Characterization of rabbit SP-B promoter region responsive to downregulation by tumor necrosis factor-α

KIFLU BERHANE, RAMGOPAL K. MARGANA, AND VIJAYAKUMAR BOGGARAM

Department of Molecular Biology, University of Texas Health Science Center at Tyler, Tyler, Texas 75708-3154

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Berhane, Kiflu, Ramgopal K. Margana, and Vijayakumar Boggaram. Characterization of rabbit SP-B promoter region responsive to downregulation by tumor necrosis factor-α. Am J Physiol Lung Cell Mol Physiol 279: L806–L814, 2000.—Surfactant protein B (SP-B) is essential for the maintenance of biophysical properties and physiological function of pulmonary surfactant. Tumor necrosis factor-α (TNF-α), an important mediator of lung inflammation, inhibits surfactant phospholipid and surfactant protein synthesis in the lung. In the present study, we investigated the TNF-α inhibition of rabbit SP-B promoter activity in a human lung adenocarcinoma cell line (NCI-H441). Deletion experiments indicated that the TNF-α response elements are located within −236 bp of SP-B 5′-flanking DNA. The TNF-α response region contained binding sites for nuclear factor-κB (NF-κB), Sp1/Sp3, thyroid transcription factor (TTF)-1, and hepatocyte nuclear factor (HNF)-3 transcription factors. Inhibitors of NF-κB activation such as dexamethasone and N-tosyl-l-phenylalanine chloromethyl ketone and mutation of the NF-κB element did not reverse TNF-α inhibition of SP-B promoter activity, indicating that TNF-α inhibition of SP-B promoter activity occurs independently of NF-κB activation. TNF-α treatment decreased the binding activities of TTF-1 and HNF-3 elements without altering the nuclear levels of TTF-1 and HNF-3 α proteins. Pretreatment of cells with okadaic acid reversed TNF-α inhibition of SP-B promoter activity. Taken together these data indicated that in NCI-H441 cells 1) TNF-α inhibition of SP-B promoter activity may be caused by decreased binding activities of TTF-1 and HNF-3 elements, 2) the decreased binding activities of TTF-1 and HNF-3α are not due to decreased nuclear levels of the proteins, and 3) okadaic acid-sensitive phosphatases may be involved in mediating TNF-α inhibition of SP-B promoter activity.

lung; respiratory distress syndrome; gene regulation; cytokines

SURFACTANT, a complex of lipids and proteins, is synthesized and secreted by type II epithelial cells of the lung. Surfactant maintains alveolar integrity through reduction of surface tension at the alveolar air-tissue interface (11) and plays important roles in host defense in the lung (36). Deficiency of surfactant is associated with the development of respiratory distress syndrome in preterm infants (2). Surfactant-associated proteins (SP), SP-A, SP-B, and SP-C, serve important roles in the biophysical properties, metabolism, and physiological function of surfactant (37). SP-B is particularly essential for the stabilization of the phospholipid monolayer formed on the alveolar surface (8). Deficiency of SP-B as a result of mutation in the SP-B gene causes fatal respiratory failure in infants with congenital alveolar proteinosis (25), and targeted disruption of the SP-B gene causes abnormalities in surfactant metabolism and respiratory failure in newborn mice (7). Heterozygous mice expressing 50% of normal SP-B levels displayed decreased lung compliance and air trapping, indicating that less than normal levels of SP-B may contribute to lung dysfunction (6). SP-B mRNA is developmentally and hormonally regulated in the lung. In the adult lung, SP-B mRNA is expressed in a cell type-specific manner by the alveolar type II and bronchiolar (Clara) epithelial cells (26, 39).

Adult respiratory distress syndrome (ARDS) is a life-threatening disease with a mortality rate of >50% (10). Abnormal surfactant function due to alterations in surfactant composition and metabolism may contribute to the pathophysiology of lung dysfunction in ARDS (19). The levels of total phospholipids, SP-A, and SP-B in bronchoalveolar lavage material are significantly reduced in ARDS patients and patients at risk for ARDS compared with those in normal individuals (12, 13). Tumor necrosis factor-α (TNF-α), an early-response cytokine, is an important mediator of lung inflammation (17, 35) and is present at high levels in the blood and alveolar lining fluid in ARDS (16).

