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*J Immunol* 2005; 175:3846-3861; doi: 10.4049/jimmunol.175.6.3846
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Epigenetic Basis for the Transcriptional Hyporesponsiveness of the Human Inducible Nitric Oxide Synthase Gene in Vascular Endothelial Cells

Gary C. Chan,* Jason E. Fish,† Intiaz A. Mawji,* Desmond D. Leung,‡ Alisa C. Rachlis,§ and Philip A. Marsden2*†‡

A marked difference exists in the inducibility of inducible NO synthase (iNOS) between humans and rodents. Although important cis and trans factors in the murine and human iNOS promoters have been characterized using episomal-based approaches, a compelling molecular explanation for why human iNOS is resistant to induction has not been reported. In this study we present evidence that the hyporesponsiveness of the human iNOS promoter is based in part on epigenetic silencing, specifically hypermethylation of CpG dinucleotides and histone H3 lysine 9 methylation. Using bisulfite sequencing, we demonstrated that the iNOS promoter was heavily methylated at CpG dinucleotides in a variety of primary human endothelial cells and vascular smooth muscle cells, all of which are notoriously resistant to iNOS induction. In contrast, in human cell types capable of iNOS induction (e.g., A549 pulmonary adenocarcinoma, DLD-1 colon adenocarcinoma, and primary hepatocytes), the iNOS promoter was relatively hypomethylated. Treatment of human cells, such as DLD-1, with a DNA methyltransferase inhibitor (5-azacytidine) induced global and iNOS promoter DNA hypomethylation. Importantly, 5-azacytidine enhanced the cytokine inducibility of iNOS. Using chromatin immunoprecipitation, we found that the human iNOS promoter was basally enriched with di- and trimethylation of H3 lysine 9 in endothelial cells, and this did not change with cytokine addition. This contrasted with the absence of lysine 9 methylation in inducible cell types. Importantly, chromatin immunoprecipitation demonstrated the selective presence of the methyl-CpG-binding transcriptional repressor MeCP2 at the iNOS promoter in endothelial cells. Collectively, our work defines a role for chromatin-based mechanisms in the control of human iNOS gene expression. The Journal of Immunology, 2005, 175: 3846–3861.

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Received for publication March 10, 2005. Accepted for publication July 6, 2005.

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1 G.C.C. and I.A.M. are recipients of Canadian Institutes of Health Research/Heart and Stroke Foundation of Canada Doctoral Research Awards. J.E.F. is the recipient of a Natural Sciences and Engineering Research Council of Canada Graduate Scholarship. P.A.M. is the recipient of a Career Investigator Award from the Heart and Stroke Foundation of Canada and is supported by Canadian Institutes of Health Research Grant MOP-36381.

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* Abbreviations used in this paper: iNOS, inducible NO synthase; AcH3, acetylated histone H3; AcH4, acetylated histone H4; 5-azaC, 5-azacytidine; ChIP, chromatin immunoprecipitation; Cyt, cytokine mixture of IFN-γ (200 U/ml), IL-1β (5 ng/ml), and TNF-α (10 ng/ml); DmMeH3K4, dimethylated histone H3 lysine 4; DmMeH3K9, dimethylated histone H3 lysine 9; DM, differentially methylated region; eNOS, endothelial NO synthase; HDAC, histone deacetylase; HIAEC, primary human iliac artery endothelial cell; HMVEC-d, primary human neonatal dermal microvascular endothelial cell; HMVEC-L, primary human lung microvascular endothelial cell; HuSv-VSMC, primary human saphenous vein smooth muscle cell; IP, immunoprecipitated; IRF, IFN regulatory factor; MHECS-T, transformed mouse heart endothelial cell; Nos2, mouse iNOS gene; NOS2A, human iNOS gene; Pol II, RNA pol- merase II; RPA, ribonuclease protection assay; TrmMeH3K9, trimethylated histone H3 lysine 9; TSA, trichostatin A.
The human and rodent iNOS genes are regulated predominantly at the transcriptional level. Characterization of the murine promoter using transient transfection of episome-based promoter/reporter constructs has identified key cis regulatory regions (e.g., regions I and II). The promoter contains, among other binding sites for transcription factors, a proximal and a distal NF-κB site, IFN-γ-activated site, and two adjacent IFN-stimulated response elements. For the human promoter, important functional contributions from numerous trans factors have been reported: NF-κB, IFN regulatory factor-1 IRF-1, AP-1, STAT3, C/EBP, hypoxia-inducible factor-1 (HIF-1), Kruppel-like factor 6, and upstream stimulatory factor-1, among others (17–19). Despite the significant homology between the human and mouse proximal 1-kb 5′-flanking sequences and the presence of additional cytokine-responsive enhancers in the human promoter at −8.3, −5.8, and −5.3 kb (20–22), the human iNOS gene is not as readily inducible as the murine counterpart. Therefore, the molecular basis for the transcriptional hyporesponsiveness of the human iNOS gene poses an interesting paradox. Multiple explanations based on classical cis element and trans factor paradigms have been offered, including nucleotide substitutions in proximal human cis elements, the absence of one or more trans factors in human cell types (9, 20), and the presence of human-specific silencer elements (23). Viewed from the perspective of the reports of iNOS expression in chronic human diseases, these mechanisms are not compelling. Importantly, it is now appreciated that the chromosomal context of classical transcriptional regulatory elements is functionally important in controlling gene expression. Although canonical cis and trans factors controlling iNOS expression are well studied, the chromatin structure of the human iNOS gene has received little consideration. In this work we investigated the transcriptional regulation of the iNOS gene in HUVEC and other human cell types from a different perspective, namely epigenetics.

DNA methylation and histone modifications represent the major epigenetic mechanisms implicated in the regulation of gene transcription in mammals. For instance, DNA methylation is a prominent feature of vertebrate genomes. This methylation occurs at carbon 5 of cytosine, primarily in the context of the dinucleotide CpG. Recent findings support the premise that hypomethylation of the DNA surrounding the proximal promoter region is a prerequisite for gene activation, whereas heavy methylation leads to gene silencing (24). Mechanistically, there are a number of ways in which DNA methylation can repress transcription. Many of the trans factors known to bind to sequences containing CpG dinucleotides (e.g., hypoxia-inducible factor-1α) do not bind when the CpG doublets are methylated (25). Alternatively, methyl-CpG-binding proteins, such as MeCP2, bind preferentially to methylated DNA and directly repress transcription, inhibiting the binding of other trans factors, structurally modify the DNA, or recruit corepressor complexes (26–28).

