of potassium and 5 M of acetate), and DNA was precipitated by isopropanol. Then the recovered DNA was incubated with RNase in TE buffer (10 mM of Tris · Cl and 1 mM of EDTA, pH 8.0) followed by phenol/chloroform extraction and precipitation by ethanol. The purity of the plasmid DNA preparations was assessed (a) by electrophoresis in 1% agarose and (b) spectrophotometrically (260 nm to 280 nm ratio). The concentrations of the DNA preparations were estimated by the optical density at 260 nm.

Transient Transfection of DNA into RASMC and Luciferase Activity Assay

Transfection with Lipofectamine was used according to a published procedure [24]. Briefly, plasmid DNA with equimolar amounts of each DNA construct was mixed with OPTI-MEM I reduced serum medium (Life Technologies) to a final volume of 400 μL. To keep the total amount of DNA (2 μg) constant, carrier DNA PGL3-Basic was cotransfected with the smaller constructs. The DNA mixture was gently added to an equal volume of diluted Lipofectamine in OPTI-MEM (22 μL of Lipofectamine + 378 μL of OPTI-MEM) and incubated at room temperature for 15 min, whereupon a further 3.2 mL of OPTI-MEM was added. This mixture was added in 1.2-mL aliquots to triplicate wells of RASMC (80% confluent) in 6-well plates, these cells being washed previously with PBS and OPTI-MEM. After incubating at 37° for 5 hr, 0.3 mL of OPTI-MEM containing 50% fetal bovine serum was added to the cells (final concentration of fetal bovine serum, 10%), and the incubation was continued for another 8–10 hr. Then the transfected cells were changed to fresh medium. Antibiotics were not present in the transfection mixture during the transfection period. This method produces reproducible transfection efficiencies and thus allows a significant number of transfections to be performed at any one time. To control for the efficiency of transfections between different constructs, a plasmid DNA containing a cytomegalovirus promoter-driven β-galactosidase gene was cotransfected. The luciferase activity of the iNOS promoter-reporter constructs was determined by the Luciferase Assay System (Promega). The transfected cells were incubated with LPS (5–10 μg/mL) or CM for the indicated time. These concentrations are similar to those used by other investigators in VSMC [18, 19]. After being washed three times with PBS, cells were lysed with 0.75 mL of 1X Cell Culture Lysis Reagent, and the luciferase activity in 20 μL of cell lysate was measured with the Luciferase Assay Substrate (Promega) in a TD 20/20 luminometer (Turner Designs). For each promoter-reporter construct, DNA from at least two individual colonies was isolated, transfected, and tested for promoter activity.

Nitrite/Nitrate Assay

Nitrite in RASMC culture supernatants was measured spectrophotometrically using the Griess reagent [25]. Nitrate was reduced enzymatically to nitrite with nitrate reductase (Boehringer Mannheim) before the Griess reaction.

Statistical Analysis

Values are reported as means ± SEM. Significant differences were estimated by *t*-test or ANOVA, as appropriate. Statistical significance was established at $P < 0.05$.

