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Epigenetic Silencing of the Human NOS2 Gene: Rethinking the Role of Nitric Oxide in Human Macrophage Inflammatory Responses

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Macrophages, including alveolar macrophages, are primary phagocytic cells of the innate immune system. Many studies of macrophages and inflammation have been done in mouse models, in which inducible NO synthase (NOS2) and NO are important components of the inflammatory response. Human macrophages, in contrast to mouse macrophages, express little detectable NOS2 and generate little NO in response to potent inflammatory stimuli. The human NOS2 gene is highly methylated around the NOS2 transcription start site. In contrast, mouse macrophages contain unmethylated cytosine-phosphate-guanine (CpG) dinucleotides proximal to the NOS2 transcription start site. Further analysis of chromatin accessibility and histone modifications demonstrated a closed conformation at the human NOS2 locus and an open conformation at the murine NOS2 locus. In examining the potential for CpG demethylation at the NOS2 locus, we found that the human NOS2 gene was resistant to the effects of demethylation agents both in vitro and in vivo. Our data demonstrate that epigenetic modifications in human macrophages are associated with CpG methylation, chromatin compaction, and histone modifications that effectively silence the NOS2 gene. Taken together, our findings suggest there are significant and underappreciated differences in how murine and human macrophages respond to inflammatory stimuli. The Journal of Immunology, 2014, 192: 000–000.

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ive macrophages are primary phagocytic cells of the innate immune system (1, 2). They are responsible, among other functions, for clearing infectious, toxic, and allergic particles from the respiratory tract. In the case of murine macrophages, the response to inflammatory stimuli is characterized by the production of large amounts of NO via NO synthase (NOS) 2 (3–5). NO produced by murine alveolar macrophages is a critical component in the removal of bacteria and foreign particles (6). It has been shown to be crucial to murine alveolar macrophage function and host survival (6–8).

The murine alveolar macrophage has been frequently used as a model for human alveolar macrophage physiology. NO production by murine macrophages has been inferred to explain human inflammatory pathophysiology. However, there is scant evidence describing NO production in human alveolar macrophages to support such an extrapolation (9, 10). What data there are consistently show that, even when stimulated, healthy human macrophages make little to no NO (9, 11–13). In murine models, inhibiting NO production impairs clearance of intracellular pathogens. Similar treatment of human alveolar macrophages has no effect (14). Further complicating the current landscape are data showing that human alveolar macrophages recovered from patients with chronic inflammatory diseases are capable of measurable, but small, amounts of NO (15–18). Thus, it remains unclear whether NO production is relevant to human macrophage physiology or macrophage-mediated pathophysiology. Given this apparent contrast with the central role NO plays in murine macrophage inflammation, we consider it essential to clarify whether these animal findings can be appropriately extrapolated to the human arena.

Epigenetic programming is one of the mechanisms that controls cell type–specific transcription of genes. Epigenetic control involves multiple regulatory mechanisms that include untranslated RNA, histone acetylation, and methylation of histones and DNA cytosine-phosphate-guanine (CpG) dinucleotide sequences (19–22). DNA methylation at the C-5 positions of cytosine (5mC) in the CpG dinucleotide is a well-characterized epigenetic modification controlling gene expression (23, 24). Methylation of CpG motifs in the promoter region has been repeatedly shown to influence DNA transcription (primarily decreasing transcription) and is an important step in both normal and malignant cell function (25–28). CpG methylation, coupled with subsequent histone deacetylation, condenses chromatin and leads to gene silencing (29–31). This mechanism relies on methyl-CpG–binding domain proteins that recognize methylated cytosines in the CpG dinucleotides. Altering gene regulation through the reversal of
DNA methylation and histone acetylation is a recent addition to clinical therapeutics. In some clinical trials, cancer chemotherapeutic agents are used specifically to promote gene transcription via CpG demethylation and histone deacetylation (32–34).

In this study, we examined the effect of classic NOS2-activating stimuli on NOS2 expression and NO production in human macrophages. We found that human macrophages failed to produce NO even after stimulation with potent inflammatory signals. Furthermore, we found that, in contrast to mouse macrophages, human macrophages did not express detectable levels of NOS protein coded by the NOS2 gene. We found that CpG motifs proximal to the transcription start site (TSS) of the human NOS2 promoter region are fully methylated, consistent with an epigenetically repressed gene. In contrast, CpG motifs proximal to the TSS in mouse macrophage NOS2 were not methylated. Assays for chromatin accessibility and histone regulatory marks were consistent with the hypothesis that the human macrophage NOS2 gene is a silenced gene. These data describe previously unrecognized differences between human and mouse macrophages and suggest that murine data be used with caution when inferring links to human disease.