The assembly of higher order chromatin structure has been linked to the covalent modification of histone tails. The combinatorial nature of histone N-terminal modifications, or the histone code, represents an additional pathway of epigenetic regulation and considerably extends the information potential of the genetic code (29). For instance, hyperacetylation of the lysine residues of H3 and H4 histones is generally associated with the promoters of actively transcribed genes, whereas hypoacetylated histones have been correlated with gene silencing (30). Intriguingly, the lysine residues on histones can be acetylated and methylated. For example, H3 lysine 4 methylation has been correlated with active gene expression (31), whereas H3 lysine 9 methylation has been linked to gene silencing and the assembly of heterochromatin (32). It is now appreciated that DNA methylation pathways and the histone code are functionally interactive. 5-Methyl-CpG dinucleotides, through the binding of MeCP2, can recruit transcriptional corepressors with histone deacetylase (HDAC) activity, providing a link between DNA methylation and histone deacetylation. MeCP2 has also been shown to associate with histone H3 lysine 9 methyltransferase activity, providing a mechanism for targeting repressive histone methylation to DNA-methylated promoters (28). In this study we present evidence that hypermethylation of CpG dinucleotides along with methylation of H3 lysine 9 at the iNOS promoter are mechanistically linked to the lack of iNOS mRNA induction in human endothelial cells. In addition, our findings suggest that differential DNA methylation of the human and murine iNOS core promoters contributes to the marked difference in inducibility in both endothelial and other cell types. Collectively, this work defines a role for chromatin-based mechanisms in the control of human iNOS gene expression.

Materials and Methods

Materials

Recombinant human IFN-γ and IL-1β were obtained from R&D Systems. Recombinant human TNF-α was a gift from Knoll Pharmaceuticals. Polyvalent acetylated histone H3 rabbit polyclonal IgG Ab (K9, K14; 06-599), polyvalent acetyl histone H4 rabbit polyclonal antiserum (K5, K8, K12, K16; 06-866), dimethyl-histone H3 K4 rabbit antiserum (07-030), and MeCP2 rabbit polyclonal IgG Ab (07-013) were purchased from Upstate Biotechnology. Dimethyl-histone H3 K9 rabbit polyclonal Ab (ab7312) and trimethyl-histone H3 K9 rabbit polyclonal Ab (ab8898) were obtained from Abcam. RNA polymerase II (Pol II) NH2-terminal (N-20) rabbit polyclonal Ab (sc-899) was purchased from Santa Cruz Biotechnology.

Cell culture

Primary HUVECs were isolated and maintained as previously described (33). Primary human iliac artery endothelial cells (HIAEC), primary human neonatal dermal microvascular endothelial cells (HMVEC-d), primary human lung microvascular endothelial cells (HMVEC-L), and primary human saphenous vein smooth muscle cells (HuSV-VSMC) were obtained from Cambrex Bio Science. Primary human hepatocytes were purchased from BD Gentest. A549 (human lung carcinoma cell line), DLD-1 (human colorectal adenocarcinoma cell line), NIH-3T3 (murine embryonic fibroblast cell line), and RAW264.7 (murine macrophage cell line) were obtained from American Type Culture Collection. MHEC5-T transformed murine endothelial cell line was purchased from DSMZ. Cells were maintained according to the suppliers’ instructions. BALB/cJ mouse primary peritoneal macrophages were obtained by lavage of the peritoneal cavity with 10 ml of RPMI 1640 supplemented with 2 mM glutamine 4 days after i.p. administration of 1.5 ml of 3% thioglycolate as previously described (34). Six-week-old female BALB/cJ mice were purchased from The Jackson Laboratory.

Sodium bisulfite genomic sequencing

Given that the nascent strand of replicating DNA is hemimethylated immediately after DNA replication, only quiescent postconfluent primary cells were used for DNA isolation. Two micrograms of genomic DNA was digested with BamHI and then subjected to sodium bisulfite treatment as previously described (35). Twenty-five nanograms of bisulfite-treated DNA was subjected to 35 cycles of PCR amplification in a volume of 50 μl using the primers described in Table I. Two microliters of the PCR product was used as template for another 35 cycles of nested PCR amplification in a volume of 50 μl using the nested primers listed in Table I. All PCR primers were specifically designed to the sodium bisulfite-modified sense strand. The final PCR products were subcloned using the TA cloning kit (Invitrogen Life Technologies) to yield individual strands, followed by sequencing. For each cell type at least 15 randomly chosen subclones were sequenced using an ABI PRISM 377 DNA sequencer (Applied Biosystems). In some experiments, as indicated in the figure legends, the final PCR products were sequenced directly to compare the methylation of cytosines.

Cytosine extension assay

Global DNA methylation status was determined using the cytosine extension assay with minor modifications (36). Briefly, 0.5 μg of genomic DNA was incubated for 16 h with 10 U of HpaII or MspI to ensure complete
Table I. Sodium bisulfite genomic sequencing primer sets

<table>
<thead>
<tr>
<th>Primer Set</th>
<th>Sequence</th>
<th>Strand</th>
<th>Location*</th>
<th>Tm (°C)</th>
<th>Size (bp)</th>
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<tr>
<td>NOS2A core promoter</td>
<td>5′-GGA AAA GTG AGA GGA TGG ATA GGG ATT A-3′</td>
<td>Forward</td>
<td>-399 to -372</td>
<td>50.7</td>
<td>600</td>
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<td></td>
<td>5′-AAG ACG TAC CTT TAC TAC AAG CAA CTT CAT C-3′</td>
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<td>+201 to +171</td>
<td>50.7</td>
<td>505</td>
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<td>5′-ATT TAT TAG AGT TGG AAA GTG AGG TTA GGT G-3′</td>
<td>Forward</td>
<td>-344 to -314</td>
<td>50.7</td>
<td>505</td>
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<tr>
<td></td>
<td>5′-TAA AAA CAA TCA ACA CAA AAA ACC TAC A3′</td>
<td>Reverse</td>
<td>+161 to +130</td>
<td>50.7</td>
<td>505</td>
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<td>NOS2A enhancer</td>
<td>5′-GAG GGT TTT TGG TGG ATA AG-3′</td>
<td>Forward</td>
<td>-5376 to -5357</td>
<td>46.6</td>
<td>413</td>
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<tr>
<td></td>
<td>5′-CTA TAA AAC TCT TAA ACC TAA A-3′</td>
<td>Reverse</td>
<td>-4964 to -4983</td>
<td>46.6</td>
<td>413</td>
</tr>
<tr>
<td></td>
<td>5′-ATT TGG GTG GGG TGG AGT TGG GTT GTA-3′</td>
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<td>-5634 to -5611</td>
<td>45.6</td>
<td>319</td>
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<tr>
<td></td>
<td>5′-AAC TCA ACA CTG TAC TAC TAA TTT-3′</td>
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<td>-5316 to -5339</td>
<td>45.1</td>
<td>351</td>
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<tr>
<td>NOS2A enhancer</td>
<td>5′-TTT TGG GGA GGG TGG ATG ATA AG-3′</td>
<td>Forward</td>
<td>-5376 to -5357</td>
<td>46.6</td>
<td>413</td>
</tr>
<tr>
<td></td>
<td>5′-CTA TAA AAC TCT TAA ACC TAA A-3′</td>
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<td>-4964 to -4983</td>
<td>46.6</td>
<td>413</td>
</tr>
<tr>
<td></td>
<td>5′-ATT TGG GTG GGG TGG AGT TGG GTT GTA-3′</td>
<td>Forward</td>
<td>-5634 to -5611</td>
<td>45.6</td>
<td>319</td>
</tr>
<tr>
<td></td>
<td>5′-AAC TCA ACA CTG TAC TAC TAA TTT-3′</td>
<td>Reverse</td>
<td>-5316 to -5339</td>
<td>45.1</td>
<td>351</td>
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<td>Nos2 core promoter</td>
<td>5′-ATT TAT TAT GGA TAG GTT AGT GTA TTA-3′</td>
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<td>-492 to -470</td>
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<td>5′-AAC CTC AAA AAT AAT CCT AAC TAC A3′</td>
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<td>+119 to +93</td>
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<td>5′-TTA GTG GAA AAA GTG TGG TTA GA-3′</td>
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<td>+97 to +75</td>
<td>49.7</td>
<td>575</td>
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</table>

