Cooperative Activation of Npr1 Gene Transcription and Expression by Interaction of Ets-1 and p300

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Abstract—The objective of the present study was to gain insight into the cooperative roles of Ets-1 and p300 in transcriptional regulation and expression of the Npr1 gene (coding for guanylyl cyclase-A/natriuretic peptide receptor-A). Overexpression of Ets-1 and p300 in mouse mesangial cells increased Npr1 promoter activity by 12-fold, natriuretic peptide receptor-A mRNA levels by 5-fold, and ANP-dependent intracellular accumulation of cGMP by 26-fold. Knockdown of Ets-1 and p300 expression by small interfering RNA inhibited Npr1 gene transcription by 90%. Sequential chromatin immunoprecipitation assay demonstrated a direct physical association between p300 and Ets-1 on binding to the Npr1 promoter, suggesting that a physical interaction between Ets-1 and p300 is important to enhance Npr1 gene transcription. Mutant p300 lacking histone acetyltransferase activity did not show a functional effect with Ets-1, suggesting that histone acetyltransferase activity of p300 is required for the cooperative interaction in modulating Npr1 gene transcription. Overexpression of wild-type adenovirus E1A significantly decreased the Npr1 promoter activity by 40%, whereas mutant E1A, which is incapable of binding to p300, did not show any effect. The results indicate that Npr1 gene transcription is critically controlled by histone acetyltransferase p300 and Ets-1. The present findings should yield important insights into the molecular signaling governing Npr1 gene transcription, an important regulator in the control of hypertension and cardiovascular events. (*Hypertension. 2009;54:172-178.*)

Key Words: atrial natriuretic peptide • guanylyl cyclase-A/natriuretic peptide receptor-A • gene transcription • gene expression • histone acetyltransferase

Atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) principally mediate natriuresis/diuresis, vasorelaxation, and antimitogenic responses, largely directed to the reduction of blood pressure and blood volume. The actions of ANP and BNP are mediated by binding to guanylyl cyclase-A/natriuretic peptide receptor-A (GC-A/NPRA), which produces the intracellular second messenger cGMP.

It has been demonstrated that Npr1 gene (coding for GC-A/NPRA) lowers arterial blood pressure and increases GC activity in a gene dose-dependent manner in Npr1 gene-targeted mice. Previous studies have also shown that disruption of the Npr1 gene leads to renal insufficiency, cardiac hypertrophy, and fibrosis in Npr1-null mutant mice. Earlier studies have provided evidence for a significant association for Npr1 gene variants with hypertensive family history and with increased left ventricular mass index, as well as with left ventricular septal wall thickness, in human essential hypertension. Extracellular osmolality and vitamin D have been shown to stimulate Npr1 gene promoter activity in inner medullary collecting duct cells. On the other hand, angiotensin II has been found to repress the Npr1 gene transcription in mouse mesangial cells. However, the mechanistic regulation of Npr1 gene transcription and expression in target cells is not well understood.

Ets-1 protein, which is expressed in a variety of cell types, including endothelial cells, mesangial cells, and vascular smooth muscle cells, has been implicated in the transcriptional regulation of several genes involved in angiogenesis and remodeling of the extracellular matrix proteins. Similarly, transcriptional coactivator p300 and CREB-binding protein (CBP) play a central role in coordinating multiple signal-development events with the transcription apparatus, allowing an appropriate level of gene activity to occur in response to diverse physiological stimuli. Many Ets family proteins, including Elk-1, Ets-1, Ets-2, and ER81, interact with coactivator CBP/p300 to stimulate gene transcription.

The glomerular mesangial cells provide an attractive model system to delineate the synergistic regulation of Npr1 gene transcription, because these cells modulate kidney function, contain functional GC-A/NPRA, and express both Ets-1 and p300 proteins. The present study demonstrates that Ets-1 and p300 play essential roles in modulating the transcription and expression of the Npr1 gene, which plays a critical role in the control of kidney function and the regulation of blood pressure and cardiovascular homeostasis.

Methods

Plasmids and Promoter Constructs

The promoter-luciferase reporter constructs were generated by cloning PCR-amplified DNA fragments of the murine Npr1 promoter upstream of the promoterless firefly luciferase gene in...
the pG3L3-basic vector (Promega), as described previously. The cloning of the construct −356/+29 bp was performed using −356 forward (5′-taaggaaggtgcggagagagaagcgcgtc-3′) and +29 reverse (5′-taaggaagratctgtgcgctcgcgcttgcccc-3′) primers. The vectors pEVR-F0-Ets-1 and pEVR-F0 were received from Dr Paul Brindle (St Jude Children’s Research Hospital, Memphis, Tenn). E1A 125Swi and E1A 12S22-36 were obtained from Dr Elizabeth Moran (University of Medicine and Dentistry of New Jersey, Newark, NJ), and pcDNA3.1-p300 WT and pcDNA3.1-p300-histone acetyltransferase (HAT)− were received from Dr Warner Greene (University of California San Francisco, San Francisco, Calif) as kind gifts.

