A Sp1 Binding Site of the Tumor Necrosis Factor α Promoter Functions as a Nitric Oxide Response Element*

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Shuibang Wang, Weihan Wang, Robert A. Wesley, and Robert L. Danner‡
From the Critical Care Medicine Department, Warren Grant Magnuson Clinical Center, National Institutes of Health, Bethesda, Maryland 20892

Regulation of gene transcription is an incompletely understood function of nitric oxide (NO). Human leukocytes produce increased amounts of tumor necrosis factor α (TNF-α) in response to NO. This effect is associated with decreases in intracellular cAMP, suggesting that NO might regulate gene transcription through promoter sequences sensitive to cAMP such as cAMP response elements (CRE) and Sp1 binding sites. Here we report that a Sp1 binding site in the TNF-α promoter conveys NO responsiveness. Human U937 cells were differentiated for TNF-α production with phorbol 12-myristate 13-acetate, NO donors and H89, an inhibitor of cAMP-dependent protein kinase increased, while dibutyryl cAMP (Bt2cAMP) decreased TNF-α promoter activity. Deletion or mutation of the proximal Sp1 site, but not the CRE site, abolished the activating effects of NO donors and H89. Further, NO- and H89-mediated increases in TNF-α promoter activity were associated with decreased Sp1 binding. The insertion of Sp1 sites into a minimal cytomegalovirus promoter conferred NO responsiveness, an effect blocked by Bt2cAMP. Mutation of these inserted Sp1 sites prevented this heterologous promoter from responding to NO, H89 and Bt2cAMP. These results identify the Sp1 binding site as a promoter motif that allows NO to control gene transcription.

Nitric oxide (NO)† regulates vascular tone (1), inflammatory responses (2–4), and gene transcription (5–7). Although NO regulates vascular tone through soluble guanylate cyclase, some actions of NO utilize alternative signal transduction pathways. Previous experiments have shown that NO donors increase tumor necrosis factor α (TNF-α) synthesis in human neutrophil (2) and peripheral blood mononuclear cell preparations (3) through a cGMP-independent mechanism. Recently, we further demonstrated that endogenously produced NO also up-regulates TNF-α production by a cGMP-independent mechanism in phorbol 12-myristate 13-acetate (PMA)-differentiated U937 cells transfected with murine, inducible NO synthase (8).

U937 cells lack soluble guanylate cyclase and do not respond to NO with a cGMP signal (8–9), suggesting that these cells might be useful for exploring cGMP-independent mechanisms by which NO regulates gene transcription. Experiments with U937 cells have demonstrated that NO augments TNF-α production through a signaling pathway dependent on decreases in intracellular cAMP (9). This finding suggests that NO might regulate TNF-α at the level of gene transcription through effects on cAMP sensitive promoter sites.

CAMP is known to regulate gene transcription through effects on cAMP response elements (CRE) (10). Furthermore, for some genes such as CYP11A and urokinase, cAMP has been shown to alter promoter activity through incompletely defined effects on Sp1 binding sites (11, 12). Similar to CRE-binding proteins, cAMP-dependent protein kinase (PKA) phosphorylation of Sp1 has been shown to enhance its DNA binding activity (13). Although Sp1 binding can activate transcription (14, 15), for some genes Sp1 functions as a repressor (16, 17). The promoter motifs or cell characteristics that determine these divergent effects of Sp1 binding are not fully understood. Therefore, CRE or Sp1 binding sites in the TNF-α promoter (18–20) may be important in mediating cAMP-dependent effects of NO on TNF-α transcription. To investigate this question, we examined the effects of NO on the human TNF-α promoter in differentiated U937 cells.

EXPERIMENTAL PROCEDURES

Reagents—Cell culture reagents were obtained from Biofluids Inc. PMA, dibutyryl cAMP (Bt2cAMP), PKA inhibitor (H89), S-nitroso-N-acetylpenicillamine (SNAP), and S-nitrosothioglutathione (SNOG) were all purchased from Calbiochem. All polymerase chain reaction (PCR) primers were synthesized by Genosys Biotechnologies, Inc.