* Relative to transcription start.

5-Azacytidine (5-azaC) and trichostatin A (TSA) treatment

DLD-1 and passage 3 HUVEC were seeded at a density of 3-4×10^5 cells/ml. Exponentially growing cells were treated with 5-azaC and/or TSA (Sigma-Aldrich) every 24 h for 7 days. Medium was changed on the fourth and sixth days of treatment. On the eighth day, cells were stimulated by the addition of vehicle or a cytokine mixture (200 U/ml IFN-γ, 5 ng/ml IL-1β, and 10 ng/ml TNF-α for 4 h; maximally effective concentrations) to induce iNOS mRNA expression.

RNase protection assay (RPA)

An [α-^32P]CTP-labeled antisense human iNOS riboprobe was synthesized using an Sp6 MAXIscript kit (Ambion) and an AgeI-linearized human iNOS pHSniNOSHI vector (37). Linearization at an internal AgeI site resulted in a 243-nucleotide complementary to exon 12 and 39 nt of exon 13 (163-nt protected fragment). RNase protection was performed with the RPA II kit (Ambion) following the manufacturer’s protocol. Gel-purified probe (2×10^6 cpm) was hybridized for 16 h at 42°C with yeast tRNA (10 μg) or human total cellular RNA (10 μg). Radioactive signals were detected with a Storm PhosphorImager and quantified with ImageQuant 2 software (Molecular Dynamics).

Quantitative TaqMan real-time RT-PCR

Five micrograms of total cellular RNA was reverse transcribed with random hexamer primers using the SuperScript II kit (Invitrogen Life Technologies) following the manufacturer’s protocol. cDNA was diluted to a final volume of 50 μl. Two microliters of the RT reaction mixture was subsequently used as a template for real-time PCR quantification. The

Table II. Real-time PCR/RT-PCR primers and TaqMan probe sets

<table>
<thead>
<tr>
<th>Primer and Probe Set</th>
<th>Sequence</th>
<th>Strand</th>
<th>Location*</th>
</tr>
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<tr>
<td>Human iNOS</td>
<td>5′-TGG AGA GGC ACC ACA CAG AGT-3′</td>
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<td>-211 to -191</td>
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<td>Real-time ChIP PCR</td>
<td>5′-TGG TTT CCA AAG GGA GTG TCC-3′</td>
<td>Reverse</td>
<td>-94 to -114</td>
</tr>
<tr>
<td></td>
<td>6FAM 5′-CAC AGG CAC CAG CAC CAG TAA-3′ TAMRA</td>
<td>Probe</td>
<td>-165 to -139</td>
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<td>Human VCAM-1</td>
<td>5′-ACT TGG CTG GTG GGT TGC ATA-3′</td>
<td>Forward</td>
<td>-116 to -97</td>
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<tr>
<td>Real-time ChIP PCR</td>
<td>5′-GAG CTG TGA AAT AGA TGC TAC A3′</td>
<td>Reverse</td>
<td>+7 to -13</td>
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<tr>
<td>Human GAPDH</td>
<td>5′-GAA GGT GAA GTG CAG GGT A-3′</td>
<td>Forward</td>
<td>+321 to +339</td>
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<tr>
<td>Real-time RT-PCR</td>
<td>5′-GAA GTG GGT GTG AGT CTA-3′</td>
<td>Reverse</td>
<td>+2270 to +2251</td>
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<td>VIC 5′-CAA GCT CCA CTC CCT CAC CC-3′ TAMRA</td>
<td>Probe</td>
<td>+2241 to +2222</td>
</tr>
<tr>
<td>Human iNOS</td>
<td>5′-CAG CAG GAC GAT TCT CAA CAG AG-3′</td>
<td>Forward</td>
<td>+273 to +292</td>
</tr>
<tr>
<td>Real-time RT-PCR</td>
<td>5′-AGG CAG AAT GTG GAC TGG CGA CTA-3′</td>
<td>Reverse</td>
<td>+347 to +328</td>
</tr>
<tr>
<td></td>
<td>6FAM 5′-CAC ACT TCC CCA TAA GGC CAA AGG TAT GTT-3′ TAMRA</td>
<td>Probe</td>
<td>+294 to +323</td>
</tr>
<tr>
<td>Human VCAM-1</td>
<td>5′-CCG AAA GGC CCA GTC GAA G-3′</td>
<td>Forward</td>
<td>+2890 to +2908</td>
</tr>
<tr>
<td>Real-time RT-PCR</td>
<td>5′-AGC AGC AGA AGC CTA CGA GGA A-3′</td>
<td>Reverse</td>
<td>+3449 to +3429</td>
</tr>
</tbody>
</table>

* Relative to transcription start.
ChIP assay using an Ab directed against the large subunit of Pol II. IP DNA was analyzed by real-time PCR using NOS2A and VCAM1 promoter-specific primers. IP DNA was calculated by subtracting the amount of INOS template present in a no Ab background control from the amount present in the IP DNA and dividing by the amount of INOS sequence in a diluted input sample. Data represent the mean ± SD of triplicate measurements from one of four independent experiments with similar results. *p < 0.05 vs vehicle.

