

Anti-inflammatory Activity of the Artificial Antioxidants 2-*Tert*-butyl-4-methoxyphenol (BHA), 2,6-Di-*tert*-butyl-4-methylphenol (BHT) and 2,4,6-Tri-*tert*-butylphenol (TBP), and their Various Combinations

YUKIO MURAKAMI, AKIFUMI KAWATA, TADASHI KATAYAMA and SEIICHIRO FUJISAWA

*Division of Oral Diagnosis, Department of Diagnostic and Therapeutic Sciences,
Meikai University School of Dentistry, Sakado City, Saitama, Japan*

Abstract. *Background/Aim:* The artificial complex phenols, 2-*tert*-butyl-4-methoxyphenol (BHA), 2,6-di-*tert*-butyl-4-methylphenol (BHT) and 2,4,6-tri-*tert*-butylphenol (TBP) exert efficient antioxidant activity; however, they are considerable toxic and potentially tumor-promoting. These phenols, particularly in combinations, have enhanced antioxidant activity due to synergistic interactions and produce bioactive intermediates such as quinone methide. We investigated the anti-inflammatory activity of BHA, BHT and TBP, and combinations of BHT/BHA (in molar ratios of 1:1, 1:2, 1:3 and 2:1), BHT/TBP (1:1), and BHA/TBP (1:1), using gene-expression systems for cyclooxygenase-2 (Cox2) and tumor necrosis factor- α (Tnfa) in RAW264.7 cells. *Materials and Methods:* The inhibitory effects of BHA, BHT and TBP on expression of Cox2 and Tnfa genes upon stimulation with *Escherichia coli* lipopolysaccharide (LPS) or *Porphyromonas gingivalis* (Pg) fimbriae were determined using real-time polymerase chain reaction. *Results:* The inhibitory effect on expression of Cox2 and Tnfa genes upon stimulation with LPS and fimbriae was greatly enhanced by the combination of two antioxidants (molar ratio 1:1), BHT/BHA. In addition, that of the Cox2 gene, but not of Tnfa gene was slightly enhanced by a combination of equimolar BHT/TBP and BHA/TBP. None of the antioxidants alone exerted any anti-inflammatory activity upon stimulation with

LPS, but a slight anti-inflammatory activity was observed upon stimulation with Pg fimbriae. The inhibitory effect of the BHT/BHA combination on expression of Cox2 mRNA upon stimulation with LPS was investigated at afferent molar ratios, and a molar ratio of 1:1 was found to have considerably less effect than a molar ratio of 1:2 or 2:1. The 1:3 combination had no effect. *Conclusion:* The combination of BHT and BHA at a molar ratio of 0.5-2 exerts potent anti-inflammatory activity. This anti-inflammatory activity on the generation of inflammatory mediators in LPS-activated RAW264.7 cells may be attributable to complex synergistic antioxidant activity of the combination of BHT and BHA. Our results suggest the potential usefulness of the BHT/BHA combination at an appropriate molar ratio as an antioxidant in foods and pharmaceuticals, whereas either antioxidant alone is unlikely to be effective.

Butylated hydroxyanisole (BHA) is a mixture of two isomeric organic compounds, 2-*tert*-butyl-4-hydroxyanisole and 3-*tert*-butyl-4-hydroxyanisole. BHA and 2,6-di-*tert*-butyl-4-methylphenol (BHT) (Figure 1) are used as antioxidant food additives, as well as for preserving fats and oil in cosmetics and pharmaceuticals. BHA and BHT are fairly heat-stable and are used in heat-processed foods. They have a number of potentially important pharmacological and toxicological properties. In animals, at high concentrations these compounds impair blood clotting, which can be explained by antagonism of vitamin K; and BHA also dose-dependently induces tumors of the forestomach in animals, whereas long-term exposure to BHT induces liver tumors (1). These adverse effects are mostly observed when high quantities are consumed.

