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*

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Mark A. Perrella‡§, Cam Patterson, Larissa Tan, Shaw-Fang Yet, Chung-Ming Hsieh, Masao Yoshizumi‡, and Mu-En Lee‡†

From the Cardiovascular Biology Laboratory, Harvard School of Public Health, the Department of Medicine, Harvard Medical School, ‡Pulmonary and Cardiovascular Divisions, Brigham and Women’s Hospital, Boston, Massachusetts 02115.

Nitric oxide (NO) is a potent vasodilator that contributes to the regulation of vascular tone (1, 2). In the presence of molecular oxygen and NADPH, NO synthase utilizes L-arginine to produce NO and citrulline (3). The inducible isofrom of NO synthase (iNOS) is present in many cell types, and the induction of iNOS is regulated at the level of gene transcription (4–6). Transcriptional regulation of the iNOS gene has been studied most extensively in macrophages after induction by lipopolysaccharide (LPS) and interferon-γ (7–10). The effect of LPS on the iNOS promoter is mediated by NF-κB (8), whereas the ability of interferon-γ to synergistically induce iNOS in the presence of LPS requires binding of interferon regulatory factor-1 to its binding site (interferon regulatory factor-E) in the iNOS promoter (10).

An important cytokine-mediated disease linked to the induction of iNOS is septic shock (1, 11, 12). The resistance of mice carrying a disrupted iNOS gene to LPS-induced death emphasizes the potential importance of NO in septic shock (13, 14).

Because vascular smooth muscle cells are critical for regulation of vascular tone, the induction of iNOS within these cells may have a particularly important role in the pathogenesis of septic shock. We have previously demonstrated that interleukin (IL)-1β and tumor necrosis factor-α, two important cytokines, downstream of endotoxin in the cascade of events leading to septic shock (15, 16), induce iNOS mRNA by increasing gene transcription in vascular smooth muscle cells (5). In addition, transforming growth factor (TGF)-β1 down-regulates NF-κB after its induction by IL-1β by decreasing the rate of iNOS gene transcription. Different from its effect on vascular smooth muscle cells, the effect of TGF-β1 on macrophage iNOS occurs post-translationally (17).

Another important difference exists between vascular smooth muscle cells and macrophages in regard to iNOS regulation by cytokines. In macrophages, an NF-κB site located in the downstream portion of the iNOS 5′-flanking sequence (−153GAGCACTTCC−76) NF-κB is necessary for induction of iNOS by bacterial LPS (8). Xie and colleagues (8) also noted that a large transcription factor complex bound to an oligonucleotide probe constructed from the iNOS promoter containing the NF-κB site plus the next 47 bp downstream. The binding of this transcription factor complex to the iNOS probe was dependent upon the synthesis of newly formed protein(s) in response to LPS. In contrast, the induction of iNOS mRNA in vascular smooth muscle cells by cytokines such as IL-1β and tumor necrosis factor-α is protein synthesis-independent (5).

This difference in protein synthesis dependence suggests that important differences exist in regulation of the iNOS gene in vascular smooth muscle cells and macrophages.

We designed the present studies to determine the role of NF-κB in the induction of iNOS by IL-1β in vascular smooth muscle cells and to determine whether TGF-β1 suppresses iNOS promoter/enhancer activity through inhibition of NF-κB.

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† To whom correspondence should be addressed: Cardiovascular Biology Laboratory, Bldg. 2, Harvard School of Public Health, 677 Huntington Ave., Boston, MA 02115. Tel.: 617-432-4994; Fax: 617-432-0031.

‡ The abbreviations used are: NO, nitric oxide; iNOS, inducible nitric oxide synthase; IL-1β, interleukin-1β; TGF-β1, transforming growth factor-β1; RASMC, rat aortic smooth muscle cells; LPS, lipopolysaccharide; bp, base pair(s); PCR, polymerase chain reaction; CAT, chloramphenicol acetyltransferase.