TNF-α is thought to cause lung injury and subsequent respiratory failure by increasing lung epithelial permeability and sequestration and activation of neutrophils (35). TNF-α also inhibits surfactant phospholipid synthesis (3) and surfactant protein gene expression (38) that can potentially contribute to lung injury and respiratory failure in ARDS patients. TNF-α decreased SP-A and SP-B mRNA levels in NCI-H441 cells (38), a human pulmonary adenocarcinoma cell line with characteristics of bronchiolar epithelial cells (Clara cells), and SP-B mRNA content in the mouse
lung (27). Mechanisms by which TNF-α decreases SP-B mRNA expression are not well understood. In NCI-H441 cells, TNF-α was found to act at the posttranscriptional level to decrease SP-B mRNA expression and the 3’-untranslated region of SP-B mRNA was implicated to contain cis-acting elements necessary for destabilization of mRNA (28). The role of transcriptional mechanisms in mediating the inhibitory effects of TNF-α on SP-B mRNA expression is not known.

In the present study, we found that TNF-α inhibited SP-B promoter activity in NCI-H441 cells, indicating that transcriptional mechanisms have important roles in the TNF-α downregulation of SP-B gene expression. The objective of our investigation was to identify and characterize cis-DNA elements and interacting transcription factors that are required for TNF-α down-regulation of SP-B promoter activity. Deletion experiments showed that the SP-B minimal promoter (−236/+39 bp) (22) was inhibited by TNF-α and further deletion of 5’ DNA to −140 bp did not alter the response of the promoter. DNA binding activities of thyroid transcription factor (TTF)-1 and hepatocyte nuclear factor (HNF)-3 but not of Sp1 elements were decreased in nuclear extracts of TNF-α-treated cells. Our studies also showed that a nuclear factor-κB (NF-κB) element (−150/−141 bp) in the SP-B promoter was not important for TNF-α inhibition of SP-B promoter activity. These data suggested that TNF-α inhibition of SP-B promoter activity is mediated through the downregulation of binding activities of TTF-1 and HNF-3 elements.

MATERIALS AND METHODS

Cell culture and transfections. NCI-H441 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml) at 37°C in a humidified atmosphere of 5% CO₂ and air.

Plasmids were amplified in Escherichia coli Top10F’ strain (Invitrogen, Carlsbad, CA) and purified by anion-exchange chromatography (QIAGEN) according to the manufacturer’s protocol. Plasmid DNA was quantified by measuring absorbance at 260 nm, and its quality was verified by agarose gel electrophoresis and ethidium bromide staining.

At least two independent preparations of plasmids were used for transfection. Plasmid DNAs were transiently transfected into cells by liposome-mediated DNA transfer using LipofectAMINE (GIBCO BRL) as described previously (22). Cells were cotransfected with a bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml) for 15 min and then lysed by the addition of 0.5% Nonidet P-40. Cell lysate was centrifuged at 3,000 rpm for 5 min to pellet the nuclei, and the cytosolic fraction was removed, adjusted to 25% with respect to glycerol, and stored at −80°C. Nuclei were extracted by incubating in 100 μl of extraction buffer (20 mM HEPES, pH 7.9, 25% glycerol, 0.4 M KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml each of leupeptin and aprotinin, and 0.5 μg/ml benamidone) for 30 min followed by centrifugation at 14,000 rpm for 10 min. After centrifugation, the supernatant was divided into aliquots into chilled tubes, frozen in liquid nitrogen, and stored at −80°C. The protein concentration of nuclear extract was determined by Bradford’s method using Bio-Rad protein assay reagent (4).

Plasmid constructions and site-directed mutagenesis. The construction of plasmids containing different lengths of rabbit SP-B 5’-flanking DNA linked to the CAT gene has been described previously (21, 22). In all SP-B-CAT promoter constructs, the 3’ end point of SP-B 5’-flanking DNA was at +39 bp.

