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TNF-α Suppresses Bone Sialoprotein (BSP) Expression in ROS17/2.8 Cells

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Abstract Tumor necrosis factor-alpha (TNF-α) is a major mediator of inflammatory responses in many diseases that inhibits bone formation and stimulates bone resorption. To determine molecular mechanisms involved in the suppression of bone formation we have analyzed the effects of TNF-α on BSP gene expression. Bone sialoprotein (BSP) is a mineralized tissue-specific protein that appears to function in the initial mineralization of bone. Previous studies have demonstrated that BSP mRNA expression is essentially restricted to fully-differentiated cells of mineralized connective tissues and that the expression of BSP is developmentally regulated. Treatment of rat osteosarcoma ROS 17/2.8 cells with TNF-α (10 ng/ml) for 24 h caused a marked reduction in BSP mRNA levels. The addition of antioxidant *N*-acetylcysteine (NAC; 20 mM) 30 min prior to stimulation with TNF-α attenuated the inhibition of BSP mRNA levels. Transient transfection analyses, using chimeric constructs of the rat BSP gene promoter linked to a luciferase reporter gene, revealed that TNF-α (10 ng/ml) suppressed expression in all constructs, including a short construct (pLUC3; nts −116 to +60), transfected into ROS17/2.8 cells. Further deletion analysis of the BSP promoter showed that a region within nts −84 to −60 was targeted by TNF-α, the effects which were inhibited by NAC and the tyrosine kinase inhibitor, herbimycin A (HA). Introduction of 2bp mutations in the inverted CCAAT box (ATTGG; nts −50 and −46), a putative cAMP response element (CRE; nts −75 to −68), and a FGF response element (FRE; nts −92 to −85) showed that the TNF-α effects were mediated by the CRE. These results were supported by gel mobility shift assays, using a radiolabeled double-stranded CRE oligonucleotide, which revealed decreased binding of a nuclear protein from TNF-α-stimulated ROS 17/2.8 cells. Further, the inhibitory effect of TNF-α on CRE DNA–protein complex was completely abolished by NAC or HA treatment. These studies, therefore, show that TNF-α suppresses BSP gene transcription through a tyrosine kinase-dependent pathway that generates reactive oxygen species and that the TNF-α effects are mediated by a CRE element in the proximal BSP gene promoter. J. Cell. Biochem. 87: 313–323, 2002.

Key words: bone sialoprotein; gene regulation; mineralized tissues; TNF-α; transcription

Abbreviations used: BSP, bone sialoprotein; TNF-α, tumor necrosis factor-alpha; LUC, luciferase; CRE, cyclic AMP response element; AP-1, activator protein-1; NFκB, nuclear factor κB; NAC, *N*-acetylcysteine; PDTC, pyrrolidine dithiocarbamate.

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Tumor necrosis factor-alpha (TNF-α) is a major cytokine of both acute and chronic inflammatory responses in many diseases, such as postmenopausal osteoporosis, rheumatoid arthritis, and periodontitis [Li and Stashenko, 1993; Gilbert et al., 2000]. TNF-α down-regulates the synthesis of bone matrix proteins and increases the production of interleukin-6 and macrophage colony-stimulating factor (M-CSF) by osteoblasts, thereby indirectly promoting differentiation of osteoclasts and enhancing bone resorption [Kurokouchi et al., 1998; Nagy et al., 2001]. Following receptor ligation, TNF-α stimulates multiple intracellular signaling pathways involving tyrosine kinase and protein kinase C activity, ADP-ribosylation, and generation of phospholipase A, phospholipase C, and reactive oxygen species, which activate NFκB [Kurokouchi et al., 1998, 2000, 2001; Sakai et al., 2001] and AP1 [Hanazawa et al., 1993; Lo and Cruz, 1995]. In osteoblasts, chondrocytes, and gingival fibroblasts TNF-α increases transcription of metalloproteinases 1 and 3 (MMP1, MMP3), intercellular adhesion molecule 1 (ICAM1), and cyclooxygenase 2 (COX2) [Kurokouchi et al., 1998; Sakai et al., 2001; Nakao et al., 2002], and down-regulates expression of collagen and insulin-like growth factor I, and suppresses 1, 25-dihydroxyvitamin D_3-stimulated osteocalcin expression in osteoblast-like cells [Nanes et al., 1989, 1991; Li and Stashenko, 1993; Gilbert et al., 2000]. TNF-α also inhibits osteoblast differentiation and bone-nodule formation by MC3T3-E1 cells [Gilbert et al., 2000]. Moreover, elevated levels of TNF-α were detected in gingival crevicular fluid during orthodontic tooth movement [Uematsu et al., 1996]. Collectively, these observations indicate that TNF-α has significant effects on bone cell metabolism.

Bone sialoprotein (BSP) is a sulfated and phosphorylated glycoprotein found almost exclusively in mineralized tissues [Oldberg et al., 1988; Ogata et al., 1995]. The temporo-spatial deposition of BSP into the extracellular matrix of bone [Chen et al., 1991, 1992] and the ability of BSP to nucleate hydroxyapatite crystal formation [Hunter and Goldberg, 1993] indicate a potential role for BSP in the initial mineralization of bone and cementum. BSP is also expressed in several pathologies in which mineralization occurs [reviewed in Ganss et al., 1999] including malignant breast cancer where it is associated with the formation of ectopic hydroxyapatite microcrystals [Ibrahim et al., 2000; Waltregny et al., 2000]. Thus, regulation of BSP gene transcription appears to be important in the differentiation of osteoblasts, for bone matrix mineralization and for pathologic mineralization. To study the transcriptional regulation of BSP gene expression, we and other groups have cloned and sequenced the rat, human, and mouse BSP gene promoters [Kerr et al., 1993; Li and Sodek, 1993; Kim et al., 1994; Benson et al., 1999], which are highly conserved within a 370 bp proximal region. This region includes an inverted TATA element [Li et al., 1995] overlapping a vitamin D response element [Kim et al., 1996] and an inverted CCAAT box (−50 to −46), which is required for basal transcriptional activity [Kim and Sodek, 1999; Shimizu and Ogata, 2002]. In addition, a fibroblast growth factor 2 response element (FRE; −92 to −85) [Shimizu-Sasaki et al., 2001], a pituitary-specific transcription factor-1 (Pit-1) motif (−111 to −105), that mediates the stimulatory effects of parathyroid hormone [Ogata et al., 2000], and a homeodomain binding element (HOX; −199 to −192) [Benson et al., 2000], have been characterized. Further upstream in the rat promoter a transforming growth factor-β activation element (−499 to −485) [Ogata et al., 1997] and a glucocorticoid response element (−920 to −906), overlapping an AP-1 site (−921 to −915) [Ogata et al., 1995; Yamauchi et al., 1996] has also been identified.