We previously investigated the radical-scavenging activity of BHA, BHT and related compounds and found that they exert a dual pro-oxidant and antioxidant action under certain conditions (2, 3). The oxidative characteristics or

Correspondence to: Dr. Yukio Murakami, Division of Oral Diagnosis, Department of Diagnostic and Therapeutic Sciences, Meikai University School of Dentistry, 1-1 Keyakidai, Sakado City, Saitama 350-0283, Japan. Tel: +81 492855511, Fax: +81 492876657, e-mail: ymura@dent.meikai.ac.jp

Key Words: Phenolic antioxidants, BHA, BHT, antioxidant combination, anti-inflammatory activity, RAW264.7 cells, cytotoxicity, COX2, TNF α .

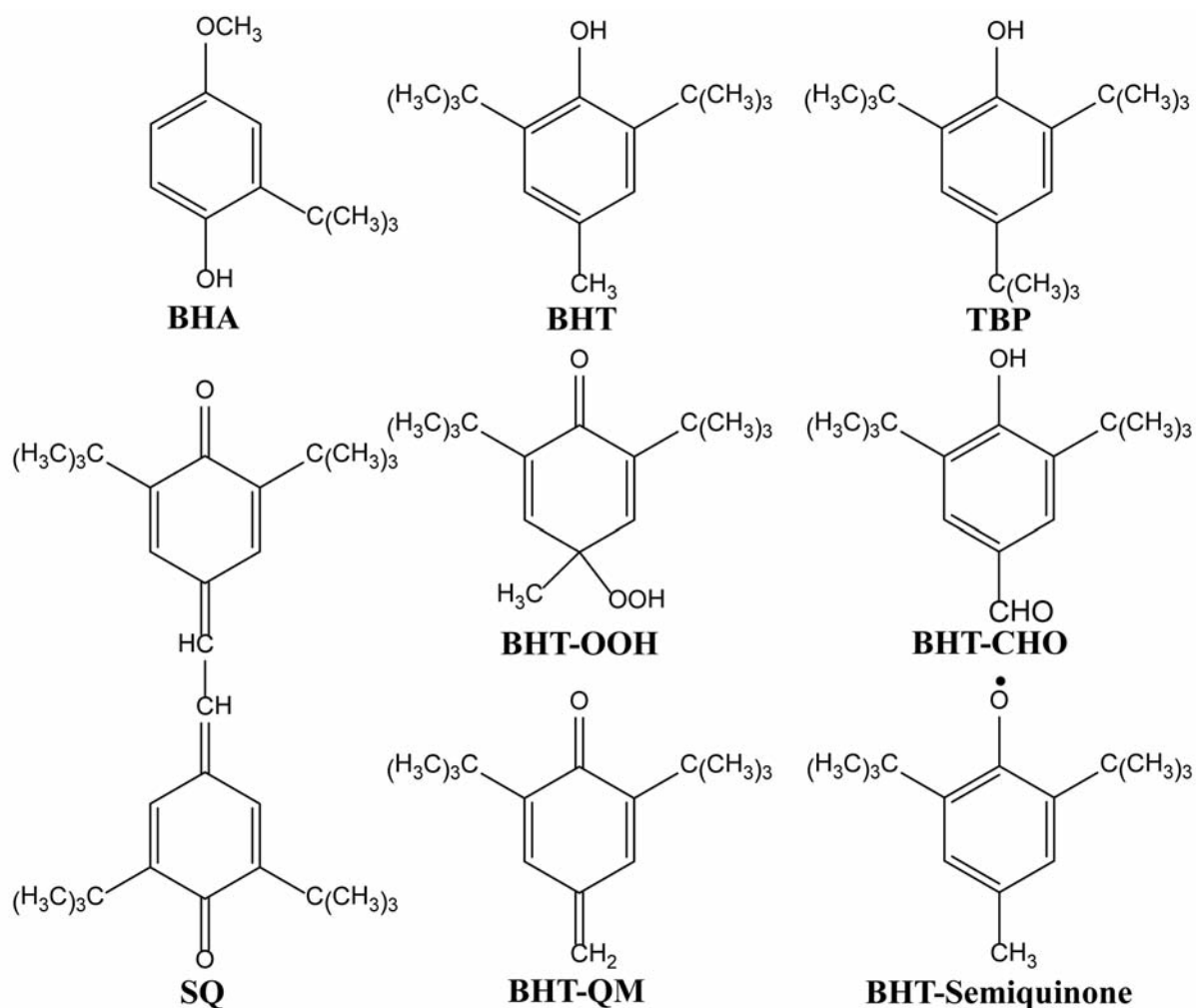


Figure 1. The chemical structures of 2-tert-butyl-4-methoxyphenol (BHA), 2,4,6-tri-tert-butylphenol (TBP), 2,6-di-tert-butyl-4-methylphenol (BHT) and BHT intermediates. BHT-Q: (BHT-quinone); BHT-QM: (BHT-quinone methide); BHT-CHO; BHT-OOH; SQ: (stilbenequinone).

