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Regulation of Inducible Nitric Oxide Synthase Expression by p300 and p50 Acetylation

Wu-Guo Deng and Kenneth K. Wu

To determine whether p300 is involved in inducible NO synthase (iNOS) transcriptional regulation, we evaluated the effect of p300 overexpression on iNOS expression and characterized p300 binding to iNOS promoter in RAW 264.7 cells. p300 overexpression increased iNOS expression which was abrogated by deletion of the histone acetyltransferase (HAT) domain (∆1472–1522). DNA-binding and chromatin immunoprecipitation assays revealed binding of p300 to several DNA-bound transactivators at basal state. Following stimulation with LPS plus IFN-γ, binding of p300, p50/p65 NF-κB, and IFN-regular factor-1 was increased by ~2-fold. Nuclear p50 was complexed with and acetylated by p300 at the basal binding state which was increased by LPS and IFN-γ stimulation. p300 overexpression resulted in increased p50 acetylation which was reduced by HAT mutation. p50 acetylation correlated with increased NF-κB binding and enhanced p300 recruitment. Co-overexpression of EI A abolished the augmentation of p50 acetylation and p50 binding induced by p300 overexpression, and a correlative suppression of p300 recruitment to the complex. We conclude that p300 is essential for iNOS transcription. Our results suggest that p300 HAT acetylates the p50 subunit of NF-κB, thereby increasing NF-κB binding and NF-κB mediated transactivation. The Journal of Immunology, 2003, 171: 6581–6588.

Inducible NO synthase (iNOS) is a calcium-independent member of the NOS gene family that catalyzes the synthesis of a large quantity of NO (1). It plays diverse pathophysiological roles including microbial killing, inflammation, septic shock, and ischemic tissue injury (2). iNOS levels in resting cells are absent or very low but are highly inducible by LPS, IFN-γ, and other cytokines (3). Many studies in murine and human cells have shown that LPS and cytokines induce iNOS expression at the transscriptional level. iNOS promoter activation by combined LPS and IFN-γ (LPS/IFN-γ) has been extensively characterized in murine RAW 264.7 cells. The 5′-flanking region of murine iNOS gene contains two clusters of cis-acting regulatory elements that are essential for iNOS transcription of which the proximal cluster is required for LPS-induced and the distal cluster is essential for IFN-γ-induced activation (4, 5). LPS/IFN-γ synergistically activates iNOS promoter by stimulating binding of several transcription activators, notably NF-κB, IFN-regulatory factor-1 (IRF-1), and C/EBPβ to their respective cognate sites in these two clusters of regulatory elements (6–8). Transmission of the transactivator message to the transcription machinery requires recruitment of interacting proteins such as coactivators (9–11). Coactivators that are involved in iNOS promoter activation have not been reported. In this study, we determined binding of p300 coactivator to iNOS promoter region and evaluated the role of p300 in regulating iNOS promoter activity. Our results indicate that LPS/IFN-γ increased p300 interaction with promoter-bound transactivators, p300 overexpression augmented LPS/IFN-γ-induced p300 binding and iNOS promoter activity. Adenoviral EI A abrogated the stimulating effects of LPS/IFN-γ and p300 overexpression. We provided evidence that p300 acetylated the p50 subunit of NF-κB thereby increasing NF-κB binding.

Materials and Methods

Cell culture

The mouse macrophage cell line RAW 264.7 was obtained from American Type Culture Collection (Manassas, VA) and cultured in DMEM supplemented with 10% FBS and 1:100 dilution of an antibiotic-antimycotic solution (Invitrogen, Grand Island, NY). For all experiments, 80–90% confluent cells were cultured in serum-free medium for 24 h, washed with PBS and incubated in fresh medium in the presence or absence of 2 μg/ml LPS (E. coli 026:B6; Sigma-Aldrich, St. Louis, MO) plus 400 U/ml IFN-γ (Sigma-Aldrich) at 37°C for 8 h. After washing with chilled PBS three times, the cells were harvested. All the tissue culture regents were obtained from Life Technologies (Grand Island, NY).