To elucidate molecular pathways through which TNF-α suppresses bone formation we have analyzed the effects of TNF-α on BSP gene expression in ROS 17/2.8 cells. These studies show that TNF-α exerts suppresses transcription of the BSP gene through a tyrosine kinase pathway that involves reactive oxygen species, the effects being mediated through a CRE located between nts −75 to −68 in the proximal promoter.

METHODS

Materials

Cell culture media, fetal bovine serum (FBS), LipofectACE, penicillin, and streptomycin, trypsin were obtained from GIBCO BRL Life Technologies (Tokyo, Japan). The pGL2-promoter vector and pSV-β-galactosidase control vector were purchased from Promega Co. (Madison, WI). *N*-acetylcysteine (NAC) was purchased from Sigma-Aldrich Japan (Tokyo, Japan), the protein kinase inhibitors H89 and

H7 were from Seikagaku Corporation (Tokyo, Japan), and herbimycin A (HA) and guanidium thiocyanate were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). PP1 was from Biomol Research Laboratories, Inc. (Plymouth Meeting, PA), and recombinant human TNF-α was from Genzyme, Techne (Minneapolis, MN). TACS apoptotic DNA laddering kits were purchased from Trevigen, Inc. (Gaithersburg, MD).

Cell Culture

The rat clonal cell line, ROS 17/2.8 (generously provided by Dr. G.A. Rodan) was used in these studies as an osteoblastic cell line that synthesizes BSP [Majeska et al., 1980; Ogata et al., 1995]. Cells grown to confluence in 60 mm tissue culture dishes in α-MEM medium containing 10% fetal calf serum (FCS) were changed to α-MEM without serum and incubated with or without 10 ng/ml TNF-α in the absence or presence of 20 mM *N*-acetylcysteine (NAC) for time periods extending over 3–24 h to determine the effects of TNF-α on the expression of BSP mRNA. RNA was isolated from triplicate cultures at various time intervals and analyzed for the expression of BSP mRNA by Northern hybridization as described below.

Northern Hybridization

Total RNA from the ROS 17/2.8 was extracted with guanidium thiocyanate and, following purification, 20 μg aliquots of RNA were fractionated on 1.2% agarose gel and transferred onto a Hybond XL membrane, as described previously [Ogata et al., 1997]. Hybridizations were performed at 42°C with a ^{32}P-labeled rat BSP, a rat osteopontin (OPN) or a rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes. Following hybridization, membranes were washed four times for 5 min each at 21°C in 300 mM sodium chloride, 30 mM trisodium citrate pH 7.0 containing 0.1% SDS. This was followed by two, 20 min washes at 55°C in 15 mM sodium chloride, 1.5 mM trisodium citrate pH 7.0, 0.1% SDS. The hybridized bands, representing the two polyadenylated forms (1.6 kb and 2.0 kb) of rat BSP mRNA, were scanned in a Bio-imaging analyzer (BAS2000, Fuji BAS 2000).

Detection of Apoptosis

Trevigen's TACS apoptotic DNA laddering kits were used to assay cells for apoptosis by determining the level of DNA degradation [Smith and Fornace, 1996].

Transient Transfection Assays

Exponentially growing ROS 17/2.8 cells were used for the transfection assays. Twenty-four hours after plating, cells at 50–70% confluence were transfected using a LipofectACE reagent. The transfection mixture included 1 μg of a luciferase (LUC) construct and 2 μg pSV-β-galactosidase control vector as an internal control [Ogata et al., 1995]. One day post-transfection, cells were deprived of serum for 12 h and 10 ng/ml TNF-α was added for 24 h prior to harvesting. The LUC assay was performed according to the supplier's protocol (PicaGene, Toyo Inki, Tokyo, Japan) using a Luminescence reader BLR20 (Aloka) to measure the LUC activity. Antioxidant, NAC (20 mM) was used to decrease the levels of reactive oxygen species. The protein kinase inhibitor H89 (5 μM) and H7 (5 μM) were used to inhibit protein kinases A and C. HA (1 μM) and PP1(10 μM) were used for tyrosine kinase and Src tyrosine kinase inhibition, respectively. Oligonucleotide-directed mutagenesis by PCR was utilized to introduce the dinucleotide substitutions using the Quickchange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA). All constructs were sequenced as described previously to verify the fidelity of the mutagenesis.

