Hypermethylation of the Inducible Nitric-oxide Synthase Gene Promoter Inhibits Its Transcription*

Received for publication, June 28, 2004, and in revised form, August 10, 2004
Published, JBC Papers in Press, August 11, 2004, DOI 10.1074/jbc.M407192200

Zhiyuan Yu† and Bruce C. Kone‡§¶**

From the Departments of Internal Medicine and of Integrative Biology, Pharmacology, and Physiology, The University of Texas Medical School, Houston, Texas 77030, §Section of Nephrology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas 77030, and the ¶Brown Foundation Institute of Molecular Medicine for the Prevention of Human Diseases, Houston, Texas 77030

Exuberant generation of nitric oxide (NO) by inducible nitric-oxide synthase (iNOS) can cause unintended injury to host cells during glomerulonephritis and other inflammatory diseases. Although much is known about the mechanisms of iNOS induction, few transcriptional repression mechanisms have been found. We explored the role of cytosine methylation in the regulation of iNOS transcription. Treatment of mesangial cells with DNA methylation inhibitors augmented cytokine induction of endogenous NO production and iNOS protein levels, as well as iNOS promoter activity. In a corresponding manner, in vitro methylation of the murine iNOS promoter was sufficient to silence its activity in mesangial cells. In contrast, antisense knockdown of DNA methyltransferase-3b expression and activity increased iNOS promoter activity and nitrite production. Bisulfite treatment and sequencing analysis of the iNOS promoter identified methylation of cytosines framing an enhancer element at −879/−871. In vitro methylation inhibited binding of NFκB p50 to this element, and deletion of the element resulted in relief of transcriptional repression. These results provide evidence for a unique molecular mechanism involved in transcriptional regulation of iNOS gene expression.

Nitric oxide (NO) is a widely expressed signaling molecule involved in numerous physiological and pathophysiological processes. NO production is governed by the activity of three NO synthase (NOS) isomorphs. Both neuronal and endothelial NOSs are generally expressed under basal conditions in selected cells and are typically calcium- and calmodulin-dependent. Inducible NOSs (iNOS) are quiescent in most tissues until it is transcriptionally activated by immune stimuli to produce large amounts of NO (1). The sustained flux of large amounts of NO produced by iNOS can result in cytotoxicity to both the host and the target cell. Accordingly, both positive and negative modulators have evolved to control tightly iNOS expression and to prevent untoward effects of excessive NO production. Although much is known about the activation of iNOS transcription by cytokines and bacterial lipopolysaccharide, relatively little is known about how iNOS transcription might be constrained.

Glomerular mesangial cells contribute to the regulation of glomerular filtration, phagocytosis of immune complexes, and the production of extracellular matrix. When activated by immunologic or inflammatory stimuli, mesangial cells generate cytokines, chemokines, and high output NO. Excessive NO production has been linked to several forms of glomerular injury (2, 3). Proinflammatory stimuli, such as IL-1β (4) and lipopolysaccharide + interferon-γ (5), activate iNOS gene transcription in mesangial cells through a complex network of signaling pathways and inducible transcription factors, including cAMP/cAMP-response element-binding protein (4), c-Jun NH2-terminal kinase and p38 mitogen-activated protein kinase (6), CCAAT/enhancer protein-β (7). In contrast, TGF-β (8), IL-13 (9), and STAT3 (10, 11) are known to limit iNOS activation in these cells.

iNOS transcription is regulated in a complex manner by several constitutive and inducible transcription factors. Studies involving deletion analysis and selective base mutation of binding sequences (12) have demonstrated that two xRE sites, positioned at −85 to −76 and −971 to −962 in the murine iNOS promoter, are critical for cytokine inducibility of the iNOS gene. Similarly, we and others have demonstrated the functional importance of two γ-interferon activated sites (GAS, consensus sequence TTCNNNTAA) at −942 to −934 (13) and at −879 to −871 (10) in activation of the iNOS gene in response to cytokines and lipopolysaccharide.