Materials and Methods

Cell lines

Human alveolar macrophages, human blood monocytes, a human monocytic leukemia cell line (THP-1), murine alveolar macrophage cell line (MH-S), and murine peritoneal macrophage cell line (RAW 264.7) were used. THP-1 and MH-S cells were cultured in RPMI 1640 supplemented with 5 ml L-glutamine, 5 ml 1 M HEPES, 5 ml 100 mM sodium pyruvate, 5 ml 250 mg/ml glucose, 5% FBS, and 5 ml Pen/Strep per liter. Human monocytes were cultured in RPMI 1640 supplemented with 40 μg/ml gentamicin and 5% FBS, and human and murine alveolar macrophages were cultured in RPMI 1640 supplemented with 40 μg/ml gentamicin.

Bronchoalveolar lavage

After informed consent was obtained under a Carver College of Medicine Institutional Review Board–approved protocol, nonsmoking volunteer research subjects performed spirometry with exclusion criteria of a forced expiratory volume in 1 s or forced vital capacity <80%. Next, they underwent standard flexible fiberoptic bronchoscopy. Premedication with i.m. morphine 10 mg injection was followed by local anesthesia with lidocaine instillation into the upper airway. Standard bronchoalveolar lavage was performed by serially instilling and suction retrieving 20-ml aliquots of 5 ml saline five times in three different lung segments. The first collection was discarded to avoid possible contamination with upper airway secretions or lidocaine. The remaining lavage was filtered through sterile gauze and centrifuged at 200 × g for 5 min. A sample of the cells was labeled with Wright stain and microscopically examined to determine the proportion of the cells that were macrophages. Aliquots of 5 × 10⁶ cells were stored at −80˚C until RNA and DNA isolation procedure was performed. The procedure generated a relatively pure population of alveolar macrophages with <5% neutrophils or lymphocytes.

Human blood monocyte isolation

As part of the bronchoscopy protocol, some subjects underwent venipuncture with 180 cc blood drawn. Mononuclear cells were isolated from the gradient interface after centrifugation with Histopaque (Sigma-Aldrich, St. Louis, MO). Monocyte isolation was performed with BigEasy Easy Sep system (StemCell, Vancouver, Canada), according to the manufacturer’s protocol and as described previously (35).

Mouse bronchoalveolar lavage

Mice were anesthetized, and the chest cavity was opened. The trachea was exposed, an incision was made, and tubing was inserted. The lungs were flushed with 3 × 1 ml PBS. Cells were pelleted. Slides were then made from the pelleted cells and determined to be >95% macrophages by Wright stain.

Murine bone marrow–derived macrophage isolation

To generate bone marrow–derived macrophages, C57BL/6 mice were euthanized and the tibias and femurs were harvested. Inside a sterile hood, bones were dipped in ethanol and submerged in DMEM supplemented with 10% FCS, l-glutamine, and penicillin/streptomycin (DMEM). The ends of the bones were cut off with a sharp scissors, and the bone marrow was flushed out with DMEM using a syringe and 25G needle (∼3 ml per tibia and 2 ml peribia). Cells were centrifuged and resuspended in DMEM (6 ml/mouse) with an added 7 ml DMEM and 2 ml L cell–conditioned medium (obtained by culture of L929 cells in DMEM for 5 d). Cells were incubated for 6 h at 37˚C and then removed from plates using 0.05% trypsin/EDTA (Life Technologies). Cells were resuspended in DMEM at 1 million/ml and seeded onto the appropriate tissue culture dishes for the proposed experiments.

DNA and RNA isolation

DNA was isolated from macrophages using QIAgen DNAeasy kit (Quagen, Valencia, CA), according to the manufacturer’s instructions. RNA was isolated using the mirVana miRNA Isolation kit (Applied Biosystems, Austin, TX). The quantity and quality of the RNA samples were assessed using an Experion Automated Electrophoresis Station (Bio-Rad, Hercules, CA). The RNA quality indicator was >8 for all samples in which values of >8 indicate primarily intact RNA on a scale of 1–10. After preparation, RNA samples were stored at −80˚C until use.

DNA methylation analysis

Determination of genome-wide methylation values was conducted under contract by the University of Minnesota BioMedical Genomics Center (Minneapolis, MN) using the Illumina Infinium 450K Human Methylation array, which contains 485,000 probes that interrogate CpG residues in 99% of RefSeq annotated genes (National Center for Biotechnology Information, Bethesda, MD). The resulting microarray data were inspected for complete bisulfite conversion of the DNA. Average β-values (i.e., average methylation) for each CpG residue were determined using the GenomeStudio V2009.2, methylation module version 1.5.5 version 3.2 (Illumina, San Diego, CA). Comparison of β-values (i.e., methylation) between cases and controls was conducted using Student t test, whereas comparisons of the relationship between overall values between individual arrays were conducted using Pearson’s correlation coefficient.