Gel Mobility Shift Assays

Confluent ROS 17/2.8 cells in four T-75 flasks incubated for 6, 12, and 24 h with 10 ng/ml TNF-α in α-MEM without serum were used to prepare nuclear extracts. Nuclear protein was extracted by the method of Dignam et al. [1993] with the addition of extra proteinase inhibitors (the extraction buffer was 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM dithiothreitol (DTT), 25% (v/v) glycerol, 0.5 mM phenylmethyl-sulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 1 μg/ml aprotinin, pH 7.9). Protein concentration was determined by Bradford [1976] method. Double-stranded oligonucleotides corresponding to the inverted CCAAT (nts −61 to −37, 5′-CCGTGACCGTG**ATTGG**CTGCTGAGA), the cAMP response element (CRE; nts −84 to −59, 5′-CCCACAGCC**TGACGTCG**CACCGGCCG), the FRE (nts −98 to −79, 5′-TTTTCT**GGTGAGAA**CCCACA), BSP-NFκB (nts −112 to −93, 5′-GTTGTAGTTA**CGGATTTTCT**) in the rat BSP gene promoter,

and CCAAT/enhancer-binding protein (C/EBP) responsive element (HS3D; 5′-TAGAGCC-TG**CGCAATCG**AAA) in the rat insulin-like growth factor-I (IGF-I) gene [Thomas et al., 1996] were prepared by Bio-Synthsis Inc. (Lewisville, TX); while consensus AP1 (5′-CG-CTTGA**TGAGTCA**GCCGGAA) was purchased from Promega. For gel shift analysis the double-stranded-oligonucleotides were end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Nuclear protein extracts (3 μg) were incubated for 20 min at room temperature (21°C) with 0.1 pM radiolabeled double-stranded oligonucleotide in buffer containing 60 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 15 mM Tris-HCl (pH 7.5), 0.5 mM DTT, 5% glycerol, and 1 μg/ml poly (dI-dC). For competition experiments unlabelled oligonucleotides for the inverted CCAAT, CRE, BSP-NFκB, HS3D, and consensus AP1 (Promega) were used at 20-, 40- and 100-fold molar excess. Following incubation, the protein–DNA complexes were resolved by electrophoresis on 5% non-denaturing acrylamide gels (38:2 acrylamide/bis acrylamide) run at 150 V at RT. Following electrophoresis, the gels were dried and autoradiograms prepared and analyzed using an image analyzer.

Statistical Analysis

Triplicate samples were analyzed for each experiment, which was replicated at least three times to ensure consistency of the responses to TNF-α. Significant differences between control and TNF-α treatment were determined using Student's *t*-test.

RESULTS

Suppression of BSP mRNA Expression in ROS 17/2.8 Cells

We studied the effect of TNF-α on BSP mRNA expression in osteoblastic ROS 17/2.8 cells [Majeska et al., 1980]. To determine the dose-response for TNF-α regulation of BSP, confluent ROS 17/2.8 cells were treated with different concentrations of TNF-α for 24 h. BSP mRNA levels were determined by Northern blot analysis. TNF-α decreased BSP transcripts at 1–100 ng/ml and had a maximal effect between 10 and 100 ng/ml (Fig. 1A). Thus, 10 ng/ml of TNF-α was used to determine a time course of BSP mRNA expression (Fig. 1B). TNF-α suppressed BSP mRNA level markedly at 24 h. In comparison, OPN mRNA was increased slightly at 12 h

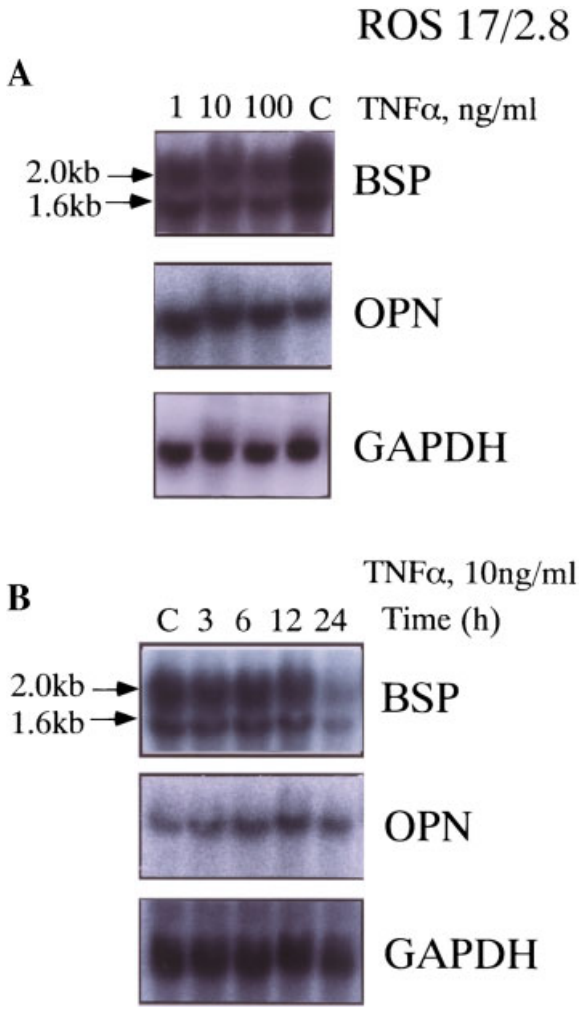

Fig. 1. Northern hybridization analysis of TNF-α effects on BSP mRNA expression. **A**: Dose–response effect of TNF-α on BSPmRNA levels in the osteoblastic cell line ROS17/2.8 treated for 24 h. At 1–100 ng/ml, TNF-α decreased BSPmRNA with a maximal effect at 10 ng/ml. This optimal level of TNF-α was used to determine a time course of BSPmRNA expression. **B**: A 24 h time-course revealed a decrease in BSP mRNA at 24 h after the administration of 10 ng/ml TNF-α to ROS17/2.8 cells. Total RNA was isolated from triplicate cultures harvested after incubation times of 3, 6, 12, and 24 h, and used for Northern hybridization analysis using a ^{32}P-labelled rat BSP DNA probe and a GAPDH DNA probe.

and returned to base line at 24 h, whereas no effect on GAPDH mRNA was observed. DNA extracted from ROS 17/2.8 cells that were cultured for 12 or 24 h in the presence of TNF-α (10 ng/ml) showed no evidence of DNA degradation into oligonucleosome fragments (data not shown).