The methylation of transcriptional control regions in the genome plays a fundamental role in the regulation of gene expression. A family of DNA methyltransferases (DNMT) that can catalyze cytosine methylation in different sequence contexts has been identified. Two methyltransferases, DNMT3a and DNMT3b, have been identified in mouse and humans and are essential for de novo methylation of DNA (15, 16). DNMT3b is expressed in adult kidney as well as heart, skeletal muscle, thymus, liver, placenta, and peripheral blood mononuclear cells (17). In this report, we examined the effects of hyper- and hypomethylation on the cytokine activation of the murine iNOS promoter in glomerular mesangial cells. We found that DNMT3b-dependent methylation of murine iNOS promoter sequences framing a functional enhancer element to which NFκB p50 binds limits iNOS transcriptional activity in response to IL-1β.
DNA Methylation Inhibits iNOS Induction in Mesangial Cells

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—Mouse mesangial cells (CRL-1297, American Type Culture Collection) were maintained in Ham's F-12 medium plus Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 5% fetal bovine serum. RAW 264.7 macrophage cells (American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum. Vehicle, IL-1β (10 ng/ml), or 5aza-2'-deoxycytidine (5-Aza-Cdr; 0.2 and 0.5 µg/ml) was added to the cells as indicated in the text and figure legends. Cell number and doubling time in complete medium were quantified by hemocytometric counting of trypsinized cells after trypan blue dye exclusion. Mouse recombinant IL-1β and interferon-γ were from R & D Systems (Minneapolis, MN) and BIOSOURCE (Camarillo, CA), respectively. Oligonucleotides were custom synthesized by Sigma Genosys (The Woodlands, TX). LipofectAMINE 2000 reagent was from Invitrogen. The Dual-Luciferase Reporter Assay System and the luciferase vectors pGL3-Basic and pRL-SV40 were from Promega. The BCA protein estimation kit was from Pierce.

Plasmids, Stable Transfections, and Reporter Gene Assays—pM-NOS-luc, which contains the murine iNOS promoter/enhancer and a portion of exon 1 (nucleotides −1486 to +145) in pGL3-Basic, has been previously characterized (18). pM-NOS-luc/Zeocin was constructed by excising the 1.6-kb murine iNOS promoter together with the firefly luciferase coding region from pM-NOS-luc and cloning it into the BamHI and EcoRI sites of pCDNA1.1-Zeo (Invitrogen). The authenticity of the DNA insert was confirmed by sequencing. For stable transfections, murine mesangial cells were seeded in 6-well plates and transfected with pcDNA1.1-NOS-luc/Zeocin using LipofectAMINE 2000 reagent (Invitrogen) according to the manufacturer's protocol. Twenty-four hours after transfection, the cells were split, and 400 µg/ml Zeocin (Invitrogen) was added for selection. Surviving clonal cell lines were expanded and tested for cytokine-stimulated firefly luciferase activity, which was measured by methods described previously in our laboratory (18). Multiple positive cell lines were expanded for subsequent experiments. Deletion constructs containing nucleotides −929 to +145 of the murine iNOS promoter or the region −863 to +145, which lacks the −879 to −871 interferon-γ activation sequence (GAS) element, were generated in pGL3-Basic. pRSV-p50, which encodes NF-κB p50, was kindly provided by Dr. Warren B. Liao, The University of Texas M. D. Anderson Cancer Center. The NF-κB reporter construct pRSV-p50/luc, which contains three tandem copies of the κB binding element (GGG-GACTCTCCC) upstream of the prolactin promoter sequence and fused with the luciferase gene, was a gift from Dr. Bharrat Agarwal, The University of Texas M. D. Anderson Cancer Center.

Antisense Experiments—High pressure liquid chromato-phyridium-puri-200 phosphorothioate oligodeoxynucleotides to target nucleotides +70 to +90 of DMNT3b (DMNT3b sense primer 5'-AATGGGAAGCTTCTAGAT-3', antisense primer 5'-CTGTCAGTCTAATGTTGTTT-3') were transfected into murine mesangial cells using LipofectAMINE 2000 reagent (Invitrogen). The cells were incubated in the presence of sense or antisense oligonucleotides for 24 h before they were processed for further analysis.

Reverse Transcriptase-PCR—Total cellular RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer's manual, and cDNA was synthesized using M-MLV Reverse Transcriptase (Promega). Amplification of cDNA was as follows: DMNT3b nucleotides +25 to +375 (sense primer 5'-AATGGAAGAAAGGGTGCCACGGC-3', antisense primer 5'-ATTCTGATTTTGAGTGCCAGC-3'), GAPDH nucleotides +238 to +757 (sense primer 5'-CACAATACATCAATGAGTGACGGC-3', antisense primer 5'-TACTGCGAGCTCCGCTTACTAGGC-3'). Amplification conditions included an initial denaturation at 94 °C for 5 min followed by 35 cycles of 1 min at 94 °C, 1 min at 55 °C, 2 min at 72 °C, and a final extension step for 7 min at 72 °C. The PCR products were electrophoresed on 0.8% agarose gels and imaged with a Kodak ImageStation 1000 (Packard).