Plasmids

A fragment of murine 1.63 kb iNOS promoter/enhancer region (~1486 to +145) was constructed into a luciferase reporter vector pGL3 as previously described (8). A full-length p300 expression construct (pCL.p300) and its histone acetyltransferase (HAT) deletion mutant (pCL.p300ΔHAT, ∆1472–1522) (12) were provided by Dr. J. Boyes (Institute of Cancer Research, London, U.K.). p300 C- and N-terminal deletion mutants were constructed by cloning the deletion mutants amplified by PCR into HindIII/XhoI sites of a mammalian expression vector pCMV-Tag2 (Stratagene, La Jolla, CA). Expression vectors for 12S E1A and its deletion mutant (∆2–36) (13) were provided by Dr. P. Raychaudhuri (University of Illinois, Chicago, IL).

Transfection and luciferase assay

The transfection procedure was performed as previously described (14). In brief, 10 μl of Lipofectamine 2000 reagent (Invitrogen) and 4 μg of DNA constructs were mixed, and the mixture was slowly added to cells in a 6-well plate and incubated for 24 h. The cells were washed, incubated in serum-free medium for 24 h, and treated with or without LPS/IFN-γ for 8 h. Luciferase activity was measured using a kit from Promega (Madison, WI) and the emitted light was determined in a luminometer (TD-20/20).
evaluate the effect of p300 overexpression, 10 μg of p300 constructs were mixed with 25 μl of Lipofectamine 2000 reagent and the mixture was added to cells cultured in a 10-cm dish. In all experiments, transfections were performed in duplicate.

Western blot analysis
Western blot analysis was performed as previously described (15). In brief, proteins were separated by electrophoresis in a 4–15% SDS-polyacrylamide gradient minigel (Bio-Rad, Hercules, CA) and electrophoretically transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ). Western blots were probed with affinity purified rabbit polyclonal IgG against iNOS, p300, p50, p65, C/EBPβ, c-Jun, or IRF-1 at 1 μg/ml each (Santa Cruz Biotechnology, Santa Cruz, CA). The protein bands were detected by ECL (Amerham Pharmacia Biotech). A number of p50 Abs were available from commercial sources. We evaluated several Abs purchased from Santa Cruz Biotechnology and Upstate Biotechnology (Boston, MA) for their sensitivity in detecting p50 proteins in RAW264.7 cell lysates. The Santa Cruz catalogue no. SC-7178, SC-114 and Upstate catalogue no. 06-866 rabbit polyclonal Abs exhibited a similar sensitivity. The Santa Cruz catalogue no. SC-8414 rabbit polyclonal Ab and catalogue no. 1192 goat polyclonal Ab had low sensitivity. We used the SC-7178 or SC-114 Ab (1 μg/ml each) in all experiments. For p65 (Rel A) immunoblots, we used a Santa Cruz rabbit polyclonal Ab catalogue no. SC-109 (1 μg/ml dilution). Another Santa Cruz Ab catalogue no. SC-372 and a polyclonal Ab from Oncogene (Boston, MA) had a similar sensitivity as SC-372.

Coimmunoprecipitation
Coimmunoprecipitation was performed as previously described (16). Nuclear extracts were prepared from 80–90% confluent RAW 264.7 treated with or without LPS/IFN-γ for 8 h and their protein concentrations were determined. Specific Abs against p300 or transactivators were added. The samples were incubated at 4°C overnight. Protein A/G-agarose beads (Santa Cruz Biotechnology) were added, incubated for 2 h, and centrifuged. The beads were washed three times with wash buffer (50 mM Tris pH 8, NaCl, 1 mM EDTA, and 0.5% Nonidet P-40). The immunoprecipitates were mixed with SDS loading buffer and analyzed by 4–15% SDS-PAGE (Bio-Rad) followed by Western blotting using a specific Ab. A rabbit nonimmune normal IgG was included as a negative control.