metabolites of BHA, BHT and 2,4,6-tri-tert-butylphenol (TBP) may contribute to carcinogenicity or tumorigenicity due to their pro-oxidant activity; however, they counteract oxidative activity when acting as an antioxidant. BHA and BHT are typically used together in combination, acting synergistically to provide greater antioxidant activity than either agent alone.

It has been proposed that increased peroxidase-dependent oxidation of BHT in the presence of BHA in ram seminal vesicle microsomes occurs *via* direct chemical interaction between the BHA phenoxy radical and BHT resulting from covalent binding of BHT [BHT quinone methide (BHT-QM) and stilbene quinone] to protein (4) (Figure 1). BHT-QM, an electrophile derived from BHT-semiquinone, has been reported to mediate tumor promotion by BHT-OOH (2,6-di-tert-butyl-4-hydroperoxyl-4-methyl-2,5-

cyclohexadienone) (5). We previously investigated the cytotoxicity and apoptosis-inducing activity of BHT, BHA and a BHA/BHT combination (molar ratio 1:1) using a human leukemia cell line (HL-60) and a human squamous carcinoma cell line (HSC-2). We found that the cytotoxicity of BHA/BHT was greater than that of either antioxidant alone, and that addition of certain antioxidants (cysteine, sodium ascorbate, and catalase) reduced this cytotoxicity, suggesting that BHT and BHA affect induction of apoptosis. Furthermore, the expression of manganese superoxide dismutase (*MnSOD*) mRNA in HL60 cells, as assayed by reverse transcriptase-polymerase chain reaction (PCR), was inhibited by the BHA/BHT combination to a greater extent than by either antioxidant alone, particularly activating caspases 3, 8 and 9 (6). The cytotoxicity and induction of apoptosis by the BHA/BHT combination may

be attributable to synergistically greater antioxidant activity and radical-induced formation of intermediates in biological systems.

Lipopolysaccharide (LPS) is well-known as one of the most powerful bacterial virulence factors in terms of its pro-inflammatory properties. LPS stimulates macrophages to release inflammatory cytokines, interleukin-1 (IL-1) β and tumor necrosis factor (TnF, also shown as TnF α), and also stimulates the production of reactive oxygen species (ROS) such as hydrogen peroxide (7). The dynamic systemic pro-inflammatory cellular response to localized periodontal bacteria, when accompanied by elevated levels of these cytokines, can sometimes lead to widespread organ damage or even death (8). Periodontitis is a multi-factorial disease linked to many factors such as oral bacteria, genetic disorders, tobacco and alcohol use, nutrition, diabetes, stress and an impaired host response (9). In oral diseases, *Porphyromonas gingivalis* is a key organism associated with periodontal destruction in patients with adult periodontitis. Toll-like receptors (TLRs) on macrophages are associated with the recognition of *P. gingivalis* components such as LPS and fimbriae, which in turn leads to aspects of periodontitis such as cytokine and chemokine production and oral bone loss (10-12).

Several investigators have demonstrated that BHA significantly inhibits cytokine-induced inflammatory responses in human and mouse cells *via* its potent antioxidant activity (13, 14). These findings suggest that artificial antioxidants such as BHA and BHT may exert potent chemopreventive activity against chronic diseases.