Nucleotides in the NF-κB binding site were mutated by site-directed mutagenesis by PCR according to Nelson and Long (24) as described previously (23). pSKCATαS containing the minimal SP-B promoter, −236/+39 bp, served as the template for mutation of the NF-κB binding sequence using mutagenic primers (mutated nucleotides are shown in bold), 1) −155-AGGGCAACAAATGCCCTGCTG-135 and 2) −161-AGGAGGCGCAACTGCCTGCTGCT-136.

Mutant DNA obtained using primer 1 was used as the template for PCR mutagenesis, with primer 2 to create mutations in the NF-κB binding sequence. TTF-1 (CTTGGAG → CAGGTG) and HNF-3 (GCAAACACT → GAGTCGCC) binding sites were mutated as described previously (23). Mutated DNA fragments were inserted upstream of CAT gene in pSKCATαS, and the sequence of the insert DNA was determined.

Electrophoretic mobility shift assays. Synthetic double-stranded oligonucleotides were annealed by heating 10 μM of single-stranded oligonucleotides in 10 mM Tris-HCl, pH 7.5, containing 10 mM MgCl₂ and 50 mM NaCl at 95°C for 5 min and then allowed to cool to room temperature over a period of 60 min. Double-stranded oligonucleotides were end labeled with [γ-³²P]ATP and T4 polynucleotide kinase. Electrophoretic mobility shift assays (EMSA) were performed by incubating 0.5 or 1.0 ng (3–10 × 10⁴ cpm) of the oligonucleotide probe with 5 μg of nuclear protein in 20 μl of binding buffer [15 mM HEPES, pH 7.9, 13% glycerol, 80 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol, 1 mM EDTA, and 0.2 or 0.5 μg of poly(dI-dC) as nonspecific competitor] for 20 min at 30°C. Nuclear proteins were incubated in binding buffer for 20 min at 30°C and then incubated with the labeled probe. Competition experiments were performed by addition of the indicated molar excess of cold wild-type or mutant oligonucleotide before addition of the labeled probe. The coding strand sequences of oligonucleotides (transcription factor binding sites are underlined and mutated nucleotides are shown in bold) used are as follows: Sp1 (−130 bp), −138-GCTGGAAAGGGGCTTTGCCTCAACAACA-114; Sp1 (−35 bp), −50-TCCCATGTCCTCCCGCCCCACGATCT-28; TTF-1 (−112 and −102 bp), −118-TCAAAACACTTGAGGAGGCTTCCAGGACAAG-90; HNF-3 (−88 bp), −96-GACAAAGGCAACACTGAGGTCT-75; NF-κB (wild type), −157-AGGGGAGAAATGCCTCGTCTG-135; NF-κB (mutant), −158-AGGGGGCAACTGGCCTG-136; and NF-κB (consensus), 5’-AGTGGAGGG-ACACTCCAGGG-3’.

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To determine the effects of antibodies on the mobility of protein-DNA complexes, nuclear extracts were incubated with 1 μg of nonimmune IgG or polyclonal antibodies to transcription factors for 1 h at room temperature or overnight at 4°C before incubation with the oligonucleotide probe. After incubation, the DNA-protein complexes were resolved by electrophoresis on a 6% non-denaturing polyacrylamide gel containing 0.5× 45 mM Tris-borate-1 mM EDTA (TBE) using 0.5× TBE as running buffer. In some experiments, 4 μl of 6× loading buffer (0.25% bromphenol blue, 0.25% xylene cyanol, and 15% Ficoll (type 400) in H2O) was added to the incubation mixtures before loading onto the gel. Electrophoresis was performed at constant current (30–35 mA) for 1.5–2 h. Gels were vacuum-dried and exposed to autoradiographic film. Polyclonal antibodies to human Sp1 and Sp3 and p50, p65, and c-Rel of NF-κB family of proteins were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibodies to forhead domains of mouse HNF-3α and HNF-3β were kindly supplied by Drs. Gunther Schutz and Wolfgang Schmid (German Cancer Research Center, Heidelberg, Germany). Polyclonal antisera to the NH₂-terminal portion of rat T/EBP (TTF-1) and rat TTF-1 were kindly supplied by Dr. Shiko Kimura (National Cancer Institute, Bethesda, MD) and Dr. Roberto di Lauro (Stazione Zoologica A. Dohrn, Naples, Italy). Antibodies against a peptide mapping at the amino-terminus (amino acids 110–122) of rat TTF-1 were purchased from the manufacturer’s protocol.