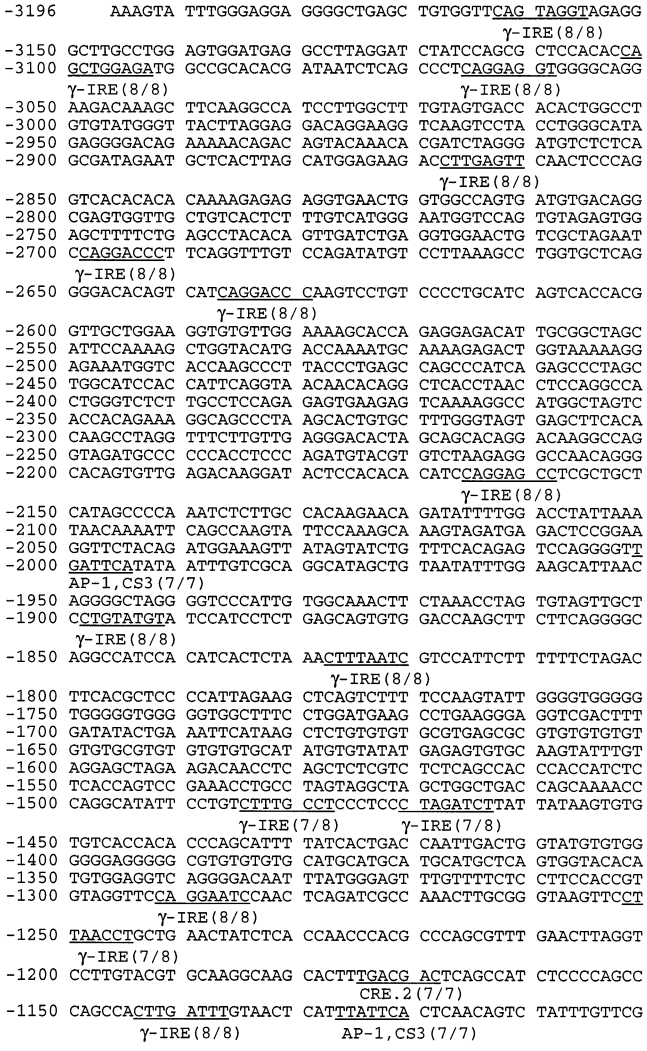
RESULTS

Cloning, Sequence, and Analysis of the 5′-Flanking Region of the Rat iNOS

Four DNA fragments with 0.404, 0.572, 3.28, and 5.2 kb of the 5′-flanking region of the rat iNOS were obtained by polymerase chain reaction amplification from *Pvu*II, *Ssp*I, *Dra*I, and *Sca*I DNA pools, respectively. The *Eco*RV DNA pool was not amplified. All DNA fragments were cloned into a luciferase containing vector, PGL3-Basic. The DNA sequences of the −3.2-kb (−3196 to +88) construct, as well as of the 404 (−316 to +88) and 572 (−484 to +88) bp, were determined by dye terminator chemistry on both strands (Fig. 1). Analysis of the −3.2-kb 5′-flanking region of the rat iNOS gene (DNASIS version 3.6) showed the presence of a TATA box, downstream from a number of elements homologous to consensus sequences for the binding of transcription factors involved in the cytokine or LPS induction of other genes. These include (among many) 29 copies of γ-IRE, 2 copies of GAS, 3 copies of AP-1, 2 copies of TNF response element (TNF-RE), 2 copies of CRE, and 2 copies of NF-κB, as shown in Fig. 1. The −3.2 rat iNOS displayed a 55% sequence homology to the human iNOS promoter [15] and 73% sequence homology to the mouse iNOS promoter [8]. The downstream 1.7 kb of the −3.2 construct showed 98% sequence homology to the published −1.7-kb 5′-flanking region of rat iNOS gene [16]. Figure 2 showed the sequence alignment analysis of two NF-κB regions of rat, human, and mouse iNOS promoter. The sequences of the downstream NF-κB binding site were well conserved among rat, human, and mouse. In rat and mouse, the sequences of the upstream NF-κB binding site were identical, but the sequence in human showed low identity compared with rat and mouse (Fig. 2).

Activation of iNOS in RASMC by Cytokines

To induce iNOS expression, RASMC were stimulated with LPS or a mixture of IL-1β and TNF-α, and iNOS activity was determined from the accumulation of the NO stable product, nitrite. The level of total nitrite was determined by the Griess reaction [25]. Unstimulated RASMC produced a small amount of nitrite. The accumulation of nitrite in the