We previously investigated the inhibitory effects of various natural and artificial phenolic compounds on activation of activator protein 1 and nuclear factor-kappa B, and expression of inflammatory cytokines and cyclooxygenase (Cox)-2 in the mouse macrophage-like cell line RAW264.7 stimulated with LPS or fimbriae from *P. gingivalis* and found that several biphenolic compounds, such as *bis*-BHA, curcumin, honokiol, *p*-hydroxytoluene dimer and *p*-hydroxyanisole dimer, exerted potent anti-inflammatory activity (15-19). It has been reported that RAW264.7 cells cultured in serum-replete α -minimum essential medium or serum-depleted medium supported the growth of co-cultured normal human osteoclasts (20). Phenolic antioxidants such as resveratrol prevent receptor activator of nuclear factor kappa-B ligand-induced osteoclast differentiation of RAW264.7 cells through inhibition of ROS production (21). The beneficial effects of phenolic compounds on chronic diseases such as periodontitis, including bone resorption, may be evaluable using our experimental model employing *P. gingivalis* fimbriae and RAW264.7 cells.

In the present study, we investigated the anti-inflammatory activity of BHA, BHT and TBP, and combinations of BHT/BHA (in molar ratios of 1:1, 1:2, 1:3 and 2:1),

BHT/TBP (1:1) and BHA/TBP (1:1), using real-time PCR, on expression of *Cox2* and *Tnfa* genes in RAW264.7 cells stimulated with LPS, and *P. gingivalis* fimbriae, respectively.

Materials and Methods

Materials. BHT, BHA and TBP were purchased from Tokyo Kasei Co. (Tokyo, Japan). The chemical structures of these phenol-related compounds and their metabolites are shown in Figure 1. Their solutions were prepared by dissolving each of them in dimethyl sulfoxide, and subsequent dilution to the indicated concentrations using serum-free RPMI-1640 (Invitrogen Co., Carlsbad, CA, USA) as test samples. Fetal bovine serum (FBS) was obtained from HyClone (Logan, UT, USA). *E. coli* O111 B4-derived LPS was obtained from List Biological Laboratories, Inc. (Campbell, CA, USA).

Cell culture. The murine macrophage-like cell line RAW264.7, obtained from Dainippon Sumitomo Pharma Biomedical Co. Ltd. (Osaka, Japan), was used. The cells were cultured at 37°C and with 5% CO₂ in air to a subconfluent state in RPMI-1640 medium supplemented with 10% FBS, washed, and then incubated overnight in serum-free RPMI-1640. They were then washed again and treated with the test samples.

Preparation of *P. gingivalis* fimbriae. *P. gingivalis* ATCC33277 fimbriae were prepared and purified from cell washings by the method of Yoshimura *et al.* (22). As documented previously, purified fimbria-induced biological activities were not attributable to LPS contaminants in the preparation (23, 24). The viability of the cells after exposure to the fimbriae at the concentrations used was over 90%, as assessed using a Cell Counting Kit-8 (CCK-8) (Dojindo Co., Kumamoto, Japan) (25). The protein content of the fimbriae was measured by the method of Smith *et al.* (26).

Preparation of total RNA and real-time PCR. The preparation of total RNA and the procedure used for real-time PCR have been described elsewhere (17). In brief, cells in NUNC 96-flat-well-type microculture plates (10⁵ cells per well) were treated with the test samples. Total RNA was isolated using an RNeasy Plus Micro Kit (Qiagen Japan Co. Ltd., Tokyo, Japan), in accordance with the instruction manual. cDNA was synthesized from total RNA (2 μ g) of each sample by random priming using a High Capacity RNA-to-cDNA Kit (Life Technologies Japan, Tokyo, Japan). Reaction mixtures without reverse transcriptase were used as a negative control. An aliquot of each cDNA synthesis reaction mixture was diluted and used for quantification by real-time PCR. An equal-volume aliquot of each cDNA was mixed, serially diluted, and used as a standard. TaqMan probes/primers for *Cox2*, *Tnfa* and *18S* rRNA and the PCR enzyme mix for real-time PCR were purchased from Life Technologies Japan. Quantification by real-time PCR was performed in triplicate using GeneAmp Sequence Detection System 5700 software (Life Technologies Japan) in accordance with the instruction manuals. The relative amount of target gene was calculated from standard curves generated in each PCR, and quantitative data with a coefficient of variance (CV) of less than 10% were used for further analyses. Each calculated amount of mRNA was standardized by reference to that of *18S* rRNA. Data are expressed as the mean of three independent experiments. Statistical analyses were performed using Student's *t*-test.

