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Transcriptional Regulation of Bone Sialoprotein Gene by *Porphyromonas gingivalis* Lipopolysaccharide

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ABSTRACT

Lipopolysaccharide (LPS) is a major mediator of inflammatory response. Periodontopathic bacterium *Porphyromonas gingivalis* LPS has quite different character from *Escherichia coli* LPS. *E. coli* LPS is agonist for Toll-like receptor 4 (TLR4), whereas *P. gingivalis* LPS worked as antagonist for TLR4. Bone sialoprotein (BSP) is an early marker of osteoblast differentiation. To investigate the effects of *P. gingivalis* LPS on BSP transcription, we used rat osteoblast-like ROS17/2.8 cells. BSP mRNA levels were decreased by 0.1 μg/ml and increased by 0.01 μg/ml *P. gingivalis* LPS at 12 h. Results of luciferase assays showed that 0.1 μg/ml decreased and 0.01 μg/ml *P. gingivalis* LPS increased BSP transcription in −116 to +60 BSP construct. The effects of *P. gingivalis* LPS were abrogated by double mutations in cAMP response element (CRE) and FGF2 response element (FRE). Tyrosine kinase inhibitor herbimycin A, ERK1/2 inhibitor and antioxidant *N*-acetylcystein inhibited effects of *P. gingivalis* LPS. Protein kinase A inhibitor and PI3-kinase/Akt inhibitor only abolished the effect of 0.01 μg/ml *P. gingivalis* LPS. Furthermore, 0.1 μg/ml LPS decreased the CRE– and FRE–protein complexes formation, whereas 0.01 μg/ml *P. gingivalis* LPS increased the nuclear protein binding to CRE and FRE. ChIP assays revealed increased binding of CREB1, JunD, Fra2, Runx2, Dlx5, and Smad1 to a chromatin fragment containing the CRE and FRE by 0.01 μg/ml *P. gingivalis* LPS. These studies therefore indicated that 0.1 μg/ml suppressed, and 0.01 μg/ml *P. gingivalis* LPS increased BSP gene transcription mediated through CRE and FRE elements in the rat BSP gene promoter. J. Cell. Biochem. 110: 823–833, 2010.

KEY WORDS: BONE SIALOPROTEIN; LIPOPOLYSACCHARIDE; *Porphyromonas gingivalis*; BONE FORMATION; OSTEOBLASTS; TRANSCRIPTION

Periodontitis is an inflammatory disease that is characterized by loss of alveolar bone supporting the tooth and is the leading cause for tooth loss [Darveau et al., 1997]. *Porphyromonas gingivalis* has been implicated as an important etiologic agent of human chronic periodontitis. It is lipopolysaccharide (LPS) as a major pathogenic component of the bacterial outer membrane, has multiple inflammatory actions and is involved in the destruction of periodontal tissues, such as alveolar bone, cementum, periodontal ligament and gingiva, in periodontal disease [Socransky and Haffajee, 1992; Kadono et al., 1999]. LPS is known to induce not only inflammatory responses but also bone resorption by enhancing osteoclastogenesis *via* osteoblast-mediated activities [Jiang et al., 2002; Nemoto et al., 2006]. LPS increases the release of inflammatory osteolytic factors from osteoblasts to stimulate alveolar bone resorption by an indirect effect through the action on osteoblasts as a direct effect on osteoclastic cells. Physiological bone remodeling is controlled by a balance between bone formation and resorption [Kadono et al., 1999]. There are important structural differences in LPS composition between different bacterial species, such as fatty acid acyl chain composition and charge that can significantly affect the host response [Darveau et al., 2002]. *P. gingivalis* LPS differs from that of other gram-negative bacteria in that the structure lacks heptose and 2-keto-3-deoxyoctonate, and *P. gingivalis* LPS shows very low endotoxic activity in classic

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endotoxin assays, although it is significantly mitogenic. Innate host recognition pathway of *Escherichia coli* starts with a series of initial binding and transfer reactions between LPS binding protein (LBP) and CD14. CD14 is located in the cell membrane of host cells (mCD14) and is also present in serum (sCD14). Transfer of *E. coli* LPS by either mCD14 or sCD14 to a cell-associated Toll-like receptor 4 (TLR4) and MD2 protein complex initiates host cell activation pathways, leading to innate host defense mediator production [Darveau et al., 2004]. While TLR4-deficient mice were unresponsive to LPS, macrophages from TLR2-deficient mice lacked the response to gram-positive bacterial cell wall, strengthening the hypothesis that TLR4 is a principal signal transducer for LPS and TLR2 is a signal transducer for other bacterial components, such as peptidoglycan and lipoprotein [Yoshimura et al., 2002; Fukusaki et al., 2007].

Bone sialoprotein (BSP) is a highly sulfated, phosphorylated, and glycosylated protein that is characterized by its ability to bind to hydroxyapatite through polyglutamic acid sequences and to mediate cell attachment through an Arg–Gly–Asp (RGD) sequence. The expression of BSP is essentially restricted in the mineralized connective tissues, and BSP mRNA is produced at high levels at the onset of bone, dentin, and cementum formation [Ganss et al., 1999; Ogata, 2008]. BSP deficiency impairs bone growth and mineralization, concomitant with dramatically reduced bone formation [Malaval et al., 2008]. The temporo-spatial deposition of BSP to nucleate hydroxyapatite crystal formation, indicates a potential role for BSP in the initial mineralization of bone [Chen et al., 1992; Hunter and Goldberg, 1993]. BSP also expressed in osteotropic cancers such as breast, lung, thyroid, and prostate cancers. Thus, regulation of the BSP gene expression is important in the differentiation of osteoblasts, bone matrix mineralization, and tumor metastasis [Waltregny et al., 2000; Ogata, 2008].

The human, mouse, and rat BSP genes have been cloned and partially characterized. These BSP promoters include a highly conserved region that extends upstream from the transcription start site to nt −370 [Li and Sodek, 1993; Kim et al., 1994; Benson et al., 1999; Kiyoshima et al., 2002]. This region includes a functional inverted TATA box [Li et al., 1995] overlapping with vitamin D response element [Kim et al., 1996], an inverted CCAAT box which is required for basal promoter transcription activity [Kim and Sodek, 1999; Shimizu and Ogata, 2002]. In addition, a cAMP response element (CRE) [Samoto et al., 2003; Araki et al., 2009; Mezawa et al., 2009], a fibroblast growth factor 2 response element (FRE) [Shimizu-Sasaki et al., 2001; Samoto et al., 2003; Nakayama et al., 2006; Shimizu et al., 2006], a pituitary-specific transcription factor-1 (Pit-1) motif [Ogata et al., 2000] and a homeodomain protein-binding site (HOX) [Benson et al., 2000; Nakayama et al., 2006] have been characterized. Further upstream, a transforming growth factor-β (TGF-β) activation element (TAE) [Ogata et al., 1997], and a glucocorticoid response element (GRE) overlapping an AP1 site [Ogata et al., 1995; Yamauchi et al., 1996; Takai et al., 2008] has also been identified.

We previously reported that *E. coli* LPS (1 μg/ml) suppressed BSP gene expression through PKA and tyrosine kinase pathways and that the effects are mediated through CRE and FRE in the proximal promoter of rat BSP gene [Kato et al., 2006]. To determine the molecular mechanism of *P. gingivalis* LPS regulation of BSP gene transcription, we have analyzed the effects of *P. gingivalis* LPS on the expression of BSP in osteoblastic ROS17/2.8 cells and rat bone marrow stromal cells (RBMC). These studies have revealed that high concentration *P. gingivalis* LPS decreased and low concentrations *P. gingivalis* LPS increased BSP transcription through CRE and FRE in the rat BSP gene promoter.