Cell Transfection and Luciferase Assay

Mouse mesangial cells (MMCs) were isolated and cultured in DMEM supplemented with 10% fetal calf serum (FCS) and insulin-transferrin-sodium selenite, as described previously. Rat thoracic aortic smooth muscle cells (RTASMCs) were cultured in DMEM supplemented with 10% FBS, as described previously. The cells (between the fourth and 15th passages) were transfected using the ChIP-IT Express kit (Active Motif). Briefly, cells (1.5 × 10^6) were treated with 5 μg of antibody (anti–Ets-1, p300, or control IgG) at 4°C with rotation overnight. Beads were pelleted and washed sequentially once with ChIP buffer 1 and twice with ChIP buffer 2. The bound protein was eluted from the beads by incubation with 10 mmol/L of dithiothreitol at 37°C for 30 minutes and again immunoprecipitated with a second antibody overnight at 4°C. After washing the magnetic beads, bound protein was eluted by gentle rotation for 15 minutes in elution buffer AM2 at 22°C. After elution of the protein/DNA complex, cross-linking was reversed at 65°C overnight to release DNA. Immunoprecipitated DNA was sequentially treated with RNase A and proteinase K and then purified. The Npr1 promoter containing Ets-1 and p300 binding sites was PCR amplified using purified DNA as a template and the forward (5′-ctcttcgtgcgctcgcgcttgcccc-3′) and reverse (5′- gggaggagcggagagagaga-3′) primers.

Immunoprecipitation of Acetylated Ets-1

Cells were transfected with the p300 expression plasmid, and cell lysate was prepared after 48 hours of transfection. Cell lysate (100 μg of proteins) was incubated with 2 μg of polyclonal Ets-1 antibody and immunoprecipitated as described above. For detection of acetylated Ets-1, membranes were incubated with anti–Ac-lys (AKL5Cl) antibody (Santa Cruz Biotechnology) and treated with antimonosial horseradish peroxidase–conjugated secondary antibody.

Transfection of Small Inhibitory RNA

Cells were cultured to 70% to 80% confluence and transfected with Ets-1 and p300 small interfering RNA (siRNA; a pool of 3 target-specific 20- to 25-nucleotide sequence siRNAs) using the Lipofectamine-2000 reagent (Invitrogen), and luciferase activity in cell lysate was measured as described previously. The results were normalized for the transfection efficiency as relative to light units per Renilla luciferase activity. Transformation efficiency was assessed using an in situ β-galactosidase staining kit (Stratagene) and achieved 80% and 85% in MMCs and RTASMCs, respectively.

Real-Time RT-PCR Assay

Total RNA was extracted from transfected cells using an RNasey Mini Kit (Quagen), and the first-strand cDNA was synthesized from 2 μg of total RNA using an RT2 First Strand Kit (SABiosciences). Real-time RT-PCRs were performed using the Mx3000P Real-Time PCR System, and data were analyzed with MxPro software (Stratagene). PCR amplification (in triplicate) was carried out in a 25-μL reaction volume using RT2 Real-Time SYBR Green/ROX PCR Master Mix. The reaction conditions were as follows: 95°C for 10 minutes, followed by 45 cycles at 95°C for 15 seconds and 60°C for 1 minute, followed by 1 cycle at 95°C for 1 minute, 55°C for 30 seconds, and 95°C for 30 seconds for the dissociation curve. β-Actin was amplified from all of the samples on each plate as a housekeeping gene to normalize expression levels of targets between different reaction samples. The gene to normalize expression levels of targets between different samples and to monitor assay reproducibility. The reaction mixture without template cDNA was used as a negative control. Threshold cycle number (Ct) was determined with MxPro QPCR Software and transformed using the change in threshold cycle number comparative method. The Npr1 gene expression values were normalized to expression values of β-actin within each sample, and relative expression of the Npr1 gene was determined by the comparative threshold cycle number analysis.

Immunoblot Assay

Forty-eight hours after transfection, cells were lysed, and immunoblot assay was performed as described previously. In brief, cell lysate (80-μg proteins) was electrophoresed for 2 hours and then transferred to a nylon membrane. The membrane was incubated with nonimmune antibody (anti–Ets-1, anti-p300, or anti-NPRA) and treated with corresponding secondary antirabbit or antimonosial horseradish peroxidase–conjugated antibodies (Santa Cruz Biotechnology). Protein bands were visualized by ChemiGlow West (Alpha Innotech).