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Using reporter gene transfection experiments in rat aortic smooth muscle cells (RASMC), we found that the majority of IL-1β-responsive activity is contained in bp – 234 to +31 of the iNOS gene, and a notable portion of iNOS inducibility is independent of NF-κB. The TGF-β1-responsive elements account for the suppression of IL-1β-induced iNOS activity exist in region – 234 to +31 of the 5′-flanking sequence, and the inhibitory effect of TGF-β1 occurred through a site(s) other than NF-κBd.

**EXPERIMENTAL PROCEDURES**

Cell Culture—RASMC were harvested from male Sprague-Dawley rats (200–250 grams) by enzymatic dissociation according to the method of Gunther et al. (18). The cells were cultured in Dulbecco’s modified Eagle’s medium (I RH Biosciences, Lenexa, KS) and supplemented with 10% fetal calf serum (HyClone, Logan, UT), penicillin (100 units/ml), streptomycin (100 μg/ml), and 25 mM Hepes (pH 7.4) (Sigma). RASMC were passaged every 4–7 days, and experiments were performed on cells 4–6 passages from primary culture. To assure the lack of endotoxin contamination within our cell culture system, assays were performed using a modified Limulus Amebocyte Lysate system (Bio-Whittaker Inc., Walkersville, MD).

Isolation of the iNOS 5′-flanking Sequence—A fragment of the 5′-flanking region of the mouse gene encoding iNOS was amplified by polymerase chain reaction (PCR). Primers were designed according to the published sequence of the mouse iNOS 5′-flanking sequence (7). The forward (5′-TCTAGACTCTGTGCCTCCCATG-3′) and reverse (5′-AGTCTCGAGTCTTCAACTCCCTGTA-3′) primers were used to amplify a 1.516-bp fragment from mouse genomic DNA. The PCR fragment was subcloned, and the sequence was verified by the dideoxy chain termination method (19).

Plasmids—Plasmids pGL2-Basic, pGL2-Control, and pGL2-Pro

moter contained the firefly luciferase gene (Promega, Madison, WI). pGL2-Basic had no promoter, pGL2-Control contained only the SV40 Basic, Plasmid pCAT-Control contained the prokaryotic chloramphenicol acetyltransferase (CAT) gene (Promega) driven by the SV40 promoter and enhancer. Reporter constructs containing fragments of the mouse iNOS 5′-flanking sequence were named according to the location of the fragment from the transcription start site in the 5′ and 3′ directions. The 1.516-bp fragment amplified from mouse genomic DNA, containing 1485 bp of the iNOS 5′-flanking sequence and the first 31 bp after the transcription start site, was named iNOS(–1485/+31). To provide localization of potential cis-acting elements responsible for IL-1β induction of smooth muscle cell iNOS, we generated a series of truncated iNOS 5′-flanking fragments using PCR techniques. Constructs containing these 5′-deleted fragments were named iNOS(–1209/+31), iNOS(–851/+31), iNOS(–518/+31), and iNOS(–234 to –31). All of the aforementioned reporter constructs were inserted into pGL2-Basic. The construct containing bp – 1485 to +31 was also inserted into pGL2-Basic in the reverse orientation, and this construct was named iNOS(–1485/+31). Another PCR-generated fragment of the iNOS 5′-flanking sequence, containing bp – 234 to –53, was inserted into pGL2-Basic to assess its effects on heterologous promoter activity in the presence of IL-1β. This construct was named iNOS(–234 to –53).

Mutagenesis—3 bp within the NF-κBd site were mutated (–85 to –83, GGG to CCT) using a PCR site-directed mutagenesis technique (20). These 3 bp were selected because they are essential for nuclear protein binding (8, 22, 23). The constructs containing the mutated NF-κBd site, iNOS(–1485/+31 NF-κBm) and iNOS(–331/+31 NF-κBm), were inserted into pGL2-Basic, and their orientation and sequence were confirmed by the dideoxy chain termination method (19).

