Characterization of Short Range DNA Looping in Endotoxin-mediated Transcription of the Murine Inducible Nitric-oxide Synthase (iNOS) Gene

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The local structural properties and spatial conformations of chromosomes are intimately associated with gene expression. The spatial associations of critical genomic elements in inducible nitric-oxide synthase (iNOS) transcription have not been previously examined. In this regard, the murine iNOS promoter contains 2 NF-κB binding sites (nt −86 and nt −972) that are essential for maximal transactivation of iNOS by LPS. Although AP-1 is commonly listed as an essential transcription factor for LPS-mediated iNOS transactivation, the relationship between AP-1 and NF-κB in this setting is not well studied. In this study using a model of LPS-stimulated ANA-1 murine macrophages, we demonstrate that short range DNA looping occurs at the iNOS promoter. This loop involves c-Jun, NF-κB p65, and STAT-1 interaction. The distal AP-1 binding site interacts via p300 with the proximal NF-κB sites (nt −1069) to form a DNA loop to initiate iNOS gene transcription. The spatial associations of critical genomic elements in iNOS transcription have not been previously examined. This reductionist model using the murine iNOS promoter indicates that while numerous transcription factors recognize and bind to various response elements, they do not equally contribute to gene transcription. Rather, a hierarchy exists among these sites that is determined by the activating factor, requirement for spatial association, and interaction among the various transcription factors and coactivators.

EXPERIMENTAL PROCEDURES

Materials—ANA-1 macrophages were a gift from Dr. George Cox (Uniformed Services University of the Health Sciences, Bethesda, MD). Wild-type RelA and its mutant expression vectors were gifts from Dr. Jeremy M. Boss (Emory University of Medicine, Atlanta, Georgia). The expression vectors containing full-length p300 and its acetyltransferase (AT) deletion derivative (m-p300) were constructed by inserting a XhoI-NotI fragment carrying the N-terminal Flag-tagged p300 open reading frame into the pCAGGS expression plasmid.
frame or its acetyltransferase deletion derivative (Δ nt 1472–1522) into pCI vector (Promega, Madison, WI) and were provided by Dr. Joan Boyes (Institute of Cancer Research, London, UK). E1A plasmid was received from Dr. Debabrata Chakravarti, University of Pennsylvania, School of Medicine.

Induction of NO Synthesis in ANA-1 Macrophages—ANA-1 macrophages were maintained in DMEM with 10% heat-inactivated fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. LPS (50 ng/ml), IFN-γ (200 units/ml), IL-1β (200 units/ml), or TNFα (2 ng/ml) were used to induce NO synthesis. After incubation at 37 °C in 5% CO₂, the supernatants and cells were harvested for further assays.

Assay of NO Production—NO released from cells in culture was quantified by measurement of the NO metabolite, nitrite. After stimulation, 50 μl of culture medium was mixed with 50 μl of 1% sulfanilamide in 0.5 n HCl. After a 5-min incubation at room temperature, an equal volume of 0.2% N-(1-naphthyl)-ethylenediamine was added. Following incubation for 10 min at room temperature, the absorbance of samples at 540 nm was compared with that of NaNO₂ standard on a MAXLINE microplate reader.

Immunoprecipitation Studies—Nuclear protein from ANA-1 cells was incubated with anti-p65 or anti-c-Jun Ab (R&D Systems, Minneapolis, MN) and protein G-agarose in Co-IP buffer (10 mmol/liter Tris–HCl, pH 7.5, 3 mmol/liter EGTA, 20 mmol/liter NaCl, 0.02% Triton X-100, 1 μg protease inhibitors mixture, 0.2 mmol/liter dithiothreitol, 1 mmol/liter phenylmethylsulfonyl fluoride at 4 °C for 4 h. Protein G-agarose beads were selected by centrifugation and washed three times with sample buffer. The immune complexes were washed in sample buffer and then loaded on 10% SDS-PAGE gels. Separated, and electrotransferred to polyvinylidene difluoride membranes for Western blot analysis. The membranes were blocked with 5% skim milk in PBS for 1 hr at room temperature. After washing three times, the membranes were incubated with anti-p65 Ab, anti-p300 Ab, or anti-p300 Ab (Upstate Biotechnology, Waltham, MA). After being washed, and incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Bound peroxidase activity was detected by the West Pico chemiluminescent kit (Pierce).