METHODS

MATERIALS

α-Minimum essential medium (α-MEM), fetal calf serum (FCS), penicillin, streptomycin, and lipofectamine were obtained from Invitrogen. PGL3-basic vector, pSV-β-galactosidase (β-Gal) control vector and ERK1/2 inhibitor U0126 were purchased from Promega Co. (Madison, WI). PI3-K/Akt inhibitor LY294002 was purchased from Calbiochem (San Diego, CA). *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 the tyrosine kinase inhibitor, herbimycin A (HA) and guanidium thiocyanate were purchased from Wako Pure Chemical Industries (Tokyo, Japan). EXScript RT reagent Kit and SYBR Premix Ex Taq were purchased from TaKaRa (Tokyo, Japan).

CELL CULTURE

Rat osteosarcoma derived ROS17/2.8 cells and RBMC [Nakayama et al., 2006; Jheon et al., 2009] were used in this study as an osteoblast-like cells that synthesize BSP. Cells were cultured at 37°C in 5% CO_2, 95% air in α-MEM containing 10% FCS. Cells were grown to confluence in 60 mm tissue culture dishes and then changed to α-MEM without serum for 12 h and incubated with or without *P. gingivalis* LPS for dose–response or for time periods extending over 3–24 h. Total RNA was isolated from triplicate cultures and analyzed for the expressions of BSP, osteopontin (OPN), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA by Northern blot or real-time PCR as described below.

NORTHERN HYBRIDIZATION

Total RNA from the ROS17/2.8 cells treated with or without *P. gingivalis* LPS (0.1 and 0.01 μg/ml, 3–24 h) was extracted with guanidium thiocyanate and following purification, 25 μg aliquots of RNA were fractionated on a 1.2% agarose gel and transferred onto a Hybond N+ membrane. Hybridizations were performed at 42°C with a ^{32}P-labeled rat BSP, OPN, and 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 rat BSP mRNA (two polyadenylated forms: 1.6 and 2.0 kb) and OPN mRNA were scanned in a Bio-imaging analyzer (Fuji BAS 2500, Tokyo, Japan).

REAL-TIME PCR

Total RNA (1 μg) was used as a template for cDNA performed with ExScript RT reagent Kit (TaKaRa). Quantitative real-time PCR was performed using the following primer sets: BSP forward,

5′-AGACCACAGCTGACGCTGGA-3′; BSP reverse, 5′-CCGTTGACGACCTGCTCATT-3′; GAPDH forward, 5′-GACAACTTTGGCATCGTGGA-3′; GAPDH reverse, 5′-ATGCAGGGATGATGTTCTGG-3′ using the SYBR Premix Ex Taq in a TP800 thermal cycler dice real time system (TaKaRa). The amplification reactions were performed in 25 μl of final reaction mixture containing: 2× SYBR Premix EX Taq (12.5 μl); 50 μM (0.1 μl) forward and reverse primers (final concentration was 0.2 μM); 25 ng (2.5 μl) cDNA for BSP and 10 ng (1.0 μl) cDNA for GAPDH. To reduce variability between replicates, PCR premixes, which contain all reagents except for cDNA, were prepare and aliquoted into 0.2 ml Hi-8-tubes (TaKaRa). The thermal profile was as follows: pre-denaturation was performed for 1 cycle at 95°C/10 s, amplification was carried out for 40 cycles at 95°C/5 s, 60°C/30 sec and final dissociation at 95°C/5 sec, 60°C/30 sec, 95°C/15 sec. Post-PCR melting curves confirmed the specificity of single-target amplification. Quantify the initial amount of RNA using a standard curve, and fold expressions of BSP relative to GAPDH were determined in quadruplicate.

WESTERN BLOT

For Western blot analyses, cell lysate from ROS17/2.8 cells were separated on 10% SDS–PAGE and transferred onto a Hybond-P membrane. The membrane was then incubated for 3 h by anti-human BSP (LF-100 provided by Dr. Larry W. Fisher) and cyclin-dependent kinase 2 (Cdk2; sc-163) (Santa Cruz Biotechnology, Inc.) antibodies. Anti-rabbit IgG conjugated with HRP (GE Healthcare UK Ltd) was used as the secondary antibody. Immunoreactivities were detected by ECL Plus Western Blotting Detection Reagents.

TRANSIENT TRANSFECTION ASSAYS

Exponentially growing ROS17/2.8 cells and RBMC were used for transfection assays. Twenty-four hours after plating, cells at 50–70% confluence were transfected using a lipofectamine reagent. The transfection mixture included 0.5 μg of a luciferase (LUC) construct (pLUC1, −18 to +60; pLUC2, −43 to +60; pLUC3, −116 to +60; pLUC4, −425 to +60; pLUC5, −801 to +60) and 2 μg of pSV-β-galactosidase (β-gal) vector as an internal control. Two days post-transfection, cells were deprived of FCS for 12 h, *P. gingivalis* LPS (0.1 or 0.01 μg/ml) were added for a further 12 h prior to harvesting. The luciferase assays were performed according to the supplier's protocol (picaGene, Toyo Inki, Tokyo, Japan) using a luminescence reader (Acuu FLEX Lumi 400; Aloka, Tokyo, Japan) to measure the luciferase activities. The tyrosine kinase inhibitor HA (1 μM), the protein kinase C inhibitor H7 (5 μM), the protein kinase A inhibitor H89 (5 μM), the ERK1/2 inhibitor U0126 (5 μM), and the PI3-kinase/Akt inhibitor LY249002 (10 μM) were used for protein kinases inhibition and an antioxidant *N*-acetylcystein (NAC; 20 mM) was used as a free radical scavenger. Oligonucleotide-directed mutagenesis by PCR was utilized to introduce the dinucleotide substitutions using the Quikchange 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 ROS17/2.8 cells in T-75 flasks incubated for 3, 6, and 12 h with *P. gingivalis* LPS (0.1 or 0.01 μg/ml) in α-MEM without FCS were used to prepare nuclear extracts, 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, 25% (v/v) glycerol, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin A, 1 μg/ml aprotinin, pH 7.9). Double stranded oligonucleotides encompassing the inverted CCAAT (nts, −61 to −37, 5′-CCGTGACCGTG**ATTGG**CTGCTGAGA), CRE (nts, −84 to −59, 5′-CCCACAGCC**TGACGTCG**CACCGGCCG), FRE (nts, −98 to −79, 5′-TTTTCT**GGTGAGAA**CCCACA), Pit-1 (nts, −115 to −96, 5′-CGTG**TTGTAGT**TACGGATTT), and HOX (nts, −204 to −179, 5′-TCCTCAGCCT**TCAATTAAAT**CCCACA) in the rat BSP promoter were prepared by Bio-Synthesis, Inc. (Lewisville, TX). 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 50 mM KCl, 0.5 mM EDTA, 10 mM Tris–HCl, pH 7.9, 1 mM dithiothreitol, 0.04% Nonidet P-40, 5% glycerol, and 1 μg of poly (dI–dC). Following incubation, the protein–DNA complexes were resolved by electrophoresis on 5% nondenaturing acrylamide gels (38:2 acrylamide/bis acrylamide) run at 200 V at room temperature. Following electrophoresis, the gels were dried and autoradiograms prepared and analyzed using an image analyzer. For competition experiments, 40 times molar unlabeled oligonucleotides for the inverted CCAAT, CRE, mutation-CRE (mCRE), FRE, mutation-FRE (mFRE), and HOX were used. Supershift experiments were performed using anti-CREB1 (p43), JunD (329: sc-74), Fra2 (Q-20: sc-604), Runx2 (PC287L-100UG), Dlx5 (AB5728), and Smad1 (A-4: sc-7965) antibodies. Antibodies were added to each reaction mixture and incubated for 4 h at 4°C before electrophoresis was performed under the same conditions as described above.