Results

Prolinflammatory cytokines do not induce iNOS in HUVEC

As shown in Fig. 1A, under basal conditions iNOS mRNA was not detected in HUVEC, even with the sensitive technique of real-time RT-PCR. Using the ChIP technique (Fig. 1B), we found that the amount of Pol II at the NOS2A promoter was not significantly different from background compared with transcriptionally quiescent intergenic regions of the genome (41). Addition of TNF-α or multiple cytokines failed to induce iNOS mRNA or Pol II loading at the proximal promoter of the NOS2A gene in HUVEC (Fig. 1). Induction of VCAM-1 by inflammatory mediators or cytokines, including LPS, IL-1β, and TNF-α, requires cis elements located in the proximal promoter of the VCAM1 gene: two tandem NF-kB sites (−72 to −63 bp, and −57 to −48 bp), a specificity protein 1 (Sp1) binding site (−46 to −42 bp), and an IRF element (−13 to +7 bp) (42, 43). Overall, the architecture of this promoter is related to that of NOS2A, where NF-kB, IRF-1, STAT-3, and other transcription factors are involved in iNOS induction by cytokines (21, 22). Unlike iNOS, however, VCAM-1 mRNA (Fig. 1A) and the loading of Pol II (Fig. 1B), a functional index of VCAM1 transcription, were markedly induced in HUVEC after addition of TNF-α or a cytokine mixture.

Human NOS2A promoter methylation correlates inversely with cytokine inducibility

Currently, very little is known about the role of epigenetic signaling pathways in human iNOS gene regulation. Therefore, we determined the DNA methylation status of six CpG doublets surrounding the human iNOS core promoter (region I and TATA box) using high resolution sodium bisulfite genomic sequencing (Fig. 2A). The bisulfite primers amplify a single copy amplicon specific for the human NOS2A promoter region, allowing for high resolution analyses and the use of ChIP-Seq methods (44). The bisulfite doublets (39 to 59) are located in promoter and exon 1 and have variable methylation levels across the NOS2A gene (Table II). The first bisulfite doublet is located about 300 bp downstream of the transcriptional start site and is rich in CpG sites (45), while the second bisulfite doublet is located at the same genomic coordinate as the first doublet and is hypomethylated (46). The degree of bisulfite methylation for the first bisulfite doublet was used to determine the relative degree of cytosine methylation at this genomic position.

Genomic structure analyses

Human genomic sequences containing iNOS, iNOS-like sequences and contiguous genomic regions were characterized using Ensembl (www.ensembl.org). VISTA plot alignment (http://genome.ucsc.edu/vista/index. shhtml) was used to compare human NOS2A with murine Nos2 and human iNOS pseudogenes, specifically human NOS2B and NOS2C (NCBI 35 assembly of the human genome, NCBI m33 mouse assembly). RepeatMasker (www.repeatmasker.org/) was used to characterize the repetitive DNA content in genomic DNA sequences.
FIGURE 2. DNA methylation status of \( NOS2A \) proximal promoter in inducible and noninducible cell types. 

A. Schematic diagram of the proximal 5'-flanking region of the human \( NOS2A \) gene analyzed by bisulfite sequencing. CpG doublets are represented by vertical lines. Numbering is with respect to transcription initiation (arrow).

B. Sodium bisulfite sequencing results of the \( NOS2A \) core promoter in iNOS-noninducible (Figure legend continues)

C. A549 (44%) and DLD-1 (39%) cell types.

D. HUVEC Vehicle (76%) and HUVEC Cytokine (83%) cell types.

E. Hepatocyte (22%) and normalized NOS mRNA Expression vs. CpG Methylation Profile of the \( NOS2A \) Promoter with \( R^2 = 0.99 \).
to NOS2A in the haploid genome. Sodium bisulfite sequencing revealed high levels of methylation of the six CpG dinucleotides surrounding the iNOS proximal promoter in DNA isolated from primary HUVEC cells (Fig. 2B). Comparable levels of DNA methylation were confirmed in five independent HUVEC cell preparations (overall NOS2A methylation profile of 76, 76, 76, 75, and 74%). Next, bisulfite genomic sequencing was used to define the methylation levels of the CpG dinucleotides in the NOS2A promoter in other human cell types. The NOS2A core promoter was also found to be densely methylated in noninducible primary dermal microvascular cells (HMVEC-d), primary lung microvascular endothelial cells (HMVEC-L), primary iliac artery endothelial cells (HIAEC), and primary saphenous vein vascular smooth muscle cells (HuSV-VSMC) (Fig. 2B). Human lung carcinoma cells A549, colon adenocarcinoma cells DLD-1, and primary hepatocytes were the best-characterized in vitro models of human iNOS induction. In contrast to murine cells, even in these human cell types the observed iNOS induction is modest and requires maximal concentrations of IFN-γ, IL-1β, TNF-α, and LPS (10, 21, 44). Intriguingly, analysis of the NOS2A promoter in these iNOS-inducible cell types revealed significantly lower levels of DNA methylation (Fig. 2C; inducible vs noninducible cell types; p < 0.05). We did not observe CpgNpG methylation or DNA methylation in asymmetric sequence contexts (CpHpH, H = A, T, C), at least in the cell types studied (45). We also failed to find DNA sequence variation in the promoter sequences of the genomic DNA alleles isolated from inducible tumor cell types.1 Importantly, the differentially methylated region (DMR) spanning −289 to +117 bp of NOS2A (Fig. 2, B and C) includes the well-characterized NF-κB and octamer-like AT-rich sequences required for iNOS induction in multiple species (Fig. 2A) (9, 21).

Little is known about the presence or functional consequence of basal levels of proximal promoter DNA methylation in cytokine-inducible genes. To determine the extent of iNOS transcriptional repression as a function of NOS2A methylation profile, real-time RT-PCR was used to accurately quantify mRNA transcript levels for human iNOS in primary HUVEC, A549, DLD-1, and primary hepatocytes stimulated with a cytokine mixture of IFN-γ, IL-1β, and TNF-α for 4 h. The overall methylation profile was defined as the average occupancy of methylcytosine at the six CpG dinucleotides surrounding the iNOS core promoter region I (Fig. 2A). It should be noted that bisulfite sequencing revealed no significant differences in the NOS2A methylation profile between resting and cytokine-activated states in the cell types examined (Fig. 2D). This is in contrast to the active DNA demethylation observed at the i22 promoter/enhancer upon transcriptional activation in stimulated T lymphocytes (46). As shown in Fig. 2E, primary HUVEC and primary hepatocytes, which represented the two ends of the NOS2A methylation profile, were shown to have null and high levels of iNOS mRNA expression, respectively. A549 and DLD-1, which had intermediate levels of iNOS promoter methylation, had iNOS mRNA levels between those of HUVEC and hepatocytes. The data suggest that the NOS2A methylation profile correlates with iNOS transcriptional repression in an exponential fashion (Fig. 2E), a finding that parallels observations made using in vitro methylated episomes (24, 47). This is in contrast to the all-or-none principle present in tumor suppressor genes (48) and cell type-specific genes (35), where promoters are either heavily methylated in transcriptionally inactive cells or completely unmethylated in active cells. Importantly, this inverse correlation supports our working model, which posits that human iNOS expression is controlled by an epigenetic mechanism.