In vitro DNA-protein binding assay
Binding of p300 protein or transactivators to iNOS promoter DNA was assayed as described previously (17). Biotin-labeled double-stranded oligonucleotide probes corresponding to iNOS promoter sequence (−1168 to −1) were synthesized by Integrated DNA Technologies (Coralville, IA). The binding assay was performed by mixing 500 ng of nuclear extract proteins, 5 μg of biotin-labeled DNA, and 50 μl of streptavidin agarose beads with 70% slurry. The mixture was incubated at room temperature for 1 h with shaking. Beads were then pelleted and washed with cold PBS for three times. The binding proteins were separated by 4–15% PAGE, followed by Western blot analysis probed with Abs against p300 or transactivators.

Chromatin immunoprecipitation (ChIP)
The ChIP assay was done as described with minor modifications (18). A total of 80–90% confluent RAW 264.7 cells were serum starved for 24 h and treated with or without LPS/IFN-γ for 8 h. Formaldehyde (1%) was added to the culture medium and after incubation for 20 min at 37°C, cells were washed twice in PBS, scraped, and lysed in lysis buffer (1% SDS, 10 mM Tris-HCl, pH 8.0, with 1 mM PMSF, pepstatin A, and aprotinin) for 10 min at 4°C. Lysates were sonicated five times for 10 s each and the debris was removed by centrifugation. One third of the lysate was used as DNA input control. The remaining two-thirds of the lysate were diluted 10-fold with a dilution buffer (0.01% SDS, 1% Triton X-100, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0, and 150 mM NaCl) followed by incubation with an anti-p300 Ab or a nonimmune rabbit IgG (Santa Cruz Biotechnology) overnight at 4°C. Immunoprecipitated complexes were collected by using protein A/G-agarose beads (Santa Cruz Biotechnology). The precipitates were extensively washed and incubated in the elution buffer (1% SDS and 0.1 M NaHCO3) at room temperature for 20 min. Cross-linking of protein-DNA complexes was reversed at 65°C for 5 h, followed by treatment with 100 μg/ml protease K for 3 h at 50°C. DNA was extracted 3 times with phenol/chloroform and precipitated with ethanol. Pellets were resuspended in TE buffer and subjected to PCR amplification using specific iNOS promoter primers (5’ primer: −498 CTGCCCAAGCTGACTTACTAC −478, 3’ primer −1 GACCCCTGCGACGACCATCAC −21). The resulting product of 498 bp for iNOS was separated by 1% agarose gel electrophoresis.

Acetylation of transactivators
p50, p65, IRF-1, C/EBPβ, or c-Jun in nuclear extracts was immunoprecipitated with a specific Ab (1 μg/ml) described above and the immunoprecipitates were collected by using protein A/G-agarose beads. After extensive washing proteins were separated by 4–15% SDS-PAGE and acetylated transactivators were detected by Western blots using a mAb against acetylated lysine (0.2 μg/ml) (Cell Signaling Technology, Beverly, MA).

Results
Regulation of iNOS expression by p300
p300 proteins were detected in resting RAW 264.7 cells which were not increased by treatment with LPS/IFN-γ for 8 h (Fig. 1a). To determine whether p300 is involved in iNOS expression, we cotransfected RAW 264.7 cells with a wild-type (WT) or a deletion mutant (∆E1A) of adenoviral E1A construct and an iNOS-luciferase expression vector. The WT E1A inhibited the basal and LPS/IFN-γ-stimulated iNOS promoter activity whereas the ∆E1A mutant had no inhibitory effect (Fig. 1b). Neither E1A nor ∆E1A overexpression alone influenced the luciferase activity. Overexpression of p300 by transient transfection (Fig. 2a) resulted in increased basal iNOS protein levels and augmented iNOS protein levels stimulated by LPS/IFN-γ (Fig. 2b). Furthermore, p300 transfection dose-dependently increased basal and LPS/IFN-γ-stimulated iNOS promoter activity, suggesting that iNOS expression is regulated by p300 levels (Fig. 3). As the p300 protein contains multiple binding domains at C- and N-terminal regions,