ChIP Assay and ChIP Quantitative PCR—ANA-1 cells were grown in 10-cm dishes and DNA–protein cross-linking by addition of 1% formaldehyde at room temperature for 10 min. Assays were performed using the ChIP assay kit (Upstate Biotechnology, Waltham, MA) following the manufacturer’s instructions. Anti-p65 Ab, anti-p300 Ab, and anti-c-Jun Ab were used for each immunoprecipitation. The DNA was recovered and subjected to analysis by PCR. The primers for the distal NF-κB binding site had the following sequence: 5′-aacagcaggtgaatttcg-3′ (sense strand) and 5′-ccaggtggctttcagttc-3′ (antisense strand) to yield a PCR product of 277 bp, and the sequences of primers for the proximal NF-κB binding site were 5′-cctagggctggctttcagttc-3′ (sense strand) and 5′-ctagggacagtttcctttcagttc-3′ (antisense strand); their product was 273 bp. The PCR program was 94 °C for 5 min, followed by 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 40 s for a total of 30 cycles, and then 72 °C for 10 min. The amplified DNA was visualized by electrophoresis on 1% agarose gel in 1× TAE (Tris acetate/EDTA) buffer after staining with ethidium bromide. For ChIP quantitative PCR analysis, anti-p65, anti-RNA Pol2, or anti-c-Jun (Santa Cruz Biotechnology) were used for each immunoprecipitation, and normal rabbit IgG was used as an endogenous control. The specific PCR primers for the iNOS promoter gene were designed using Primer 3 and mfold software. Forward primer: 5′-ttaaatcaagacagttcagtttga-3′ and reverse primer: 5′-tgtctacgacagttcagtttga-3′ were for the middle AP-1 binding site. Forward primer: 5′-tcctagggctggcttttcctttc-3′ and reverse primer: 5′-cctagggacagtttcctttccacagttc-3′ were for the distal NF-κB and AP-1 binding sites, forward primer: 5′-tatggaaggctttcctttcctttc-3′ and 5′-attagcagtttcctttcctttc-3′ were for proximal NF-κB and AP-1 binding sites. Real-time PCR was performed with iQ SYBR Green super mix (Bio-Rad), using iCycler iQ Real-time PCR Detection System (Bio-Rad) with the following amplification parameters: denaturation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 s and 55 °C for 30 s. Quantification was normalized using endogenous control and calculated using the ΔΔCt method (11).

Chromosomal Interaction Capture (3C) Assay—ANA-1 cells (1×10⁶) were cultured in 10-cm dishes and fixed with 1% formaldehyde for 10 min at room temperature. The reaction was quenched by the addition of 0.125 M glycine for 5 min at room temperature. The cells were washed three times with 1X PBS and lysed in cold lysis buffer (10 mmol/liter Tris–HCl, 0.5 mm sodium chloride, 0.2% Igepal, 0.8% NP-40, 0.2% deoxyribonuclease, 20 μg/ml leupeptin, 10 μg/ml pepstatin A, 0.5 mmol/liter thiotimamide oxalate, 5 μmol/liter camptothadin, and 5 μmol/liter microcystin-LR (microcystin-leucine arginine)). The nuclei were harvested and suspended in digestion buffer with 0.1% SDS and 1% Triton X-100. The DNA was digested with restriction enzymes SspI and/or Rsal overnight at 37 °C. Samples were then diluted with ligation buffer with 0.1% SDS and 1% Triton X-100. T4 DNA ligase was added with incubation at 16 °C overnight, followed by overnight incubation at 65 °C in the presence of 10 μg/ml proteinase K to reverse the cross-links. The DNA was isolated by phenol-chloroform extraction and ethanol precipitation. The purified DNA concentration was determined and was used as a PCR template with the following primer pair: 5′-ctgcatcagcagtttgaatttcg-3′ and 5′-ccaggtggctttcagttc-3′. PCR was performed in 25-μl reaction mixtures with an initial denaturing step for 4 min at 94 °C and then 30 cycles including 30 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C. The PCR products were run on 1% agarose gels. 3C analyses were performed in three independent experiments. PCR products were cloned and sequenced to confirm the presence of the predicted junction in the iNOS DNA SspI site.