Plasmid Construction—The two-plasmid reporter gene system chosen for these experiments lacks cryptic CRE sites that might lead to spurious CAMP-dependent effects (21). The first plasmid was made from plasmid pTet-off (CLONTECH), designated here as pCMV-tTA because it uses the strong immediate cytomegalovirus (CMV) promoter to express tetracyclin (tet)-responsive transcriptional activator (tTA). tTA is a fusion protein of the first 207 amino acids of the tet repressor and of the C-terminal activation domain of the herpes simplex virus VP16 protein. The tTA protein transactivates expression of a reporter gene, chloramphenicol acetyltransferase (CAT), by binding to a tet-responsive element in the promoter of a second plasmid, pHUG10.3CAT (21). Promoterless pT TA vector was generated from pCMV-tTA by removing the whole CMV promoter region using a Xhol/EcoRI digest. The 1311-bp human TNF-α promoter, cut from pXPI-1311TTPY by Xhol/HindIII (22), was subcloned into the Xhol/EcoRI site of pT TA to generate pT NF-tTA. Either the CRE sequence (5'-TGAAGCTCA-3' at position +107 to +100) or the Sp1 binding site (5'-CCCCGGC-3' at position +52 to +45) was removed from pTNF-tTA and replaced with a 6-bp EcoRV restriction site (5'-GATATC-3') using ExSite® (Stratagene) to generate the non-CRE constructs pTNF-CRE-tTA and pTNF(GE)-tTA, respectively. Furthermore, a pT NF(mSp1)-tTA construct was made from pTNF-tTA by mutating the Sp1 binding site (from 5'-CCCCGGC-3' to 5'-CCCCGTTT-3') using Chameleon™ (Stratagene). This mutation prevents the binding of Sp1(23). The other Sp1 site in the TNF-α promoter
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(transport 173 to 166) was not studied. It overlaps with an Egr-1 motif and has been shown to bind Egr-1, not Sp1 protein in PMA or lipopolysaccharide-stimulated U937 and THP1 cells (20, 24).

Next, a pCMVmut-tTA construct devoid of enhancer elements was created from pCMV-tTA by PCR. A 35-bp double-stranded oligonucleotide containing three copies of the Sp1 sequence (5′-CCCCTCCCGCCCTGTGAGCTACCTTGAGCCAACTCCCCTGGCC-3′) or the mutated Sp1 sequence (5′-CCCCTGCCCTGTGAGCTACCTTGAGCCAACTCCCCTGGCC-3′) was inserted into pCMVmut-tTA at a XhoI site to create pCMVmut(Sp1)−tTA and pCMVmut(mSp1)−tTA, respectively. All vectors were partially sequenced to confirm the correct sequences and orientations.

Cell Transfections and CAT Assay—U937 cells (ATCC) in RPMI 1640 complete medium (10% fetal bovine serum, 50 μg/ml poly(dI-dC)) for 20 min at room temperature. Samples were subjected to electrophoresis through 6% DNA-retardation gels (Novex) in 0.25× Tris-borate-EDTA buffer at 4 °C. In competition and antibody supershift experiments, 200-fold molar excess of cold Sp1 oligonucleotides or 2 μg mouse monoclonal anti-Sp1 (Santa Cruz Biotechnology, Inc.), respectively, were added to each sample prior to addition of hot Sp1 probe.

Statistical Analysis—For Figs. 1 and 2 comparisons were made using two-tailed Student’s t tests adjusted with Holm’s procedure. Analysis of variance followed by post-hoc tests using Fisher’s least significant difference method was employed to analyze the data in Figs. 4 and 5. Differences were considered to be significant at p < 0.05.

RESULTS AND DISCUSSION

Effect of NO and PKA Activation or Inhibition on TNF-α Promoter Activity—TNF-α promoter activity was undetectable in naive, undifferentiated U937 cells under all test conditions (data not shown). Two NO donors, SNAP and SNOG, increased wild type TNF-α promoter activity in PMA-differentiated U937 cells by 128 ± 56% (Fig. 1A, p < 0.05) and 92 ± 4% (Fig. 1B, p < 0.04), respectively. If this increase in TNF-α promoter activity (Fig. 1A and B) is occurring through the cAMP-lowering effect of NO, then Bt2cAMP would be expected to decrease TNF-α promoter activity and a PKA inhibitor would mimic the effect of NO by increasing TNF-α promoter activity. As shown in Fig. 1A and B, respectively, Bt2cAMP decreased TNF-α promoter activity by 53 ± 4% (p < 0.01), and SNOG, an inhibitor of PKA, increased TNF-α promoter activity by 67 ± 6% (p < 0.04).