Sequencial Chromatin Immunoprecipitation Assay

Chromatin immunoprecipitation (ChIP) was performed using the ChIP-IT Express kit (Active Motif). Briefly, cells (1.5 × 10^7) were cross-linked in 1% formaldehyde for 10 minutes at 22°C, and the reaction was quenched with 0.1 mol/L of glycine. Cells were scraped, resuspended in 1 mL of lysis buffer on ice, and homogenized with a dounce homogenizer. The homogenate was centrifuged at 5000 rpm for 10 minutes at 4°C to pellet the nuclei. The pellet was resuspended in 1 mL of digestion buffer and 50 μL of enzymatic shearing mixture and incubated at 37°C for 10 minutes. The reaction was stopped by adding 20 μL of 0.5-mol/L EDTA followed by chilling on ice for 10 minutes. Sheared DNA was centrifuged at 13 000 rpm at 4°C for 10 minutes, and supernatant was collected. Immunoprecipitation was performed using protein G magnetic beads and 5 μg of antibody of Ets-1, p300, or control IgG at 4°C with rotation overnight. Beads were pelleted and washed sequentially once with ChIP buffer 1 and twice with ChIP buffer 2. The bound protein was eluted from the beads by incubation with 10 mmol/L of dithiothreitol at 37°C for 30 minutes and again immunoprecipitated with a second antibody overnight at 4°C. After washing the magnetic beads, bound protein was eluted by gentle rotation for 15 minutes in elution buffer AM2 at 22°C. After elution of the protein/DNA complex, cross-linking was reversed at 65°C overnight to release DNA. Immunoprecipitated DNA was sequentially treated with RNase A and proteinase K and then purified. The Npr1 promoter containing Ets-1 and p300 binding sites was PCR amplified using purified DNA as a template and the forward (5′-ctcttcgtgcgctcgcgcttgcccc-3′) and reverse (5′- gggaggagcggagagagaga-3′) primers.
highly conserved in mice, rats, and humans (Figure 1B). Overexpression of Ets-1 and p300 plasmids with construct −356/+55 enhanced Npr1 promoter activity by ≈12-fold (Figure 2A). A construct having mutations in both Ets-1 binding sites showed only 4-fold stimulation in luciferase activity with overexpression of Ets-1 and p300 as compared with the untransfected controls. The deletion of p300 sites also showed only 7-fold stimulation in luciferase activity with overexpression of Ets-1 and p300 as compared with the wild-type construct (Figure 2A). The construct −356/−46 (lacking Ets-1 and p300 binding motifs) failed to respond to Ets-1 and p300 protein expression, further confirming that Ets-1 and p300 use their consensus sites to activate Npr1 gene transcription. A significant expression of Ets-1 and p300 proteins (2-fold) in MMCs was confirmed by Western blot analysis using anti–Ets-1 and anti-p300 antibodies (Figure 2B). Cotransfection of p300 and Ets-1 siRNA, along with the Npr1 promoter, reduced the luciferase activity by 50% and 75%, respectively, as compared with untransfected cells; however, transfection of control siRNA showed no change in

![Figure 1. Luciferase activity of 3′ deletion constructs of Npr1 promoter. A, Left, Schematic representation of the deletion construct of the Npr1 promoter. Right, Transcriptional activity of these constructs in MMCs and RTASMCs. B, A direct interspecies comparison of Ets-1 and p300 sites in a mouse, rat, and human Npr1 gene promoter. Sequence in bold shows the consensus-binding sites of Ets and p300. Bars represent the mean±SE of 4 independent experiments in triplicates. *P<0.01; ††P<0.01; ***P<0.001; †††P<0.001.](image1)

![Figure 2. Effects of overexpression and knockdown of Ets-1 and p300 on Npr1 promoter activity. A, Luciferase activity of 3′ deletion constructs (−356 to +55 bp) of the Npr1 promoter cotransfected with Ets-1, p300, or empty vector (−Ets-1/−p300) in MMCs. B, Western blot analysis of Ets-1 and p300 in transfected MMCs. C, Luciferase activity of the Npr1 promoter cotransfected with Ets-1, p300, and control (ctrl) siRNA. D, Western blot analysis of knockdown effects of Ets-1, p300, and control siRNA in MMCs. β-Actin was used as a loading control. Data represent the mean±SE of 4 independent experiments. UT indicates untransfected; WB, Western blot. *P<0.05; **P<0.01; ***P<0.001.](image2)
luciferase activity (Figure 2C). Transfection of Ets-1 and p300 siRNA together repressed luciferase activity by \( \frac{90}{11022} \). Western blot analysis showed that Ets-1 and p300 protein expressions were markedly reduced in siRNA-transfected cells as compared with control siRNA–transfected cells (Figure 2D).