ChIP Loop Assay—ANA-1 cells were cross-linked for 10 min with 1% formaldehyde at room temperature. After cell lysis, chromatin was sheared and digested by the restriction enzyme SspI. Chromatin fragments were immunoprecipitated using normal rabbit IgG, anti-p65 Ab, anti-c-Jun Ab, or anti-p300 Ab with protein G-agarose beads at 4 °C overnight, followed by overnight ligation with T4 ligase at 16 °C. After reverse cross-linking with proteinase K digestion at 65 °C overnight, the DNA was isolated by phenol-chloroform extraction and ethanol pre-

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cipation. PCR and real-time PCR were performed as described above.

**Immunoprecipitation of Acetylated Proteins**—ANA-1 cells were transfected with p300 wild-type or m-p300 expression vectors using JetPEI-Macrophage reagent (Genesee Sci, San Diego, CA), and incubated at 37 °C, 5% CO2 for 24 h. The cells were incubated in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA) containing 50 ng/ml LPS for 30 min before the cells were collected for nuclear protein. The nuclear extract was immunoprecipitated overnight at 4 °C with acetylated-lysine antibody (Cell Signaling, Danvers, MA) cross-linked to agarose beads. After washing the beads with cold TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl), protein was eluted from the beads by heating for 10 min at 95 °C, followed by SDS-PAGE (4–20% polyacrylamide) and fluorography. Western blot analysis was performed using Ab directed against NF-κB p65, AP-1, c-Jun, or p300 (Upstate Inc, Billerica, MA).

**Statistical Analysis**—All data are presented as mean ± S.D. Analysis was performed using a Student’s t test. Values of *p* < 0.05 were considered significant.

**RESULTS**

**AP-1 and NF-κB Promote LPS-mediated iNOS Gene Transcription**—The human iNOS promoter contains five putative NF-κB binding sites upstream of ~4.7 kb that contribute to human iNOS expression (12). In contrast, the murine iNOS promoter contains only two requisite NF-κB binding sites, thus, was chosen as a simpler model for investigating DNA looping that may occur among the various promoter sites (1). The relevant AP-1 and NF-κB sites in the murine iNOS promoter are depicted in Fig. 1a. To determine the role of AP-1 and NF-κB in the regulation of LPS-mediated iNOS transcriptional activity at the murine iNOS promoter (GenBank™ accession no. L09126) after 2 h of incubation. Purified chromatin was immunoprecipitated using anti-RNA polymerase II (RNA Pol II) or as a control, rabbit immune serum. Murine iNOS promoter DNA immunoprecipitation with RNA Pol II was then quantified by real-time PCR using primers specific for the promoter region inclusive of the TATA start site (Fig. 1b). In selected instances, siRNA to AP-1, c-Jun, and/or NF-κB p65 was administered. Our results show that LPS (50 ng/ml) treatment increased iNOS transcription activity. When c-Jun-siRNA or p65-siRNA was added 24 h prior to administration of LPS, the extent of iNOS transcription was decreased by 50 and 75%, respectively (Fig. 2a) (AT) deletion. Western blot analysis confirmed that siRNAs ablated of c-Jun, p65, or p300 in DNA looping at the iNOS promoter, loss-of-function studies were performed using siRNA to c-Jun, p65, and/or p300. Additional negative controls included transient transfection of S276A, a p65 mutant in which serine 276 in the Rel homology domain has been mutated to an alanine, E1A, a direct inhibitor of p300, and/or m-p300, a p300 containing an acetyltransferase domain has been mutated to an alanine. Ssp1 and T4 ligase. In Fig. 2a, 3C assays were performed (Fig. 2b) in ANA-1 cells that were exposed to LPS (50 ng/ml), IFN (200 units/ml), TNF (2 ng/ml), or IL-1 (1000 units/ml) for periods of 10, 30, and 60 min. The resulting gels demonstrate that DNA looping was detected in LPS- and IL-1-treated cells as early as 10 min, peaking at 30 min, and then significantly diminished by 90 min.