Primer design

We used Genome Browser (http://genome.ucsc.edu/index.html) to obtain the sequence of the NOS2 gene promoter region from −2000 bp upstream of TSS to the second exon. We used the publicly available online program Meth Primer (http://www.urogene.org/methprimer/index1.html) to obtain predicted bisulfite-converted sequence. Primers were designed based on this sequence (Supplemental Table I).

Bisulfite sequencing

DNA was bisulfite modified and then amplified using EZ DNA Methylation Kit (Zymo Research), according to the manufacturer’s instructions. The DNA samples were amplified using a touchdown nested PCR protocol (Supplemental Table I) with primers designed for the human and murine NOS2 promoter regions (Supplemental Table II). The PCR product was gel purified and extracted using QIAquick Gel Extraction Kit according to the manufacturer’s instructions. PCR products were then sequenced at the University of Iowa DNA Facility.

Western analysis

Whole-cell protein was obtained by lysing the cells on ice for 20 min in 200 μl lysis buffer (0.05 M Tris [pH 7.4], 0.15 M NaCl, 1% Nonidet P-40) with added protease and phosphatase inhibitors, as follows: 1 protease minitab (Roche Biochemicals)/10 ml and 100 μl 100× phosphatase inhibitor mixture (Calbiochem)/10 ml. The lysates were sonicated for 30 s, kept at 4˚C for 30 min, and spun at 16,000 × g for 10 min, and the supernatant was saved. Protein determinations were made using the Bradford protein assay from Bio-Rad. Cell lysates were stored at −70˚C until use.

Western analysis was performed on whole-cell proteins. A total of 30 μg protein was mixed 1:1 with 2× sample buffer (20% glycerol, 4% SDS, 10% 2-ME, 0.05% bromphenol blue, and 1.25 M Tris [pH 6.8], all chemicals from Sigma-Aldrich), heated to 95˚C for 5 min, loaded onto a 10% SDS-PAGE gel, and run at 100 V for 90 min. Cell proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA) by semidy transfer (Bio-Rad). Equal loading of the protein groups on the blots was evaluated using Ponceau S, a staining solution to confirm protein quantification or by stripping and reprobing with Abs to β-actin or GAPDH. The polyvinylidene difluoride was dried and then incubated with the primary Ab overnight in 5% milk. The blots were washed four times...
with TTBS and incubated for 1 h with horseradish peroxidase–conjugated anti-rabbit or mouse IgG Ab as a control. Immuneactive bands were developed using a chemiluminescent substrate (ECL Plus; Amersham, Arlington Heights, IL). An autoradiograph was obtained, with exposure times of 10 s to 2 min.

**Griess assay of NO production**

Measurement of NO production was performed using the Griess reaction, as described previously (13, 36), using Griess Reagent Kit for Nitrite Determination (Invitrogen), according to the manufacturer’s protocol.

**Chromatin accessibility assay**

Cells were cultured with or without stimulation with IFN-γ and LPS, harvested, and pelleted by centrifugation. Cells were washed with ice-cold PBS and then lysed with lysis buffer (cold radiolabeling standard buffer + 0.10% Nonidet P-40). Nuclei were isolated by centrifugation. Nuclear DNA was digested with increasing concentrations of DNase (0, 2.5, 5, and 10 U) for 10 min at 37°C. DNA was isolated with Qiagen DNAeasy Kit, as described above. DNA was then purified and used for quantitative PCR (qPCR). We designed primers that cover genomic sequences proximal to the TSS for GAPDH (a housekeeping gene), RPL30, a transcriptionally active gene; ICAM1, an inflammatory response gene; β-actin (HBB) gene, a known transcriptionally silent gene (silent in nonerythroid cells); TNF-α, an inflammatory response gene; and NOS2 (Supplemental Table II).

**Chromatin immunoprecipitation assay**

Chromatin immunoprecipitation (ChIP) assay was performed using a ChIP assay kit from Cell Signaling Technology (Beverly, MA). Briefly, macrophage proteins and DNA were cross-linked with formaldehyde. Cells were lysed in SDS-lysis buffer and then sonicated. For sonication, the amplitude setting was 20%. The time setting was as follows: 1 s sonicate, 1 s off for a total of 10 each or 20 s total, rest 30 s on ice, and then repeat for a total of three times. Sonication was performed using a Sonics Vibra Cell Model CV18, Sonics and Materials, and 50 μl sheared chromatin was used to analyze chromatin digestion, with most of DNA sample found to be in the appropriate 500- to 600-bp size range. A total of 10 μl purified chromatin was stored as input sample. The remaining chromatin was diluted with provided ChIP buffer, and then immunoprecipitation was performed using anti–trimethyl-histone H3 (Lys27) (H3K27me3) polyclonal rabbit IgG (Cell Signaling Technology), anti–trimethyl-histone H3 (Lys4) (H3K4me3) polyclonal rabbit IgG (Millipore, Temeluca, CA), or a negative control Ab (monoclonal mouse IgG isotype control or mouse IgG fraction). Chromatin cross-linked with formaldehyde at the incubation mix. After bead separation with a magnetic separation rack, chromatin was eluted from the Ab/protein G beads with ChIP elution buffer. DNA was purified and used for qPCR.