**Human NOS2A DMR is confined to the proximal promoter**

Because additional human genomic regions are functionally important in transcriptional regulation of iNOS, we comprehensively examined the DNA methylation status of regions spanning these domains from −6226 to −4964 bp (Fig. 3A). This upstream sequence encompasses NF-κB and AP-1 sites in the −5.8 and −5.3 kb cytokine-responsive enhancers (20–22). Although we noted patterns of methylation, these regions did not display important differences in methylation between iNOS-noninducible and -inducible cell types (Fig. 3, B and C). We conclude that the human NOS2A DMR is confined to the proximal core promoter.

**5-azacytidine induces NOS2A promoter demethylation and enhances iNOS mRNA expression**

An inverse correlation between methylation of the proximal NOS2A promoter and the inducibility of iNOS does not definitively establish that DNA methylation controls iNOS transcription. Arguably, the observed inverse relationship could be interpreted as a footprint of iNOS transcription activity. In this scenario, active Pol II loading and assembly of the transcriptional apparatus on the iNOS core promoter could prevent methylation by DNA methyltransferases, resulting in less methylation in an actively transcribed iNOS gene. If this model is correct, iNOS transcription would not be directly affected by changes in promoter methylation status. To address this issue, 5-azacytidine was used to assess the functional

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1 When evaluating iNOS promoter methylation status, a previously unreported single nucleotide polymorphism in the 5'-untranslated region of the human NOS2A gene was identified. This +3/C/G polymorphism in exon 1 was typed by PCR-restriction fragment length polymorphism with the Exog restriction enzyme. The allele frequencies of C (Exog-) and G (Exog+) in 100 unrelated Caucasians were 0.63 and 0.37, respectively (mean heterozygosity, 0.47; polymorphism information content, 0.36). Interestingly, this +3/C/G single nucleotide polymorphism results in the absence or the presence, respectively, of a CpG dinucleotide at +37 bp with respect to the transcription start site. No important differences were noted across the two alleles in the methylation status of other CpGs of the human iNOS promoter in HUVEC or other cell types. Clearly, an extra CpG dinucleotide was evident in the Exog- allele, but otherwise, important allelic differences in methylation status were not evident.

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primary human cell types: neonatal HMVEC-d, HUVEC, HMVEC-L, HIAEC, and HuSV-VSMC. The average occupancy of methylcytosine at single CpG dinucleotides is shown as the percent methylation on the y-axis. Location of the CpG sites is indicated on the x-axis. Numbers in parentheses represent the overall methylation profile of the six CpGs in this genomic region. C, NOS2A bisulfite sequencing results in inos-inducible human cell types: A549 pulmonary adenocarcinoma cell line, DLD-1 colon adenocarcinoma cell line, and primary hepatocytes. D, Bisulfite sequencing results of the NOS2A core promoter in vehicle- and cytokine-treated (200 U/ml IFN-γ, 5 ng/ml IL-1β, and 10 ng/ml TNF-α; 4 h) cells. Similar conclusions were reached either by cloning and sequencing individual molecules (HUVEC; top panel) or by direct sequencing of the PCR products (HUVEC; middle panel). Direct PCR sequencing identifies partial methylation of a cytosine residue in the population of DNA molecules as a mixture of cytosine signal (methylated cytosine) and thymine signal (unmethylated cytosine) at the same position. The degree of methylation can be estimated by comparing the cytosine signal relative to the thymine signal. Treatment with cytokines did not change NOS2A methylation, as shown by similar C to T signal ratios (compare Vehicle and Cytokine). E, Postconfluent cells were stimulated with a cytokine mixture of IFN-γ (200 U/ml), IL-1β (5 ng/ml), and TNF-α (10 ng/ml) for 4 h, after which total cellular RNA was isolated and examined by real-time RT-PCR. The iNOS mRNA expression was normalized to the GAPDH mRNA level. An x y scatter plot is shown, with NOS2A methylation profile as the x values and the normalized level of cytokine-induced iNOS mRNA on the y-axis (log scale). An exponential curve (straight line) was fitted through the data points. The iNOS expression data points represent the mean ± SEM of at least three independent experiments. Where error bars are not evident, the SEM are below the figure resolution.
Figure 3. The DMR is confined to the NOS2A proximal promoter. A. Schematic diagram of the −5.8 kb cytokine-responsive enhancer of the human NOS2A gene analyzed by bisulfite sequencing. B and C, NOS2A bisulfite sequencing results in iNOS-noninducible primary HUVEC cells (B) and iNOS-inducible A549, DLD-1, and primary hepatocytes (C).
Inhibition of DNA methyltransferase activity enhances iNOS mRNA induction. Cells were treated with vehicle or 5-azaC every 24 h for 7 days, after which DNA and RNA were isolated. Four hours before extraction, postconfluent cells were stimulated with vehicle or a cytokine mixture (Cyt) of IFN-γ (200 U/ml), IL-1β (5 ng/ml), and TNF-α (10 ng/ml). A and B, Sodium bisulfite sequencing results of the NOS2A core promoter in vehicle- or 5-azaC-treated DLD-1 (A) or HUVEC (B). Bisulfite data represent one of two (DLD-1) or four (HUVEC) independent experiments with similar results. C, RPA of iNOS mRNA using a 243-nt probe complementary to exons 12 and 13 of iNOS. The protected fragment is 163 nt. Radioactive signals were detected with a Storm PhosphorImager and were quantified with ImageQuant 2 software. The inset is a quantitation of this representative gel. D, Real-time RT-PCR of iNOS and GAPDH mRNA. The iNOS mRNA expression was normalized to the GAPDH mRNA level (in arbitrary units). Data represent the mean ± SD of triplicate measurements from one of three or more independent experiments, each with similar results. *p < 0.01 vs vehicle Cyt.