FIGURE 1. Role of p300 in iNOS promoter activation. a, p300 protein levels in RAW 264.7 cells treated with or without LPS/IFN-γ for 8 h. The densitometric analysis shows mean ± SD of three experiments. b, iNOS promoter activity determined by transfecting an iNOS-luciferase construct. Cotransfection with E1A or E1A deletion mutant (ΔE1A) construct was performed as described in Methods section. Each bar is mean ± SD of three independent experiments.
we evaluated the influence of C- and N-terminal deletion mutations on iNOS promoter activity. Deletion of 714 and 1220 aa from the C terminus or 671 aa from the N terminus resulted in a complete abrogation of the stimulatory activity of p300 (Fig. 4). Mutation of p300 HAT (ΔHAT) also abrogated the p300 stimulatory activity, consistent with the involvement of p300 HAT in iNOS transactivation. Roscovitine, an indirect inhibitor of p300 HAT (19), inhibited basal and LPS/IFN-γ-stimulated iNOS promoter activity in a concentration dependent manner, confirming the importance of p300 HAT in iNOS promoter activation (Fig. 5).

**Regulation of p300 binding to iNOS promoter**

Several transactivators, notably NF-κB and IRF-1, have been shown to be required for iNOS transcriptional activation. By using a streptavidin-agarose pull-down assay, we detected p50 and p65 NF-κB isoforms, IRF-1, C/EBPβ, and c-Jun in the ternary complex in resting cells (Fig. 6). There was a ~2-fold increase in
NF-κB and IRF-1 binding in cells stimulated with LPS/IFN-γ for 8 h (Fig. 6). Neither C/EBPβ nor c-Jun binding was influenced by LPS/IFN-γ treatment (Fig. 6). p300 was also detected in the complex at the basal state which was increased by ~2-fold after stimulation with LPS/IFN-γ for 8 h (Fig. 6). Results from the ChIP assay also show binding of p50/p65, IRF-1 and p300 to chromatin iNOS promoter region in resting cells, which was significantly increased by LPS/IFN-γ treatment (Fig. 7).

Complex of p300 with transactivators in nuclear extracts

To provide evidence for a direct interaction between p300 and NF-κB or IRF-1, we prepared nuclear extracts from resting and LPS/IFN-γ-treated cells and performed immunoprecipitation with Abs specific for p50/p65 or IRF-1. p300 in the immunoprecipitate was resolved by Western blots. p300 complexed with p50/p65 NF-κB and IRF-1 was significantly increased by LPS/IFN-γ treatment (Fig. 8a). A control IgG did not precipitate a p300-containing complex. We next immunoprecipitated the nuclear extracts with anti-p300 Abs and detected transactivators in the precipitate by Western blots using Abs specific for p50, p65 and IRF-1. The levels of transactivators in the precipitate were significantly increased by LPS/IFN-γ (Fig. 8b). Thus, p300 binds p50/p65 and IRF-1 in nucleus at basal cellular state and the binding is up-regulated by LPS/IFN-γ.

Augmentation of NF-κB binding and p300 recruitment by p50 acetylation

We observed that p50 binding to the iNOS promoter probe was augmented by WT p300 but not HAT mutant overexpression in resting and LPS/IFN-γ-treated cells (Fig. 9). Like p50, p65 binding was also augmented by WT p300, but not HAT. By contrast, IRF-1 binding was not affected by WT or HAT p300 (Fig. 9). To determine whether p300-mediated p50 binding might be regulated by p50 acetylation, we prepared nuclear extracts from resting and LPS/IFN-γ-treated cells, immunoprecipitated the nuclear extract proteins with anti-p50, anti-p65 or other transactivator Abs. Trans-activator acetylation was detected with an anti-acetyl-lysine Ab. Acetylated p50 (Ac-p50) was detectable at basal cell state which

**FIGURE 5.** Effect of roscovitine on iNOS promoter activity. Each concentration point represents mean ± SD of triplicates.