Nitrite Assays—Mesangial cells, wild type or transfected, were seeded in 96-well plates and stimulated with IL-1β (20 ng/ml) for 24 h. The medium was then collected, and the nitrite concentration was determined with the Griess Reagent System (Promega) according to the manufacturer's protocol.

Western Blotting—Cytoplasmic and nuclear extracts from mouse mesangial cells were prepared using the Nuclear/Cytoplasm Fractionation Kit (BioVision) according to the manufacturer's manual. Twenty-µg samples of nuclear, cytoplasmic, or whole cell extracts were resolved by SDS-PAGE, and the proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Hybond ECL, Amersham Biosciences). The blots were probed with an anti-iNOS antibody (0.2 µg/ml) or an anti-α-tubulin antibody (0.2 µg/ml) overnight at 4 °C. The blots were washed extensively with a solution containing 50 mM Tris, pH 8.0, 138 mM NaCl, 2.7 mM KCl, and 0.05% Tween 20. The antibody-antigen complexes were detected by the ECL protocol using horseradish peroxidase-conjugated donkey anti-rabbit IgG as secondary antibody.

Sodium Bisulfite Modification and Genomic Sequencing—Genomic DNA was extracted from mouse mesangial cells or RAW 264.7 cells that had been treated with or without 5-Aza-CdR for 24 h using the DNAeasy kit (Qiagen) according to the manufacturer's protocol. Two µg of DNA was denatured in 50 µl of 0.3 M NaOH for 15 min at 37 °C. For the chemical modification of DNA, 520 µl of 3 M sodium bisulfite (Sigma) and 30 µl of 10 µM hydroquinone (Sigma) were added to the DNA solution and the samples were mixed, overlaid with mineral oil, and incubated at 50 °C overnight. Modified DNA was purified with the Wizard DNA Clean-up system (Promega) and eluted in water. As a final step, 1 µg of DNA was denatured in 50 µl of 0.3 M NaOH for 15 min at 37 °C. The methylated DNA was purified by the Wizard DNA Clean-up kit (Promega) into PCR-ready form. Clones with appropriate sized inserts were sequenced.

In Vitro DNA Methylation and Transient Transfection—The methylated plasmids (Met-pM-NOS-luc and Met-pRL-Basic) were generated by incubating 40 µg of plasmid DNA with 100 units SgII and 10 units Tris-HCl, 10 mM MgCl2, 1 mM dithiothreitol, pH 7.9, and 180 µM S-adenosylmethionine according to the manufacturer’s instructions (Stratagene, La Jolla, CA). Reactions were carried out at 37 °C overnight. Complete methylation was verified by digestion with the methylation-sensitive restriction enzyme HpaII. Only plasmids that showed a complete protection with double strand probes or competitors as described (18).

Nuclear extracts were prepared, quantified, and used for EMSA assays—Electrophoretic Mobility Shift Assays (EMSA) and Supershift Assays—Nuclear extracts were prepared, quantified, and used for EMSA with double strand probes or competitors as described (18). The methylated −885 to −866 oligonucleotide was prepared by incubating 20 µg of unmethylated −885 to −866 oligonucleotide (−855 to −866, 5'-AATGCTCTTATGGAAGCTTCTGAGGTCAG-3') with 80 units of SsSI methylase at 37 °C overnight. The reaction was then heated at 65 °C to inactivate the methylase, purified by PAGE, and concentrated with Centricron 3 microconcentrators. Nuclear extracts were incubated for 20 min on ice in the presence or absence of unlabeled competitor oligonucleotides followed by the addition of the end-labeled probe and 15 min incubation on ice. Supershifted complexes were performed by incubating 1 µg of antibodies against STAT1, STAT3, NF-κB p50, NF-κB p65, and AP-2 isoforms (2 µg) or IgG as a negative control as described.