Results

Cox2 and *Tnfa* inhibition by antioxidants alone and in equimolar combinations. The inhibitory effects of BHT-related compounds on LPS- or *P. gingivalis*-fimbria induced expression of the *Cox2* or *Tnfa* genes in RAW264.7 cells were investigated at a non-cytotoxic concentration of 10 μ M using real-time PCR. LPS-induced gene expression of *Cox2* was inhibited more dramatically by the BHT/BHA combination than by either antioxidant alone (Figure 2). The 1:1 BHT/BHA combination induced a 50% decrease in gene expression of *Cox2*. Similarly, equimolar BHT/TBP and BHA/TBP combinations inhibited the expression of *Cox2* weakly. LPS-induced expression of *Tnfa* was weakly but significantly suppressed by the 1:1 BHT/BHA combination, whereas the other antioxidant combinations had no suppressive effect (Figure 3). By contrast, fimbria-induced expression of the *Cox2* gene was slightly but significantly inhibited by BHA, BHT and TBP, and by the BHA/TBP combination. The equimolar BHT/BHA and BHT/TBP combinations, particularly the former, induced a 50% decrease in the expression of *Cox2* ($p < 0.01$; Figure 4). BHA, BHT, and TBP did not inhibit the expression of *Tnfa* mRNA induced by *P. gingivalis* fimbriae, whereas each combination, particularly BHT/BHA, induced a 50% decrease in the expression of *Tnfa* mRNA ($p < 0.01$; Figure 5). These results indicate that the BHT/BHA combination exerted a strong inhibitory effect on the expression of both *Cox2* and *Tnfa* mRNA.

Cox2 and *Tnfa* inhibition by different combinations of two antioxidants at different molar ratios. The results are shown in Figures 6 and 7, respectively. The inhibitory effects of the BHT/BHA combination on LPS-stimulated expression of the *Cox2* and *Tnfa* genes were investigated using different BHA concentrations and time periods. The BHT/BHA combination at molar ratio of 1:2 and 2:1 inhibited LPS-stimulated gene expression of *Cox2* more markedly than did their 1:1 combination. However, treatment with an excess of BHA, as in the 1:3 BHT/BHA combination had no inhibitory effect (Figure 6). LPS-induced gene expression of *Tnfa* was more greatly suppressed by the 1:2 and 2:1 BHT/BHA combinations, than by the 1:1 combination ($p < 0.05$) (Figure 7). The 1:2 and 2:1 BHT/BHA combinations induced a 50% decrease in LPS-induced gene expression of *Tnfa*. By contrast, treatment with excess BHA in the 1:3 BHT/BHA combination induced a more marked expression of *Tnfa* gene than did stimulation with *P. gingivalis* fimbriae alone. These results indicate that a combination of BHT/BHA at 1:2 and 2:1 molar ratios has a more effective anti-inflammatory activity than that at 1:1 or of either antioxidant alone.

Table I. Cited calculated quantum chemical parameters for 2-*t*-butyl-4-methoxyphenol (BHA), 2,6-di-*t*-butyl-4-methylphenol (BHT) and 2,4,6-tri-*t*-butylphenol (TBP). The lowest unoccupied molecular orbital (LUMO) energy (E_{LUMO}) and highest occupied molecular orbital (HOMO) energy (E_{HOMO}) were calculated using Spartan 6 software (Wavefunction Inc., Irvine, CA, USA) using DFT (B3LY/6-31G*).

Phenol	E_{LUMO} (eV)	E_{HOMO} (eV)	BDE (kJ/mol)	IP (eV)	HOMO-LUMO gap eV
BHA	-5.30	0.109	325	5.30	5.409
BHT	-5.517	0.258	319	5.52	5.775
TBP	-5.542	0.255	320	5.54	5.797

HOMO-LUMO gap: Energy difference between the HOMO and LUMO; IP: ionization potential by Koopman's theorem, absolute HOMO energy value; BDE: phenolic O-H bond-dissociation enthalpy. These values were taken from Fujisawa and Kadoma (40).