CAT and β-galactosidase assays. CAT activity of cell extracts was determined by the liquid scintillation counting assay [33] using [14C]chloramphenicol and n-butylryl coenzyme A as described previously [22]. β-Galactosidase activity was determined by the chemiluminescent assay using Ga lacto-Light Plus (TROPIX, Bedford, MA) substrate according to the recommended protocol. CAT activities were normalized to cotransfected β-galactosidase activity or protein content of cell extracts.

Immunoblotting. Nuclear and cytosolic proteins were separated by SDS-PAGE on 10% gels and electrophoretically transferred to nitrocellulose membranes. The blots were probed with polyclonal antibodies to TTF-1, HNF-3α, and Sp1 proteins and developed with an enhanced chemiluminescence detection system kit (Amersham Life Sciences, Piscataway, NJ).

Measurement of lactate dehydrogenase activity. The effect of TNF-α on cell toxicity was determined by measuring the lactate dehydrogenase (LDH) activity of culture medium. LDH activity was measured with CytoTox nonradioactive cytotoxicity assay kit (Promega, Madison, WI) according to the manufacturer’s protocol.

RESULTS

Effect of TNF-α on SP-B promoter activity. We determined the effect of TNF-α on SP-B promoter activity by transient transfection of SP-B-CAT promoter construct containing −2,176/+39 bp of SP-B 5′-flanking DNA into NCI-H441 cells. Results showed that TNF-α acted in a dose-dependent manner to decrease SP-B promoter activity (Fig. 1). At a concentration of 5 ng/ml and higher, TNF-α decreased SP-B promoter activity by 60–70% after 48 h of incubation. Measurement of LDH in cell culture medium indicated that TNF-α at concentrations of 5 and 25 ng/ml did not have any significant cytotoxic effects on the cells.

Deletion mapping of SP-B 5′-flanking region for identification of TNF-α response sequence. We used deletion mapping to identify SP-B genomic regions that are important for TNF-α inhibition of SP-B promoter activity. SP-B-CAT constructs containing SP-B 5′-flanking DNA deleted at the 5′-end were transiently transfected into NCI-H441 cells, and the effect of TNF-α (25 ng/ml) on CAT activity was determined after 48 h of incubation (Fig. 2). Results showed that the minimal SP-B promoter, −236/+39 bp, was inhibited by TNF-α and further deletion of 5′-flanking DNA to −140 bp did not alter response of the promoter. These data indicated that TNF-α-responsive elements are located within −236 bp of SP-B 5′-flanking DNA.

Functional role of NF-κB binding site in TNF-α inhibition of SP-B promoter activity. Deletion experiments showed that the minimal SP-B promoter was inhibited by TNF-α, and further deletion to −140 bp did not alter response of the promoter. These data suggested that the SP-B promoter regions −236/+39 and −140/+39 bp contain cis-DNA elements necessary for TNF-α inhibition. TNF-α regulation of a variety of genes is mediated through activation of the transcription factor NF-κB [9]. We sought to determine the role of NF-κB in TNF-α inhibition of SP-B promoter activity.