DNA Methylation Transferase Activity Assay—DNA methyltransferase activity was determined in protein extracts by using the assay developed by Adams et al. (19) with minor modifications. Briefly, cells were lysed in buffer containing 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 0.01% sodium azide, 1 mM phenylmethylsulfonyl fluo-
Results
DNA Methylation Inhibitors Augment IL-1β Induction of Endogenous NO Production, iNOS Protein, and iNOS Promoter Activity—To determine whether the state of DNA methylation contributes to the regulation of iNOS-generated NO production in mesangial cells, IL-1β-stimulated nitrite production and iNOS protein abundance were measured in the presence of vehicle or 5-Aza-CdR, a potent and specific inhibitor of DNA methylation, iNOS is the only NOS isoform appreciably expressed in these cell types and induced by these stimuli (20). Mesangial cells that had been treated with 5-Aza-CdR and stimulated with IL-1β exhibited ~150% greater nitrite levels compared with IL-1β-stimulated controls (Fig. 1A). Similarly, mesangial cells that had been treated with 5-Aza-CdR exhibited higher levels of IL-1β-stimulated protein expression compared with vehicle and IL-1β-treated controls (Fig. 1B). Cell counting experiments indicated that under these cell culture conditions, the cells exhibited a sigmoidal growth curve with a mean doubling time of ~11.5 h before confluence.

To determine whether the stimulatory effect of 5-Aza-CdR included effects on iNOS transcription, vehicle or 5-Aza-CdR was added to mesangial cell lines that had been stably transfected with a mammalian expression vector containing the murine iNOS promoter fused to the firefly luciferase gene. In this context, the iNOS promoter-luciferase is stably integrated into the genome. The transfected cells were then treated with medium or IL-1β, and the induction of iNOS reporter gene activity was measured. As seen in Fig. 1C, mesangial cells treated with IL-1β in the absence of 5-Aza-CdR exhibited the expected induction of iNOS promoter-luciferase activity. In keeping with the nitrite and iNOS protein responses, mesangial cells treated with 5-Aza-CdR exhibited a ~135% increase in the fold-induction of iNOS promoter activity in response to IL-1β (Fig. 1C). These data suggest that hypomethylation increases (and hypermethylation decreases) cytokine induction of

DNA Methylation Inhibitors Augment IL-1β Induction of Endogenous NO Production, iNOS Protein, and iNOS Promoter Activity
iNOS promoter activity in mesangial cells. The restoration of iNOS induction by 5-Aza-CdR treatment could be related to a direct demethylation of the iNOS gene or another gene, whose expression is required for iNOS activation.

Antisense Knockdown of DNMT3b Activity Augments IL-1β Induction of Endogenous NO Production and iNOS Promoter Activity in Mesangial Cells—To address more specifically the effects of methylation status on iNOS promoter induction, DNMT3b was specifically suppressed by antisense methods in mesangial cells stably expressing iNOS promoter-luc, and the consequences on iNOS promoter-reporter gene activity were measured. As seen in Fig. 2A, antisense oligonucleotides directed against DNMT3b suppressed DNMT3b mRNA expression but not the control gene GAPDH as monitored by semi-quantitative reverse transcriptase-PCR, whereas sense oligonucleotides had no effect. This antisense blockade of DNMT3b corresponded to a ~70% decrease in DNMTase activity (Fig. 2B), indicating the functional efficacy of the blockade and the fact that DNMT3b is the principal isoenzyme contributing to DNMTase activity in these cells. In agreement with the results of pharmacologic inhibition of methylation (Fig. 1C), antisense knockdown of DNMT3b resulted in enhanced levels of nitrite production (Fig. 2C) and iNOS promoter activity (Fig. 2D) in response to IL-1β compared with similarly treated sense controls. DNMT3b protein has a relatively short half-life (21), and the doubling time of the mesangial cells was sufficiently rapid to allow at least one replication cycle to occur after antisense knockdown of DNMT3b.

In Vitro Methylation Inhibits iNOS Promoter Activity—To
DNA Methylation Inhibits iNOS Induction in Mesangial Cells

Fig. 4. Sodium bisulfite modification and genomic sequencing of the iNOS promoter reveals cytosine methylation at −881 and −869. DNA from mesangial cells treated in the presence and absence of 5-Aza-CdR was treated with sodium bisulfite to modify methylated cytosines to uracil. DNA sequencing was then performed. Representative sequencing graphs of the −885−866 region are presented. Modified and unmodified cytosines are indicated by arrows.

determine whether methylation of the iNOS promoter is sufficient to silence its expression in mesangial cells, the iNOS promoter-luciferase plasmid piNOS-luc was methylated in vitro by SssI, an enzyme that methylates cytosines, and transiently transfected into mouse mesangial cells. In parallel experiments, unmethylated piNOS-luc was transfected as a control. Compared with full stimulation of unmethylated piNOS-luc, methylated piNOS-luc was only marginally stimulated by IL-1\(_\beta\) when transiently transfected (Fig. 3).