CHROMATIN IMMUNOPRECIPITATION (ChIP) ASSAY

ChIP assays were carried out using a ChIP-IT^{TM} Express Enzymatic kit (Active Motif) according to the manufacturer's protocol. ROS17/2.8 cells and RBMC were grown to confluence in 100 mm dishes (six dishes for each chromatin preparation, approximately 4.5×10^7 cells) and cultured in serum-free α-MEM for 12 h before treatment with (six dishes) or without (six dishes) *P. gingivalis* LPS (0.01 μg/ml) for 6 h. The cells were fixed for 10 min at RT using 1% formaldehyde and then chromatin was prepared using the ChIP-IT Express Enzymatic Kit protocol. Cell pellets were homogenized by dounce homogenizer and centrifuged to pellet the nuclei. The nuclei pellet was digested by the enzymatic shearing cocktail (200 U/ml) to shear the chromatin at 37°C for 5 min and the reaction was stopped with the addition of cold EDTA. The equivalent of 6.3 μg of DNA (sheared chromatin) was used as starting material (input) in each ChIP reaction with 2 μg of the appropriate antibody (CREB, JunD, Fra2, c-Jun, c-Fos, Runx2, Dlx5, Smad1, and rabbit IgG were used as control) and protein G magnetic beads at 4°C overnight. Place tube on magnetic stand to pellet beads on the tube side and wash the beads extensively. Elute chromatin from the beads by elution buffer and reverse cross-link buffer, and then the samples were treated with proteinase K for 1 h at 37°C. The purified DNA was subjected to PCR amplification (1 cycle, 94°C for 5 min; amplification was carried out for 30 cycles, denature 94°C for 30 sec, anneal 58°C for 30 sec, and

extend 72°C for 30 sec; final extension at 72°C for 10 min) for the CRE and FRE site within the rat BSP promoter using For; 5′-GCCTCTCACCCATTCACTCGCTCTTGC-3′, Rev; 5′-CGACCTCTT-GGCAGCAGGCGCGCCTTTC-3′ primers. The PCR products were separated on 2% agarose gels and visualized with ultraviolet light. All ChIP assays were repeated at least three times and with triplicate samples for each antibody used in ChIP reactions.

STATISTICAL ANALYSIS

Triplicate or quadruplicate samples were analyzed for each experiment, and experiments were replicated to ensure consistency of the responses to drugs. Significant differences between control and treatment were determined using unpaired Student's *t*-test.

RESULTS

REGULATION OF BSP AND OPN mRNA LEVELS BY *P. gingivalis* LPS

To study the regulation of BSP expression by *P. gingivalis* LPS, we used osteoblast-like ROS17/2.8 cells to express BSP mRNA constitutively. Firstly, a dose–response effect of *P. gingivalis* LPS on BSP expression was established by treating the ROS17/2.8 cells with different concentrations of *P. gingivalis* LPS for 12 h. BSP mRNA levels were increased by 0.01 μg/ml *P. gingivalis* LPS and were decreased by 0.1 and 1 μg/ml *P. gingivalis* LPS (Fig. 1A). Then 0.01 and 0.1 μg/ml *P. gingivalis* LPS were used to determine the time course effects of LPS on BSP and OPN mRNA expressions. 0.01 μg/ml *P. gingivalis* LPS increased BSP mRNA level at 12 h and down regulated at 24 h, and induced OPN mRNA levels at 12 and 24 h (Fig. 1B). 0.1 μg/ml *P. gingivalis* LPS suppressed BSP mRNA levels at 3 h and reached maximal at 24 h, while increased OPN mRNA expression at 24 h (Fig. 1C).

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P. gingivalis LPS (0.01 μg/ml) induced BSP protein expression at 3 h and reached maximal at 12 h in ROS17/2.8 cells. Cdk2 was used as loading control [Barnouin et al., 2002; Takai et al., 2008] (Fig. 2).

P. gingivalis LPS REGULATED RAT BSP GENE TRANSCRIPTION

To determine how *P. gingivalis* LPS regulates BSP transcription, transient transfection analyses were performed using chimeric constructs encompassing different regions of the rat BSP gene promoter ligated to a luciferase reporter gene (pLUC1–5) transfected into ROS17/2.8 cells and RBMC. Luciferase activity of BSP promoter construct (pLUC3, nts −116 to +60) was decreased by 0.1 μg/ml *P. gingivalis* LPS, whereas increased by 0.01 μg/ml *P. gingivalis* LPS. PLUC4 (nts −425 to +60) and pLUC5 (nts −801 to +60) activities were suppressed by 0.1 μg/ml *P. gingivalis* LPS, while 0.01 μg/ml *P. gingivalis* LPS had no effect (Fig. 3A). When transcriptional activities in response to *P. gingivalis* LPS were analyzed in normal RBMC, the transcriptional activities of pLUC3, pLUC4, and pLUC5 were decreased by 0.1 μg/ml *P. gingivalis* LPS, whereas increased by 0.01 μg/ml *P. gingivalis* LPS (Fig. 3B). Within the DNA sequence unique to pLUC3, an inverted CCAAT box (nts −50 to −46), a CRE (nts −75 to −68), a putative Runx2 (nts −84 to −79), a FRE (nts −92 to −85), and a Pit-1 motif (nts −111 to −105) are present (Fig. 4). To more closely define the regulatory elements between nts −116 and −43 utilized by *P. gingivalis*. LPS, we prepared a series of 5′-deletion constructs. The transcriptional activities of −108 and −116 BSPLUC were suppressed by 0.1 μg/ml *P. gingivalis* LPS, while only −116 BSPLUC were increased by 0.01 μg/ml *P. gingivalis* LPS (Fig. 5). After introduced 2 bp mutations in the putative response elements within pLUC3 and pLUC4, the basal activities of M-CCAAT, M-CRE, M-FRE and M-Pit1 in pLUC3 and M-FRE and M-HOX in pLUC4 were lower than the basal transcriptional activities of pLUC3 and pLUC4. The transcriptional suppression by 0.1 μg/ml

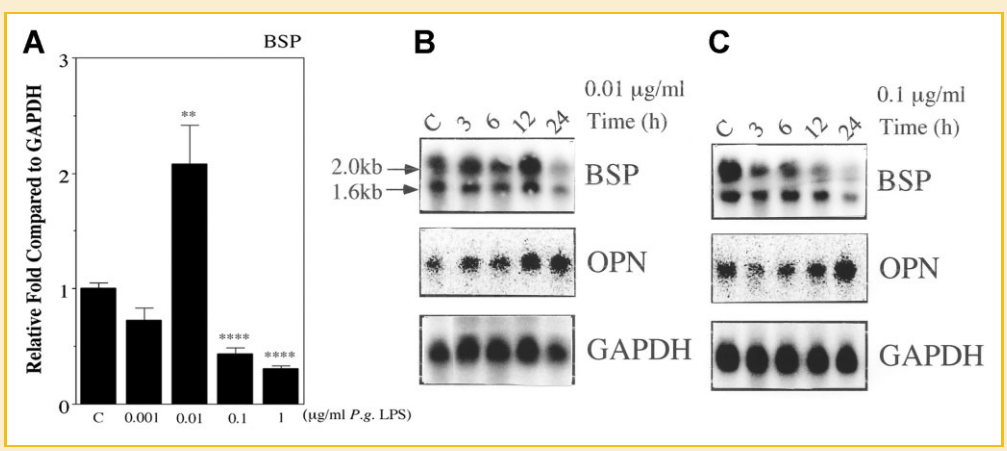

Fig. 1. Effects of *P. gingivalis* LPS on BSP and OPN mRNA levels in ROS17/2.8 cells. A: Dose–response effects of *P. gingivalis* LPS on BSP mRNA levels in ROS17/2.8 cells (real-time PCR). B,C: Time course effects of 0.01 μg/ml (B) and 0.1 μg/ml (C) *P. gingivalis* LPS on BSP and OPN expressions. Total RNA was extracted with guanidium thiocyanate and Northern hybridizations (B,C) were performed with rat BSP, OPN, and GAPDH cDNA probes. The experiments were performed in triplicate (quadruplicate for real-time PCR) for each data point. Results of a representative hybridization analysis for control and *P. gingivalis* LPS treated cells are shown ($^{**}P < 0.05$; $^{****}P < 0.01$).

