Abstract. Butylated hydroxyanisole (BHA; a mixture of 2- and 3-BHA) is widely used as a potent antioxidant, but is reported to have adverse effects, such as carcinogenesis and pro-inflammatory activity, possibly due to the pro-oxidant property of this compound. 2-Methoxyphenol dimers derived from ferulic acid were recently demonstrated to inhibit the expression of lipopolysaccharide-stimulated cyclooxygenase-2 (COX-2) via redox-sensitive transcription factors such as nuclear factor kappa B or activator protein-1 (AP-1), due to a weakening of its pro-oxidant property by dimerization. To develop anti-inflammatory and/or anticancer drugs for the prevention of oral diseases, such as leukoplakia and destructive chronic periodontitis, whether 2-BHA (2-tert-butyl-4-methoxyphenol) and its synthetic ortho dimer, bis-BHA (3,3’-di-tert-butyl-5,5’-dimethoxy-1,1’-biphenyl-2,2’-diol) can inhibit AP-1 transcriptional activity stimulated by Porphyromonas gingivalis fimbriae was examined. The fimbria-stimulated AP-1 activation of RAW 264.7 murine macrophages was markedly inhibited by bis-BHA. However, BHA showed slight inhibition. Furthermore, bis-BHA significantly inhibited fimbria-induced COX-2 gene expression, which is closely involved with inflammation and carcinogenesis. These findings suggest that bis-BHA may possess a potent anti-inflammatory effect against oral diseases.

Butylated hydroxyanisole (BHA; 2-t-butyl-4-methoxyphenol), an artificial phenolic antioxidant, is widely used in the food industry as an efficient antioxidant. BHA (2- and 3-BHA) has a number of pharmacological and toxicological properties. Several investigators have demonstrated that BHA significantly inhibits cytokine-induced inflammatory responses in human and mouse cells via its potent antioxidant activity (1, 2). Although BHA is an effective antioxidant, it has been reported to promote carcinogenesis and inflammatory activity in animals (3-5), possibly due to its pro-oxidant activity and consequent formation of reactive quinone methide intermediates (6, 7). Therefore, to weaken the potent oxidative activity of 2-BHA, bis-BHA (3,3’-di-t-butyl-5,5’-dimethoxy-1,1’-biphenyl-2,2’-diol), an ortho dimer of 2-BHA, was recently synthesized and its antioxidant activities and cytotoxicity were investigated. Actuarially, bis-BHA showed less cytotoxicity and less oxidation activity (8,9), suggesting that bis-BHA might act as a potent inhibitor of inflammatory and allergic responses.

Porphyromonas gingivalis is an oral anaerobic bacterium causing chronic periodontal diseases characterized by serious gingival tissue inflammation and tooth loss due to alveolar bone resorption. P. gingivalis fimbriae are an especially important bacterial component that regulate adhesion to host cells of the organisms affected by these diseases. Our previous studies (10-12) demonstrated that the fimbriae actually induced the expression of inflammatory cytokines, such as IL-1 beta, IL-6 and neutrophil chemo-attractant KC (murine CXC chemokine) in several kinds of host cells, and stimulated bone resorption in murine embryonic calvarial bone cells. These findings suggest that the fimbria function not only as a component
for binding to host cells, but also play an important role as a key virulence factor by promoting the various biological actions of inflammatory cytokines in the initiation and development of chronic oral diseases.

Activator protein-1 (AP-1) is a well-known and important transcriptional factor for the expression of inflammatory cytokines, cell survival and carcinogenesis (13-15). Recently, Naganuma et al. demonstrated that AP-1 plays a functional role in fimbria-stimulated bone resorption via endogenous IL-1 beta in the mouse calvarial system (16). These findings suggest that AP-1 inhibition may be connected with the prevention of inflammatory bone resorption. The promoter region of the IL-1 beta IL-6 and cyclooxygenase (COX)-2 genes contains the consensus sequence for the AP-1 binding site, the tetradecanoyl phorbol acetate-responsive element (TRE) (17-19). Therefore, in order to explore the possible use of BHA as a potent chemopreventive agent for chronic oral diseases, the ability of 2-BHA and its synthetic dimer bis-BHA to inhibit fimbria-stimulated AP-1 activity and also induced COX-2 expression in the mouse macrophage-like cell line RAW 264.7 was investigated.

**Materials and Methods**

**Reagents.** 2-BHA was purchased from Tokyo Kasei Co. (Tokyo, Japan). bis-BHA was synthesized from 2-BHA monomers by the CuCl (OH)-catalyzed coupling reaction described previously (8). The chemical structures of BHA and bis-BHA are shown in Figure 1. The megaprime DNA labeling system, 5'-end labeling system, 5'-[alpha-32P]dCTP and [gamma-32P]ATP were purchased from Amersham Biosciences Co. (Piscataway, NJ, USA). Mouse COX-2 cDNA probes of approximately 1.2 kbp length were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). The beta-actin oligonucleotide (single-stranded DNA) probe was purchased from Genedetect.com Ltd. (Bradenton, FL, USA) and RPMI 1640, Opti-MEM and Lipofectamine™ were purchased from Invitrogen Corp. (Carlsbad, CA, USA). FBS was from HyClone (Logan, UT, USA).