**Bisulfite Treatment and Sequencing Analysis Identifies Methylation of the iNOS Promoter—** iNOS promoter methylation at cytosine nucleotides was investigated. Sodium bisulfite deaminates unmethylated cytosine to uracil in single-stranded DNA under conditions in which the 5-methylcytosine remains nonreactive. Thus, all cytosine residues remaining at the time of sequencing represent cytosines that were methylated in the original DNA sequence. The methylated cytosines were distinguished from the unmethylated cytosines within the sequenced fragment because they were resistant to sodium bisulfite treatment and remained unchanged. Genomic DNA from murine mesangial cells in the presence and absence of 5-Aza-CdR and genomic DNA from RAW 264.7 macrophage cells were analyzed. Sequencing analysis showed that cytosines at −881, −869, −648, and −601 were methylated in the iNOS promoter from both cell lines and were demethylated in the presence of 5-Aza-CdR. Cytosines at −196, −652, −654, −656, −828, −830, −845, −919, and −927 of the iNOS promoter were methylated in mesangial cells (and demethylated in the presence of 5-Aza-CdR) but not in RAW 264.7 cells. In contrast RAW 264.7 cells but not mesangial cells were methylated at −644. We placed particular emphasis on the cytosines methylated in both cell lines and noted that the methylated cytosines at −881 and −869 framed a functional enhancer containing a GAS consensus sequence at −879 to −871 (Fig. 4). In our previous work, we showed that mutation of this GAS consensus site in the iNOS promoter resulted in a 65% reduction in maximal IL-1\(_\beta\) induction of promoter activity (10).

**Cytosine Methylation Bordering the −879/−871 GAS Element Inhibits DNA Binding of NF\(\kappa\)B p50—** Cytosine methylation in the promoter region, when present within regulatory elements, could potentially interfere with binding of specific transcription factors that bind these motifs. To determine whether such methylation within the −879/−871 putative GAS element interferes with transcription factor binding, we compared the binding abilities in EMSA reactions of a 20-mer oligomer (nucleotides −885 to −866), which contained the GAS element and neighboring cytosines that we had identified as methylated by sodium bisulfite (Fig. 4), in unmethylated and methylated forms. First we examined the abilities of unmethylated and methylated −885/−866 oligomers to compete with the unmethylated −885/−866 oligomer probe in binding to nuclear proteins from IL-1\(_\beta\)-treated mesangial cells. As seen in Fig. 5A, two sequence-specific gel shift complexes were observed in the absence of competitors. In competition EMSA reactions, addition of a 100-fold excess of unlabeled unmethylated −885/−866 oligomers inhibited complex formation, suggesting that the unmethylated −885/−866 oligomer binds activated protein as strongly as the −879/−871 GAS sequence (Fig. 5A). However, the addition of the same amount of unlabeled methylated −885/−866 or of the unrelated AP-1 oligomers resulted in no inhibition of DNA-protein complex formation (Fig. 5A).

Then we compared the abilities of unmethylated and methylated −885/−866 oligomers to bind nuclear proteins from IL-1\(_\beta\)-treated mesangial cells in EMSA reactions using \(^{32}\)P-labeled unmethylated −885/−866 or methylated −885/−866 probes prepared to have equal specific activities. The two major DNA-protein complexes were observed to form with unmethylated −885/−866, but formation of Complex II and to a much lesser degree Complex I with methylated −885/−866 was significantly inhibited (Fig. 5B). Therefore, methylation of cytosines at −881 and −869 significantly inhibited binding of proteins to the −885/−866 element.

To determine the identity(is) of the protein components of the iNOS promoter-protein complexes identified by EMSA, supershift analysis was performed with antibodies directed against STAT1, STAT3, NF\(\kappa\)B p65 and p50, and AP-2 isoforms (Fig. 5C). The antibody to NF\(\kappa\)B p50 dramatically inhibited the DNA-nuclear protein binding of both complexes, whereas the other antibodies did not suppress or supershift the complexes. To verify this protein-DNA association within the living cell, we performed ChIP assays using anti-NF\(\kappa\)B p50 antibodies to immunoprecipitate complexes from chromatin and PCR primers flanking the −879 to −871 GAS element. As seen in Fig. 5D, NF\(\kappa\)B p50 was immunoprecipitated and associated with this DNA fragment to a very limited degree in the presence of IL-1\(_\beta\) or 5-Aza-CdR alone but to a much greater degree in cells treated with both of these agents. Under basal conditions in the absence of either agent, or when immunoprecipitations were performed with nonimmune IgG, no association was observed.