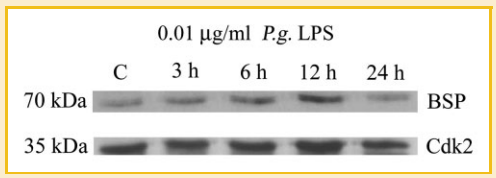

Fig. 2. Effect of *P. gingivalis* LPS on BSP protein levels in ROS17/2.8 cells. ROS17/2.8 cells were treated with or without *P. gingivalis* LPS (0.01 μg/ml) for 24 h. The expression of BSP protein was analyzed by Western blotting using anti-BSP antibody. Anti-cyclin-dependent kinase 2 (Cdk2) antibody was used for loading control.

P. gingivalis LPS was abrogated in the M-CRE (pLUC3) and M-FRE (pLUC3 and pLUC4) constructs, whereas the stimulatory effects of 0.01 μg/ml *P. gingivalis* LPS were abrogated in the M-CRE and M-FRE (pLUC3) (Fig. 6). To confirm the functional elements, we also performed double mutation analyses. Double mutation in FRE and CRE (M-FRE/CRE) completely abolished the effects of 0.1 and 0.01 μg/ml *P. gingivalis* LPS (Fig. 6). To determine the signaling pathways mediating the effects of *P. gingivalis* LPS, we used several protein kinase inhibitors and antioxidant on *P. gingivalis* LPS regulated BSP transcription. The tyrosine kinase inhibitor HA, the ERK1/2 inhibitor U0126 and the antioxidant NAC completely

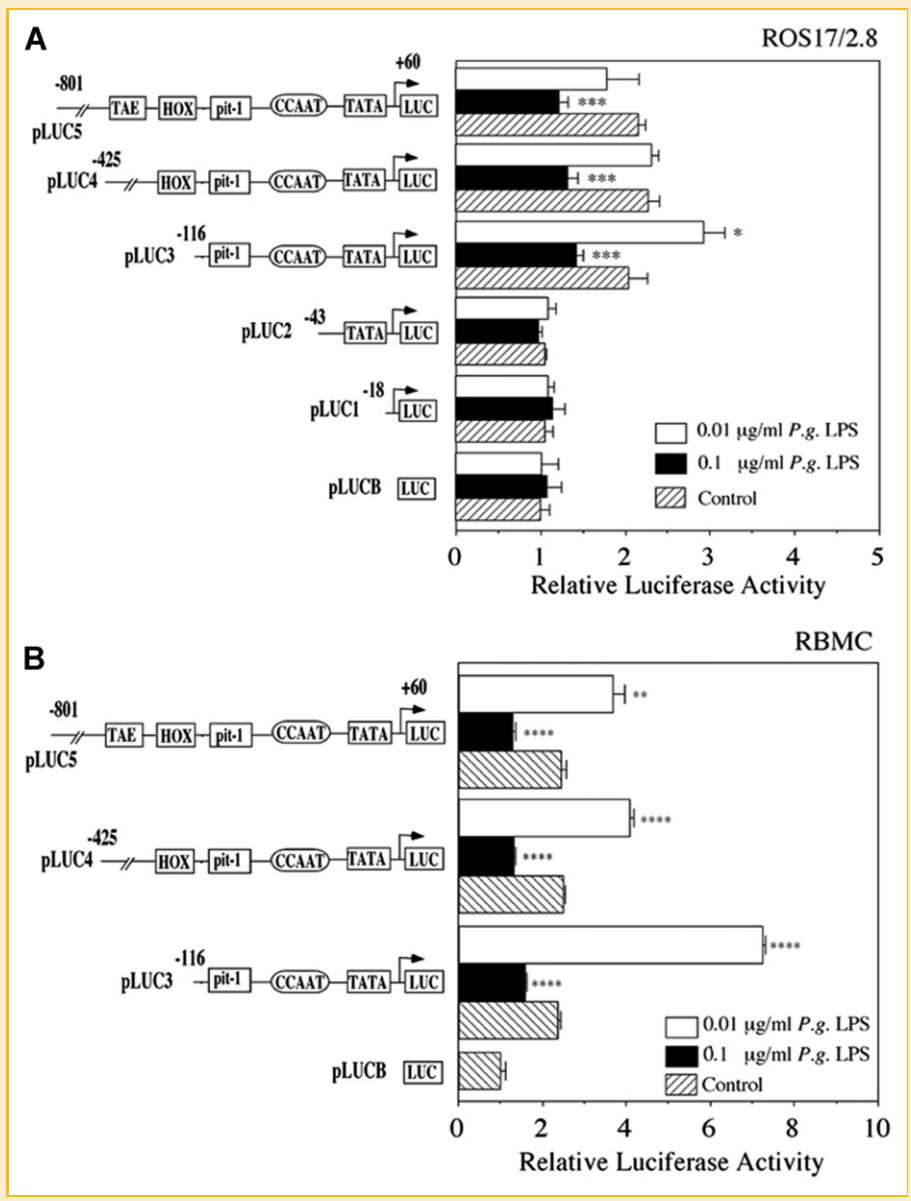

Fig. 3. *P. gingivalis* LPS regulates BSP promoter activities in ROS17/2.8 cells and RBMC. Transient transfections of ROS17/2.8 cells (A) and RBMC (B) in the presence or absence of 0.1 and 0.01 μg/ml *P. gingivalis* LPS for 12 h were used to determine transcriptional activities of chimeric constructs that included various regions of the rat BSP promoter ligated to a luciferase reporter gene. The results of transcriptional activity obtained from three (A) or four (B) separate transfections with constructs, pLUC basic (pLUCB) and pLUC1 to pLUC5, have been combined, and the values are expressed with standard errors ($^{*}P < 0.1$; $^{**}P < 0.05$; $^{***}P < 0.02$; $^{****}P < 0.01$).