**FIGURE 6.** Binding of transcriptional activators and p300 coactivator to a biotinylated iNOS probe. Nuclear extracts from cells treated with and without LPS/IFN-γ were incubated with the biotinylated probe and the complex was pulled down with streptavidin-agarose beads. After extensive washing, proteins in the complex were analyzed by Western blotting using Abs to p300 or the indicated transactivators. Control probe is a biotinylated 22-bp nonrelevant DNA sequence. a, A representative binding profile. b, Densitometric analysis of three separate experiments. Each bar is mean ± SD of three experiments.

**FIGURE 7.** Binding of p300, p50/p65, and IRF-1 to chromatin iNOS promoter region determined by ChIP. Chromatin was immunoprecipitated with Abs to p300 (α p300), p50/p65 (α p50/p65), IRF-1 (α IRF-1) or a normal rabbit IgG. a, A representative ChIP gel. b, Densitometry of three experiments showing mean ± SD.
was increased by ~2-fold in LPS/IFN-γ treated cells (Fig. 10). Traces of Ac-p65 and Ac-c-Jun were detected and there was no detectable Ac-IRF-1 or Ac-C/EBPβ (Fig. 10). The level of Ac-p50 increase correlated with that of p50 binding (Fig. 9). We next evaluated the effects of WT and ΔHAT p300 overexpression on p50 acetylation. WT p300 overexpression resulted in an almost 2-fold increase in Ac-p50, whereas ΔHAT overexpression did not induce an increase in Ac-p50 (Fig. 11a). Total p50 protein levels were unaltered by p300 or ΔHAT overexpression (Fig. 11b). It is interesting to note that p300 binding was enhanced by ~2-fold by WT p300 transfection but not the ΔHAT mutant (Fig. 12). These results suggest a relationship between p50 acetylation and p50/p300 binding to iNOS promoter.

Inhibition of p50 acetylation and binding by E1A

To ascertain the role of p300 in p50 acetylation, we cotransfected cells with p300 and E1A and determined Ac-p50 in the presence or absence of LPS/IFN-γ stimulation. E1A overexpression resulted in a reduced level of Ac-p50 at basal state and in cells stimulated with LPS/IFN-γ (Fig. 13a) without an effect on p50 levels (Fig. 13b). We next determined the effect of E1A overexpression on p50 binding by the streptavidin pull-down assay. p50 binding at the basal state and in the presence of LPS/IFN-γ was severely suppressed by E1A (Fig. 14a). Reduced p50 binding was correlated with a diminished p300 level in the binding complex (Fig. 14b).

Discussion

Results from this study provide new insight into the role of p300 in iNOS transcriptional activation. Data from E1A overexpression experiments indicate that p300 is essential for iNOS promoter activity induced by LPS/IFN-γ. The action of E1A is specific for p300 as WT E1A abrogates the iNOS promoter activity stimulated by LPS/IFN-γ whereas a N-terminal deletion mutant of E1A has no effect on LPS/IFN-γ-stimulated iNOS promoter activity. Results from p300 binding experiments provide additional support for the p300 requirement. p300 binding to iNOS promoter-bound transactivators is enhanced by LPS/IFN-γ stimulation which is abrogated by E1A overexpression. Our findings further indicate that iNOS transcriptional activation is regulated by p300 protein levels. Overexpression of p300 by transient transfection of RAW 264.7 cells with various concentrations of p300 constructs elicits a concentration-dependent increase in iNOS promoter activity stimulated by LPS/IFN-γ. These data are in agreement with a general concept that p300 proteins in most cells are expressed in low abundance and are a limiting factor in gene transcription. Another potential factor that regulates p300 transactivation is its posttranslational modification by phosphorylation.
CBP phosphorylation has been demonstrated during cell differentiation, cell cycle progression and cell signaling via the protein kinase C pathway (19–22). It has been shown that CBP is phosphorylated by cyclin E-Cdk2 during cell cycle progression and the phosphorylated CBP exhibits an enhanced HAT activity (19). Roscovitine, an inhibitor of cyclin E-Cdk2, abrogates the increase in HAT activity. We, therefore, chose to evaluate the effect of roscovitine on iNOS promoter activity. Our data indicate that roscovitine dose-dependently blocks not only the iNOS promoter activity induced by LPS/IFN-γ but also the basal promoter activity. These results suggest that p300 phosphorylation by cyclin E-Cdk2 may play a critical role in regulating iNOS transcriptional activation. Further studies are needed to provide direct evidence for this.