To determine the functional importance of this region to IL-1\(_\beta\) inducibility of the iNOS promoter, the promoter activities of constructs containing +145/−929 or +145/−863, the
latter of which lacks the −885/−866 region, fused to the firefly luciferase gene were compared by transient transfection. The iNOS promoter construct exhibited enhanced IL-1β-stimulated iNOS promoter activity compared with the +145/−929 construct, consistent with a repressive effect of methylation of cytosines at −881 and −869 (Fig. 6). Overexpression of SssI methylase in vitro with S-adenosylmethionine as detailed under “Experimental Procedures.” Each probe was end-labeled under identical conditions, diluted to have equal specific activities, and used in an amount equivalent to 20,000 counts/min in each reaction. Results are representative of three such experiments. C, supershift analysis of DNA-protein complex. Nuclear proteins from IL-1β-treated mesangial cells were incubated with antibodies against the indicated transcription factors, and then EMSA reactions were performed with a radiolabeled −885/−866 oligomer. Autoradiograph representative of three independent experiments. Some of the samples were duplicated on the gel to enhance reproducibility of the data. D, ChIP analysis in mesangial cells, which had been treated in the presence or absence of 5-Aza-CdR, of the iNOS gene region −978 to −818 using NFκB p50 antibodies (or nonimmune IgG as a negative control) to immunoprecipitate following vehicle or IL-1β stimulation. One of three representative experiments is shown. UM., unmethylated; Met., methylated.
expression of NFκB p50 did not by itself enhance the activity of the iNOS promoter-luciferase construct compared with cells transfected with the parent vector. The functional efficacy of the expression plasmid was verified by the fact that it activated the NFκB reporter construct p36B(−3)(NFκB)3-luc reporter construct in these cells (data not shown).

**DISCUSSION**

The regulation of the iNOS gene is extremely complex and involves the interactions of both basally expressed and inducible transcription factors, interactions with corepressors, and physical and functional interactions among these classes of proteins. Our results provide evidence for cytokine methylation as a unique molecular mechanism involved in the transcriptional regulation of iNOS gene expression in mesangial cells. First, we demonstrated that inhibition of DNA methylation pharmacologically or by antisense gene knockdown of DNMT3b in mesangial cells accentuates IL-1β-stimulated NO production, iNOS protein expression, and iNOS promoter activity. Second, we showed that methylation of the iNOS promoter in vitro dramatically reduces its IL-1β-inducibility. Third, sequence analysis of the bisulfite-modified iNOS promoter demonstrated that cytosine residues flanking a functional GAS element at −879/−871 are methylated. Fourth, methylation reduced the ability of NFκB p50 to complex with the −885/−866 region in the iNOS promoter in vitro by EMSA and supershift assays and in vivo in ChIP assays. Finally, deletion of this region from the iNOS promoter restores its inducibility. In the aggregate our results indicate that hypermethylation of cytosine residues flanking the −879/−871 element impairs the ability of NFκB p50 to bind the promoter and partially silences the cytokine induction of the iNOS promoter.

DNA methylation can repress gene transcription either by directly inhibiting the interaction of transcription factors with their regulatory sequences or by attracting methylated DNA binding proteins that in turn recruit histone deacetylases and histone methyltransferases, resulting in an inactive chromatin structure (22). Promoter regions without CpG islands are variably methylated in normal cells often in a cell-specific manner that reflects the transcriptional activity of the gene. In these promoter regions, methylation of specific cytosine residues in or near transcription regulatory motifs can block accessibility of the transcription factor. For example, hypermethylation of a CpG-response element in the promoter regions of BRCA1 and NF1 inhibits binding of CpG-response element binding protein (23). Cytosine methylation in the NFκB recognition sequence has been shown to inhibit binding of this transcription factor to the E-selectin promoter (24) and the human immunodeficiency virus, type 1 long terminal repeat (25). Recent studies have also shown that DNMT3b has functions beyond DNA methylation that include the ability to interact with histone deacetylase 1 (HDAC1), HDAC2, HP1 proteins, Suv39h1, and the ATP-dependent chromatin remodeling enzyme hSNF2H to act as transcriptional corepressors (26). Further studies will be needed to determine whether these other mechanisms are operative as well in controlling iNOS transcription.