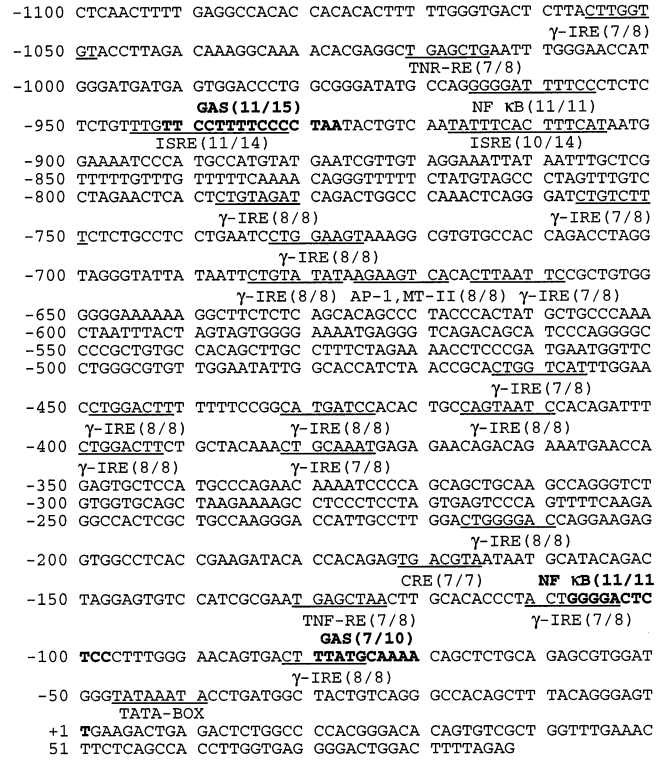


FIG. 1. Nucleotide sequence of the rat iNOS 5′-flanking region. The putative transcriptional site [16] is denoted as nucleotide position +1 (bold). Positions of potential binding sites for transcription factors are underlined and labeled with the homology ratios in parentheses. The overlapping sites are labeled as bold letters.

medium could be detected at 6 hr after stimulation. At 24 hr, significant amounts of nitrite had accumulated in response to LPS (72 μmol/L) and CM (158 μmol/L), respectively (Table 1). Nitrite accumulation was inhibited significantly by the NOS inhibitor L-NAME (Table 1).

Stimulation of Rat iNOS Promoter

The rat type II NOS promoter (3.2 kb)-luciferase gene construct was transfected and induced in RASMC. The promoter activity was induced in response to CM or LPS in RASMC 24 hr post-transfection and continued to increase for the next 48–72 hr. Figure 3 shows that the luciferase activity could be detected after a 3-hr exposure to CM or LPS; peak activation of iNOS promoter occurred after a 6-hr exposure to CM or LPS and declined significantly by 24 hr. This pattern of stimulation mirrors the time course of accumulation of cyclicGMP, an indicator of iNOS induction in RASMC exposed to LPS [26]. Promoter activity remained longer at peak levels following exposure to CM than to LPS, and luciferase activity in response to CM was also significantly higher than that in response to LPS at 12 and 24 hr. The higher promoter activity at 24 hr following exposure to CM may explain the higher levels of nitrite after exposure to CM for 24 hr (Table 1).

Localization of Cytokine and LPS Response Elements

To study the transcriptional regulation of the rat iNOS gene promoter, constructs containing different lengths of the 5′-flanking region of iNOS were prepared and transfected into RASMC. Promoter activities were studied after 6 hr of exposure to CM or LPS. The PGL3-Basic DNA construct (without any promoter and enhancer) exhibited 38 ± 9 light units of luciferase activity, and all five iNOS promoter-reporter constructs exhibited similar basal activities (41–59 light units). The basal activities of PGL3-Basic and five iNOS promoter-reporter constructs were not statistically different from each other ($P = 0.45$ by ANOVA). The PGL3-Basic DNA construct did not respond to stimulation by CM or LPS, and the −316 bp and −484-bp constructs containing a single NF-κB site, with sequences identical to the corresponding regions of the −3.2-kb construct, responded only slightly to either CM or