p300 contains multiple domains at the N- and C-terminal regions that interact with myriad DNA-bound transcription activators including p65 subunit of NF-κB (9–11). Results from our p300 deletion mutant transfection experiments suggest that multiple domains are required for stable binding of p300 to iNOS promoter-bound transactivators. As expected, the core HAT domain is also required for p300-mediated iNOS promoter activity. p300 phosphorylation by cyclin E-Cdk2 during cell cycle progression and the phosphorylated CBP exhibits an enhanced HAT activity (19). Roscovitine, an inhibitor of cyclin E-Cdk2, abrogates the increase in HAT activity. We, therefore, chose to evaluate the effect of roscovitine on iNOS promoter activity. Our data indicate that roscovitine dose-dependently blocks not only the iNOS promoter activity induced by LPS/IFN-γ but also the basal promoter activity. These results suggest that p300 phosphorylation by cyclin E-Cdk2 may play a critical role in regulating iNOS transcriptional activation. Further studies are needed to provide direct evidence for this.

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The p50 NF-κB isoform joins several transcriptional activators including p53, GATA-1 whose DNA binding activities have been shown to be enhanced by p300-catalyzed acetylation (12, 24). p50 has been reported to be acetylated in lymphocytes infected by HIV-1 (25). The viral Tat protein appears to be a requisite cofactor for p50 acetylation by p300. Several lysine residues of p50 were proposed to be potential acetylation sites (25). It is unclear whether p50 acetylation by p300 in RAW264.7 cells also requires a cofactor. Rel A (p65) binding to iNOS promoter is also up-regulated by LPS and IFN-γ. However, we detected only very low level of Ac-p65 in resting cells and there was no apparent induction of Ac-p65 by LPS and IFN-γ or p300. Thus, binding of p65 to iNOS promoter may not be directly regulated by acetylation in RAW264.7 cells in response to stimulation with LPS and IFN-γ. However, it is possible that its binding is enhanced via Ac-p50 by forming heterodimers with p50.

It has been reported that Rel A (p65) is acetylated by p300 at lysine 218, 221 and 310 (26). Acetylation at K221 in Rel A enhances DNA binding and inhibits IkBα interaction, thereby retaining Rel A in the nucleus (27). Acetylation of K310 is required for transcription without a direct effect on DNA binding or IkBα interaction. In those studies, Rel A acetylation in 293T or COS-7 cells was enhanced by treatment with TNF-α. It is unclear why we detected only a very low level of Ac-p65 in RAW264.7 cells which was not induced by LPS and IFN-γ. One potential shortcoming of our study is that we had not determined p65 acetylation with [3H]acetate which may be more specific than acetyl-lysine Ab. We were also concerned whether our results might be influenced by cross-reactivity of NF-κB Abs. We, therefore, evaluated several p50 and p65 Abs and found variations in sensitivities in detecting p50 on Western blot analysis. Three p50 and p65 Abs each exhibited a similar degree of specificity and sensitivity in recognizing p50 and p65 proteins, respectively and consistently show induced p50 protein overexpression by E1A. Each bar is mean ± SD of three experiments.

In summary, p300 plays a major role in iNOS transcriptional activation. Our results suggest that it interacts with p50, and acetylates p50 in nucleus, thereby increasing NF-κB binding to its corporate sites. It also complexes with IRF-1 but does not acetylate IRF-1 or C/EBPβ. IRF-1 and C/EBPβ binding may thus depend on different cell types under stimulation by different agonists. It should be emphasized that several lysine residues in p50 have been identified as potential sites for p300-catalyzed acetylation (25). Thus, both p50 and p65 comprise lysine residues for acetylation by p300. Their acetylation and the functional consequences may depend on the signaling from external stimuli and the cellular program.

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