To examine whether Ets-1 and p300 coexist within the same protein complex resident in the Npr1 promoter, a sequential ChIP assay was performed. The initial ChIP assay was carried out with Ets-1 antibody, and the sequential ChIP showed the occupancy of p300 in the same protein-DNA complex (Figure 3A). Conversely, initial ChIP with p300 antibody, followed by a second immunoprecipitation with the Ets-1 antibody, further confirmed the coexistence of Ets-1 and p300 in the protein-DNA complex binding to the Npr1 promoter (Figure 3B). To investigate the effect of overexpression of Ets-1 and p300 on the endogenous Npr1 gene expression, we analyzed the mRNA levels of NPRA by real-time RT-PCR assay. Experiments were performed in MMCs and RTASMCs to determine the effects of Ets-1 and p300 in a cell-type specific manner. There was an \( \approx 5 \)-fold induction in NPRA mRNA levels in Ets-1– and p300-transfected MMCs, as compared with untransfected controls (Figure 4A). The Western blot analysis exhibited an enhanced level of NPRA protein expression in Ets-1/p300-transfected MMCs as compared with untransfected control cells (Figure 4B). The treatment of Ets-1–transfected RTASMCs and MMCs with 100 nM ANP showed an increase in intracellular accumulation of cGMP by 7-fold and 12-fold, respectively, compared with untransfected control cells (Figure 4C and 4D). Coexpression of Ets-1 and p300 and treatment with 100 nM ANP significantly increased cGMP levels by \( \approx 19 \)-fold in RTASMCs and 26-fold in MMCs as compared with untransfected controls.

We examined the cooperative interactive roles of Ets-1 and p300 in the regulation of Npr1 gene transcription and expression in MMCs and RTASMCs, which were transfected with Ets-1 alone or wild-type or mutant p300 construct lacking HAT activity. Cotransfection of Ets-1 and mutant p300(-HAT) construct did not show any significant increase in promoter activity as compared with Ets-1–transfected MMCs (Figure 5A). We confirmed the overexpression of the Ets-1 and p300-HAT mutants in MMCs by Western blot analysis (Figure 5B). Immunoprecipitation assay showed that endogenous Ets-1 exhibited measurable levels of lysine acetylation.
in untransfected MMCs (Figure 5C). However, there was a significant increase in the level of Ets-1 acetylation in cells transfected with the p300 expression plasmid. The bottom panel in Figure 5C shows the direct Western blot analysis of total Ets-1 on a different gel. We further confirmed the role of p300 and its HAT activity in cooperation with Ets-1 on total Ets-1 detected by direct Western blot of the lysate. Representative blots of 3 experiments are shown. Bars represent the mean±SE of 4 independent experiments in triplicate. UT indicates untransfected; IP, immunoprecipitation; WB, Western blot; Ac, acetylated. ∗P<0.05.

Figure 5. Effect of p300 HAT activity on Npr1 transcription in MMCs. A, Cells were transiently transfected with Npr1 promoter construct −356/+55 bp, and Ets-1 expression plasmid (250 ng) with wild-type p300 or mutant p300 (250 ng) plasmid and luciferase activity was measured. B, Western blot analysis of Ets-1 and p300-HAT in transfected MMCs. C, After immunoprecipitation, acetylated Ets-1 and total Ets-1 were determined. The complex formation was not detected in lysates immunoprecipitated with control mouse IgG. Bottom, Total Ets-1 detected by direct Western blot of the lysate. bars represent the mean±SE of 4 independent experiments in triplicate. UT indicates untransfected; IP, immunoprecipitation; WB, Western blot; Ac, acetylated. ∗P<0.05, **P<0.01.

Figure 6. Effect of adenovirus E1A on Npr1 gene transcription. Luciferase activity of Npr1 promoter construct −356/+55 with wild-type or mutant adenovirus E1A expression plasmid was measured. Wild-type or mutant adenovirus E1A plasmids (100 ng) were transfected alone or with Ets-1 (250 ng) and p300 (250 ng) expression vector with Npr1 promoter construct in MMCs. Bars represent the mean±SE of 4 independent experiments. Ad indicates adenovirus. ∗P<0.05, **P<0.01.