Transfection and Luciferase Assay—RASMC were transfected by a DEAE-dextran-mediated method. In brief, 500,000 cells were plated onto 100-mm tissue culture dishes and allowed to grow for 48–72 h (until 80–90% confluent). 5 μg each of luciferase plasmid DNA and pCAT-Control (to correct for differences in transfection efficiency) were added to RASMC in a solution containing 500 μg/ml of DEAE-dextran. RASMC were subsequently shocked with a 5% MeSO solution for 1 min and then allowed to recover in medium containing 10% fetal calf serum. 12 h after transfection, RASMC were placed in 2% fetal calf serum. The RASMC were stimulated with vehicle, human recombinant IL-1β (10 ng/ml) (Collaborative Biomedical, Bedford, MA), or a combination of IL-1β and simian recombinant TGF-β1 (10 ng/ml) (gift of Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA) for 48 h. We chose these doses of IL-1β and TGF-β1 based on data from our previous studies (5). Cell extracts were prepared by a detergent method (Promega, Madison, WI), and luciferase activity was measured in duplicate for all samples using an ECL AutoLum LB953 luminometer (Gaithersburg, MD) and the Promega Luciferase Assay system. The CAT assay was performed with a modified two-phase fluor diffusion method as described previously (24, 25). The ratio of luciferase to CAT activity in each sample served as a measure of normalized luciferase activity.

Electrophoretic Mobility Shift Assay—Electrophoretic mobility shift assay was performed as described previously (21, 26). The probe consisted of annealed synthetic complementary oligonucleotides encoding two tandem NF-κB sites (GGGAGTCTCCCTCCTCAGG). Prior to annealing, the oligonucleotides were labeled with [γ-32P]ATP using polynucleotide kinase (Boehringer Mannheim). A typical binding reaction contained 20,000 cpm DNA probe, 0.1 μg of poly(dI-dC):poly(dI-dC), 25 μM Hepes (pH 7.9), 40 mM KCl, 0.1 mM EDTA, 1 mM diethiothreitol, 10% glycerol, and 9 μg of nuclear extract in a final volume of 25 μl. The reaction mixture was incubated at room temperature for 20 min and fractionated on a 5% native polyacrylamide gel electrophoresis in a 1× TAE (40 mM Tris base, 20 mM acetic acid, 2 mM EDTA) recirculating buffer system. To determine the specificity of the DNA-protein complexes, competition assays were performed using 100-fold molar excess of an unlabeled double-stranded oligonucleotide encoding NF-κB (specific inhibitor), or a 100-fold molar excess of an unrelated double-stranded oligonucleotide of comparable length (nonspecific inhibitor).

Statistics—Comparisons between groups were made by factorial analysis of variance followed by Fisher’s least significant difference test when appropriate.

**RESULTS**

Promoter/Enhancer Activity of the 5′-Flanking Sequence of the iNOS Gene—IL-1β (10 ng/ml) increased the luciferase activity of plasmid iNOS(–1485/+31) by more than 10-fold (p < 0.05) in primary cultured RASMC (Fig. 1). The luciferase activity produced by iNOS(–1485/+31) in the presence of IL-1β is comparable with that produced by pGL2-Control, which is driven by the potent SV40 enhancer and promoter. The plasmid iNOS(31/+1485 R), containing the iNOS 5′-flanking sequence inserted into pGL2-Basic in the reverse orientation, produced minimal luciferase activity, and it was not responsive to IL-1β stimulation.

Localization of IL-1β-responsive Elements within the iNOS 5′-Flanking Sequence—We next transfected RASMC with a series of 5′-truncated promoter/enhancer constructs to localize the activity produced by iNOS(–1485/+31) or reverse (iNOS(+31/+1485 R)) orientations and in the presence (+) or the absence (–) of IL-1β (n = 4 in each group). The white bar represents the promoter/enhancer activity of pGL2-Control (n = 6). All constructs were cotransfected with pCAT-control correct for transfection efficiency, and luciferase activity was expressed as a percentage of iNOS(–1485/+31) in the absence of IL-1β (mean ± S.E.). Significant differences are noted in the text.