Since TNF-α is known to stimulate reactive oxygen species we analyzed the effects of an antioxidant on the suppressive effect of TNF-α in ROS 17/2.8 cells. The cells were treated with

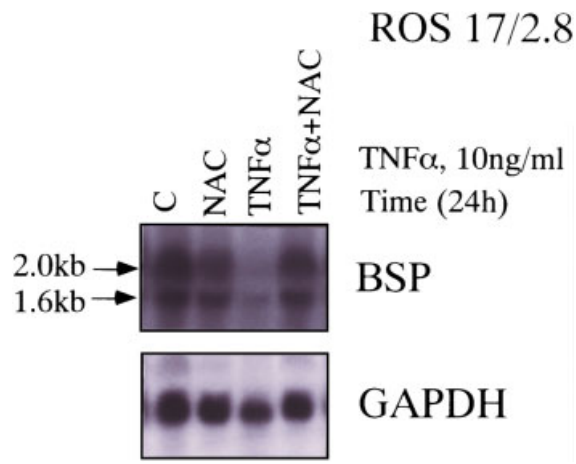

Fig. 2. Effect of antioxidant on the suppressive effect of TNF-α in ROS17/2.8 cells. The cells were treated with 20 mM *N*-Acetylcystein (NAC) for 30 min and then incubated with TNF-α together with NAC for 24 h. NAC markedly attenuated the TNF-α-dependent suppression of BSP mRNA.

20 mM NAC for 30 min and then incubated with TNF-α together with NAC for 24 h. NAC markedly attenuated the TNF-α-dependent suppression of BSP mRNA (Fig. 2).

Transient Transfection Analysis of Rat BSP Promoter Constructs

To determine how TNF-α regulates BSP expression sequences from the 5′-flanking region of the BSP gene were tested for transcriptional activity and responsiveness to TNF-α by transient transfection of chimeric constructs in ROS 17/2.8 cells. The constructs used (pLUC1–pLUC5) and their responsiveness to TNF-α are shown in Figure 3A. The transcriptional activity of pLUC3, which encompasses nucleotides −116 to +60, was reduced ∼0.59 (0.59 ± 0.02)-fold and almost the same level of decrease were observed in constructs pLUC4 and pLUC5. However, deletion of the sequence between −116 and −43 abolished the TNF-α-mediated reduction (pLUC2 and pLUC1). Notably, the suppressive effects of TNF-α on transcriptional activity (pLUC3) were completely inhibited by the antioxidant NAC (Fig. 3B). Within the DNA sequence of pLUC3 that is unique in this region (nts −116 and −43), is an inverted CCAAT box (ATTGG; nts −50 and −46), a cAMP response element (CRE; nts −75 to −68), a fibroblast growth factor 2 response element (FRE; nts −92 and −85), a putative NFκB site (BSP-NFκB; between nts −93 and −102) and a Pit-1 motif (between nts −111 and −105), which is a target of parathyroid hormone stimulation (Fig. 4). To

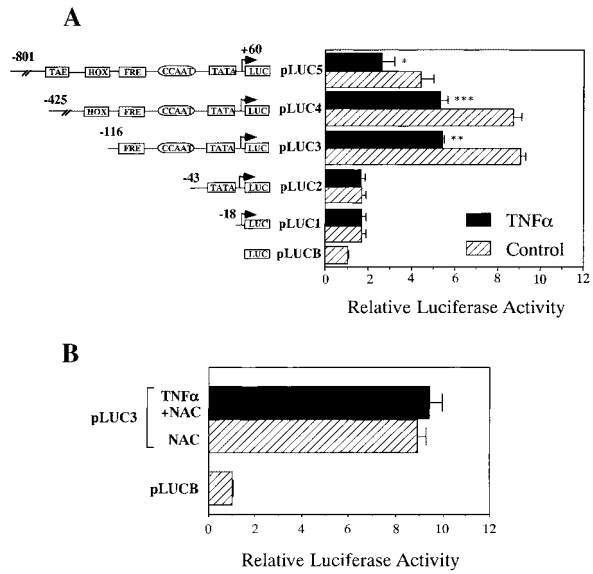

Fig. 3. **A**: TNF-α down-regulates BSP promoter activity. Transient transfections of ROS 17/2.8 cells, in the presence or absence of TNF-α (10 ng/ml) for 24 h, were used to determine transcriptional activity of chimeric constructs that included various regions of the BSP promoter ligated to a LUC reporter gene. The results of transcriptional activity obtained from three separate transfections with constructs; pLUC basic (pLUCB) and pLUC1 to pLUC5 have been combined and the values expressed with standard errors. Significant differences from control: *($P < 0.1$); **($P < 0.05$); ***($P < 0.02$). **B**: The suppressive effect of TNF-α on BSP promoter activity (pLUC3) is inhibited by antioxidant NAC. Transiently transfected ROS 17/2.8 cells were pre-treated with 20 mM NAC for 30 min and then incubated with TNF-α together with NAC for 24 h.