**Preparation of P. gingivalis fimbriae.** P. gingivalis (ATCC 33277) fimbriae were prepared and purified from cell washings by the method of Yoshimura et al. (20). Purified fimbria-induced biological activities were not attributed to lipopolysaccharide contaminants in the preparation, as documented previously (10). The protein content of the fimbriae was measured by the method of Bradford (21).

**Cell culture.** Cells of the murine macrophage-like cell line RAW 264.7, obtained from the Riken Cell Bank, were used. The cells were cultured to a subconfluent state in RPMI 1640 medium supplemented with 10% FBS at 37°C and 5% CO2 in air, washed and then incubated overnight in serum-free RPMI 1640. The cells were then washed further and treated with the test samples.

**Gel mobility shift assay.** Cells in Falcon 15-cm-diameter dishes (104 cells per dish) (Becton Dickinson Labware, Franklin Lakes, NJ, USA) were treated with test samples. Their nuclei were then isolated and the extracts were prepared for the gel mobility shift assay as described previously (22). The protein concentration was measured by the method of Smith et al. (23). Binding reactions were performed for 20 min at room temperature with 10 μg of the nuclear proteins in 20 μL of binding buffer [2 mM HEPES (pH 7.9) containing 8 mM NaCl, 0.2 mM EDTA, 12 % (v/v) glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride and 1 μg poly (dI-dC)] containing 20,000 cpm 32P-labeled oligonucleotide. The poly (dI-dC) and nuclear extracts were incubated at 4°C for 10 min before addition of the labeled oligonucleotide. The double-stranded oligonucleotide (30-mer), containing a tandem repeat of the consensus sequence for the AP-1 binding site TGGACTCA (Oncogene Science, Inc. Manhasset, NY, USA), was end-labeled by the T4 polynucleotide kinase-[gamma-32P]ATP

![Figure 1. Chemical structures of 2-BHA and bis-BHA.](image-url)

2-BHA

bis-BHA
method (Amersham Biosciences Co., Piscataway, NJ, USA). Reaction mixtures for the binding were incubated for 15 min at room temperature after addition of the labeled oligonucleotide. DNA-protein complexes were electrophoresed on native 5% polyacrylamide gel in 0.25 x TBE buffer (22 mM Tris, 22 mM boric acid, 0.5 mM EDTA [pH 8.0]). The gel was dried and then exposed to Kodak X-ray film at –70°C.

Plasmid construction and transient expression assay. The plasmid pAP-1-Luc (Clontech Laboratories, Inc., Palo Alto, CA, USA) was constructed by inserting a synthetic oligonucleotide containing multiple copies of the AP-1 enhancer sequence into the corresponding sites of pTA-Luc (Clontech), which contains the HSV thymidine kinase (HSV-TK) promoter enhancer region located upstream of the firefly luciferase gene, pRL-TK, which contains the HSV-TK promoter located in the region upstream of Renilla luciferase (Promega Co., Madison, WI, USA). pRL-TK was also used. Transient expression was assayed as described previously (24). Briefly, cells in Falcon 5-cm-diameter dishes (10^6 cells per dish) were incubated for 1 h in serum-free Opti-MEM. They were then transfected with the reporter plasmid at 2 µg and pRL-TK at 0.2 µg using Lipofectamine™ (Invitrogen). After incubation for 24 h, the transfected cells were treated with test samples in serum-free RPMI 1640. The cellular extracts were subsequently prepared with reporter passive lysis buffer (Promega) and were examined for firefly luciferase activity after determination of the Renilla luciferase activity (pRL-TK). The latter was used as an internal control to normalize for variations in transfection efficiency.

Northern blot analysis. After cells (10^6 cells) in Falcon 5-cm-diameter dishes had been treated with the test samples, the total cellular RNA was extracted by the acid guanidine phenol chloroform (AGPC) procedure (25). As described earlier (22), the RNA was electrophoresed in 1% agarose gels with 0.2 M sodium phosphate as a running buffer and blotted onto nylon membranes (Micron Separations, Inc., Westboro, MA, USA). The membranes were then hybridized with the COX-2 cDNA probe labeled with 5’-[alpha-32P] dCTP by use of the megaprime DNA labeling system or the beta-actin oligonucleotide probe, which had been labeled with [gamma-32P] ATP by use of the 5’-end labeling system (Amersham Biosciences Co.). After hybridization, the membranes were washed, dried and exposed overnight to Kodak X-ray film (Eastman Kodak Co., Rochester, NY, USA) at –70°C. Beta-actin was used as an internal standard for quantification of the total RNA in each lane of the gel.