A search of the SP-B promoter sequence, −236/+39 bp, identified two potential NF-κB binding sequence motifs at positions −150/−141 (5′-AGGAATGCC-3′) and −9/+1 (5′-AGGACACC-3′) bp that are nearly identical to the consensus NF-κB binding sequence (5′-GGGRRNYYC-3′; R = A/G; Y = T/C, N = R/Y). EMSA indicated that only the distal NF-κB site (−150/−141 bp) formed a complex that was markedly induced in cells incubated with TNF-α (Fig. 3), whereas the proximal NF-κB site did not form an inducible complex.
We further examined the role of NF-κB activation by investigating the effects of mutation of the NF-κB element on TNF-α downregulation of SP-B promoter activity. EMSA demonstrated that mutation of the NF-κB site prevented binding of NF-κB, and as such, investigation of the effects of TNF-α on SP-B promoter containing a mutation within the NF-κB site would provide conclusive evidence for the functional role of NF-κB in TNF-α inhibition of SP-B promoter activity. Cells were transiently transfected with a SP-B promoter construct containing a mutation within the NF-κB site (AGGAA → CACGC), and the effect of TNF-α on SP-B promoter activity was determined after 48 h of incubation. Results showed that the degree of inhibition of SP-B promoter activity in cells transfected with wild-type or mutant SP-B promoters was similar (Fig. 4), indicating that mutation of the NF-κB element had no effect on TNF-α inhibition of SP-B promoter activity. These results are in agreement with the data of the effects of inhibitors of NF-κB activation on SP-B promoter activity and suggested that NF-κB does not have a functional role in TNF-α inhibition of SP-B promoter activity.

Functional role of Sp1, TTF-1, and HNF-3 elements in TNF-α inhibition of SP-B promoter activity. Our studies have shown that Sp1, TTF-1, and HNF-3 elements are necessary for SP-B promoter function and act in a combinatorial manner to maintain promoter activity (23). Because we found that the NF-κB element does not have a functional role in TNF-α inhibition of SP-B promoter activity, we investigated
whether alterations in the binding activities of Sp1, TTF-1, and HNF-3 elements are important for TNF-α inhibition of SP-B promoter activity.

NCI-H441 cells were incubated with and without TNF-α for 48 h, and nuclear extracts were prepared. The effect of TNF-α on the binding activities of Sp1, TTF-1, and HNF-3 elements was determined by EMSA. Results showed that whereas binding activities of Sp1 elements were unaltered in TNF-α-treated cells (data not shown), binding activities of TTF-1 and HNF-3 elements were reduced in nuclear extracts from TNF-α-treated cells (Fig. 5). We used antibodies in EMSA to assess the levels of transcription factors bound to their DNA elements in nuclear extracts from control and TNF-α-treated cells. Quantification of antibody-shifted bands by densitometric scanning showed that TNF-α treatment reduced TTF-1 binding activity by 40 ± 2.6% (n = 5). We were unable to accurately quantify the intensities of HNF-3α antibody-shifted bands because under the conditions of EMSA the antibody-shifted band was diffuse and did not completely migrate into the gel. We also were unable to determine whether TNF-α alters the binding of HNF-3β to its DNA element because the HNF-3β antibody inhibited protein-DNA complex formation rather than producing a supershifted protein-DNA complex (22). However, densitometric quantification of protein-DNA complex showed that binding to the HNF-3 element was reduced by 40 ± 3.4% (n = 4) in nuclear extracts from TNF-α treated cells.

**Effects of mutations in TTF-1 and HNF-3 binding sites on TNF-α inhibition of SP-B promoter activity.** TNF-α reduced DNA-binding activities of TTF-1 and HNF-3 elements in nuclear extracts of NCI-H441 cells, suggesting that TTF-1 and HNF-3 elements have important roles in TNF-α inhibition of SP-B promoter activity. To further assess the role of these elements in TNF-α inhibition of SP-B promoter, we determined the effects of mutations of these elements on TNF-α inhibition of SP-B promoter activity. Consistent with our previous findings, mutations of TTF-1 and HNF-3 elements caused 90% inhibition of SP-B promoter activity (23). Results showed that whereas TNF-α inhibited wild-type SP-B promoter by >50% (control = 100, treated = 48 ± 4.17, n = 4; one-tailed P = 0.0007 by one-sample t-test), it had significantly less effect on the mutant promoter (control = 9.9 ± 2.94, treated = 7.1 ± 1.4, n = 4; two-tailed P > 0.4 by unpaired t-test and Mann-Whitney test).