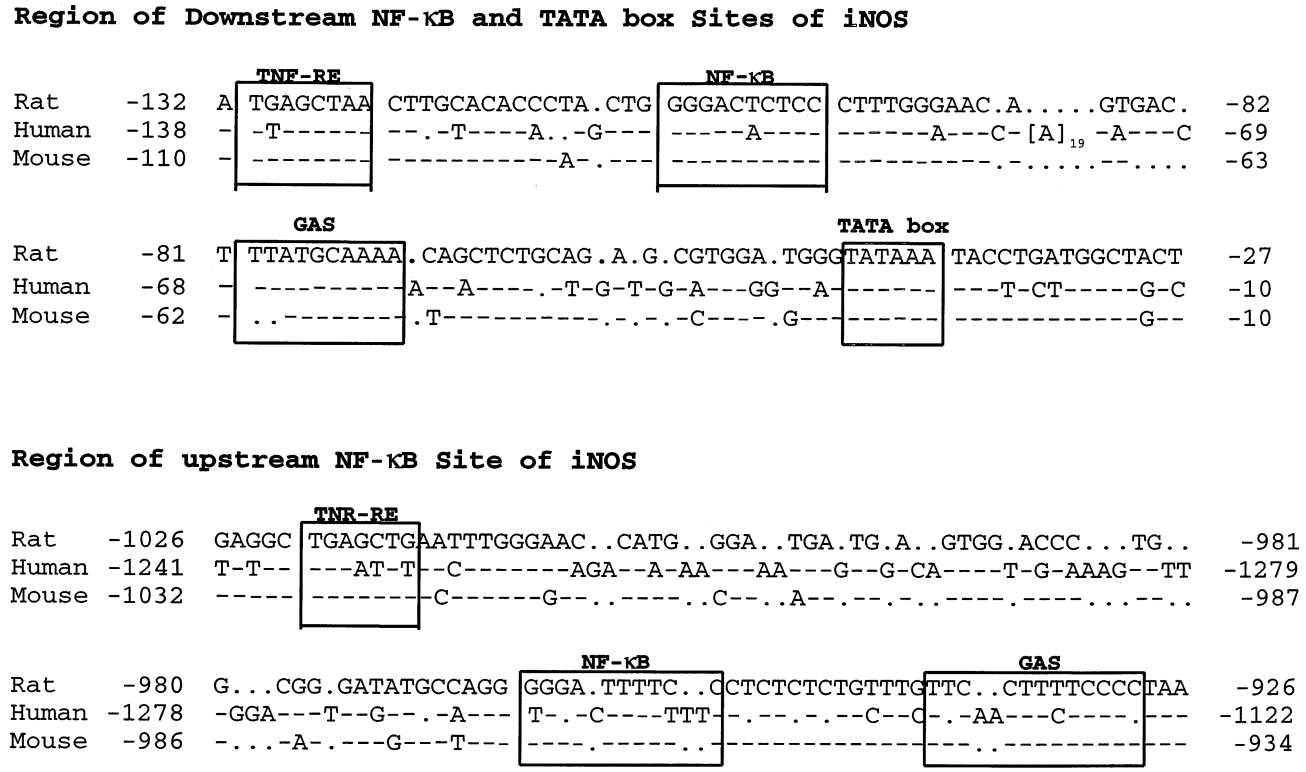


FIG. 2. Sequence alignment analysis of two NF-κB regions of rat, human, and mouse iNOS promoter. Numbers indicate the relative position to the +1 transcription start site. The homologous nucleotides are indicated by *dashes*, and the missing bases are indicated by *dots*. The putative transcriptional binding sites are *boxed*.

LPS. However, the −1.7-kb construct exhibited a 13-fold increase in luciferase activity in response to LPS or CM, whereas both the −3.2-kb and −5.1-kb constructs produced a 24-fold induction in luciferase activity in response to CM or LPS (Fig. 4). To control the efficiency of the transfection between different lengths of constructs, galactosidase gene driven by CMV promoter was cotransfected. Very similar results were obtained from two additional experiments and are shown in Fig. 5. These data suggest that the cytokine and LPS responsive region or elements are located in −0.484 bp to −3.2 kb upstream from the putative transcription site in rat iNOS gene.

TABLE 1. NO production by RASMC and effect of L-NAME on NO production

	Nitrite levels (μmol/L)	
	−L-NAME	+L-NAME
Control	0.60 ± 0.40 (7)	1.18 ± 0.73 (5)
LPS	72.29 ± 3.91* (6)	1.88 ± 1.31† (4)
CM	158.35 ± 21.32*‡ (6)	12.68 ± 2.84† (4)

Confluent RASMC were incubated with LPS (10 μg/mL) or CM in the presence or absence of 3 mmol/L of L-NAME for 24 hr. NO production was determined from analysis of total nitrite accumulation. Values are means ± SEM with the number of determinations given in parentheses.

*$P < 0.001$, compared with control.

†$P < 0.001$, compared with the corresponding group without L-NAME.

‡$P < 0.01$, compared with LPS.

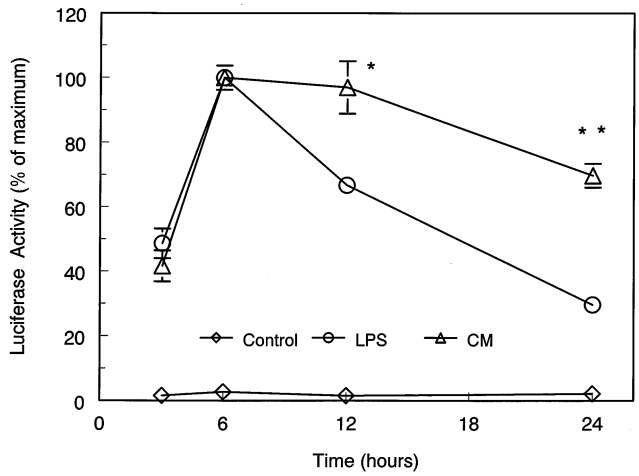
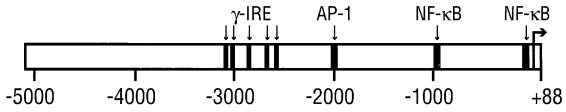