**Fig. 1. Activity of the iNOS 5′-flanking sequence in RASMC.** The black bars represent the promoter/enhancer activity of plasmids containing the 5′-flanking sequence of the mouse iNOS gene in the forward (iNOS(–1485/+31)) or reverse (iNOS(+31/+1485 R)) orientation and in the presence (+) or the absence (–) of IL-1β (n = 4 in each group). The white bar represents the promoter/enhancer activity of pGL2-Control (n = 6). All constructs were cotransfected with pCAT-control correct for transfection efficiency, and luciferase activity was expressed as a percentage of iNOS(–1485/+31) in the absence of IL-1β (mean ± S.E.). Significant differences are noted in the text.
Deletion analysis of iNOS promoter/enhancer activity in response to IL-1β. Plasmids containing variable lengths of the iNOS 5′-flanking sequence and the luciferase reporter gene were transfected into RASMC in the presence (+, black bars) or the absence (−, white bars) of IL-1β (n = 10 in each group). All constructs were cotransfected with pCAT-Control to correct for transfection efficiency, and luciferase activity was expressed as a percentage of iNOS(−1485/+31) in the absence of IL-1β (mean ± S.E.).

Element(s) Other Than NF-κB Are Important for Activation of the iNOS Promoter/Enhancer by IL-1β in Vascular Smooth Muscle Cells—To determine the importance of the NF-κB site located in the downstream portion of the iNOS 5′-flanking sequence (NF-κBd, −85 to −76) and to determine if sites other than NF-κB may be important for the activation of iNOS by IL-1β in vascular smooth muscle cells, we mutated three bp (−85 to −83, GGG to CTC) within the NF-κBd site that are required for nuclear protein binding (B, 22, 23). This plasmid (iNOS(−1485/+31 NF-κBd)) was transfected into RASMC, and its ability to induce luciferase activity after IL-1β stimulation was compared with iNOS(−1485/+31), which contained an intact NF-κBd site. IL-1β (10 ng/ml) induced an 11-fold increase in luciferase activity in cells transfected with iNOS(−1485/+31) (Fig. 3A). When the NF-κBd site was mutated, luciferase activity after IL-1β stimulation was reduced compared with iNOS(−1485/+31) (p < 0.05); however, a large portion of iNOS inducibility remained independent of NF-κBd (45%) in the transfected vascular smooth muscle cells.

To exclude the possibility that the upstream NF-κB site (−971 to −962) adopted the activity of the mutated NF-κB site, we generated a second NF-κBd mutant that had the upstream portion of the iNOS 5′-flanking sequence deleted (iNOS(−331/+31 NF-κBd)). When compared with iNOS(−1485/+31 NF-κBd), iNOS(−331/+31 NF-κBd) demonstrated no difference in luciferase activity after IL-1β stimulation (p = 0.154) (Fig. 3B). These data suggest that neither the upstream NF-κB site (−971 to −962) nor the 5′-flanking sequence (bp −1485 to −332) were responsible for the iNOS promoter/enhancer activity remaining after mutation of NF-κBd. Thus, a site(s) other than NF-κBd within the −331 to +31 region of the iNOS gene is important for iNOS induction by IL-1β in vascular smooth muscle cells.

TGF-β1 Suppresses iNOS Promoter/Enhancer Activity in Vascular Smooth Muscle Cells—To examine the effect of TGF-β1 on iNOS promoter/enhancer activity and to localize the site of TGF-β1-responsive element(s), we transfected RASMC with the same 5′-truncated constructs as described for Fig. 2. The constructs were stimulated with IL-1β (10 ng/ml) in the presence or the absence of TGF-β1 (10 ng/ml). TGF-β1 suppressed luciferase activity by 60% in cells transfected with plasmid iNOS(−331/+31) (p < 0.05), which is not different from the reduction in luciferase activity noted in all of the more 5′-flanking constructs (Fig. 4). These data suggest that TGF-β1-responsive element(s) responsible for the suppression of IL-1β-induced iNOS promoter/enhancer activity exist between bp −234 and +31 in the iNOS 5′-flanking region. In addition, TGF-β1 did not suppress iNOS promoter/enhancer activity in the absence of IL-1β (data not shown), confirming that the response of reporter genes to TGF-β1 was not a nonspecific effect of TGF-β1 on reporter activity.