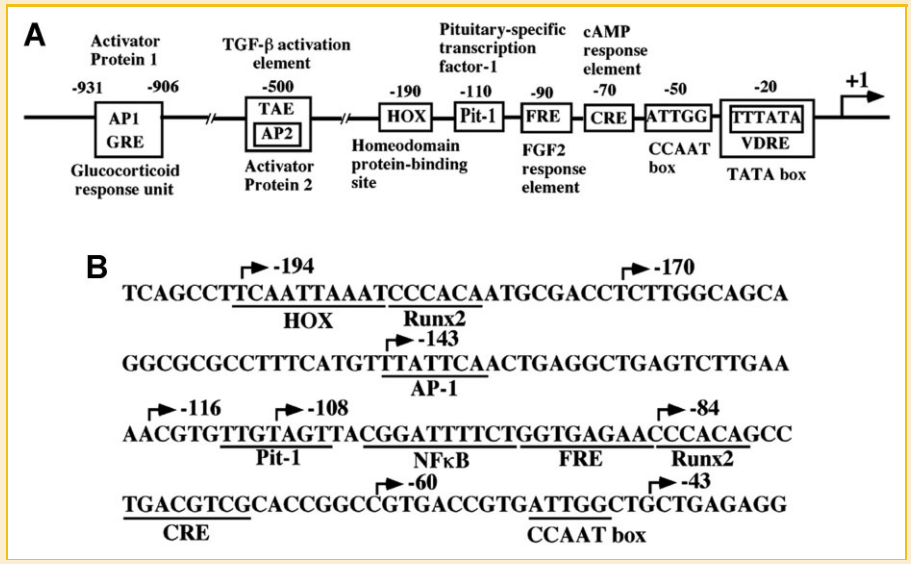

Fig. 4. Regulatory elements in the proximal promoter of rat BSP gene. A: The positions of the inverted TATA and CCAAT boxes, vitamin D response element (VDRE) that overlaps the inverted TATA box, a CRE (cAMP response element), a FRE (FGF2 response element), Pit-1 (mediates the stimulatory effects of parathyroid hormone, PTH), homeodomain protein-binding site (HOX), TAE (TGF-β activation element) overlaps with AP2 and GRE (glucocorticoid response element) overlapping with AP1 are shown. B: In the nucleotide sequence of the rat BSP gene proximal promoter from −194 to −43, Two Runx2 (CCCACA) elements exist in juxtaposed to FRE and HOX.

inhibited effects of *P. gingivalis* LPS on BSP transcription. On the other hand, the protein kinase A inhibitor H89 and the PI3-kinase/Akt inhibitor LY249002 abolished only the effect of 0.01 μg/ml *P. gingivalis* LPS on BSP transcription (Fig. 7).

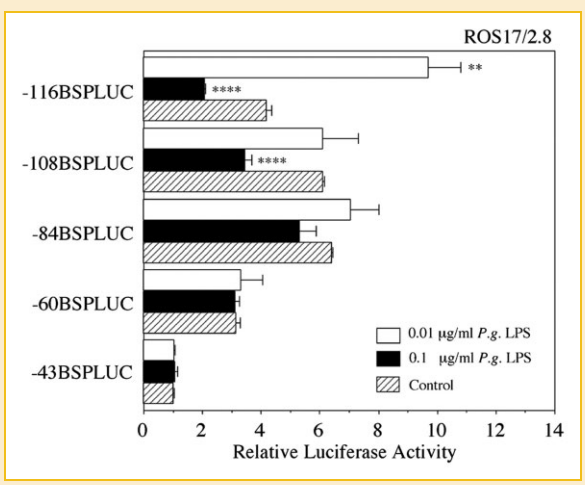

Fig. 5. Fine 5′ deletion mapping of the nts −116 to −43 element in the BSP promoter. A series of rat BSP promoter 5′ deletion constructs were analyzed for relative promoter activity after transfection into ROS17/2.8 cells and examined for regulation in the presence or absence of 0.1 and 0.01 μg/ml *P. gingivalis* LPS for 12 h. The results of transcriptional activity obtained from three separate transfections with constructs, −43BSPLUC (−43 to +60), −60BSPLUC (−60 to +60), −84BSPLUC (−84 to +60), −108BSPLUC (−108 to +60), and −116BSPLUC (−116 to +60), have been combined, and the values are expressed with standard errors ($^{**}P < 0.05$; $^{****}P < 0.01$).

P. gingivalis LPS REGULATES DNA BINDING ACTIVITIES OF NUCLEAR PROTEINS THAT RECOGNIZED THE CRE AND FRE

To identify nuclear proteins that bind to the CCAAT, CRE, FRE, Pit-1, and HOX elements and mediate the *P. gingivalis* LPS effects on transcription, double-stranded oligonucleotides were end-labeled and incubated with equal amounts (3 μg) of nuclear proteins extracted from confluent ROS17/2.8 cells that were either not treated (control) or treated with 0.1 or 0.01 μg/ml *P. gingivalis* LPS for 3, 6, and 12 h. When we used the inverted CCAAT, Pit-1, and HOX elements as probes, the DNA–protein complexes did not change after stimulation by *P. gingivalis* LPS (0.1 and 0.01 μg/ml) (Figs. 8 and 9, lanes 1–4, 13–16, and 17–20). When the CRE was used as a probe, 0.1 μg/ml *P. gingivalis* LPS increased nuclear proteins binding to CRE at 3 and 6 h and decreased at 12 h (Fig. 8, lanes 5–8), whereas 0.01 μg/ml *P. gingivalis* LPS increased nuclear proteins binding to CRE at 3–12 h (Fig. 9, lanes 5–8). When the FRE was used as a probe, 0.1 μg/ml decreased and 0.01 μg/ml *P. gingivalis* LPS increased FRE–protein complexes formations (Figs. 8 and 9, lanes 9–12). That the DNA–protein complexes represent specific interactions were indicated by competition experiments in which CRE double-stranded oligonucleotide reduced the amount of CRE-protein complexes formation (Fig. 10, lane 3), while mCRE, FRE, and CCAAT did not compete with CRE binding activities (Fig. 10, lanes 4–6). FRE and HOX double-stranded oligonucleotides reduced the amount of FRE–protein complexes formation (Fig. 10, lanes 9 and 11), while mFRE and CCAAT did not compete with FRE binding activities (Fig. 10, lanes 10 and 12). To further characterize the proteins in the complexes formed with the CRE and FRE, we used antibodies for several transcription factors. CREB1, JunD, and Fra2 antibodies disrupted the formation of the CRE-protein complexes

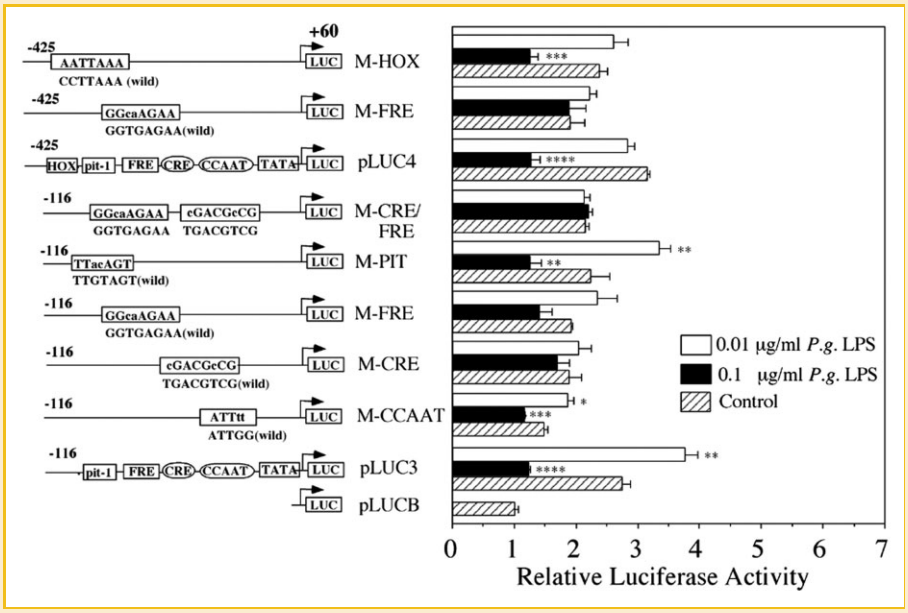

Fig. 6. Site mutation analyses of luciferase activities. Dinucleotide substitutions were made within context of the pLUC3 BSP promoter fragment. mCCAAT (ATTtt), mCRE (cGACGcCG), mFRE (GGcaAGAA), mPit-1 (TTacAGT), mHOX (aaTTAAA), and mCRE/FRE constructs were analyzed for relative promoter activity after transfection into ROS17/2.8 cells, and examined for regulation after treatment with 0.1 and 0.01 μg/ml *P. gingivalis* LPS for 12 h. The results of transcriptional activity obtained from three separate transfections with constructs were combined and the values expressed with standard errors ($^{*}P < 0.1$; $^{**}P < 0.05$; $^{***}P < 0.02$; $^{****}P < 0.01$).