FIG. 3. Time study of rat iNOS promoter induction in response to LPS and CM in RASMC. Luciferase activity was determined in RASMC transfected with a −3.2 kb iNOS-luciferase construct DNA and incubated with LPS (5 μg/mL) or CM for the indicated time. Data are expressed as the percentage of the highest luciferase activity induced by LPS or CM at 6 hr in each set of determinations (mean ± SEM) from three separate experiments. The highest luciferase activity at 6 hr ranged from 584 to 2120 light units/20 μL lysate for LPS, and from 636 to 1778 light units/20 μL lysate for CM. *P < 0.04 and **P < 0.00002, compared to LPS.



	Luciferase Activity (fold induction)		
	Control	LPS	CM
PGL_3-Basic Luciferase	1.00 ± 0.03 (4)	0.81 (2)	0.79 ± 0.04 (4)
-0.32 kb Luciferase	1.00 ± 0.01 (4)	1.65 ± 0.17 (4)[a]	1.51 ± 0.04 (4)[b]
-0.48 kb Luciferase	1.00 ± 0.02 (4)	1.53 ± 0.07 (4)[b]	1.72 ± 0.13 (4)[b]
-1.7 kb Luciferase	1.00 ± 0.02 (16)	13.13 ± 0.42 (16)[c]	13.23 ± 0.58 (4)[c]
-3.2 kb Luciferase	1.00 ± 0.03 (24)	24.07 ± 0.71 (24)[c,d]	24.31 ± 0.95 (7)[c,d]
-5.1 kb Luciferase	1.00 ± 0.05 (7)	24.68 ± 1.16 (7)[c,d]	25.73 ± 1.94 (7)[c,d]

FIG. 4. Inducibility of different length rat iNOS promoter constructs in response to LPS or CM in RASMC. The constructs containing different lengths of the 5′-flanking region of iNOS are shown on the left with certain restriction sites and transcription factor binding sites identified. Each construct DNA was transfected into RASMC. Luciferase activity was determined in RASMC incubated with LPS (5 μg/mL) or CM for 6 hr following the transfection of the construct. Data are expressed as fold induction from the respective unstimulated controls. Values are means ± SEM (number of determinations). (a) $P < 0.05$ vs controls; (b) $P < 0.01$ vs controls; (c) $P < 0.0002$ vs controls; and (d) $P < 0.0004$ vs the −1.7-kb construct.

Regulation of the Rat iNOS Promoter by Endogenous NO

It has been reported that NO may affect iNOS gene expression [27–29]. To test this possibility, RASMC were transfected with the −3.2-kb iNOS promoter-luciferase construct. The cells were preincubated with 3 mmol/L of L-NAME or 2 mmol/L of L-NMMA for 1 hr and then stimulated by CM or LPS in the presence of the inhibitor for 6 or 24 hr. Subsequently, the luciferase activities were analyzed. L-NAME significantly inhibited NO production (Table 1); however, neither L-NAME nor L-NMMA affected the CM- or LPS-up-regulation of rat iNOS promoter activity (Table 2).

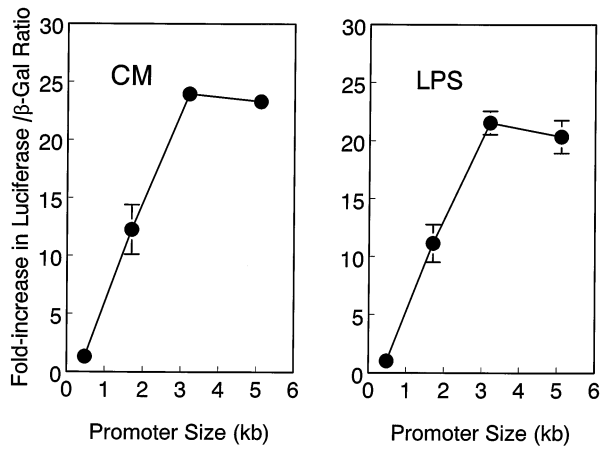


FIG. 5. Cytokine and LPS induction in RASMC cotransfected with β-galactosidase and different length rat iNOS promoter constructs. Constructs containing different lengths of 5′-flanking region of rat iNOS were transfected into RASMC. β-galactosidase gene was cotransfected to correct for transfection efficiency. Luciferase and β-galactosidase activities were determined in RASMC incubated with LPS (5 μg/mL) or CM for 6 hr following the transfection of the constructs. Data are expressed as the ratio of fold induction of luciferase to β-galactosidase from the respective unstimulated controls. Values are means ± SEM from 3–5 determinations from two experiments.