**Demethylation and histone deacetylation treatment of therapy-resistant melanoma patients**

DNA samples were obtained from conventional treatment-resistant metastatic melanoma patients enrolled in an experimental protocol at the University of Iowa Hospitals and Clinics. The research treatment protocol included 5-azacytidine (a DNA methyltransferase inhibitor), a histone deacetylase (HDAC) inhibitor, and an oral alkylating agent. Patients consented and blood was drawn at designated time points (day 0 and 2–3 wk following the second treatment cycle) (Fig. 7A). Demethylation of DNA was monitored by measuring serum hemoglobin F (HbF) levels with high power liquid chromatography, as described previously (37). For an analysis of the histone acetylation state, DNA was isolated from subject blood mononuclear cells and bisulfite was converted. CpG methylation was determined at the NOS2 locus by sequencing the region proximal to the TSS (Supplemental Table I).

**Results**

**Human macrophages do not increase NO production or NOS2 expression in response to inflammatory stimuli**

We studied primary human alveolar macrophages, the human macrophage cell line THP-1, bone marrow–derived murine macrophages, and the murine macrophage cell line MHS. Cells were stimulated with IFN-γ and LPS for 24 h. Nitrite levels were measured in the cell culture supernatants. In human macrophages, only minimal nitrite levels were released at baseline, with no sign of a response to the stimuli (Fig. 1A). In stark contrast, murine macrophages made large amounts of NO in response to stimulation (Fig. 1A). Next, we measured NOS2 protein levels in the cell lysates of human alveolar macrophages, human THP-1 cells, murine MHS macrophages, and mouse bone marrow–derived macrophages after stimulation with IFN-γ and LPS. NOS2 protein amounts were in line with our findings with NO showing a robust increase with stimulation in murine macrophages and no detectable NOS2 protein in human macrophages (Fig. 1B). NO measurements also correlated with NOS2 mRNA levels determined by quantitative RT-PCR (qRT-PCR). There was an impressive increase in NOS2 mRNA with stimulation in bone marrow–derived mouse macrophages (500-fold) and in the murine MHS cell line (10,000-fold). There were barely detectable levels of NOS2 mRNA present in human alveolar macrophages at baseline, with almost no increase following stimulation (Fig. 1C).

**Human alveolar macrophages have an intact LPS/IFN-γ inflammatory pathway when stimulated**

Having found that human alveolar macrophages lacked NOS2 expression, we next asked whether the signaling cascade downstream of the LPS and IFN-γ receptors was intact. To evaluate signaling events downstream of the LPS (TLR4) and IFN-γ receptors, we evaluated STAT1 activation. STAT1 is a transcription factor that is activated via phosphorylation synergistically induced by the combination of LPS and IFN-γ (38). Fig. 2A demonstrates substantial activation of STAT1 by LPS/IFN-γ reflected in the rapid increase in phosphorylated STAT1. To examine whether proteins other than NOS2 known to be induced by LPS/IFN-γ were upregulated in stimulated human alveolar macrophages, we examined ICAM1. ICAM1 is a transmembrane protein that is expressed upon cytokine stimulation and facilitates macrophage migration past the endothelium (37). Stimulation of human alveolar macrophages with LPS/IFN-γ led to significant upregulation of ICAM1 mRNA (Fig. 2B); the murine macrophage cell line MHS showed a similar response. Stimulation of human alveolar macrophages also led to significant increase in ICAM1 protein (Fig. 2C). These data demonstrate that there is not a global defect in LPS and IFN-γ signaling in human macrophages and that the signaling cascade downstream of the LPS/IFN-γ receptors in human alveolar macrophages is intact and upregulates inflammatory genes other than NOS2.

**CpG motifs in the NOS2 promoter region in human alveolar macrophages and monocytes are uniformly methylated**

There is convincing evidence indicating that CpG methylation at the promoter region and first exon is an important epigenetic mechanism regulating the activation of gene transcription (39). Because we had found that intracellular signaling and downstream expression of other inflammatory genes were intact in stimulated human macrophages, we hypothesized that DNA methylation might be responsible for the lack of transcription of the NOS2 gene in human alveolar macrophages.