The NFκB transcription factor family includes the p50 (NFκB1), p52 (NFκB2), p65 (Rel A), Rel B, c-Rel, v-Rel, dorsal, and Dif proteins. These proteins share at their N-terminal region a conserved NFκBrel/dorsal homology region responsible for DNA binding, dimerization, and nuclear localization as well as redox modulation of DNA binding activity (27). In previous work, we demonstrated the basal expression of NFκB p50/p50 homodimers and the IL-1β-inducible expression of NFκB p65/p65 homodimers in murine mesangial cells (10). The region of the iNOS promoter implicated in the present report contains a consensus GAS sequence at −879/−871 and IL-1β-mediated enhancer activity (10), and it binds NFκB p50/p50 homodimers in mesangial cells, although it lacks a consensus κB element binding sequence. This result may indicate that this sequence represents an alternative recognition site for NFκB, perhaps in complex with another regulatory protein. Because NFκB p50/p50 homodimers typically produce a single gel shift band, we were somewhat surprised to find two sequence-specific gel shift complexes, both of which were completely interrupted by NFκB p50 antibodies, suggesting that they represented NFκB p50/p50 homodimers. It may be that other proteins that we did not probe for in the supershift assays are involved with one of the complexes (altering its mobility) or that a posttranslational modification alters mobility. Further studies will be needed to determine the complete composition of both complex I and complex II gel shift bands.

The fact that NFκB p50 binding and iNOS promoter activity were enhanced during hypomethylation yet that deletion of the binding region also enhanced iNOS promoter activity may reflect the fact that in the latter case a neighboring E-box at −893/−888 (to which upstream stimulatory factor proteins are known to bind and trans-repress the iNOS promoter (28)) was also deleted in the +145/−863 iNOS promoter construct. Moreover, the fact that NFκB p50 when overexpressed alone did not alter iNOS promoter activity may reflect the complex participation of this transcription factor in iNOS transcriptional regulation. NFκB p50 is known to bind to other κB elements on the iNOS promoter and to complex with coregulatory proteins [e.g., p300 histone acetyltransferase (29)] and transcription factors (i.e. STAT3 (10), high mobility group-I(Y) protein (30)] that affect iNOS transcriptional control. Teasing out the specific contributions of methylation status on these other regions requires additional analysis.

This is the first time that promoter methylation has been demonstrated to regulate the expression of iNOS, and it implicates DNMT3b as a key enzyme in controlling iNOS synthesis. Mutations in the DNMT3b gene are responsible for most cases of the immunodeficiency, centromeric region instability, and facial anomalies (ICF) syndrome, a rare autosomal recessive disease (31). It will be interesting to determine whether such patients exhibit enhanced iNOS expression following pro-inflammatory stimulation as the current data would predict. Because NO itself has been shown to activate DNA methyltransferases by a posttranscriptional mechanism to induce DNA methylation and repress expression of several genes, including FMR1 and HPRT (14), it will be interesting to test the hypothesis that methylation of the iNOS promoter repre-
sents a feedback inhibition mechanism by which the end-prod-
uct NO silences the gene (iNOS) responsible for its production.
Such a mechanism might serve to terminate high output NO
production and prevent host cell damage.