DISCUSSION

Type II NOS can be induced in many species and cell types, and the molecular regulation of this gene appears to differ among cell types. For example, in a mouse macrophage cell line, RAW 264.7, the promoter region that responds to LPS contains only a single downstream NF-κB binding site [11]. However, in the VSMC cell line A7r5, it is the −890-to −1002-bp region (also containing another NF-κB site) that best responds to stimulation by cytokines [18]. Still, in RASMC it is the −234 to +31 region that appears crucial in the induction of iNOS in response to cytokines [19]. These data demonstrate that with the same mouse iNOS promoter, different cell types control iNOS induction differently. Since the induction of iNOS in VSMC may be an important factor in the hypotension, vascular hyporesponsiveness, and death associated with septic shock, VSMC are an appropriate model for the study of iNOS promoter regulation. Pharmacological control of iNOS promoter activity may prove important for the study and management of these pathologies. Primary cultures of VSMC were used in this study since it has been reported that VSMC cell line A7r5 does not possess the p50 subunit that, together with p65, forms NF-κB [30]. We further employed a homologous system (rat iNOS promoter transfected into RASMC) to eliminate possible species differences in the regulation of iNOS.

LPS responsive elements are located exclusively within −300 bp upstream from the transcriptional site of non-mammalian iNOS gene in a chicken macrophage cell line, HD11 [31]. In mice, however, −85 bp upstream of the transcriptional start site is crucial in the response to LPS in a transfected macrophage cell line [11]. An enhancer, also

TABLE 2. Effect of NOS inhibitors on iNOS promoter activity in RASMC

	Luciferase activity (light units)	
	−NOS inhibitor	+NOS inhibitor
Experiment I (6-hr exposure in the presence or absence of L-NAME)		
Control	44.3 ± 3.2 (3)	43.6 ± 3.9 (3)
LPS	2120.0 ± 205.1*	2096.7 ± 177.3*
CM	1774.7 ± 62.3*	1801.3 ± 89.6*
Experiment II (6-hr exposure in the presence or absence of L-NMMA)		
Control	25.8 ± 2.1 (3)	26.7 ± 1.3 (3)
LPS	584.2 ± 18.1*	631.8 ± 20.8*
CM	635.5 ± 28.8*	660.2 ± 35.2*
Experiment III (24-hr exposure in the presence or absence of L-NMMA)		
Control	20.4 ± 1.12 (3)	22.1 ± 3.6 (3)
LPS	182.6 ± 8.6*	171.5 ± 5.9*
CM	450.4 ± 50.2*	361.4 ± 19.2*

Luciferase activity was determined in RASMC transfected with the −3.2-kb iNOS-luciferase construct and incubated with LPS (5 μg/mL) or CM in the presence or absence of NOS inhibitors. Values are means ± SEM, $N = 3$.
*$P < 0.01$, compared with the corresponding control.

located upstream, mediates the synergistic effect of IFN-γ and LPS [12]. The responsive regions in both chicken and mouse iNOS promoters contain a downstream NF-κB binding site, suggesting that this factor may play an important role in iNOS induction. Still, in humans, up to −3.8 kb of the promoter transfected in AKN-1 (a human liver epithelial cell line) is unable to confer inducibility to LPS, even though this region contains two putative NF-κB binding sites. In humans, a full-length −16-kb promoter is required for induction by a cytokine mixture even though the full sequence and elements of this promoter are still unknown [14]. In the present study, we found that −3.2 and −5.1 kb of the rat iNOS promoter confer a full and equal activity in response to LPS and cytokines. The −484- and −316-bp constructs show a slight inducibility even though they contain one NF-κB binding site that is identical to the mouse iNOS promoter (Fig. 2). Thus, in RASMC, the elements responsive to LPS and cytokines in the rat iNOS promoter require the region upstream of −484 bp. It is possible that the induction of rat iNOS promoter requires the coexistence of two NF-κB sites and/or other additional elements. Further studies are needed to locate these regions and elements. The −1.7-kb construct containing two NF-κB binding sites is well induced by both LPS and cytokines. However, the inducibility of the construct is only 54% of the full promoter, indicating that the 1.5 kb upstream from −1.7 kb contains elements important in the regulation of iNOS induction. Computer analysis reveals the presence of many putative elements in this 1.5-kb region, including one copy of AP-1 and 9 copies of γ-IRE. Our data differ from those of Eberhardt *et al.* [16] in which the −1.7-kb rat iNOS promoter shows only three-fold induction in response to cytokines. The most likely reason for this difference may lie in the Swiss 3T3 fibroblasts used by Eberhardt *et al.* to study the induction of the rat iNOS construct, and this cell line may exhibit different mechanisms of inducibility from those of RASMC and may not be suitable for studying iNOS induction.