First, we assessed methylation levels at the NOS2 gene promoter region in data from an Illumina 450K array. The Illumina array allows for a broad analysis of methylation levels in >450,000 probes across the genome. The Illumina array includes a number of probes upstream of and leading into the NOS2 gene. In examining β values (average methylation of seven probes covering −1500 bp upstream of TSS to the end of first exon) from an Illumina array (alveolar macrophages from five normal subjects), we found high levels of methylation at probes that clustered around the TSS of the NOS2 gene (Fig. 3A). This was in stark contrast with the low methylation levels found at probes around the TSS of the LPS/IFN-γ-inducible gene, ICAM1 (Fig. 3B).
Given the results from the Illumina methylation array, we next examined in greater detail the CpG motifs around the NOS2 TSS. To do this, we compared bisulfite sequencing of macrophage DNA from human (alveolar macrophages obtained by bronchoscopy or THP-1 monocytic cell line) and murine sources (bone marrow–derived macrophages [BMDMs] or the mouse alveolar macrophage cell line, MHS) were put in culture at 1 million cells/ml and treated with and without LPS (100 ng/ml) and IFN-γ (100 ng/ml) for 18 h. Cells were cultured for 18 h, and supernatants were collected for NO measurement (Greiss reaction). Data represent mean ± SE for three separate experiments. Significant differences from unstimulated control were evaluated using a Student’s t test (Fig. 3C). For the murine cells, the data were distinctly different. By contrast, we found that CpGs in the region 400 bp upstream of the TSS and in exon 1 contained many unmethylated CpG motifs (Fig. 3D).

Based on previous data (40), we asked whether stimulation with cytokines could reverse CpG methylation. We exposed THP-1 cells to a combination of cytokines and found methylation of CpG motifs in the NOS2 promoter region unchanged after stimulation (data not shown).

Chromatin immunoprecipitation assay reveals silencing histone modification at the NOS2 gene in human alveolar macrophages

A ChIP assay generates data on whether a particular gene region is associated with repressive or transcriptionally active histone marks; this, in part, determines whether a given promoter is open or closed...
to transcription factors. Methylation of lysines on histones can result in either promotion or inhibition of transcription, based on the level of methylation and position of the lysine. It has been previously shown that, near gene TSS, trimethylation of lysine H3K4 is linked to active transcription, whereas trimethylation of lysine H3K27 is linked to transcriptional silencing (41).

First, we established the efficacy of our immunoprecipitation using Abs to H3K4me3 and H3K27me3 in lysates of THP-1 cells (Fig. 4A). Next, a ChIP assay was performed on formaldehyde-fixed THP-1 cells and human alveolar macrophages. We used Abs to H3K4me3 and H3K27me3 to immunoprecipitate histone H3 protein. We then isolated DNA from the precipitated protein and performed qPCR using primers for NOS2, ICAM1, housekeeping gene GAPDH, RPL30, TNFα, and a known silent gene, HBB. In both human alveolar macrophages and THP-1 cells, there was association of actively transcribed genes (GAPDH, ICAM1, RPL30, and TNFα) with the activating histone mark H3K4me3 and association of NOS2 and HBB with the silencing H3K27me3 modification (Fig. 4A, 4B). When these same studies were performed using mouse bone marrow–derived macrophages and the murine MHS cell line, NOS2 DNA was found associated with the activating histone modification H3K4me3, as would be expected of an accessible gene (Fig. 4C, 4D). Thus, NOS2 has characteristics of a silenced gene in human macrophages and looks more like an open gene in mouse cells.

**Association of the human NOS2 gene with H3K27me3 is resistant to inflammatory stimuli**

In mice, macrophage NO expression increases dramatically with inflammatory stimuli. To determine whether the association of the human NOS2 gene with the gene-silencing histone modification, H3K27me3, was changed by inflammatory stimuli, human macrophages were stimulated with IFN-γ and LPS regardless of stimulation (Fig. 5A). Stimulation did not increase activating histone marks associated with the known silenced gene HBB or NOS2 (Fig. 5B); the activating/silencing mark ratio remained negative after stimulation. In comparison, mouse macrophages (MHS) stimulated with IFN-γ and LPS demonstrated an increase in the association of the NOS2 gene with H3K4me3 (Fig. 5C). Thus, NOS2 behaves like a closed gene that cannot be modulated by acute inflammatory signaling in human macrophages.