(Fig. 11, lanes 4–6). Runx2, Dlx5, and Smad1 antibodies disrupted the FRE–protein complexes formation (Fig. 11, lanes 10–12).

ChIP ASSAY

We next examined whether CREB1, JunD, Fra2, Runx2, Dlx5, and Smad1 transcription factors are able to interact directly with rat BSP gene promoter and how *P. gingivalis* LPS influences these transcription factors interaction with the CRE and FRE. To clarify this issue further, we used ChIP assays to examine the in vivo association of these transcription factors with proximal CRE and FRE elements in ROS17/2.8 cells or RBMC. For this experiments, cells were treated with 0.01 μg/ml *P. gingivalis* LPS for 6 h to induce BSP expression and cross-linked with formaldehyde. After enzymatic shearing, soluble chromatins were immunoprecipitated with either antibodies or control IgG. The PCR bands amplified and corresponding to DNA–protein complexes immunoprecipitated with antibodies revealed that Runx2, Dlx5, CREB1, JunD, Fra2, and Smad1 interacted with a chromatin fragment containing the CRE and FRE sites were increased in ROS17/2.8 cells (Fig. 12A) and RBMC (Fig. 12B) after stimulation by 0.01 μg/ml *P. gingivalis* LPS. When we used normal RBMC for ChIP analyses, the increase bindings of Dlx5, Fra2, and Smad1 to the promoter sequence after stimulation by 0.01 μg/ml *P. gingivalis* LPS were weaker and the increase bindings of CREB1 was stronger than the results of ROS17/2.8 cells.

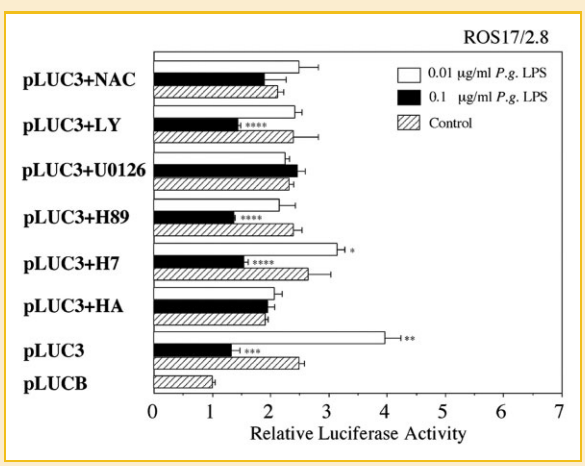

Fig. 7. Effects of kinase inhibitors and antioxidant on transcriptional regulation by *P. gingivalis* LPS. Transient transfection analysis of pLUC3 in the presence or absence of 0.1 and 0.01 μg/ml *P. gingivalis* LPS for 12 h in ROS17/2.8 cells is shown together with the effects of HA (1 μM), H7 (5 μM), H89 (5 μM), U0126 (5 μM), LY294002 (10 μM), and NAC (20 mM). The results of transcriptional activity obtained from three separate transfections with constructs were combined and the values expressed with standard errors ($^{**}P < 0.05$; $^{***}P < 0.02$; $^{****}P < 0.01$).

DISCUSSION

In this study, we have shown that 0.1 μg/ml *P. gingivalis* LPS suppressed and 0.01 μg/ml *P. gingivalis* LPS enhanced BSP gene transcription by targeting CRE and FRE elements in the proximal promoter of rat BSP gene. The expression of BSP is essentially specific to mineralized connective tissues and is expressed by newly formed osteoblasts coincident with mineralization, provides a valuable marker for osteogenic differentiation and bone formation [Ganss et al., 1999; Ogata, 2008]. Since LPS is one of the virulent

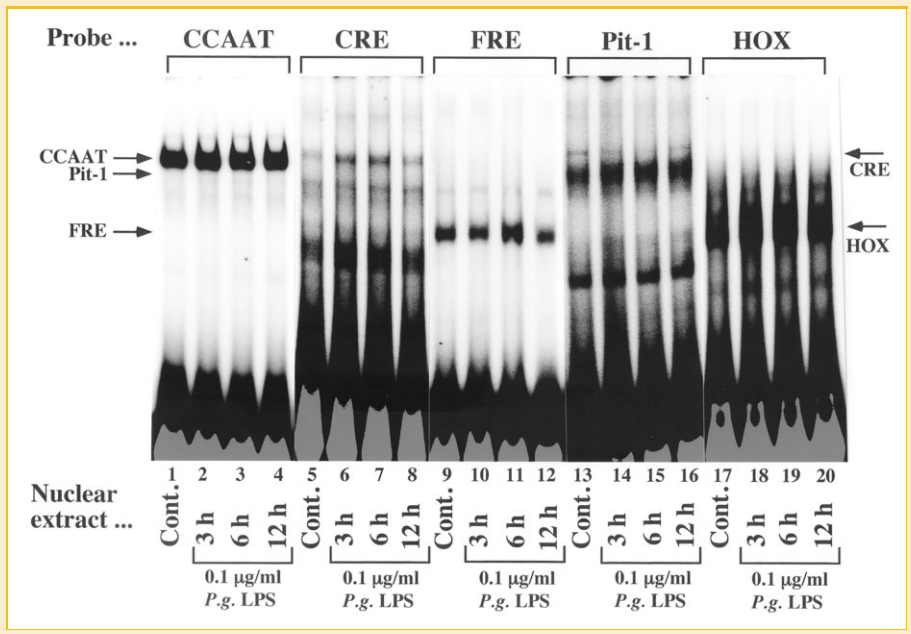

Fig. 8. Gel mobility shift assays using inverted CCAAT, CRE, FRE, Pit-1, and HOX. Radiolabeled double-stranded inverted CCAAT (−61 CCGTGACCGTG**ATTGG**CTGCTGAGA −79), CRE (−84 CCCACAAGCC**TGACGTCG**CACCGGCCG −59), FRE (−98 TTTTCT**GGTGAGAA**CCCACA −79), Pit-1 (−115 CGTG**TTGTAGT**TACGGATTT −96), and HOX (−204 TCCTCAGCCT**TCAATTAAAT**CCCACA −179) oligonucleotides were incubated with nuclear protein extracts (3 μg) obtained from ROS17/2.8 cells stimulated without (lanes 1, 5, 9, 13, and 17) or with 0.1 μg/ml *P. gingivalis*. LPS for 3 h (lanes 2, 6, 10, 14, and 18), 6 h (lanes 3, 7, 11, 15, and 19), and 12 h (lanes 4, 8, 12, 16, and 20). 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 image analyzer.