To determine the role of AP-1, c-Jun, NF-κB p65, and p300 in DNA looping at the iNOS promoter, loss-of-function studies were performed using siRNA to c-Jun, p65, and/or p300. Additional negative controls included transient transfection of S276A, a p65 mutant in which serine 276 in the Rel homology domain has been mutated to an alanine, E1A, a direct inhibitor of p300, and/or m-p300, a p300 containing an acetyltransferase (AT) deletion. Western blot analysis confirmed that siRNAs directed against c-Jun, p65, and p300 effectively ablated expression of the respective proteins in LPS-stimulated cells (data not shown). 3C assays were performed (Fig. 2c). In each instance, ablation of c-Jun, p65, or p300 resulted in loss of DNA looping in the iNOS promoter of LPS-stimulated ANA-1 macrophages. Mutation of the AT domain in p300 resulted in the loss of DNA
FIGURE 1. a, schematic representation of NF-κB and AP-1 sites in the murine iNOS promoter with relevant target sequences for Rsa1 and Ssp1 restriction endonucleases. b, ChIP assay of RNA Pol II binding to the iNOS promoter. Murine iNOS promoter DNA coimmunoprecipitating with RNA Pol II were quantified by real-time PCR using primers specific for the promoter region inclusive of the TATA start site. In selected instances, siRNA to AP-1, c-Jun, and/or NF-κB p65 was administered. Mismatch siRNA served as a control. LPS (50 ng/ml) was administered to induce iNOS transcription. Data are presented as the mean ± S.D. of three experiments. *, p < 0.01 versus control, MM-c-Jun, MM-p65, and si-c-Jun + si-p65; **, p < 0.01 versus control, MM-c-Jun, and MM-p65. c, assay of NO production. NO released from cells in culture was quantified by measurement of the NO metabolite, nitrite. LPS (50 ng/ml) was administered to induce iNOS transcription. Data are presented as mean ± S.D. of three experiments. *, p < 0.01 versus control, MM-c-Jun, MM-p65, and si-c-Jun + si-p65; **, p < 0.01 versus control, MM-c-Jun, and MM-p65.
looping. These data suggest that AP-1, c-Jun, NF-κB p65, and p300 with intact AT activity are all required for DNA looping and subsequent iNOS expression in LPS-stimulated ANA-1 macrophages.

We performed IP assays to determine whether p65, c-Jun, or p300 were potential targets of p300 AT activity in LPS-stimulated ANA-1 macrophages.

**AP-1, c-Jun, and NF-κB p65 Interact with p300**—The transcriptional coactivator, p300, has been demonstrated to be essential for iNOS expression. It interacts with NF-κB and AP-1 and is a key scaffolding protein in the formation of enhanceosomes (9). Examination of p300 indicates the presence of individual binding sites for NF-κB p65 at the CREB-binding domain and AP-1, c-Jun at the N-terminal zinc finger domain; this would suggest that p300 does not interact with two NF-κB (or two AP-1) proteins simultaneously (9). In this regard, we examined the potential interactions between c-Jun, p65, and p300, using IP-Western blot studies. (Fig. 3a) Our IP-Western blot studies of nuclear protein demonstrate that a c-Jun, p65, and p300 protein complex exists during LPS stimulation of ANA-1 macrophages. We then performed ChIP-Loop Q PCR
for NF-κB p65, AP-1, c-Jun, and p300 binding using PCR primers previously described as specific for iNOS DNA looping (Fig. 3b). Using 2-ΔΔCt analysis, the relative binding of NF-κB/AP-1/p300 within the DNA loop was 2.3:2.3:1. These results support the engagement of the NF-κB p65, AP-1, c-Jun, and p300 complex in DNA loop formation during LPS-mediated iNOS gene transcription.