We next transfected RASMC with plasmid iNOS(−1485/+31 NF-κBd) and assessed luciferase activity after stimulation with vehicle, IL-1β alone (10 ng/ml), or a combination of IL-1β (10 ng/ml) and TGF-β1 (10 ng/ml). As shown previously (Fig. 3), IL-1β increased luciferase activity in the presence of a mutated NF-κBd site (p < 0.05) (Fig. 5). When TGF-β1 was administered in combination with IL-1β, the induction of luciferase activity was completely inhibited. Thus, inhibition of lucifer-
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FIG. 4. Deletion analysis of iNOS promoter/enhancer activity in response to TGF-β1. Plasmids containing variable lengths of the iNOS 5′-flanking sequence and the luciferase reporter gene were transfected into RASMC and stimulated with IL-1β in the presence (+, white bar) or the absence (−, black bar) of TGF-β1 (10 ng/ml, white bar). All constructs were cotransfected with pCAT-Control to correct for transfection efficiency, and luciferase activity was expressed as a percentage of iNOS(−1485/+31) (mean ± S.E.). Significant differences are noted in the text.

FIG. 5. Effect of TGF-β1 on iNOS promoter/enhancer activity. The NF-κB site in plasmid iNOS(−1485/+31) was mutated (as denoted by ×), and this construct was transfected into RASMC (n = 4 in each group) and exposed to vehicle alone (+/−, black bar) or a combination of IL-1β and TGF-β1 (+/+, striped bar). All groups were cotransfected with pCAT-Control to correct for transfection efficiency, and luciferase activity was expressed as a percentage of vehicle alone (−/−) (mean ± S.E.). Significant differences are noted in the text.

TGF-β1 Does Not Inhibit Binding of Nuclear Proteins to the NF-κB Site. To determine if TGF-β1 could inhibit nuclear protein binding to the NF-κB site, we performed electrophoretic mobility shift assay using a probe made from annealed synthetic complementary oligonucleotides encoding NF-κB. Incubation of the labeled NF-κB probe with nuclear extract prepared from RASMC stimulated with IL-1β (10 ng/ml) resulted in the formation of a DNA-protein complex indicated by the arrow in Fig. 6. The intensity of this complex was much greater than the barely detectable band noted in nuclear extract from RASMC exposed to vehicle. This DNA-protein complex was specific because a 100-molar excess of unlabeled oligonucleotide encoding NF-κB, but not an unrelated oligonucleotide competed for nuclear protein binding and abolished the presence of the retarded complex. Incubation of the labeled NF-κB probe with nuclear extract from RASMC stimulated with IL-1β (10 ng/ml) and TGF-β1 (10 ng/ml, a dose shown to suppress iNOS promoter/enhancer activity) also resulted in the formation of a specific DNA-protein complex. This complex was of similar intensity as the band produced by nuclear extract from RASMC stimulated with IL-1β alone.

FIG. 6. Effect of TGF-β1 on nuclear protein binding to the NF-κB site. Electrophoretic mobility shift assay was performed using a double-stranded, 32P-labeled, 20-bp oligonucleotide probe containing tandem NF-κB sites from the iNOS promoter. Nuclear extracts from RASMC stimulated with vehicle alone, IL-1β alone, or a combination of IL-1β and TGF-β1 were added, resulting in a retarded DNA-protein complex (arrow) in the latter two groups. These reactions were also performed in the presence of a specific (S) and a nonspecific (NS) competitor.