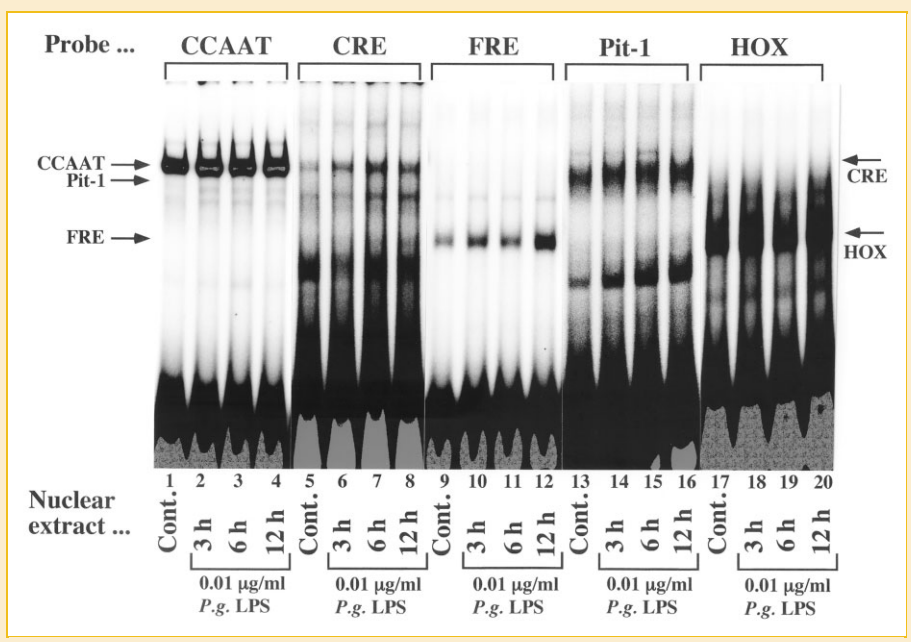

Fig. 9. Gel mobility shift assays using inverted CCAAT, CRE, FRE, Pit-1, and HOX. Radiolabeled double-stranded inverted CCAAT (−61 CCGTGACCGTG**ATTGG**CTGCTGAGA −79), CRE (−84 CCCACAAGCC**TGACGTCG**CACCGGCCG −59), FRE (−98 TTTTCT**GGTGAGAA**CCCACA −79), Pit-1 (−115 CGTG**TTGTAGT**TACGGATTT −96), and HOX (−204 TCCTCAGCCT**TCAATTAAAT**CCCACA −179) oligonucleotides were incubated with nuclear protein extracts (3 μg) obtained from ROS17/2.8 cells stimulated without (lanes 1, 5, 9, 13, and 17) or with 0.01 μg/ml *P. gingivalis*. LPS for 3 h (lanes 2, 6, 10, 14, and 18), 6 h (lanes 3, 7, 11, 15, and 19), and 12 h (lanes 4, 8, 12, 16, and 20). 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 image analyzer.

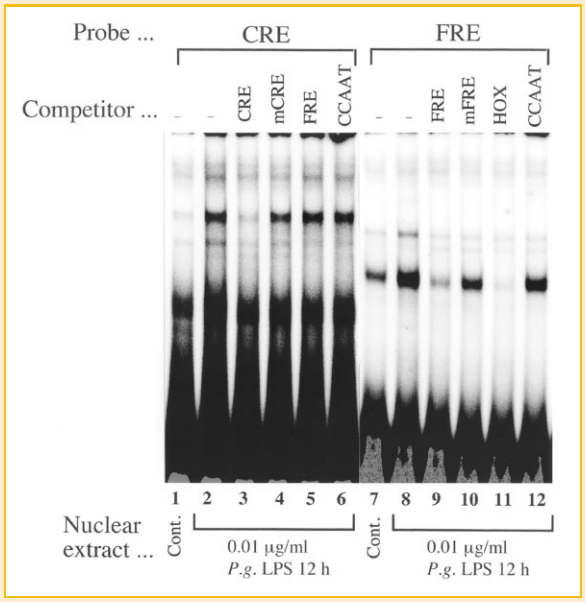

Fig. 10. Specific binding of nuclear proteins to CRE and FRE. Radiolabeled double-stranded CRE (−84 CCCACAAGCC**TGACGTCG**CACCGGCCG −59) and FRE (−98 TTTTCT**GGTGAGAA**CCCACA −79) oligonucleotides were incubated with nuclear protein extracts (3 μg) obtained from ROS17/2.8 cells treated without (lanes 1 and 7) or with 0.01 μg/ml *P. gingivalis* LPS for 12 h (lanes 2–6, 8–12). Competition reactions were performed using a 40-fold molar excess of unlabeled CCAAT (lanes 6 and 12), CRE (lane 3), mCRE (lane 4), FRE (lanes 5 and 9), mFRE (lane 10), and HOX (lane 11) oligonucleotides.

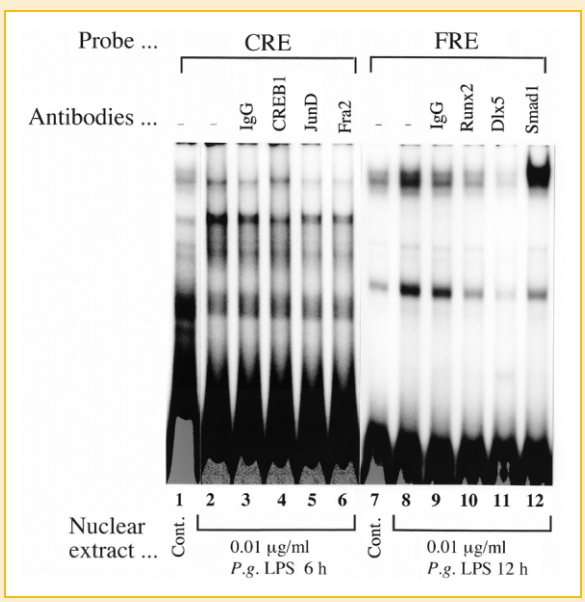

Fig. 11. Specific binding of nuclear proteins to the CRE and FRE. Radiolabeled double-stranded CRE and FRE were incubated with nuclear protein extracts (3 μg) obtained from ROS17/2.8 cells stimulated without (lanes 1 and 7) or with 0.01 μg/ml *P. gingivalis* LPS (lanes 2–6, 8–12) for 6 or 12 h. Supershift experiments were performed with 0.4 μg of antibodies against IgG (lanes 3 and 9), CREB1 (lane 4), JunD (lane 5), Fra2 (lane 6), Runx2 (lane 10), Dlx5 (lanes 11), and Smad1 (lanes 12) added separately to each gel shift reaction.