**Proximal NF-κB and Distal AP-1 Binding Sites Engage with p300 for DNA Loop Formation**—Analysis of the various AP-1 and NF-κB binding sites in the iNOS promoter suggest a variety of combinations to form the relevant DNA loop. These include d-AP-1 and p-NF-κB, m-AP-1 and p-NF-κB, and p-AP-1 and d-NF-κB. Our ChIP Q-PCR results indicate that binding is skewed toward the d-AP-1 and p-NF-κB sites. To confirm that these relevant sites combine to form the DNA loop, we performed 3C assays using combinations of Ssp1 and Rsa1 restriction endonucleases. Primer 1 was chosen to be complementary to the distal iNOS promoter DNA between the Rsa1 and Ssp1 sites upstream from the d-NF-κB site; primer 2 was at the proximal end of the iNOS promoter downstream from the p-NF-κB site (Fig. 4a). If DNA looping occurred between p-AP-1 and d-NF-κB sites, a 3C product would be detected; this is represented as Option 1. However, if the interaction occurred between d-AP-1 and p-NF-κB sites, no 3C product would be formed as shown in Option 2. When genomic DNA from LPS-treated ANA-1 macrophages was exposed to Ssp1 and Rsa1 during the 3C assay, there were no 3C products (Fig. 4b). This result indicates that short range DNA looping in LPS-treated ANA-1 cells occurs via interactions between the d-AP-1 and p-NF-κB sites.

**DISCUSSION**

In LPS-mediated sepsis and inflammation, pro-inflammatory cytokines are elaborated, and iNOS is systemically expressed in multiple cell types, including macrophages (13–16). The sustained production of NO in high concentration regulates multiple cellular and biochemical functions, including inotropic and chronotropic cardiac responses, systemic vasmotor tone, intestinal epithelial permeability, endothelial activation, and microvascular permeability (17–19). The cellular and biochemical consequences of NO production in the setting
of sepsis and inflammation are exemplified by DNA single-strand breakage, ADP-ribosylation of nuclear proteins, nitrosative stress-mediated alteration in gene transcription, and inhibition of mitochondrial respiration. In experimental endotoxemia, NO production results in cytotoxicity, inhibition of mitochondrial respiration, platelet aggregation, neutrophil adhesion, hypotension, and vasoplegia (13, 20–25). In systemic inflammation induced by LPS, the macrophage is responsible for the majority of the circulating NO metabolites. Macrophage iNOS expression is central to many of the systemic effects associated with LPS stimulation. However, while the molecular pathways which regulate iNOS transcription have been extensively studied in multiple cell types, including the macrophage, little is known of epigenetic pathways in which secondary and tertiary structural issues are considered.

In this study using a model of LPS-stimulated ANA-1 murine macrophages, we demonstrate that short range DNA looping occurs at the iNOS promoter. This looping requires the presence of AP-1, c-Jun, NF-κB p65, and p300-associated acetyltransferase activity. The distal AP-1 binding site interacts via p300 with the proximal NF-κB binding site to create this DNA loop to participate in iNOS transcription. Although AP-1, NF-κB, and p300 are commonly listed as essential transcription factors and cofactors for LPS-mediated iNOS transactivation, the potential epigenetic relationship among these three entities in this setting has not been well studied. Other geographically distant AP-1 and NF-κB sites are certainly occupied, but selected sites are critical for iNOS transcription and the formation of the c-Jun, p65, and p300 transcriptional complex. This multiprotein complex is not unique and has been previously described by Brockmann et al. (26) in their description of an AP-1, p300, NF-κB complex that is critical to expression of major histocompatibility complex genes. Interestingly, although not addressed by these authors, they describe the crit-