Effect of Site Deletions on NO Responsiveness of the TNF-α Promoter—Substitution of the CRE sequence or the Sp1 binding site with an unrelated 6 bp sequence reduced basal TNF-α promoter activity by 43 ± 6% (p < 0.003) and 48 ± 10% (p < 0.006), respectively. If NO and H89 up-regulate the TNF-α promoter by release of the CRE sequence (Fig. 2A and 2B, C, p < 0.05 for all). These results demonstrated that NO and H89 similarly altered gene transcription independent of the CRE site. Likewise, Bt2cAMP repressed promoter activity despite release of the CRE site (Fig. 2B, p < 0.02). In contrast, Sp1 binding site replacement completely abolished the activating effects of NO donors and H89 (Fig. 2B, C, and p > 0.8 for all) and partially blunted the inhibitory effect of Bt2cAMP (Fig. 2B, p < 0.1 for inhibition). These data suggested that the Sp1 binding site, not the CRE sequence, mediates induction of the TNF-α promoter by both NO and the PKA inhibitor H89. The borderline effect of Bt2cAMP on the promoter lacking a Sp1 site suggests that elevated cAMP concentrations may affect TNF-α transcription through sites other than Sp1, such as CRE (10) or NF-κB (21).

Effect of NO and PKA Activation or Inhibition on Sp1 DNA Binding—As reported previously (26), nuclear extract from PMA-differentiated U937 cells formed two specific complexes (C1 and C2) with the hot Sp1 binding site of the human TNF-α promoter (Fig. 3A). Anti-Sp1 antibody supershifted only the highest order EMISA complex (C1) demonstrating that it contained Sp1 or a closely related protein (Fig. 3A, lane 4). Incubation with either SNAP or SNOG similarly decreased formation of the C1 complex compared with nuclear extract from control cells (Fig. 3B). Conversely, Bt2cAMP increased the binding interaction at C1. Furthermore, in the presence of Bt2cAMP, SNAP had no effect on formation of the C1 complex (Fig. 3B, lane 5).

Effect of Sp1 Site-directed Mutation on NO Responsiveness of the TNF-α Promoter—If NO and H89 up-regulate the TNF-α promoter by reducing Sp1 binding, then the proximal Sp1 site would be functioning as a repressor element. However, deleting the 8-bp Sp1 site and replacing it with a 6-bp EcoRV sequence had decreased overall TNF-α promoter activity. To test the possibility that this decrease in promoter activity was created by steric hindrance between flanking AP1 and AP2 motifs, we mutated the Sp1 site to maintain proper spacing (see “Exper-

FIG. 1. Regulation of TNF-α promoter activity by NO. A, U937 cells were transfected with wild type TNF-α promoter construct pTNF-α-tTA, differentiated with PMA (100 nM), and incubated with medium alone, the NO donor SNAP (500 μM), or the cell permeable cAMP analog Bt2cAMP (100 μM). B, cells were incubated with medium alone, the NO donor SNOG (500 μM), or the PKA inhibitor H89 (30 μM). Values represent the mean ± S.E. of four experiments performed in duplicate. 0.004), respectively (Fig. 2A). The NO donors SNAP and SNOG, which reduce cAMP concentrations below basal levels, and H89, which mimics this effect of NO by inhibiting PKA (9), still enhanced activity of the TNF-α promoter after replacement of the CRE sequence (Fig. 2, B and C, p < 0.05 for all). These results demonstrated that NO and H89 similarly altered gene transcription independent of the CRE site. Likewise, Bt2cAMP repressed promoter activity despite release of the CRE site (Fig. 2B, p < 0.02). In contrast, Sp1 binding site replacement completely abolished the activating effects of NO donors and H89 (Fig. 2B, C, and p > 0.8 for all) and partially blunted the inhibitory effect of Bt2cAMP (Fig. 2B, p < 0.1 for inhibition). These data suggested that the Sp1 binding site, not the CRE sequence, mediates induction of the TNF-α promoter by both NO and the PKA inhibitor H89. The borderline effect of Bt2cAMP on the promoter lacking a Sp1 site suggests that elevated cAMP concentrations may affect TNF-α transcription through sites other than Sp1, such as CRE (10) or NF-κB (21).
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**Fig. 2.** Effect of site deletions on the NO responsiveness of the TNF-α promoter. A, U937 cells were transfected with wild type TNF-α promoter construct pTNF-tTA, the CRE site deletion mutant pTNF(dCRE)-tTA, or the Sp1 site deletion mutant pTNF(dSp1)-tTA, and differentiated with PMA (100 nM). B, U937 cells were transfected with pTNF(dCRE)-tTA or pTNF(dSp1)-tTA, differentiated with PMA (100 nM), and incubated with medium alone, the NO donor SNAP (500 μM), or Bt2cAMP (100 μM). C, cells were incubated with medium alone, the NO donor SNOG (500 μM), or the PKA inhibitor H89 (30 μM). Values represent the mean ± S.E. of four experiments performed in duplicate.