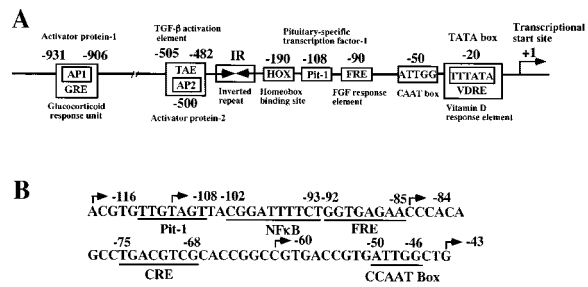

Fig. 4. **A**: Regulatory elements in the proximal rat BSP promoter. The inverted TATA and CCAAT boxes, FGF2 response element (FRE), pituitary-specific transcription factor-1 (Pit-1) motif, homeodomain binding site (HOX), inverted repeat (IR), TGF-β activation element (TAE) overlapping with AP2 and glucocorticoid response element (GRE) overlapping the AP1, a vitamin D response element (VDRE) that overlaps the inverted TATA box are present within a proximal promoter are shown. **B**: The nucleotide sequences of proximal promoter region of the rat BSP gene are shown from nucleotides −116 to −43. The inverted CCAAT box, cAMP response element (CRE), nuclear factor-kappa B (NFκB), Pit-1, and FGF response element (FRE) are present.

more closely define the regulatory element between nts −116 and −43 that is utilized by TNF-α, we prepared a series of 5′ deletion constructs. Construct −84BSPLUC and longer constructs suppressed by TNF-α. The results showed that the element responding to TNF-α was present between nt −84 and the transcription start site in the BSP promoter (Fig. 5). Next, we introduced two base pair mutations in the pLUC3 construct. Whereas mutations in the FRE had no effect on TNF-α suppression and mutation of the CCAAT box essentially abolished basal expression, mutation of the CRE significantly reduced the TNF-α effects on the transcriptional activity (Fig. 6).

Since protein kinases mediate TNF-α signaling, we also investigated the effects of the protein kinase C inhibitor H7, the protein kinase A inhibitor H89, the tyrosine kinase inhibitor HA, and the Src tyrosine kinase inhibitor PP1 on TNF-α-mediated transcription. Whereas TNF-α suppression of −116BSPLUC promoter activity was inhibited by HA, no effects were observed for PKC, PKA, and PP1 (Fig. 7), indicating an involvement of tyrosine kinase in the signaling pathway.

Gel Mobility Shift Assays

To clarify nuclear proteins that bind to the CRE and mediate the TNF-α effects on transcription, double-stranded oligonucleotides were end-labeled and incubated with equal amount (3 μg) of nuclear proteins extracted from confluent ROS 17/2.8 cells that were either not treated (control) or treated with 10 ng/ml TNF-α for 6, 12, and 24 h. When we used the inverted CCAAT sequence as a probe, the DNA–NF–Y protein complex [Tezuka et al., 1996; Kim and Sodek, 1999; Shimizu and Ogata, 2002] did not

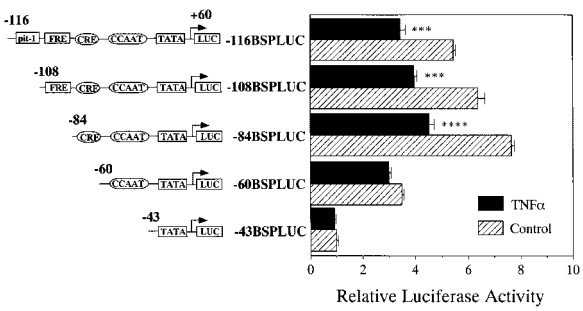

Fig. 5. Fine 5′-deletion mapping of the nts −116 to −43 element in the BSP promoter. A series of rat BSP promoter 5′ deletion constructs were analyzed for relative promoter activity after transfection into ROS17/2.8 cells and examined for reduction in the presence of TNF-α (10 ng/ml) for 24 h. The results demonstrated that the TNF-α response region exists in between −84 and transcriptional start site in the BSP promoter. The results of transcriptional activity obtained from three separate transfections with constructs; −43 BSPLUC (−43 to +60), −60 BSPLUC (−60 to +60), −84 BSPLUC (−84 to +60), −108 BSPLUC (−108 to +60), −116 BSPLUC (−116 to +60) have been combined and the values expressed with standard errors. Significant differences from control: ***($P < 0.02$); ****($P < 0.01$).

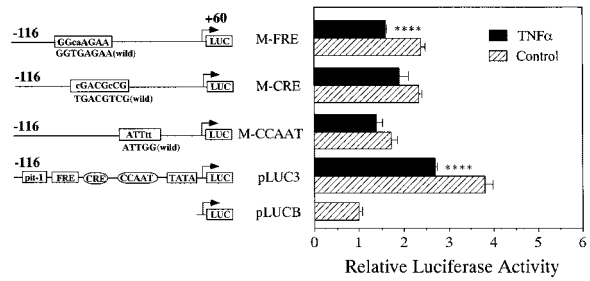

Fig. 6. CRE in the rat BSP promoter are necessary for suppressive effect of TNF-α. Dinucleotide substitutions were made within context of the homologous −116 to +60 BSP promoter fragment (pLUC3). M-CCAAT (ATTtt), M-CRE (cGACGcCG), and M-FRE (GGcaAGAA) constructs were analyzed for relative promoter activity after transfection into ROS17/2.8 cells and examined the effects of TNF-α for 24 h. The results of transcriptional activity obtained from three separate transfections with constructs; pLUCB, pLUC3, M-CCAAT, M-CRE, and M-FRE were combined and the values expressed with standard errors. Significant differences from relative LUC activity of pLUC3: ****($P < 0.01$).