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** Inhibition of DNA methyltransferase activity enhances iNOS mRNA induction. Cells were treated with vehicle or 5-azaC every 24 h for 7 days, after which DNA and RNA were isolated. Four hours before extraction, postconfluent cells were stimulated with vehicle or a cytokine mixture (Cyt) of IFN-γ (200 U/ml), IL-1β (5 ng/ml), and TNF-α (10 ng/ml). A and B, Sodium bisulfite sequencing results of the NOS2A core promoter in vehicle- or 5-azaC-treated DLD-1 (A) or HUVEC (B). Bisulfite data represent one of two (DLD-1) or four (HUVEC) independent experiments with similar results. C, RPA of iNOS mRNA using a 243-nt probe complementary to exons 12 and 13 of iNOS. The protected fragment is 163 nt. Radioactive signals were detected with a Storm PhosphorImager and were quantified with ImageQuant 2 software. The inset is a quantitation of this representative gel. D, Real-time RT-PCR of iNOS and GAPDH mRNA. The iNOS mRNA expression was normalized to the GAPDH mRNA level (in arbitrary units). Data represent the mean ± SD of triplicate measurements from one of three or more independent experiments, each with similar results. *p < 0.01 vs vehicle Cyt.

consequences of inhibiting DNA methyltransferase activity in DLD-1 human colorectal cancer cells. The efficacy of the 5-azaC treatments in DLD-1 was confirmed using the cytosine extension assay. 5-AzaC (1 μM, 7 days) induced DLD-1 global demethylation, reducing the relative global CmCGG methylation content from a basal level of 62 to 41%. Bisulfite sequencing revealed that
the NOS2A promoter in DLD-1 was completely demethylated from the basal level of 39 to 0% (Fig. 4A). The effects of NOS2A demethylation on the ability of a cytokine mixture (IFN-γ, IL-1β, and TNF-α) to induce iNOS mRNA expression were evaluated. 5-AzaC treatment (1 μM; 7 days) of DLD-1 enhanced iNOS mRNA induction by an average of 3.1-fold (Fig. 4C) and 2.9-fold (Fig. 4D), as demonstrated by RPA and real-time RT-PCR, respectively. Taken together, these findings provide evidence for a causal relationship between DNA methylation at the NOS2A promoter and the transcriptional response of iNOS to cytokines.

Given the potent effect of 5-azaC on NOS2A methylation and iNOS induction in DLD-1, we considered whether the iNOS gene could be reactivated in 5-azaC-treated HUVEC. Unexpectedly, RPA and real-time RT-PCR failed to detect significant amounts of iNOS transcript in cytokine-stimulated HUVEC after the addition of maximally tolerated concentrations of 5-azaC for prolonged periods of time (5 μM; 7 d; Fig. 4, C and D). 5-AzaC (5 μM; 7 days) reduced the global methylation content in HUVEC from 85.1 ± 4.9 to 48.0 ± 8.2% (n = 3; mean ± SEM; vehicle-treated vs 5-azaC-treated, p < 0.05). Surprisingly, bisulfite sequencing repeatedly confirmed that the iNOS promoter was still significantly hypermethylated (Fig. 4B) despite a 37% decrease in global methylation in 5-azaC-treated HUVEC. It is not likely that bisulfite sequencing overestimated the level of methylation in 5-azaC-treated HUVEC. This is because this method detected 5-azaC-induced complete NOS2A demethylation in DLD-1 (Fig. 4A). Therefore, the lack of substantial iNOS promoter demethylation in 5-azaC-treated HUVEC cannot be construed as a methodological artifact. That specific regions of the genome are selectively resistant to drug-induced DNA demethylation has previously been reported in mammals (50, 51). We posit that the NOS2A promoter hypermethylation that is preserved in 5-azaC-treated endothelial cells is functionally important and considered whether the hypermethylated iNOS promoter in HUVEC has an additional epigenetic silencing mark.

MeCP2 and the NOS2A proximal promoter in endothelial cells

MeCP2 is known to play an important role in gene repression by recruiting histone deacetylases and H3 lysine 9 methyltransferases to CpG hypermethylated promoters, thereby mediating nucleosomal histone deacetylation and H3 lysine 9 methylation, respectively (26, 28). MeCP2 can also influence chromatin structure independently of DNA methylation (27). As shown in Fig. 5, MeCP2 was highly enriched at the NOS2A proximal promoter in HUVEC. In contrast, MeCP2 was not basally present at the cytokine-inducible VCAM1 proximal promoter in HUVEC. This suggests that MeCP2 may play a role in repressing iNOS expression in HUVEC.

Histone modifications at the NOS2A proximal promoter

It is known that histone deacetylation is involved in methylation-induced transcriptional repression. ChIP analysis was conducted using polyvalent anti-acetylated H3 Ab and anti-acetyl H4 antisera. Under basal conditions, we observed no differences in the acetylation status of histones H3 and H4 at the NOS2A core promoter between HUVEC and DLD-1 (Fig. 6, A and B). This is contrary to what would be expected if deacetylation was indeed the additional epigenetic constraint on iNOS induction in HUVEC. After the addition of multiple cytokines to HUVEC, the NOS2A promoter underwent 4.2- and 1.8-fold increases in acetylation at histones H3 and H4, respectively (Fig. 6, C and D). Remarkably, however, these increases in H3 and H4 acetylation did not correlate with the recruitment of Pol II at NOS2A (Fig. 1B; vehicle vs Cyt); the iNOS promoter was still transcriptionally silent. Thus, H3 and H4 acetylation levels are not predictive of iNOS mRNA inducibility.

MeCP2 can induce transcriptional repression in part through the recruitment of HDAC activity (26). To examine whether HDAC activity is required for the maintenance of transcriptionally silent state, HUVEC were treated with the HDAC inhibitor TSA. Mammalian pericentric heterochromatin is specifically responsive to prolonged treatment with TSA (52), and the NOS2A genomic locus is near the centromere at 17q11.2–12 (37). Moreover, some genes can be activated by 5-azaC and some by HDAC inhibition (e.g., TSA), whereas others require the combined addition of both 5-azaC and TSA. For other genes, the addition of both inhibitors leads to a synergistic activation compared with the effect of either agent alone (48, 53). However, as shown in Fig. 6E, TSA (100 nM; 7 days), alone or in combination with 5-azaC (5 μM; 7 days), failed to affect iNOS mRNA expression in response to cytokines (IFN-γ, IL-1β, and TNF-α; 4 h) in HUVEC or DLD-1. Furthermore, TSA failed to modify the methylation status of the iNOS promoter in HUVEC in either the presence or the absence of 5-azaC (Fig. 6F). Overall, inhibiting HDAC activity had no discernible effect on iNOS promoter activation in HUVEC or DLD-1. These findings argue that DNA methylation-induced NOS2A repression operates through pathways other than histone deacetylation, at least in the cell types studied.