REFERENCES

1. Kone, B. C., and Baylis, C. (1997) Am. J. Physiol. 272, F561–F578
2. Furusu, A., Miyazaki, M., Abe, K., Tsukasaki, S., Shishita, K., Sasaki, O., Miyazaki, K., Ozono, Y., Kaji, T., Harada, T., Sakai, H., and Kohno, S. (1998) Kidney Int. 53, 1760–1768
3. Narita, I., Border, W. A., Ketteler, M., and Noble, N. A. (1995) Lab. Invest. 72, 17–24
4. Eberhardt, W., Pluss, C., Hummel, R., and Pfeilschifter, J. (1998) J. Immunol. 160, 4961–4969
5. Sharma, K., Danoff, T. M., DePiero, A., and Ziyadeh, F. N. (1995) Biochem. Biophys. Res. Commun. 207, 80–88
6. Guan, Z., Baier, L. D., and Morrison, A. R. (1997) J. Biol. Chem. 272, 8083–8089
7. Beck, K. F., and Sterzel, R. B. (1996) FEBS Letters 394, 263–267
8. Pfeilschifter, J., and Vosbeck, K. (1991) Biochem. Biophys. Res. Commun. 175, 372–379
9. Saura, M., Martinez Dalmau, R., Minty, A., Perez-Sala, D., and Lamas, S. (1996) Biochem. J. 315, 641–646
10. Yu, Z., Zhang, W., and Kone, B. C. (2002) Biochem. J. 367, 97–105
11. Yu, Z., and Kone, B. C. (2004) J. Am. Soc. Nephrol. 15, 585–591
12. Xie, Q. W., Kashiwabara, Y., and Nathan, C. (1994) J. Biol. Chem. 269, 4705–4708
13. Gao, J., Morrison, D. C., Parmely, T. J., Russell, S. W., and Murphy, W. J. (1997) J. Biol. Chem. 272, 1226–1230
14. Hmadcha, A., Bedoya, F. J., Sobrino, F., and Pintado, E. (1999) J. Exp. Med. 190, 1595–1604
15. Xie, S., Wang, Z., Okano, M., Nagami, M., Li, Y., He, W. W., Okumura, K., and Li, E. (1999) Gene (Amst.) 236, 87–95
16. Okano, M., Bell, D. W., Haber, D. A., and Li, E. (1999) Cell 99, 247–257
17. Robertson, K. D., Uzvolgyi, E., Liang, G., Talmadge, C., Sumegi, J., Gonzales, F. A., and Jones, P. A. (1999) Nucleic Acids Res. 27, 2291–2298
18. Gupta, A. K., and Kone, B. C. (1999) Am. J. Physiol. 276, F599–F605
19. Adams, R. L., Rinaldi, A., and Seivwright, C. (1991) J. Biochem. Biophys. Methods 22, 19–22
20. Kuncewicz, T., Balakrishnan, P., Snuggs, M. B., and Kone, B. C. (2001) Am. J. Physiol. 281, F326–F336
21. Leu, Y. W., Rahmatpanah, F., Shi, H., Wei, S. H., Liu, J. C., Yan, P. S., and Huang, T. H. (2003) Cancer Res. 63, 6110–6115
22. Geiman, T. M., and Robertson, R. D. (2002) J. Cell. Biochem. 87, 117–125
23. DiNardo, D. N., Butcher, D. T., Robinson, D. P., Archer, T. K., and Rodenhisier, D. I. (2001) Oncogene 20, 5331–5340
24. Smith, G. M., Whelan, J., Pescini, R., Gherva, P., DeLamarter, J. F., and Hseif van Huijnduijn, R. (1999) Biochem. Biophys. Res. Commun. 194, 215–221
25. Bednarik, D. P., Duckett, C., Kim, S. U., Perez, V. L., Griffin, K., Guenther, P. C., and Folks, T. M. (1991) New Biol. 3, 969–976
26. Fujita, N., Watanabe, S., Ichimura, T., Tsuzuki, S., Shinkai, Y., Tachibana, M., Chiba, T., and Nakao, M. (2003) J. Biol. Chem. 278, 24132–24138
27. Li, X., and Stark, G. R. (2002) Exp. Hematol. 30, 285–296
28. Gupta, A. K., and Kone, B. C. (2002) Am. J. Physiol. 283, C1065–C1072
29. Deng, W. G., and Wu, K. K. (2003) J. Immunol. 171, 6581–6588
30. Ferrella, M. A., Pellacani, A., Wiesel, P., Chin, M. T., Foster, L. C., Ibanez, M., Hsieh, C. M., Reeves, R., Yet, S. F., and Lee, M. E. (1999) J. Biol. Chem. 274, 9045–9052
31. Hansen, R. S., Wijmenga, C., Luo, P., Stanek, A. M., Canfield, T. K., Weemaes, C. M., and Gartler, S. M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 14412–14417
Hypermethylation of the Inducible Nitric-oxide Synthase Gene Promoter Inhibits Its Transcription
Zhiyuan Yu and Bruce C. Kone

J. Biol. Chem. 2004, 279:46954-46961. doi: 10.1074/jbc.M407192200 originally published online August 11, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M407192200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 28 references, 11 of which can be accessed free at http://www.jbc.org/content/279/45/46954.full.html#ref-list-1