**Chromatin accessibility assay indicates a closed chromatin conformation at the NOS2 locus in human alveolar macrophages**

Because the ChIP assay suggested closed chromatin at the human NOS2 gene, we went on to directly measure chromatin accessibility. Chromatin accessibility assays provide data regarding the closed or open state of chromatin proximal to a particular gene. The assay tests the ability of DNase to cleave and degrade the relevant DNA. Data are outlined as an accessibility index (accessibility index = 2^[Ct DNase treated] − [Ct Unseen]]). Thus, a higher number implies more degradation by DNase and, therefore, a more accessible gene. Previous research has shown strong inverse correlation between DNA methylation and chromatin accessibility (42). qPCR performed on DNase-treated human THP-1 cell nuclei revealed striking differences in chromatin accessibility between known actively transcribed genes (GAPDH, RPL30, ICAM1) and a known transcriptionally silent gene, HBB. In a series of three separate experiments, the NOS2 gene index was similar to the silent HBB gene (Fig. 6A). To ask whether stimulation would change chromatin accessibility at the NOS2 locus, we stimulated human alveolar macrophages with IFN-γ and LPS, followed by a chromatin accessibility assay. qPCR performed for ICAM1 and TNFα revealed even higher indices of accessibility after stimulation, whereas the NOS2 gene remained inaccessible regardless of stimulation (Fig. 6B). To ask whether the murine NOS2 gene was more accessible than the human gene, we performed a chromatin accessibility assay on treated mouse MHS.
macrophages. In contrast with the human NOS2 gene, murine NOS2 gene segregated with known actively transcribed genes GAPDH and ICAM1 (Fig. 6C). These data collectively demonstrate that the difference between the mouse and human macrophage NO response to inflammatory signaling is due to epigenetic silencing of the gene in human cells.

The 5-aza-2'-deoxycytidine and trichostatin A induce changes in NOS2 promoter methylation profile in murine MHS cells

We tested the ability of the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (5-aza-dC) and the HDAC inhibitor trichostatin A (TSA) to alter CpG methylation at the human NOS2 locus in vitro. The NOS2 TSS in THP-1 cell was fully methylated (except for two CpGs at +117 and +165), and we were unable to change the methylation profile with drug exposure (Fig. 7A); these data were consistent with previous findings that showed an inability to demethylate the heavily methylated NOS2 promoter in human endothelial cells (43). This is consistent with our hypothesis that genes that are heavily methylated and associated with histone-silencing marks are resistant to the effects of demethylating agents.

To ask whether an already open locus (mouse NOS2) was sensitive to DNA methyltransferase inhibition, we treated the murine macrophage cell line, MHS, with 5-aza-dC and TSA. At baseline, sequencing of MHS DNA revealed a string of demethylated CpGs at the beginning of NOS2 promoter and upstream of it. Subjecting these cells to a protocol of 5-aza-dC and TSA treatment resulted in further demethylation of CpG targets upstream and downstream of the NOS2 promoter region (Fig. 7A). This, again, supports our observations that the NOS2 gene is open for regulation in the mouse, whereas it is closed to manipulation in human macrophages.
FIGURE 4. Human NOS2 gene is associated with silencing H3K27me3 marks; mouse NOS2 segregates with the activating histone mark H3K4me3. (A) THP-1 cells were processed for ChIP-Seq. Chromatin-associated DNA was pulled down with Abs to either the activating histone modification, H3K4me3, or the silencing histone modification, H3K27me3. Shown is a Western analysis of proteins from immunoprecipitations with the two Abs, demonstrating specific pulldown of either H3K4me3 or H3K27me3. DNA was isolated and qRT-PCR was performed using primers specific for the region proximal to the TSS of the GAPDH, RPL30, ICAM1, TNFα, HBB, and NOS2. The graph on the left shows data from a representative experiment (of three) presented as a ChIP index (\(2^{-\Delta\Delta CT}\)). The graph on the right shows a comparison of the H3K4me3 pulldown values and the H3K27me3 pulldown values (H3K4me3 ChIP index/H3K27me3 ChIP index). If the ratio was <1, data were converted to a fold change ([1/value][1]). (B–D) Identical experiments to those shown in Fig. 5A were performed using human alveolar macrophages (B), bone marrow–derived macrophages from C57BL/6 mice (C), and the murine MHS macrophage cell line (D). The data are presented in the same format as (A).
The 5-aza-dC and TSA do not induce demethylation changes in NOS2 promoter methylation profile in human blood DNA during in vivo treatment