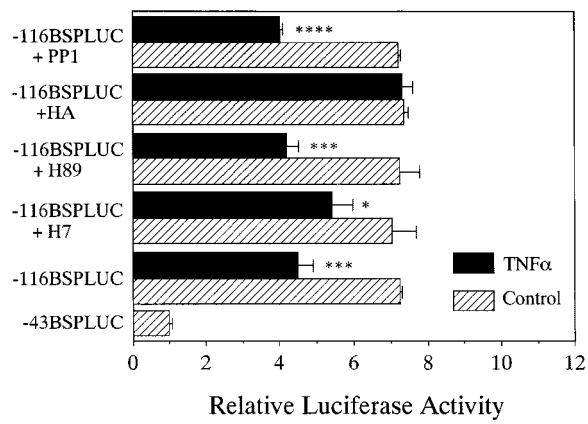

Fig. 7. Effect of kinase inhibitors on transcriptional regulation by TNF-α. Transient transfection analysis of −116BSPLUC in the presence or absence of TNF-α (10 ng/ml) in ROS 17/2.8 cells is shown together with the effects of the PKC inhibitor (H7, 5 μM), PKA inhibitor (H89, 5 μM), tyrosine kinase inhibitor (HA, 1 μM), and Src kinase inhibitor (PP1, 10 μM). H7, H89, and PP1 had no effect on the TNF-α suppression effect. On the other hand, HA completely abolish the TNF-α effect. The results obtained from three separate transfections were combined and the values expressed with standard errors. Significant differences from control: *($P < 0.1$); ***($P < 0.02$); ****($P < 0.01$).

change after TNF-α stimulation (Fig. 8, lanes 1–4). With nuclear extracts from confluent, control cultures of ROS 17/2.8 cells, a shift of a diffuse CRE DNA–protein complex was evident (Fig. 8, lane 5). After stimulation by TNF-α (10 ng/ml) for 12 and 24 h, DNA binding activity was decreased (Fig. 8, lanes 7, 8). That the DNA–protein complex represents a specific interaction was indicated by competition experiments in which an excess of CRE reduced the amount of complex formed in a dose-dependent manner (20-, 40-, and 100-fold molar excess) (Fig. 9, lanes 2–4). In contrast, inverted CCAAT, AP1, and BSP-NFκB oligonucleotides (20–100-fold excess) did not compete with complex formation (Fig. 9, lanes 5–7, 8–10, and 11–13). Notably,

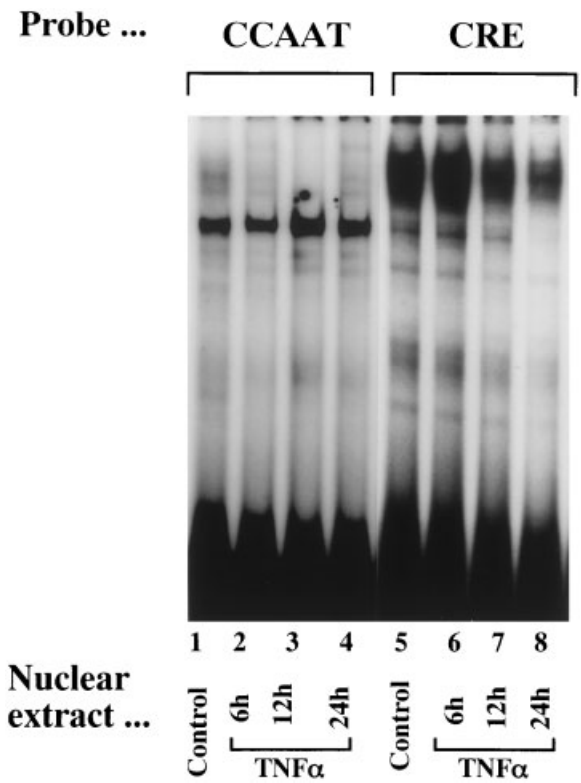

Fig. 8. TNF-α decreases the DNA binding activity recognized by CRE sequence. Radiolabeled double-stranded inverted CCAAT (−61 CCGTGACCGTG**ATTGG**CTGCTGAGA −37) and CRE (−84 CCCACAGCC**TGACGTCG**CACCGGCCG −59) oligonucleotides were incubated for 20 min at 21°C with nuclear protein extracts (3 μg) obtained from ROS 17/2.8 cells incubated without (**lanes 1** and **5**) or with TNF-α (10 ng/ml) for 6 h (**lanes 2** and **6**), 12 h (**lanes 3** and **7**), and 24 h (**lanes 4** and **8**). DNA–protein complexes were separated on 5% polyacrylamide gel in low-ionic-strength Tris-borate buffer, dried under vacuum, and exposed to an imaging plate for quantitation using an imaging analyzer.

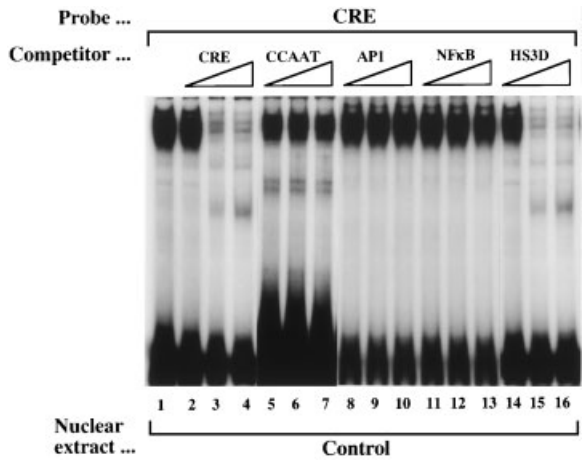

Fig. 9. Specific binding of a nuclear protein to the CRE. Radiolabeled double-stranded CRE was incubated for 20 min at 21°C with nuclear protein extracts (3 μg) obtained from control confluent ROS 17/2.8 cells (**lane 1**). Competition reactions were performed using a 20-, 40-, 100-fold molar excess of unlabelled CRE (−84 CCCACAGCC**TGACGTCG**CACCGGCCG −59; **lanes 2–4**), inverted CCAAT (−61 CCGTGACCGTG**ATTGG**CTGCTGAGA −37; **lanes 5–7**), consensus AP1 (CGCTTGA**TGAGTCA**GCCGGAA; **lanes 8–10**), BSP-NFκB (−112 GTTGTAGTTA**CCGGATTTTCT** −93; **lanes 11–13**), and HS3D (TAGAGCCTG**CGCAATCG**AAA; **lanes 14–16**). DNA–protein complexes were separated on 5% polyacrylamide gel in low-ionic-strength Tris-borate buffer, dried under vacuum, and exposed to an imaging plate for quantitation using an imaging analyzer.