**Effect of TNF-α on the intracellular translocation of TTF-1 and HNF-3.** It was recently reported that phorbol ester inhibition of SP-B promoter activity in NCI-H441 cells is caused by decreased nuclear levels of TTF-1 and HNF-3 factors as a result of cytosolic trapping (34). To determine whether TNF-α inhibition of SP-B promoter activity is caused by changes in the intracellular translocation of TTF-1 and HNF-3, we investigated TTF-1 and HNF-3 DNA binding activities...
in the cytosolic and nuclear extracts of cells treated with and without TNF-α. Results showed that TTF-1 and HNF-3 binding activities were reduced in nuclear extracts of treated cells and no binding activity could be detected in cytosolic fractions (Fig. 6). This suggested that decreased DNA binding activities are not caused by changes in the intracellular translocations of transcription factors.

**Effects of TNF-α on the levels of TTF-1, HNF-3, and Sp1 proteins.** To determine whether reduced DNA binding activities of TTF-1 and HNF-3 are the result of reduced levels of TTF-1 and HNF-3 proteins, we determined the levels of TTF-1, HNF-3, and Sp1 in H441 cells treated with and without TNF-α by immunoblotting analysis. Analysis of the levels of the transcription factors in the nuclear and cytosolic fractions of cells showed that TNF-α treatment did not alter the levels of TTF-1, HNF-3α, and Sp1 in the nuclear or cytosolic fractions (Fig. 7). In contrast to TTF-1, which was not detected in the cytosolic fraction, HNF-3α and Sp1 were detected in the cytosolic fractions of control and treated cells.

**Effect of okadaic acid on TNF-α inhibition of SP-B promoter activity.** TNF-α decreased DNA binding activities of TTF-1 and HNF-3 elements without altering the levels of immunoreactive TTF-1 and HNF-3 proteins. This suggested that the reduced DNA binding activities of TTF-1 and HNF-3 could be due to factors that influence the binding affinities of the proteins. Phosphorylation is a widely recognized mechanism by which DNA binding activities of transcription factors are regulated (15). TTF-1 contains seven serine phosphorylation sites and is phosphorylated in vitro by protein kinase C (42). cAMP-dependent protein kinase A phosphorylation of TTF-1 has been implicated in the activation of SP-B (40) and SP-A (20) gene expression in lung epithelial cells. To determine whether TNF-α inhibition of SP-B promoter activity is the result of...
alterations in the phosphorylation status of key transcription factors, we investigated the effect of okadaic acid, a protein phosphatase inhibitor, on TNF-α inhibition of SP-B promoter activity. Results indicated that pretreatment of cells with okadaic acid for 2 h nearly reversed TNF-α inhibition of SP-B promoter activity (Fig. 8).

**DISCUSSION**

TNF-α has been implicated to have a major role in the pathogenesis of ARDS (17, 35). Reduced levels of surfactant, including surfactant proteins that occur in ARDS, could be a contributing factor leading to the development of lung dysfunction in ARDS (19). TNF-α decreases expression of SP-B mRNA in NCI-H441 cells in vitro (39) and in mouse lung in vivo (27). Decreased SP-B mRNA stability in TNF-α-treated NCI-H441 cells has been implicated to result in decreased mRNA levels (28). In the present study, we found that TNF-α decreased SP-B promoter activity, indicating that transcriptional mechanisms also contribute to the downregulation of SP-B gene expression by TNF-α.