We next asked whether in vivo exposure to a demethylating agent and inhibitor combination would alter CpG methylation at the NOS2 locus. The 5-aza-dC and HDAC inhibitors are being used in clinical trials for some malignancies, including melanoma. The hope is that the combined drug therapy will reverse methylation of tumor suppressor genes and slow the progression of the cancer. In a trial at the University of Iowa, patients with advanced melanoma are treated with a protocol that includes 5-aza-dC and a HDAC inhibitor (Fig. 7A). We have collected blood before and after treatment and examined DNA methylation of the NOS2 gene. The clinical effect of the demethylating agents was confirmed by examining HbF levels. During fetal development, HbF is the dominant hemoglobin isoform. From ∼6 mo postpartum through adulthood, it is gradually replaced by hemoglobin A. This process of hemoglobin switching is controlled epigenetically by methylation of the HbF (γ-globin) gene promoter in bone marrow erythroblasts (44). We followed four patients enrolled in the current trial. Two of these had a rise in HbF, whereas the other two had unchanged levels of HbF during the course of treatment (Fig. 7B). We analyzed bisulfate-converted DNA for methylation of the NOS2 promoter in all four patients prior to treatment and then later post-treatment, as outlined in Materials and Methods. We found two CpGs (+3 and +165) changed in one patient who showed a HbF response after treatment (Fig. 7C). All other CpGs remained methylated. The second patient who responded to treatment with increase in HbF, along with two nonresponders, showed no changes in methylation pattern. These data suggest that the human NOS2 gene is resistant to the effects of a demethylating agent and HDAC inhibitor in vivo as well as in vitro, making mouse very different than man. (Fig. 8).

**Discussion**

The major conclusion of our study is that human macrophages do not produce NO in response to acute inflammatory stimuli as a result of epigenetic regulation of the NOS2 gene (Fig. 8). This conclusion is based on several complementary lines of experimental evidence. First, we demonstrate that, despite an otherwise intact inflammatory response, human alveolar macrophages do not make NO or increase expression of NOS2 when stimulated. Next, we demonstrate uniform methylation of CpGs in the NOS2 promoter region in human macrophages. Consistent with the CpG methylation data, ChIP assays demonstrate that the human macrophage NOS2 gene is associated with a silencing histone mark and has tightly compacted chromatin, rendering it inaccessible to transcription factors. Stimulation of human macrophages with
LPS and IFN-γ does not alter the NOS2, the ChIP assay results, or chromatin accessibility. Finally, we show that this epigenetic silencing of the NOS2 region in human macrophages is resistant to the effects of demethylating agents both in circulating leukocytes in vivo and in a human macrophage cell line in vitro. Collectively, this experimental evidence strongly supports the overall concept that human macrophages, including primary alveolar macrophages, do not produce NO in response to inflammatory stimuli because of epigenetic silencing of the NOS2 gene.

Our human findings stand in stark contrast to the physiology of normal mouse macrophages. We find that in mouse macrophages the CpG motifs proximal to the NOS2 TSS are unmethylated. Furthermore, murine macrophage NOS2 segregates with open genes on chromatin accessibility assay and ChIP analysis reveals murine NOS2 is associated with H3K4me3, a histone mark that correlates with active transcription. Thus, it appears that the regulation of NO as an inducible inflammatory response in the mouse mononuclear phagocyte does not extend to human cells.

**FIGURE 6.** In human macrophages, NOS2 is a closed gene that remains inaccessible even after inflammatory stimulation. (A) Human THP-1 cells were treated with varying amounts of DNase. DNA was isolated and qRT-PCR was performed for GAPDH, RPL30, ICAM1 as representative actively transcribed open genes, HBB as a prototypical closed gene, and NOS2 to determine DNase sensitivity at the loci. (B) The effect of LPS/IFN-γ exposure on chromatin accessibility was determined in THP-1 cells exposed to LPS (100 ng/ml) and IFN-γ (100 ng/ml) for 18 h. Pelleted cells were exposed to increasing levels of DNase and qRT-PCR performed for ICAM1, TNF-α, and NOS2. Data are presented as accessibility index = 2[(Ct DNase treated) − (Ct Uncut)]. The data are mean ± SE of three separate experiments. Significance was evaluated using Student’s t-test. (C) Mouse MHS cells were treated with varying amounts of DNase. DNA was isolated and qRT-PCR was performed for GAPDH, ICAM1, HBB, and NOS2 associated DNA to determine DNase sensitivity at the loci. In human macrophages, NOS2 has a chromatin accessibility pattern similar to the closed gene HBB and does not become more accessible with stimulation.
Our studies are consistent with a larger body of work on the overall differences in inflammation-related gene expression between mice and humans (45). The literature is full of conflicting reports on the role of NO in human inflammation. Schneeman et al. (11) performed a comprehensive analysis of human monocytes in 1993. They found that human mononuclear phagocytes exposed to an array of potent stimuli did not produce nitrite, consume L-arginine, produce L-citrulline, or display NOS activity. Weinberg et al. (46) found minimal immunoreactive NOS2 protein or NOS2 mRNA in human macrophages stimulated for 3 d in culture; secreted NO was not detected. This is similar to our studies demonstrating induction of neither NOS2 mRNA nor protein with...
creased induction of the CpG methylation contributes to decreased NO response in human macrophages. Our conclusion that differential methylation upstream of the (43). Extending this observation, we find that differential methylation of CpGs proximal to the TSS are methylated in mouse macrophages, whereas there is no CpG island in the NOS2 promoter, we have identified a number of CpGs proximal to the TSS that are differentially methylated between mouse and human macrophages. Recent literature supports the importance of these TSS-proximal, nonisland CpGs in regulation of gene expression (66–70). These particular studies identified the role of CpG methylation (proximal to the TSS without being in an island) in transcription of the IL-2, IFN-γ, MMP13, IL-1β, and TNF-α genes.