Two reports have suggested that NO may influence iNOS gene expression in liver and CNS glial cells [27, 28]. Weisz *et al.* [29] reported that L-NMMA, a competitive inhibitor of NOS, significantly increases promoter activity in a mouse macrophage cell line, RAW 264.7, transfected with the mouse iNOS promoter. In the present study, rat iNOS promoter appeared not to be feedback-regulated by the enhanced levels of NO in RASMC because L-NAME completely abolished NO production, but did not affect promoter activity (Table 2). It is thus possible that VSMC may have different regulatory mechanisms controlling iNOS expression from those of RAW 264.7 cells.

This work was supported by Grant HL52958. We thank Dr. Richard C. Venema for helpful discussion and suggestions and Ms. Aretha Bogan for preparing the manuscript.

References

1. Harald HH, Schmidt W and Walter U, NO at work. *Cell* **78:** 919–925, 1994.
2. Moncada S, Palmer RM and Higgs EA, Nitric oxide: Physiology, pathophysiology, and pharmacology. *Pharmacol Rev* **43:** 109–142, 1991.
3. Nathan C and Xie Q-W, Nitric oxide synthase: Roles, tolls, and controls. *Cell* **78:** 915–918, 1994.
4. Kilbourn RG, Jubran A, Gross SS, Griffith OW, Levi R, Adams J and Lodato RF, Reversal of endotoxin-mediated shock by N^G-methyl-L-arginine, an inhibitor of nitric oxide synthesis. *Biochem Biophys Res Commun* **172:** 1132–1138, 1990.
5. Wright CE, Rees DD and Moncada S, Protective and patho-