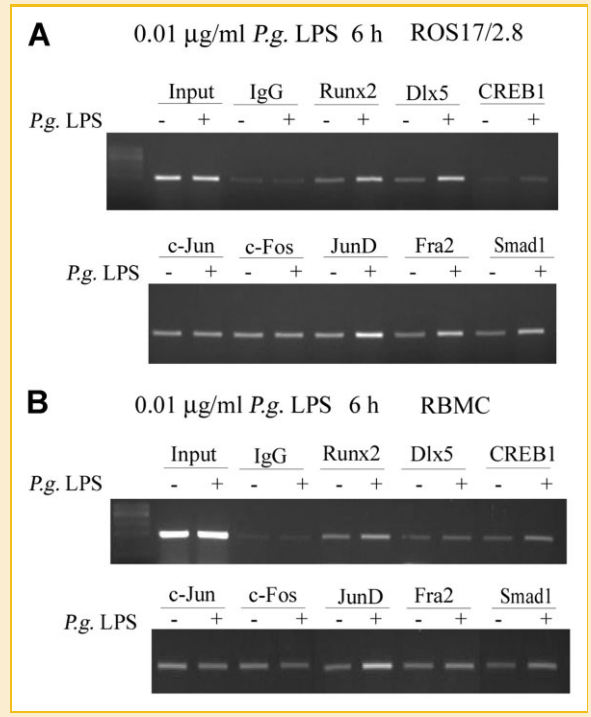

Fig. 12. ChIP analyses of Runx2, Dlx5, CREB1, c-Jun, c-Fos JunD, Fra2, and Smad1 binding to CRE and FRE sites in the rat BSP promoter in ROS17/2.8 cells and RBMC. ROS17/2.8 cells (A) or RBMC (B) (100 mm plates) were treated without (−) or with (+) 0.01 μg/ml *P. gingivalis* LPS for 6 h, before cells were cross-linked with formaldehyde for ChIP analysis. Three independent IP reactions were carried out with antibodies (Runx2, Dlx5, CREB1, c-Jun, c-Fos, JunD, Fra2, and Smad1) and control reactions with normal IgG. Ethidium bromide stained agarose gels of the PCR products obtained with ChIP DNAs using the rat BSP promoter primers (CRE/FRE). Input DNA was also used as control in PCR analysis.

factors for periodontitis and stimulates alveolar bone resorption [Kadono et al., 1999]. In this study, we analyzed the regulation of BSP gene transcription by LPS derived from *P. gingivalis* that has been identified as a major pathogenic microorganism in the development and progression of periodontitis [Socransky and Haffajee, 1992]. BSP mRNA levels were suppressed by 0.1 μg/ml *P. gingivalis* LPS and induced by 0.01 μg/ml *P. gingivalis* LPS at 12 h in ROS17/2.8 cells (Fig. 1). These results are same as the effects of *E. coli* LPS on BSP mRNA levels in our previous study [Kato et al., 2006]. Furthermore, 0.01 μg/ml *P. gingivalis* LPS increased BSP protein levels in ROS17/2.8 cells (Fig. 2). These results indicate *P. gingivalis* LPS regulates BSP gene expression as well as protein expression. An antioxidant NAC abolished the effects of *P. gingivalis* LPS (Fig. 7). NAC is an inhibitor of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and reactive oxygen species production considered as second messengers in the inflammatory response [Antonicelli et al., 2004; Peng et al., 2005; Lin et al., 2006; Wang et al., 2007], therefore the effects of *P. gingivalis* LPS on BSP transcription might be mediated through reactive oxygen species.

From transient transfection assays we initially located the *P. gingivalis* LPS responsive region to the proximal promoter

(−116 to −43) of the BSP gene (Fig. 3), which encompasses an inverted CCAAT box, CRE, Runx2, FRE, and Pit-1 motifs (Fig. 4). Transcriptional regulation by *P. gingivalis* LPS was abrogated completely when CRE and FRE were double mutated in pLUC3 (Fig. 6). The involvement of the CRE and FRE elements is further supported by gel shift analyses in which nuclear proteins formed complexes with the CRE and FRE elements that were decreased by 0.1 μg/ml and increased by 0.01 μg/ml *P. gingivalis* LPS (Figs. 8 and 9). Luciferase activities of BSP promoter construct (pLUC3, nts −116 to +60) were decreased by 0.1 μg/ml and increased by 0.01 μg/ml *P. gingivalis* LPS in ROS17/2.8 cells and RBMC. Interesting differences were observed in the luciferase analyses performed in the ROS17/2.8 cells compared to the RBMC. Although in ROS17/2.8 cells the deletion of sequence between −425 and −116 was necessary to permit the increased transcription after stimulation by 0.01 μg/ml *P. gingivalis* LPS (Fig. 3A). In RBMC, the enhanced transcription was already observed using the luciferase construct (pLUC4) containing the sequence between −425 and −116 (Fig. 3B). These findings suggest that suppressor elements may exist in the sequence between −425 and −116, which interact with ROS17/2.8-specific nuclear proteins after stimulation by 0.01 μg/ml *P. gingivalis* LPS. While the tyrosine kinase inhibitor HA, the ERK1/2 inhibitor U0126 and the antioxidant NAC completely inhibited effects of *P. gingivalis* LPS on BSP transcription, the protein kinase A inhibitor H89 and the PI3-kinase/Akt inhibitor LY249002 abolished the effect of 0.01 μg/ml *P. gingivalis* LPS (Fig. 7). These findings suggest that high (0.1 μg/ml) and low (0.01 μg/ml) concentrations of *P. gingivalis* LPS regulates BSP transcription via different signaling pathways. Results of gel shift (Fig. 11) and ChIP assays (Fig. 12) suggest that 0.01 μg/ml *P. gingivalis* LPS induced BSP transcription through CREB1, JunD, and Fra2 targeting to the CRE, and through Runx2, Dlx5, and Smad1 targeting to the FRE in the rat BSP gene promoter. And also, results of ChIP analyses suggest that the regulatory mechanism of BSP gene transcription in osteosarcoma-derived ROS17/2.8 cells and normal RBMC are different.

We previously reported *E. coli* LPS (1 μg/ml) decreased FRE–protein complexes formation at 3 h, and did not change nuclear proteins binding to CRE [Kato et al., 2006], suggesting the regulatory mechanisms of *E. coli* and *P. gingivalis* LPS on BSP transcription are quite different. While several studies reported that *E. coli* LPS is recognized by TLR4–LBP–CD14–MD2 complexes [Wang and Ohura, 2002], *P. gingivalis* LPS worked as antagonist for TLR4 [Yoshimura et al., 2002]. *P. gingivalis* LPS used TLR2 to activate epithelial cells and TLR4 to activate endothelial cells, suggesting the idea that the same *P. gingivalis* LPS preparation can act as a TLR2 or TLR4 agonist depend on the TLR expression of the host cells [Kocgozlu et al., 2009].

In this study, we could not conclude which TLR was activated by *P. gingivalis* LPS, despite of 0.01 μg/ml *P. gingivalis* LPS induced both TLR4 and TLR2 mRNA expression in ROS17/2.8 (data not shown). TLR4 as a transmembrane protein contains a conserved intracellular domain and occurs in a series of intracellular adaptors including mitogen activated protein kinase (MAPK) family (p38, ERK1/2, JUNK) signaling pathways, which play a critical role in the LPS-induced inflammation and periodontal bone loss [Darveau et al., 2002; Wang and Ohura, 2002; David et al., 2005; Cohn et al., 2006; Kirkwood et al., 2007]. Since BSP transcription is regulated mainly by tyrosine kinase, cAMP, and MAPK [Shimizu-Sasaki et al., 2001; Nakayama et al., 2006; Ogata, 2008], we investigated the effects of several kinase inhibitors on *P. gingivalis* LPS regulated BSP gene transcription. The results suggest that PKA and PI3-K/Akt pathways mediated 0.01 μg/ml *P. gingivalis* LPS targeting to the CRE, on the other hand tyrosine kinase, MAPK (ERK1/2) and NADPH oxidase (ROS products) mediated 0.01 and 0.1 μg/ml *P. gingivalis* LPS targeting to the FRE element in the rat BSP gene promoter.

In summary, we have shown that 0.1 μg/ml *P. gingivalis* LPS suppressed and 0.01 μg/ml *P. gingivalis* LPS enhanced BSP transcription via different signaling pathways mediated through CRE and FRE elements in the rat BSP gene promoter, and could involve CREB1, JunD, Fra2, Runx2, Dlx5, and Smad1 transcription factors in *P. gingivalis* LPS regulated BSP gene transcription.

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