HS3D (C/EBP responsive element) was able to compete for CRE DNA–protein complex formation (Fig. 9, lanes 14–16). Since the results of northern hybridization and transient transfection assays indicate the effects of TNF-α are mediated through a reactive oxygen species and a tyrosine kinase-dependent pathway, we used NAC and HA together with TNF-α to prepare the nuclear extracts. While NAC and HA had no effect on the CCAAT DNA–NF–Y protein complex (Fig. 10, lanes 3–6), both drugs completely abolished the inhibitory TNF-α on the formation of CRE DNA–protein complex (Fig. 10, lanes 9–12).

We also used gel shift analyses to evaluate the potential effects of TNF-α on the nearby NFκB and AP1 sites. Although a BSP-NFκB DNA–protein complex was formed it did not change after TNF-α stimulation (Fig. 11, lanes 1–6), whereas AP1 binding was increased by TNF-α (Fig. 11, lane 8). The effects of TNF-α on the AP1 DNA–protein complex did not influence by antioxidant NAC (Fig. 11, lane 10) and abolished by tyrosine kinase inhibitor, HA Fig. 11, lane 12).

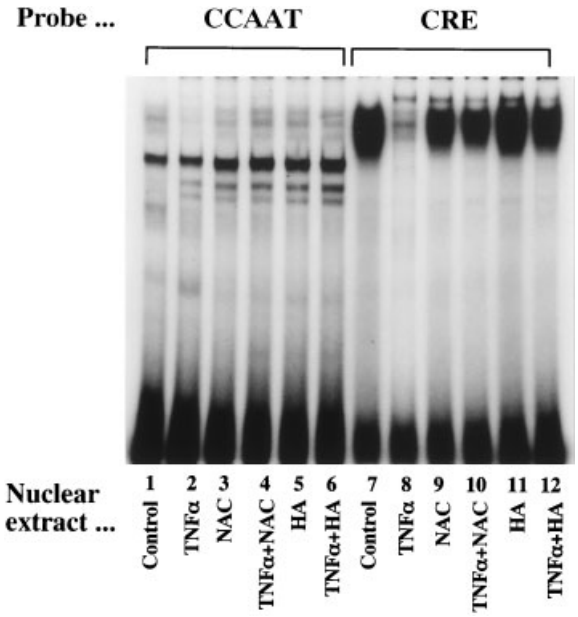

Fig. 10. NAC and herbimycin A (HA) completely blocked the suppressive effects of TNF-α on CRE DNA–protein complex formation. Radiolabeled double-stranded inverted CCAAT and CRE oligonucleotides were incubated for 20 min at 21°C with nuclear protein extracts (3 μg) obtained from ROS 17/2.8 cells treated without (**lanes 1** and **7**) and with TNF-α (**lanes 2** and **8**), NAC (**lanes 3** and **9**), HA (**lanes 5** and **11**) for 24 h, and preincubated with 20 mM NAC 30 min or HA 12 h and then incubated with TNF-α together with NAC (**lanes 4** and **10**) or HA (**lanes 6** and **12**) for 24 h. DNA–protein complexes were separated on 5% polyacrylamide gel in low-ionic-strength Tris-borate buffer, dried under vacuum, and exposed to an imaging plate for quantitation using an imaging analyzer.

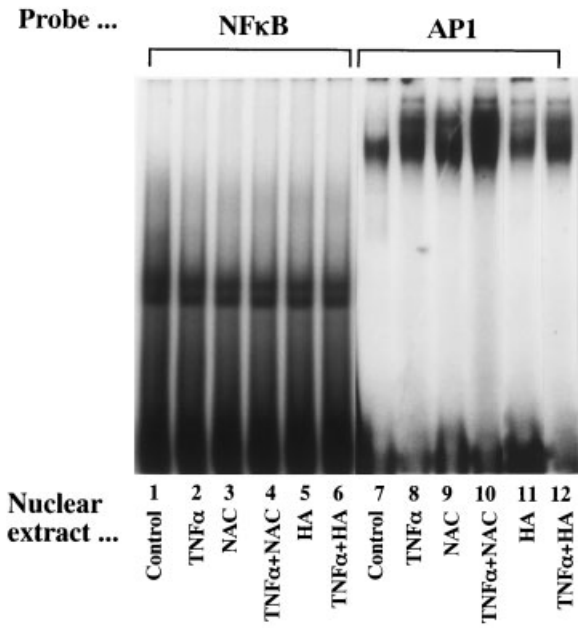

Fig. 11. Gel mobility shift assays using NFκB and AP1 oligonucleotides. Radiolabeled double-stranded BSP-NFκB and consensus AP1 oligonucleotides were incubated for 20 min at 21°C with nuclear protein extracts (3 μg) obtained from ROS 17/2.8 cells treated without (**lanes 1** and **7**) and with TNF-α (**lanes 2** and **8**), NAC (**lanes 3** and **9**), HA (**lanes 5** and **11**) for 24 h, and preincubated with 20 mM NAC 30 min or HA 12 h and then incubated with TNF-α together with NAC (**lanes 4** and **10**) or HA (**lanes 6** and **12**) for 24 h. DNA–protein complexes were separated on 5% polyacrylamide gel in low-ionic-strength Tris-borate buffer, dried under vacuum, and exposed to an imaging plate for quantitation using an imaging analyzer.