These data, together with the transfection studies, indicate that TGF-β1 did not decrease nuclear protein binding to the NF-κB site and that TGF-β1 inhibited iNOS promoter/enhancer activity through a site(s) other than NF-κB.

DISCUSSION

An important aspect of our study was to investigate the regulation of the iNOS 5′-flanking sequence in primary cultured vascular smooth muscle cells, which may differ from iNOS regulation in cell lines. Previous studies have demonstrated that the downstream NF-κB site (−85 to −76) was necessary for iNOS inducibility by LPS in a macrophage-like cell line (RAW 264.7) (7–9). Recently, cytokine regulation of the iNOS promoter/enhancer has also been investigated (27) in a clonal cell line originating from the thoracic aorta of embryonic rats (A7r5) (28). Using multiple cytokine stimulation (IL-1β, tumor necrosis factor-α, and interferon-γ), these investigators (27) reported the upstream NF-κB site (−971 to −962) may play a role in the regulation of the iNOS 5′-flanking sequence in A7r5 cells. However, vascular smooth muscle cells differ from macrophage-like cells in regard to iNOS regulation (5), and primary cultured smooth muscle cells differ markedly from A7r5 cells in their response to cytokines (e.g. A7r5 cells are resistant to iNOS mRNA induction by IL-1β). Our data support the significance of the NF-κB site, but more importantly the data suggest that a site(s) other than NF-κB in the downstream portion of the iNOS 5′-flanking sequence (−234 to +31) is critical for iNOS induction in primary cultured vascular smooth muscle cells.

We have shown previously that TGF-β1 down-regulated iNOS mRNA after its induction by IL-1β in vascular smooth muscle cells (5). This down-regulation occurred at the transcriptional level, resulting in a 65% reduction in the rate of iNOS transcription by TGF-β1. We demonstrate in the present study a comparable 60% reduction in IL-1β-induced iNOS promoter/enhancer activity by TGF-β1 (Fig. 4). Kerr and colleagues (29) have identified a specific sequence within the 5′-flanking region of several TGF-β1-inhibited genes that is required for TGF-β1 to have its inhibitory actions (29). This sequence, GNNNTGAGTA, is known as the TGF-β1 inhibitory element. Within the mouse iNOS 5′-flanking sequence, one complete TGF-β1 inhibitory element site (−1232 to −1223) exists. However, after deletion of the upstream 5′ portion of the flanking sequence (−1485 to −1210), cells transfected with plasmid iNOS(−1209/+31) continued to respond to TGF-β1.

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(Fig. 4), demonstrating that this TGF-β1 inhibitory element site is not responsible for the inhibition of iNOS by TGF-β1. Our data indicate that the TGF-β1-responsive elements accountable for the suppression of IL-1β-induced iNOS activity exist in region −234 to +31 of the 5′-flanking sequence, and this inhibitory effect of TGF-β1 occurs through a site(s) other than NF-κBd in vascular smooth muscle cells. Because the NF-κBd site contributes to the induction of iNOS by IL-1β, TGF-β1 was not able to completely inhibit iNOS promoter/enhancer activity in the presence of an intact NF-κBd site. Our results are consistent with studies by Mauviel et al., which showed that TGF-β1 did not alter NF-κBd-driven promoter activity, nor did it suppress cytokine-induced NF-κB binding in fibroblasts (30).

The inducible isoform of NO synthase is a complex gene that is regulated differently in vascular smooth muscle cells and macrophages (5). These differences may occur at a transcriptional level and may depend upon the usage of different cis-acting elements by vascular smooth muscle cells and macrophages. Further studies to specifically localize the element(s) responsible for the action of IL-1β (up-regulation) and TGF-β1 (down-regulation) on iNOS promoter/enhancer activity in vascular smooth muscle cells will add to our understanding of iNOS gene regulation, particularly as it relates to the regulation of vascular tone. Moreover, these elements involved in the regulation of iNOS by IL-1β and TGF-β1 may be targets for smooth muscle cell-specific inhibition of the iNOS gene in a cytokine-driven disease process such as septic shock.

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