**Fig. 3.** Effect of NO on Sp1 DNA binding measured by EMSA. A, nuclear extract from PMA (100 nM)-differentiated U937 cells was combined with radiolabeled Sp1 binding site probe. Site specific DNA-protein complexes are designated C1 and C2. The addition of anti-Sp1 antibody (lane 4) supershifted C1, and C2. B, nuclear extract for EMSA was prepared from differentiated cells incubated in medium alone, the NO donor SNAP (500 μM), the PKA inhibitor H89 (30 μM), the cell permeable cAMP analog Bt2cAMP (100 μM), or both SNAP (500 μM) and Bt2cAMP (100 μM).

**Fig. 4.** Effect of Sp1 site-directed mutation on NO responsiveness of the TNF-α promoter. U937 cells were transfected with wild type TNF-α promoter construct pTNF-tTA or Sp1 site-directed mutant pTNF(mSp1)-tTA, differentiated with PMA (100 nM), and incubated with medium alone, NO donors SNAP (500 μM) or SNOG (500 μM), the PKA inhibitor H89 (30 μM), the cell-permeable cAMP analog Bt2cAMP (100 μM) or both SNAP (500 μM) and Bt2cAMP (100 μM). Values represent the mean ± S.E. of three experiments performed in duplicate.

**Fig. 5.** Functional analysis of Sp1 binding sites in a heterologous, enhancerless CMV promoter. U937 cells were transfected with pCMVmin-tTA, pCMVmin(Sp1)3-tTA, or pCMVmin(mSp1)3-tTA, differentiated with PMA (100 nM), and incubated with medium alone, the NO donor SNAP (500 μM), the cell permeable cAMP analog Bt2cAMP (100 μM), or both SNAP (500 μM) and Bt2cAMP (100 μM). Values represent the mean ± S.E. of three experiments performed in duplicate.
by insertion of both Sp1 and mutated Sp1 binding sites was primarily due to inclusion of a spacer sequence between the second and third Sp1 regions (see “Experimental Procedures”) that was later found to unintentionally bind AP1 (data not shown). These results indicate that the addition of Sp1 binding sites into a heterologous promoter can convert it from NO unresponsive to responsive. Collectively, our findings demonstrate that the 8 bp Sp1 sequence (5′-CCCGGCCC-3′) can function as an essential part of a NO response element in the TNF-α promoter.

Modulation of promoter activity by NO has been reported to occur by either cGMP-dependent or -independent mechanisms and can result in either up-regulation or down-regulation of gene transcription (5–7, 27, 28). Work in this area has focused on relatively large promoter regions that appear to be involved in these NO mediated responses (6–7, 27). Other studies have identified transcription factors including NF-κB (3, 28), AP1 (22), and can result in either up-regulation or down-regulation of transcription factors to Sp1 flanking sequences which include AP1 and AP2 sites. This latter scenario would suggest that Sp1 is only part of a larger NO response complex.

Transcriptional regulation of TNF-α through the Sp1 binding site of its promoter may represent an important mechanism by which NO regulates inflammatory responses. Furthermore, Sp1 binding sites exist in many promoters (11–17, 23, 26). Thus, this Sp1-based regulatory system could account for the effects of NO on the expression of other gene products.

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ginger finger protein, by releasing zinc from thiol groups (32). NO up-regulation of TNF-α may also involve effects on the binding of transcription factors to Sp1 flanking sequences which include AP1 and AP2 sites. This latter scenario would suggest that Sp1 is only part of a larger NO response complex.

Transcriptional regulation of TNF-α through the Sp1 binding site of its promoter may represent an important mechanism by which NO regulates inflammatory responses. Furthermore, Sp1 binding sites exist in many promoters (11–17, 23, 26). Thus, this Sp1-based regulatory system could account for the effects of NO on the expression of other gene products.