Discussion

The results of the present study demonstrate that Npr1 promoter activity is regulated by cooperative interaction of Ets-1 and p300, which activate Npr1 gene transcription through the consensus sites present in the region −46 to +55 bp of the Npr1 promoter. Sequential ChIP assay further demonstrated that Ets-1/p300 activation of the Npr1 gene transcription involves a direct physical association between these 2 factors. Overexpression of Ets-1 and p300 greatly stimulated NPRA mRNA and protein levels and increased intracellular accumulation of second-messenger cGMP in both MMCs and RTASMCs. Ets-1 seems to be essential for normal development of mammalian kidneys and for maintenance of glomerular integrity.30,31 It is implicated that Ets-1 is critical in hematopoiesis and angiogenesis during the earliest stages of embryogenesis, and in later stages it is important in organ formation and tissue remodeling in kidneys, liver, and vasculature.32,33 In the nephritic kidney, Ets-1 has been shown to decrease extracellular matrix deposition and type I collagen expression.26 Similarly, endogenous p300 seems to be critical in the integration of signal transduction pathways in mesangial cells, and interference with p300 alters apoptotic signals.25 Several lines of evidence suggest that p300 is critical for the development of the embryonic heart and plays a central role in quantitative control of physiologically adaptive cardiac hypertrophy that depends on its HAT activity.13,14 The present results indicate that the Npr1 gene is under tight control of Ets-1 and p300 transcription factors. The overexpression of the Ets-1 gene in human colon cancer cell lines has been shown to reduce the rate of anchorage-independent growth in a dose-dependent manner.26 On the other hand, Ets-1 expression has been suggested to regulate endothelial cell proliferation during angiogenesis and is essential for normal coronary and myocardial development.37–39 On the basis of those previous findings and the present results, it is possible that Ets-1 plays a dual role during pathological conditions and upregulates Npr1 signaling as a protective regulatory mechanism in renal and cardiovascular disease states.
HATs, including p300, are able to acetylate several transcription factors, including p53 and GATA-4, to enhance their transcriptional activity and regulate gene expression patterns by affecting chromatin structure. Our present results demonstrate that HAT activity of p300 is required for the cooperative interaction of Ets-1, and p300 in regulating the Npr1 gene. Detection of acetylated Ets-1 in the immunoprecipitated complex from p300-transfected cells showed that p300 hyperacetylates endogenously expressed Ets-1 protein. It has been shown that, under in vivo conditions, acetylated Ets-1 preferentially associates with p300 complexes. Our data demonstrate that adenovirus E1A disrupted the Ets-1/p300-dependent transcription, whereas mutant E1A lacking the p300-binding domain showed no significant effect on Npr1 gene transcription. These results suggest that E1A inhibition is exclusively dependent on its binding to p300. Apart from its HAT activity, p300/CBP provides a link between specific transcription factors and general transcriptional machinery; thus, E1A may inhibit p300/CBP and thereby the Npr1 promoter either by inhibiting intrinsic HAT activity of p300/CBP or by diminishing the interaction between p300 and Ets-1. The present results are compatible with the findings that E1A interferes in the association of p300/CBP and inhibits acetylation of transcription factors p53 and GATA-4.

Our previous studies have shown that angiotensin II represses whereas Ets-1 stimulates Npr1 promoter activity. It has also been suggested that angiotensin II increases the expression of Ets-1 in mesangial cells, as well as in vascular smooth muscle cells, and Ets-1 thereby provokes gene activation in response to vascular inflammation. Furthermore, Ets-1 induces the expression of caspase 1, which plays a prominent role in the apoptotic induction of inflammatory response cells. Because Ets-1 is stimulated by proinflammatory mediators and also enhances Npr1 gene transcription, it is implicated that Ets-1 may have a dual function involving p300 in cardiovascular disease states. On one hand, it plays a role in inflammatory responses downstream of the angiotensin II signaling pathway, and on the other hand, in cooperative interaction with p300, it stimulates Npr1 gene transcription and expression, which seem to inhibit proinflammatory responses in hypertension and cardiovascular disease states.

Perspectives

The present findings provide direct evidence that Ets-1 and p300 are essential for Npr1 gene transcription and expression. The functional and physical interactions between Ets-1 and p300 are necessary for their action in regulating the optimum level of Npr1 gene transcription. Additional understanding of the regulation of DNA binding ability and turnover of these transcription factors in the regulation of NPARA expression would provide the framework to develop strategies to regulate the expression levels of NPRA. The results of this present study will greatly enhance our understanding of the pathway involved in the transcriptional regulation of the Npr1 gene, an important locus in the control of hypertension and cardiovascular events.

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Disclosures

None.

References


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