FIGURE 4. a, schematic representation of potential interactions between the NF-κB and AP-1 sites in the murine iNOS promoter with p300 in DNA loop formation. b, 3C assay using SspI and RsaI in the murine iNOS promoter. ANA-1 macrophages were treated with LPS (50 ng/ml). The presence of a band at ~300 bp indicates the presence of DNA looping. This band was sequenced and found to be identical to the region of interest. The gel is representative of three experiments.
iNOS Promoter DNA Looping

ical need for two geographically separated NF-κB and AP-1 binding sites for maximal cytokine inducible function of their enhancer. In a manner similar to that seen with our iNOS data, DNA looping could certainly be relevant to their observations. Given that transcription supersedes the linear order of elements to emphasize the critical nature of secondary and tertiary structure, our results confirm the importance of DNA loop formation for LPS-mediated iNOS expression. These observations have not been previously described for iNOS transcription.

DNA looping refers to a conformation of DNA in which cis elements of a DNA strand are physically in proximity to one another to bring a locally high concentration of transcription factors, cofactors, and chromatin-modifying factors near the transcriptional start site of genes and activate transcription (27). This mechanism has been used to explain why enhancer elements located hundreds to thousands of base pairs away from the transcriptional start site can contribute to gene activation. Using the 3C assay, looping of this nature has been demonstrated to provide proximity between the β-globin locus control region and the β-major globin promoter, which are located ~50 kilobase pairs apart (28). However, in a setting similar to ours, Babu et al. (29) study of insulin gene regulation indicates that looping can occur over shorter spans of hundreds of base pairs in relatively nucleosome-free segments of DNA. In this context, our results indicate that p300 acetyltransferase activity is critical for preferential targeting of distinct transcription factors at specific locations along the iNOS promoter. DNA looping results in a transcriptional protein complex. Beyond this, our results indicate that p300 acetyltransferase activity regulates LPS-induced iNOS expression by increasing the potential candidates are histones and NF-κB.

NF-B activity is currently unknown, but our data suggest that among the potential candidates are histones and NF-κB p65. Our data provide a framework for modeling the molecular events that lead to LPS-induced iNOS gene activation in ANA-1 murine macrophages. Central to this model is the formation of a DNA loop that brings into proximity a distal region at about ~1000 bp near the coding region of the gene. Certainly, our data support the mechanism that ensures that iNOS remains in an “off” until the correct activation signal is received. The off-state is maintained by an inaccessible chromatin structure at the promoter region. Separation of the distal and proximal regulatory regions allows the iNOS gene to maintain two distinct chromatin structures: an accessible structure that can bind the requisite transcription factors and initiate transactivation and a closed structure to prevent the random binding of RNA polymerase or a factor that can recruit RNA polymerase. The differentiation between these two states depends upon a variety of signals including acetylation of targets such as histones and the interacting transcription factors.

The spatial arrangement of critical genomic elements in iNOS transcription, previously examined. The simplified view of the iNOS promoter indicates that while p65, a classic NF-κB activator, recognizes and may bind tovariable scales do not equally contribute to the hierarchy exists among these factors. The activating factor, requirement for interaction among the various transcription factors, further application of this concept may increase the more “complex” model of the human iNOS promoter. The iNOS promoter will be challenging. Functionally important NF-κB binding sites have been identified at −5.2, −5.5, −5.8, −6.1 kb, and −8.2 kb in the human iNOS promoter (12,15). In addition, inducible AP-1 binding sites have been reported at −5.1 and −5.3 kb in the hiNOS promoter. The potential exists for a multitude of DNA loops to exist in this context. Nevertheless, consideration of the relationship between regulatory elements and genes must go beyond the linear order of elements to account for secondary and tertiary considerations. Local structural properties and spatial conformations clearly play a major role in regulation of gene expression.

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