DISCUSSION

Our studies show that TNF-α suppresses the expression of BSP, a major protein of the bone matrix that is involved in osteogenic differentiation and the formation of mineral. Treatment of ROS 17/2.8 cells with TNF-α decreased the steady-state level of BSP mRNA (Fig. 1B), reflecting a reduction of gene transcription measured by transient transfection assays. The effects of TNF-α were mediated by tyrosine kinase activity and the formation of reactive oxygen species. From a combination of transcription and gel mobility shift assays the site of transcriptional regulation by TNF-α could be identified as a CRE element, which is located close to the CCAAT box in the highly conserved proximal promoter of the BSP gene. When we used rat stromal bone marrow cells (SBMC) [Pitaru et al., 1993] for LUC assays to see the effect of TNF-α on BSP transcription, TNF-α suppressed BSP transcription [H. Samoto, E. Shimizu and Y. Ogata, unpublished results]. Therefore, TNF-α suppresses BSP expression not only in transformed ROS 17/2.8 cells but also in normal osteoprogenitors.

TNF-α is a pro-inflammatory cytokine that stimulates bone resorption [Tashjian et al., 1987]. In addition to direct effects on osteoclasts, TNF-α also increases the synthesis of Interleukin-6 (IL-6) [Passeri et al., 1994], IL–8 [Chaudhary et al., 1992], and M-CSF [Felix et al., 1989], while inhibiting collagen [Bertolini et al., 1986], proteoglycan [Saklatvala, 1986] and osteocalcin synthesis [Nanes et al., 1991] by osteoblastic cells. Although TNF-α rapidly induces the activation of NFκB, and increases expression of IL-6 and intercellular adhesion molecule-1 (ICAM-1) within 3 h in ROS17/2.8 cells [Kurokouchi et al., 1998], suppression of BSP mRNA expression was maximal at 24 h (Fig. 1B), suggesting that TNF-α regulates BSP transcription indirectly. It has been proposed that oxygen radicals participate in NFκB

activation pathways, and compounds that have antioxidant activities, such as NAC and pyrrolidine dithiocarbamate (PDTC), block a reaction required for release of IκB from NFκB [Schreck et al., 1992]. The ability of the NAC (Figs. 2 and 3B) and tyrosine kinase inhibitor HA (Fig. 7) to block the TNF-α effects on the BSP mRNA expression and promoter activity implicated NFκB activation and tyrosine phosphorylation as early events in the signaling pathway of TNF-α. However, BSP-NFκB binding did not change after stimulation by TNF-α (Fig. 11), demonstrating that TNF-α does not regulate BSP via NFκB.

That the CRE element mediates the effects of TNF-α on BSP was initially determined by transient transfection assays in which promoter constructs spanning the proximal promoter (−116 and −43; Fig. 3A) of the BSP gene were used. This region encompasses an inverted CCAAT box (nts −50 and −46), an FGF response element (FRE; nts −92 and −85), a putative NFκB (nts −102 and −93), and a Pit-1 motif (nts −111 and −105), as well as the CRE (nts −75 and −68) (Fig. 4). Mutation of the CRE was used to demonstrate that the effects of TNF-α could be blocked in the absence of a functional CRE element (Fig. 6). However, the possible involvement of the inverted CCAAT box was more difficult to ascertain since basal transcriptional activity is lost when this element is mutated [Kim and Sodek, 1999]. Thus, to further establish the site of BSP gene regulation, gel mobility shifts were used to identify proteins in nuclear extracts of ROS 17/2.8 cells that selectively bound to the CRE and inverted CCAAT sequences (Fig. 8). CRE-binding proteins were down-regulated by TNF-α in association with decreased transcription. In contrast, the protein binding to the inverted CCAAT did not change after stimulation with TNF-α, while the CRE DNA–protein complex was unaffected by an inverted CCAAT, and consensus AP1 and BSP-NFκB (Fig. 9), confirming the involvement of the CRE.

CRE was first identified as an inducible enhancer of genes that can be transcribed in response to an increased cAMP level. Multiple CRE-binding proteins such as CREB [Alberts et al., 1994], ATFs [Chen et al., 1994], and C/EBPs [Poli et al., 1990] have been identified. All of these proteins have a basic region adjacent to a leucine zipper motif (bZip domain) [Laoide et al., 1993]. The leucine zipper is responsible for the dimerization of the protein and dimerization is a prerequisite for DNA binding [Dwarki et al., 1990]. The CRE is bound by CREB homodimers, ATF2 homodimers, ATF3 homodimers, C/EBP family members, and Jun-Fos family heterodimers [Benbrook and Jones, 1994]. A consensus CRE is constituted by an 8 bp palindromic sequence (TGACGTCA) [Comb et al., 1986]. The CRE in BSP promoter is identical in sequence (TGACGTCG) to the consensus CRE except for a single, A to G, nucleotide change [Ogata et al., 2000]. Interestingly, the protein binding to CRE was competed with HS3D (C/EBP responsive element), suggesting that the CRE binding protein might be a member of the C/EBP family.

Basal transcription of the BSP and OPN genes is mediated by an inverted CCAAT box that can be stimulated by v-Src [Tezuka et al., 1996; Kim and Sodek, 1999]. The major transcription factor that binds the inverted CCAAT box is NF-Y, which is essential for the expression of the class II genes of the major histocompatibility complex (MHC) and is involved in the regulation of albumin, and type I collagen genes [Maity et al., 1992; Caretti et al., 1999]. Also, activation of BSP gene transcription by flavonoids is mediated through an inverted CCAAT box in ROS17/2.8 cells [Shimizu and Ogata, 2002]. However, in gel shift assays, the CCAAT-NF-Y complex did not change after stimulation with TNF-α. TNF-α has been suggested to regulate apoptosis of osteoblast [Jilka et al., 1998]. However, the lack of cell death and DNA laddering in our cultures makes an apoptotic mechanism less likely.

In this study, therefore, we have shown that TNF-α suppresses BSP expression through a protein kinase signaling pathway that involves the generation of reactive oxygen species. A CRE element, which is conserved in mammalian promoters of BSP, has been identified as the target of TNF-α-mediated regulation of BSP gene transcription.

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