DNA methylation can also silence a promoter by inducing a heterochromatic state through histone H3 lysine 9 methylation (28, 32). Intriguingly, the unstimulated NOS2A promoters in HUVEC and DLD-1 were found to be differentially methylated at histone H3 lysine 9. As shown in Fig. 7, A and B, histone H3 of the proximal iNOS promoter was modified by di- and trimethylation of lysine 9 in HUVEC. In contrast, the relatively CpG hypomethylated NOS2A promoter in DLD-1 (Fig. 2C) was minimally methylated at H3 lysine 9 (Fig. 7, A and B). Lysine methylation was previously thought to be a relatively stable and irreversible histone modification compared with acetylation. Recently, however, a subset of inducible inflammatory gene promoters was demonstrated to be basally methylated at H3 lysine 9. Intriguingly, this methylation was erased before Pol II loading and was restored concurrently with Pol II release, thereby generating a time window during which gene transcription could occur (54). We considered whether basal H3 lysine 9 methylation was a characteristic feature of inducible genes in vascular endothelium, and whether active lysine 9 demethylation/remethylation directed their transcriptional induction. Specifically, we asked whether dynamic modulation of lysine 9 methylation in endothelial cells could be observed at the iNOS or VCAM-1 promoters. An H3 lysine 9 methyl mark was not detected.

FIGURE 5. MeCP2 is differentially recruited to the proximal promoters of iNOS vs VCAM-1 in endothelial cells. ChIP was used to assess the binding of MeCP2 to the proximal promoters of iNOS and VCAM-1 in HUVEC. The iNOS primers and probe reside in the same proximal promoter region analyzed by the bisulfite method. Data represent the mean ± SEM of three independent experiments.
basally or after cytokine treatment at the promoter of the VCAM1 gene (Fig. 7, C and D). Similar findings have recently been reported for another cytokine-inducible gene in the vascular endothelium, namely E-selectin (55). As noted above, ChIP assays revealed basal levels of lysine 9 methylation at the iNOS promoter in HUVEC. Cytokine stimulation did not decrease lysine 9 methylation at the NOS2A promoter in HUVEC (Fig. 7, C and D). We take these data to indicate that nucleosomes of the HUVEC iNOS promoter are specifically modified by H3 lysine 9 methylation, a mark of silent chromatin.

Methylation of histone H3 lysine 9 is believed to repress transcription by compacting chromatin. In contrast, methylation of H3 lysine 4 is associated with gene transcription (31). To further dissect the role of histone methylation in NOS2A regulation, ChIP assays were conducted using a dimethylated H3 lysine 4 antiserum. Methylated lysine 4 was not detected at the unstimulated NOS2A promoters in HUVEC or DLD-1 (Fig. 8A). In contrast to NOS2A, the VCAM1 promoter in HUVEC exhibited basal H3 lysine 4 methylation, a histone modification indicative of an open chromatin structure (Fig. 8B). After 4 h of stimulation of HUVEC with TNF-α or a cytokine mixture, the VCAM1 promoter underwent 1.9- and 2.4-fold increases in lysine 4 methylation, respectively. In contrast, the HUVEC NOS2A promoter remained unmethylated at H3 lysine 4 (Fig. 8B).
Intravenous correlation between mouse Nos2 promoter methylation and iNOS induction

To compare the regulation of human and murine iNOS we assessed the DNA methylation status of the iNOS promoter across species. The promoters for the human iNOS (NOS2A) and mouse iNOS (Nos2) genes are not CpG islands (56). Using bisulfite genomic sequencing we examined all seven proximally located CpG doublets surrounding the highly homologous mouse basal promoter region (57). Using bisulfite genomic sequencing we examined all seven proximally located CpG doublets surrounding the highly homologous mouse basal promoter region (56). Using bisulfite genomic sequencing we examined all seven proximally located CpG doublets surrounding the highly homologous mouse basal promoter region (56). Using bisulfite genomic sequencing we examined all seven proximally located CpG doublets surrounding the highly homologous mouse basal promoter region (56).

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Epigenetic divergence at the human and mouse iNOS orthologs

We compared the CpG methylation status of the iNOS promoter in human and murine endothelial cell types. An analysis of MHEC5-T (mouse heart endothelial cell line) showed hypomethylation at the Nos2 promoter (Fig. 9C) compared with that at the NOS2A promoter in HUVEC, representing a 4.5-fold difference (17 vs 76%, respectively; Fig. 2B). Coupled with findings by us and others, namely, that Nos2 is readily inducible in these and other nonhuman endothelial cells (2, 4), but notoriously resistant to induction in human endothelial cells (7, 59), and the knowledge that the human and mouse iNOS promoters are highly homologous, our present findings strongly suggest that differential methylation of the human and mouse iNOS core promoters contributes to the marked difference in inducibility in both endothelial and other cell types.

An unresolved issue is what determines the divergence in iNOS epigenetic states between humans and rodents. Relatively little is known about the relatedness of epigenetic pathways between species, but this is an active area of study, with results of genome-based assessments of DNA methylation and histone modifications starting to appear. For instance, histone H3 lysine 4 methylation patterns at orthologous loci are strongly conserved between human and mouse, at least for human chromosomes 21 and 22 (60). A sequence comparison of the human and mouse iNOS genes is shown in Fig. 10A. Notably, there is a high degree of sequence conservation between the human and mouse iNOS genes at the ~5.8-~5.3 kb enhancer-like region, the proximal promoter, and all exonic sequences. Compared with other orthologous gene pairs, iNOS is not unique (61). However, three specific features are noteworthy when comparing the human and murine iNOS genes: proximity to pericentric heterochromatin, gene copy number, and repetitive DNA content.

Human NOS2A is pericentrially juxtaposed on chromosome 17q11.2-q12, ~0.9 Mb from the centromere, whereas mouse Nos2 is located in chromosome 11 band B5, ~78.5 Mb away from the centromere. Transcriptionally inert pericentric heterochromatin is characterized by DNA hypermethylation and histone H3 K9 trimethylation (52, 62). Importantly, this heterochromatinization or epigenetic silencing can spread to neighboring pericentric euchromatic genes. In disease, the reverse can occur. For example, satellite regions can become hypomethylated, and CpG islands of active genes within pericentric DNA can become paradoxically hypermethylated (63). Although the NOS2A gene is located in a pericentricromeric location, it is closely juxtaposed to transcription units that are not repressed. LGALS9, KSR, and WSB1 are located proximal to NOS2A with respect to the centromere, at...
In addition to human, exists as a single copy gene in the haploid genome. In contrast, in mental duplications and pseudogenes (64). The murine meres (e.g., minor and major satellites), pericentric DNA is char-
epigenetic silencing of human vs murine iNOS.
mouse iNOS does not represent the sole molecular basis for the
alysis of established sequence tag databases and Northern blot hy-
distances of 0.3, 3.3, and 12.6 Mb, respectively. Importantly, anal-
B
NOS2C
resides 91 kb proximal to
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and exhibits a tail-to-tail
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like), and
NOS2B
shares
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promoter.