DNA methylation depends on an array of methyltransferases, enzymes capable of de novo methylation and demethylation of genomic DNA, along with maintenance of methylation in daughter cells (71, 72). Human and mouse macrophages express DNA methyltransferases (DNMT) 1, 3A, and 3B that can be inhibited with 5-azacytidine with resultant reduced methylation in replicating daughter cells. Our efforts to demethylate the NOS2 TSS met with only modest success. In murine cells that already had many demethylated CpG motifs at baseline, in vitro inhibition of DNA methyltransferase was successful. Human cells, both in vivo and in vitro, proved more resistant to the demethylation agent. In some melanoma patients who received a DNA methylation inhibitor on an experimental treatment protocol, a rise in blood HbF levels signaled demethylation at the bone marrow erythroblast level. The dose or duration of chemotherapeutic agents may not have been adequate to demethylate the NOS locus in circulating leukocytes or the NOS2 gene may be more resistant to such manipulation. Toxicity of the DNA methyltransferase inhibitors can impair cell viability in vitro, and demethylation agents work best on rapidly dividing cells. Our work is consistent with previous reports in which 5-aza-dC and TSA failed to alter DNA structure on rapidly dividing cells. Our work is consistent with previous reports in which 5-aza-dC and TSA failed to alter DNA structure on rapidly dividing cells. Our work is consistent with previous reports in which 5-aza-dC and TSA failed to alter DNA structure on rapidly dividing cells. Our work is consistent with previous reports in which 5-aza-dC and TSA failed to alter DNA structure on rapidly dividing cells. Our work is consistent with previous reports in which 5-aza-dC and TSA failed to alter DNA structure on rapidly dividing cells. Our work is consistent with previous reports in which 5-aza-dC and TSA failed to alter DNA structure on rapidly dividing cells.

In this study, we demonstrate silencing at the NOS2 gene at a number of levels, as follows: 1) CpG motifs proximal to the human TSS are methylated. 2) Histone modifications consistent with gene silencing are found at the NOS2 gene (H3K27me3). 3) Histone modifications consistent with active gene activity (H3K4me3) are in low abundance at the NOS2 promoter region. 4) The NOS2 locus is relatively protected from DNase treatment, suggesting closed chromatin. Our conclusion from these studies is that the NOS2 gene in human macrophages is silenced. Alternatively, production from this locus may be below the sensitivity of present measurement techniques or there may be transcription of a very unstable transcript. At this time, we cannot definitively rule out these possibilities, but consider them unlikely.

There are a number of potential weaknesses in this study. One of these is the lack of data showing that with demethylation of the NOS2 proximal CpGs, NOS2 is transcribed. Another weakness is the limited number of human cell types that are analyzed. It would be nice to know whether the silencing is true for macrophages alone or is also true in other cell types such as airway epithelial cells. For the patient studies, these are only preliminary data, and future trials with more patients and higher doses of the DNMT and HDAC inhibitors may be more fruitful.

In future studies, we will be examining more cell types for silencing of the NOS2 locus. We will be asking whether this locus is dynamically regulated and further testing whether demethylation with a DNMT inhibitor is possible. This project was started because of our interest in human inflammation and its links to both chronic and acute diseases. There are some papers suggesting that mycobacterial infection generates NO from human macrophages (75, 76). There are also suggestions that NO plays an important role in chronic obstructive pulmonary disease, adult respiratory distress syndrome, sarcoidosis, asthma, and viral infections (47–49, 77, 78).
In light of the present work, it will be important to determine the conditions under which macrophage NOS2 expression might change and how that might affect disease outcome. Our findings demonstrate that the susceptibility to epigenetic modification is gene specific and may be predictable from methylation sequencing analysis. Future clinical trials utilizing treatments aimed at demethylation and/or histone modifications should consider the susceptibility of the target gene(s) of interest. In this study, we show that human macrophages, including primary alveolar macrophages, do not make NO at baseline or in response to acute inflammatory stimuli. The lack of NOS2 expression and subsequent NO secretion is explained by epigenetic changes that are consistent with silencing of the NOS2 transcription locus. We believe this study points out the pitfalls of cross-species extrapolation in understanding inflammatory mechanisms. Future studies exploring how human macrophages kill pathogens and respond to inflammation should focus on alternative pathways to NOS2 and NO. To do otherwise is to risk having the best-laid schemes of mice and men go awry (with apologies to Robert Burns).

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Disclosures
The authors have no financial conflicts of interest.

References