Figure 2. bis-BHA inhibited the fimbria-stimulated AP-1 binding in RAW 264.7 cells. A) The cells were untreated or pretreated for 30 min with 2-BHA or bis-BHA at 10 µM and were then untreated or treated with the fimbriae at 2 µg/ml for 1 h. Next, the nuclear proteins were prepared for a gel mobility shift assay, which was performed in the presence of the nuclear proteins with 32P-labeled oligonucleotide containing the AP-1 consensus sequence. An identical experiment, independently performed three times, gave similar results. B) Quantification of AP-1 binding in (A) was carried out by densitometry and the data are expressed as the relative signal intensity (percentage of the maximum). Bars represent the mean for three independent experiments. Standard errors <15%. There was a significant difference between 2-BHA and bis-BHA (p<0.01).
Results and Discussion

AP-1 is an important transcriptional factor for the expression of inflammatory cytokines, cell survival and carcinogenesis (13-15). Since AP-1 plays a functional role in fimbria-stimulated bone resorption via endogenous IL-1 beta in the mouse calvarial system (16), we focused on the inhibitory effects of bis-BHA and BHA on AP-1, as a possible explanation for the anti-inflammatory action of these compounds in chronic periodontal disease.

Using a gel mobility shift assay, whether bis-BHA or BHA were capable of inhibiting fimbria-stimulated AP-1 in RAW cells was examined. As shown in Figure 2 A and B, fimbria-stimulated AP-1 binding to TRE was strongly inhibited by bis-BHA at 10μM, but was only slightly inhibited by 2-BHA. This finding strongly suggests that AP-1 inhibition by bis-BHA results from suppression of its transcriptional activity.

To clarify the inhibitory effect of bis-BHA on AP-1, RAW cells were transfected with a luciferase reporter gene under the control of a promoter containing AP-1 sites prior to stimulation of the cells. The luciferase assay was subsequently performed. As expected, bis-BHA clearly inhibited the fimbria-stimulated transcriptional activity of AP-1 in the cells (Figure 3). However, 2-BHA showed only slight inhibition in this system. From these findings, it was clear that bis-BHA functioned as an effective inhibitor of fimbria-stimulated AP-1 activation.

COX-2 is one of the rate-limiting enzymes in the conversion of arachidonic acid to prostanoids that is induced by growth factors, cytokines and lipopolysaccharide (LPS) in a variety of cells closely involved in inflammation and mutagenesis (19, 26, 27). We investigated whether BHA and bis-BHA could inhibit the fimbria-induced COX-2 gene in the cells studied. The fimbria-induced gene expression of COX-2 was clearly inhibited by bis-BHA at the low concentration of 10μM (Figure 4 A, B). However, BHA did not inhibit COX-2 expression. These findings suggest that bis-BHA exerts potent anti-inflammatory and anticarcinogenic activities via suppression of AP-1 activity and, subsequently, COX-2 expression.

We recently demonstrated that three synthetic dimers of o-methoxyphenols, bis-eugenol (3,3’-dimethoxy-5,5’-di-2-propenyl-1,1’-biphenyl-2,2’-diol), dehydrodiisoeugenol (2(3-methoxy-4-hydroxyphenyl)-3-methyl-5-(1-propenyl)-7-methoxy-2,3-dihydrobenzofuran) and bis-ferulic acid (4-cis,8-cis-bis(4-hydroxy-3-methoxyphenyl)-3,7-dioxabicyclo[3.3.0]octane-2,6-dione), all designed for decreasing the pro-oxidant activity derived from eugenol (4-allyl-2-methoxyphenol), isoeugenol (4-propenyl-2-methoxyphenol) and ferulic acid (4-hydroxy-3-methoxycinnamic acid), components of clover oil or rice bran, all inhibited LPS-induced expression of inflammatory cytokines and/or COX-2 in macrophages (28-31). These findings suggest that dimer compounds derived from various phenolic compounds may possess potent anti-inflammatory activity. In the present study, bis-BHA strongly inhibited fimbria-stimulated AP-1 activation and, consequently, expression of the COX-2 gene. However, 2-BHA did not show such inhibitory activity, possibly due to its own oxidation activity. Some free radical intermediates, i.e., cyclic BHA compounds such as bis-BHA, are generated during the peroxidative oxidation of 2-BHA (32). The peroxidative oxidation of 2-BHA in biological systems may be affected by fimbria-stimulated AP-1 activation.

In conclusion, since bis-BHA exhibited an inhibitory effect on AP-1 activation, it might be applicable to the chemoprevention of oral diseases, such as leukoplakia and destructive chronic periodontitis.
References


Received March 10, 2006
Accepted April 10, 2006