Other forms of repetitive DNA elements, such as transposable elements and viruses, also attract DNA methylation. Methylation of cytosines in these highly repetitive sequences silences transcriptional activity and serves as a genome-based defense mechanism guarding against recombination of these parasitic elements (72). However, parasitic DNA integrated into promoters of host genes could lead to de novo methylation and change the expression of endogenous genes. Despite the relatively similar repeat content of the human and mouse genomes (47.6 vs 41.2%, respectively) (61), NOS2A and Nos2 differ significantly in their repetitive DNA contents. RepeatMasker analysis of 10 kb of sequence encompassing −9 to +1 kb of the iNOS gene, a region that is not duplicated, revealed that repetitive DNA comprised 43.9% of human NOS2A and 24.6% of mouse Nos2. Likewise, 32.9% of the NOS2A gene sequence (exon 1–27, introns included) was identified as highly repetitive DNA compared with 22.4% of equivalent Nos2 genomic sequences. Therefore, the human iNOS gene has accumulated more high copy repetitive sequences than the mouse gene, which may also be relevant to the DNA hypermethylation evident at the NOS2A promoter vs the Nos2 promoter.

Discussion
The contribution of chromatin-based mechanisms to the regulation of gene expression in vascular endothelial cells is a newer area of study. We have recently shown that epigenetics may provide a new paradigm for understanding endothelial cell-specific endothelial NO synthase (eNOS) expression. Transient transfection of eNOS promoter/reporter constructs into endothelial and nonendothelial cell types revealed high levels of transcription regardless of whether the native chromatin-based eNOS gene was active (35). A
similar finding has been reported for human iNOS promoter/reporter episome-based studies across a variety of cell types (20–23). The contribution of chromatin-based mechanisms cannot be adequately assessed using transient transfection of episomal-based vectors. Despite nonendothelial cell activity of the eNOS promoter in transient transfection studies, the expression of eNOS promoter/reporter genes in insertional transgenic mice demonstrated endothelial-specific reporter activity (73). By analyzing a large portion of the eNOS/NOS3 locus for cell-specific DNA methylation patterns, we found a DMR that was highly localized to the proximal promoter (35). Although nonendothelial cells were highly methylated at the eNOS proximal promoter sequences, endothelial cells lacked DNA methylation in this same region. This DMR was evident in vivo in the mouse aorta and was functionally relevant in vitro (35). In contrast to eNOS, the work presented in this study indicates that genes can also be actively repressed in vascular endothelium by epigenetic pathways.

The major finding of the current work is that the human NOS2A gene is epigenetically silenced/repressed by DNA methylation and histone H3 lysine 9 methylation. In human cell types notoriously resistant to iNOS induction (e.g., primary endothelial cells and vascular smooth muscle cells), the NOS2A proximal promoter was densely methylated at CpG dinucleotides. In contrast, human primary cell types and transformed cell lines capable of iNOS induction (i.e., hepatocytes, A549, and DLD-1) had a lower density of methylated CpGs at the NOS2A proximal promoter. The studies reported in this study argue that DNA methylation is functionally important in iNOS regulation. Prolonged inhibition of DNA methyltransferase activity in human DLD-1 cells resulted in DNA demethylation of the iNOS promoter and a 3-fold increase in cytokine-induced iNOS mRNA expression. In contrast, endothelial cells were resistant to DNA demethylation, and iNOS remained silenced even after repeated 5-azaC treatments. The finding that iNOS could not be induced in 5-azaC-treated HUVEC suggested that additional repressive epigenetic mechanisms were contributing to DNA methylation-dependent NOS2A silencing. Genes that are silenced by epigenetic mechanisms can respond differentially to inhibition of repressive chromatin-based transcriptional pathways. Recent evidence from the model organisms Neurospora crassa and Arabidopsis thaliana indicates that maintenance of
DNA methylation is dependent upon histone H3 lysine 9 methylation (74, 75). We identified histone H3 lysine 9 methylation as an additional epigenetic modification responsible for maintaining the silenced state at the NOS2A promoter in HUVEC. Accordingly, we propose a model in which the high density of methylated CpG residues, binding of MeCP2, and H3 lysine 9 methylation of the nucleosomes at the proximal iNOS promoter establish a permanent repressive epigenetic state in endothelial cells. Consistent with our model, recent studies have reported that human iNOS promoter/reporter murine insertional transgenes robustly express the reporter in chromatin-based expression assays. These studies found that the human iNOS promoter is transcriptionally activated by inflammatory...
stimuli (e.g., LPS) in a wide variety of tissues and cell types when the transgene is randomly inserted into the genome. Clearly the relevant cis elements in the human iNOS promoter are functional when the gene is placed in a permissive chromatin context (19).

Intriguingly, iNOS promoter DNA methylation status varied across species. We found that the murine iNOS proximal promoter was hypomethylated relative to human sequences. For example, in endothelial cells the murine iNOS promoter possessed 17% CpG methylation compared with the 76% methylated human iNOS promoter. Our results suggest that differential DNA methylation of the human and mouse iNOS promoters contributes to the marked difference in cytokine-induced gene expression. Moreover, our comparative genomic analyses suggest that human NOS2A gene duplication and the accumulation of high copy repetitive sequences at the NOS2A locus may be relevant to the divergence in iNOS epigenetic states between humans and rodents.

Concepts of how de novo methylation of human NOS2A might have arisen should be distinguished from conjecture about why the hypermethylation status was maintained in humans throughout evolution. It has been estimated that 5% of the mammalian genome is under evolutionary selection. This evolutionary divergence is especially evident for classes of proteins implicated in reproduction, olfaction, and immunity (61). From an evolutionary perspective, the expression of a functional iNOS is one example of the known discrepancies in innate immunity between these two species. Such differences should not be surprising considering that humans and mice have evolved in quite different ecological niches for ~75 million years. NO is a double-edged sword, whose anti-microbial and antitumor activities are accompanied by host cell cytotoxicity. In humans, it may be advantageous to limit the expression of iNOS to chronic inflammation or infected cells. In contrast, mice evolved in a quite different environment from humans and have been exposed to a relatively higher Ag load. Accordingly, it may be selectively advantageous for mice to have a robust functional iNOS as part of their innate immune repertoire. With respect to vascular endothelial cells specifically, it is relevant that these cells already have a constitutive NOS isoform, namely eNOS. To fully comprehend the relevance of iNOS to human disease, it is essential that the discrepancy between acute human iNOS inducibility in vitro and chronic inducibility in vivo be mechanistically understood. The studies reported in this paper suggest that changes in the epigenetic regulation of iNOS should now be considered in the pathogenesis of iNOS expression in vivo. Understanding the epigenetic mechanisms limiting human NOS2A induction is relevant from a clinical perspective, given that modulators of epigenetic processes could be useful for therapeutic iNOS inhibition.

Disclosures

The authors have no financial conflict of interest.

References