logical roles of nitric oxide in endotoxin shock. *Cardiovasc Res* **26:** 48–57, 1992.
6. Moritz T, Niederle N, Baumann J, May D, Kurschel E, Osieka R, Kempeni J, Schlick E and Schmidt CG, Phase I study of recombinant human tumor necrosis factor α in advanced malignant disease. *Cancer Immunol Immunother* **29:** 144–150, 1989.
7. Hibbs JB Jr, Wesrenfelder C, Taintor R, Vavrin Z, Kablitz C, Baranowski RL, Ward JH, Menlove RL, McMurry MP, Kushner JP and Samlowski WEJ, Evidence for cytokine inducible nitric oxide synthesis from L-arginine in patients receiving interleukin-2 therapy. *J Clin Invest* **89:** 867–877, 1992.
8. Xie Q-W, Whisnant R and Nathan C, 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, 1993.
9. Xie Q-W and Nathan C, The high-output nitric oxide pathway: Role and regulation. *J Leukoc Biol* **56:** 576–582, 1994.
10. Lowenstein CJ, Alley EW, Raval P, Snowman AM, Snyder SH, Russell SW and Murphy WJ, Macrophage nitric oxide synthase gene: Two upstream regions mediate induction by interferon γ and lipopolysaccharide. *Proc Natl Acad Sci USA* **90:** 9730–9734, 1993.
11. Xie Q-W, Kashiwabara Y and Nathan C, Role of transcription factor NF-κB/Rel in induction of nitric oxide synthase. *J Biol Chem* **269:** 4705–4708, 1994.
12. Martin E, Nathan C and Xie Q-W, Role of interferon regulation factor 1 in induction of nitric oxide synthase. *J Exp Med* **180:** 977–984, 1994.
13. Kleinert H, Euchenhofer C, Ihrig-Biedert I and Förstermann U, Glucocorticoids inhibit the induction of nitric oxide synthase II by down-regulating cytokine-induced activity of transcription factor nuclear factor-κB. *Mol Pharmacol* **49:** 15–21, 1996.
14. De Vera ME, Shapiro RA, Nussler AK, Mudgett JS, Simmons RL, Morris SM Jr, Billiar TR and Geller DA, Transcriptional regulation of human inducible nitric oxide synthase (NOS2) gene by cytokines: Initial analysis of the human NOS2 promoter. *Proc Natl Acad Sci USA* **93:** 1054–1059, 1996.
15. Spitsin SV, Koprowski H and Michaels FH, Characterization and functional analysis of the human inducible nitric oxide synthase gene promoter. *Mol Med* **2:** 226–235, 1996.
16. Eberhardt W, Kunz D, Hummel R and Pfeilschifer J, Molecular cloning of rat inducible nitric oxide synthase gene promoter. *Biochem Biophys Res Commun* **223:** 752–756, 1996.
17. MacMicking JD, Nathan C, Nom G, Chartrain N, Fletcher DS, Trumbauer M, Stevens K, Xie Q-W, Sokol K, Hutchinson N, Chen H and Mudgett JS, Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. *Cell* **81:** 641–650, 1995.
18. Spink J, Cohen J and Evans TJ, The cytokine responsive vascular smooth muscle cell enhancer of inducible nitric oxide synthase. *J Biol Chem* **270:** 29541–29547, 1995.
19. Perrella KA, Patterson C, Tan L, Yet S-F, Hsieh C-M, Yoshizumi M and Lee M-E, Suppression of interleukin-1β-induced nitric-oxide synthase promoter/enhancer activity by transforming growth factor-β1 in vascular smooth muscle cells. Evidence for mechanisms other than NF-κB. *J Biol Chem* **271:** 13776–13780, 1996.
20. Geinsterfer AAT, Peach MJ and Owens GO, Angiotensin II induces hypertrophy but not hyperplasia of cultured rat aortic smooth muscle cells. *Circ Res* **62:** 749–756, 1988.
21. Siebert PD, Chenchik A, Kellogg DE, Lukyanov KA and Lukyanov SA, An improved PCR method for walking in uncloned genomic DNA. *Nucleic Acids Res* **23:** 1087–1088, 1995.
22. Geng YJ, Almqvist M and Hansson GK, cDNA cloning and expression of inducible nitric oxide synthase from rat vascular smooth muscle cells. *Biochim Biophys Acta* **1218:** 421–424, 1994.
23. Sambrook J, Fritsch EF and Maniatis T, *Molecular Cloning: A Laboratory Manual*, I.38-I.41. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
24. Zhang H, Henderson H, Gagne SE, Clee SM, Miao L, Liu G and Hayden MR, Common sequence variants of lipoprotein lipase: Standardized studies of *in vitro* expression and catalytic function. *Biochim Biophys Acta* **1302:** 159–166, 1996.
25. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS and Tannenbaum SR, Analysis of nitrate, nitrite and [^{15}N]nitrate in biological fluids. *Anal Biochem* **126:** 131–138, 1982.
26. Marczin N, Go CY, Papapetropoulos A, Hattori R and Catravas JD, Induction of nitric oxide synthase by protein synthesis inhibition in aortic smooth muscle cells. *Br J Pharmacol*, **123:** 1000–1008, 1998.
27. Luss H, DiSilvio M, Litton AL, Vedia LM, Nussler AK and Billiar TR, 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, 1994.
28. Park SK, Lin HL and Murphy S, Nitric oxide limits transcriptional induction of oxide synthase in CNS glial cells. *Biochem Biophys Res Commun* **201:** 762–768, 1994.
29. Weisz A, Cicatiello L and Esumi H, Regulation of the mouse inducible-type nitric oxide synthase gene promoter by interferon-γ, bacterial lipopolysaccharide and N^G-monomethyl-L-arginine. *Biochem J* **316:** 209–215, 1996.
30. Saunders LA and Spink JM, Differential response of VSMC preparations to LPS is mediated via members of the Rel family of transcriptional activators. *Endothelium* **3:** S50, 1995.
31. Lin AW, Chang CC and McCormick CC, Molecular cloning and expression of an avian macrophage nitric-oxide synthase cDNA and the analysis of the genomic 5′-flanking region. *J Biol Chem* **271:** 11911–11919, 1996.