Deletion mapping of SP-B 5′-flanking region showed that SP-B promoter constructs containing −236/+39 and −140/+39 bp were inhibited by TNF-α, indicating that TNF-α response sequences are located within −236 or −140 bp of SP-B 5′-flanking DNA. TNF-α has both stimulatory and inhibitory effects on gene expression, and its effects are often mediated through the activation of transcription factor NF-κB (9). A number of studies have shown that specifically the NF-κB p50/p65 homodimer functions as a transcriptional repressor (5, 18, 31). The minimal SP-B promoter contained a NF-κB binding site (−150/−141 bp) that is recognized by c-Rel, p50, and p65 proteins. Analysis of the functional role of NF-κB in TNF-α downregulation of SP-B promoter activity through the use of inhibitors of NF-κB activation such as dexamethasone and TPCK and mutation of the NF-κB site showed that NF-κB does not have a role in TNF-α downregulation of the SP-B promoter. These data are consistent with recent findings that showed TNF-α and phorbol ester inhibition of SP-A and SP-B mRNA accumulation in NCI-H441 cells occurs independently of NF-κB activation (29). The function of the NF-κB binding site in SP-B promoter function is unclear. Mutation of the NF-κB binding site caused an ~40% decrease in SP-B promoter activity (21), suggesting that it may be important for the basal activity of the SP-B promoter.

Our data indicated that TNF-α inhibited SP-B promoter activity independently of NF-κB activation, suggesting the involvement of other factors. We investigated the involvement of Sp1, TTF-1, and HNF-3 factors in TNF-α inhibition of the SP-B promoter. Sp1, TTF-1, and HNF-3 are essential for SP-B promoter activity and function in a cooperative or combinatorial manner to activate the promoter (23). We found that the binding activities of TTF-1 and HNF-3 elements were reduced by 40% in TNF-α-treated cells, suggesting that TNF-α inhibition of SP-B promoter activity might be caused by decreased binding activities of TTF-1 and HNF-3 elements.

TNF-α treatment did not alter the nuclear levels of immunoreactive TTF-1 and HNF-3 proteins. This suggested that the decreased binding activities of TTF-1 and HNF-3 elements in TNF-α-treated cells could be the result of alterations in the binding affinities rather than the levels of factors. These results indicated that the action of TNF-α to decrease SP-B promoter activity is different from that of phorbol ester. Phorbol ester inhibition of human SP-B promoter activity in H441 cells was suggested to be caused by cytosolic trapping of TTF-1 and HNF-3 proteins (34).

Okadaic acid nearly reversed TNF-α inhibition of SP-B promoter activity, implying that okadaic acid-sensitive phosphatases may have important roles in TNF-α inhibition of SP-B gene expression. It remains to be determined whether TTF-1 and HNF-3 proteins serve as targets for phosphatases activated by TNF-α. TNF-α treatment of neonatal rat cardiac myocytes resulted in a concentration-dependent increase in type 2A protein phosphatase activity, indicating the potential role of TNF-α in the control of protein function by phosphorylation (41). TTF-1 contains seven serine/threonine phosphorylation sites and is phosphorylated by protein kinase C in vitro (42). cAMP induction of SP-A phosphorylation sites and is phosphorylated by protein kinase C in vitro (42). cAMP induction of TTF-1 and HNF-3 proteins (34).

Fig. 8. Effect of okadaic acid on TNF-α inhibition of SP-B promoter activity in NCI-H441 cells. SP-B-CAT plasmid containing minimal SP-B promoter (−236/+39 bp) was transiently transfected into cells. After overnight incubation, cells were treated with varying concentrations of okadaic acid for 2 h. Afterward, medium containing okadaic acid was removed and cells were incubated in medium containing TNF-α (5 ng/ml) for 48 h. CAT activity in cell extracts was determined and normalized to cotransfected β-galactosidase activity. The data are means ± SE of 4 independent experiments. *Significantly different from cells treated with TNF-α alone, P < 0.01.
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HNF-3 without affecting their abundance. Pretreatment of cells with okadaic acid reversed TNF-α inhibition of SP-B promoter activity, implying that alterations in the phosphorylation status of key transcription factors may be necessary for inhibition of promoter activity. Our studies have also shown that TNF-α downregulation of SP-B promoter activity occurs independently of NF-κB activation. Although our results have indicated that TNF-α inhibition of SP-B promoter activity is the result of decreased binding activities of TTF-1 and HNF-3 elements, the involvement of other unidentified factors cannot be ruled out.

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