Discussion

Horswill and Ingold reported that oxidized 2- or 2,6-*tert*-butylphenols such as BHA, BHT and TBP produce certain reactive intermediates, as the stoichiometric factor (n , number of free radicals trapped by one mole of phenolic moiety of antioxidant) of these compounds is 2.0 or less (27). We previously investigated the radical-scavenging activity of various phenolic compounds and found that in general, monophenolic compounds with an n value of about 2 suggests the formation of quinone, whereas those with an n value near 1 suggests the formation of dimers (3). Highly hindered TBP and BHT, with two bulky *tert*-butyl groups at the *ortho* position, have reduced access to free radicals such as the superoxide radical (O_2^-), free hydroxy radicals (OH^-), nitric oxide ($.NO$) and peroxy radical (ROO^\bullet) to the phenolic hydrogen atom. In contrast, less hindered BHA, with one bulky *tert*-butyl group at the *ortho* position, is easily attacked by free radicals at the phenolic hydrogen atom. Therefore, there exists a large difference of radical-scavenging activity between BHA, a less-hindered phenol, and BHT and TBP, hindered phenols. On the other hand, since the ionization potential (IP) of BHA is the smallest among the three antioxidants (Table I), in a mixture of BHT (or TBP) with BHA, BHA is believed to be easily and initially oxidized in the presence of ROS, including ROO^\bullet radicals from unsaturated fatty acids in biological systems. In the synergistic antioxidant mechanism of mixtures with BHA, BHT or TBP interact with the phenoxy radical of BHA. This then removes a hydrogen from the hydroxy group of BHT (or TBP). BHT and TBP thus act as a hydrogen replenishers of BHA, allowing recovery of its antioxidant activity. On the other hand, the bond dissociation enthalpy for the phenolic O-H and the IP values for BHT and TBP are almost identical to each other.

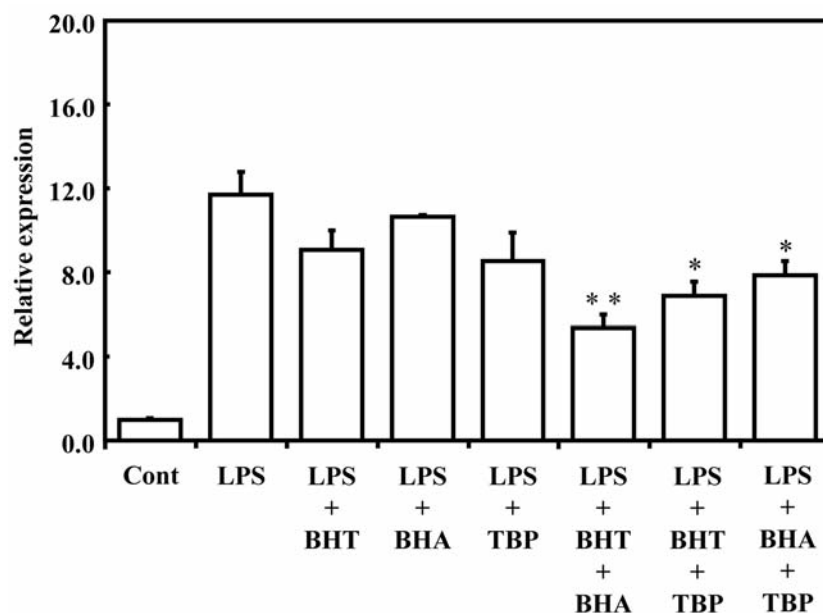


Figure 2. Effect of 2,6-di-tert-butyl-4-methylphenol (BHT), 2-tert-butyl-4-methoxyphenol (BHA) and 2,4,6-tri-tert-butylphenol (TBP) on lipopolysaccharide (LPS)-stimulated expression of the cyclooxygenase-2 (Cox2) gene in RAW264.7 cells. The cells were pre-treated for 30 min with each of these BHT-related compounds alone at 10 μ M, or in three combinations at the same concentration. They were then incubated for 3 h with or without LPS at 100 ng/ml, and their total RNAs were then prepared. Each cDNA was synthesized, and the levels of expression of Cox2 mRNA were determined by real-time polymerase chain reaction and standardized against the expression of 18S rRNA. The results are presented as means \pm SE of three independent experiments. SE<15%. Significantly different at * p <0.05 and ** p <0.01 vs. LPS.

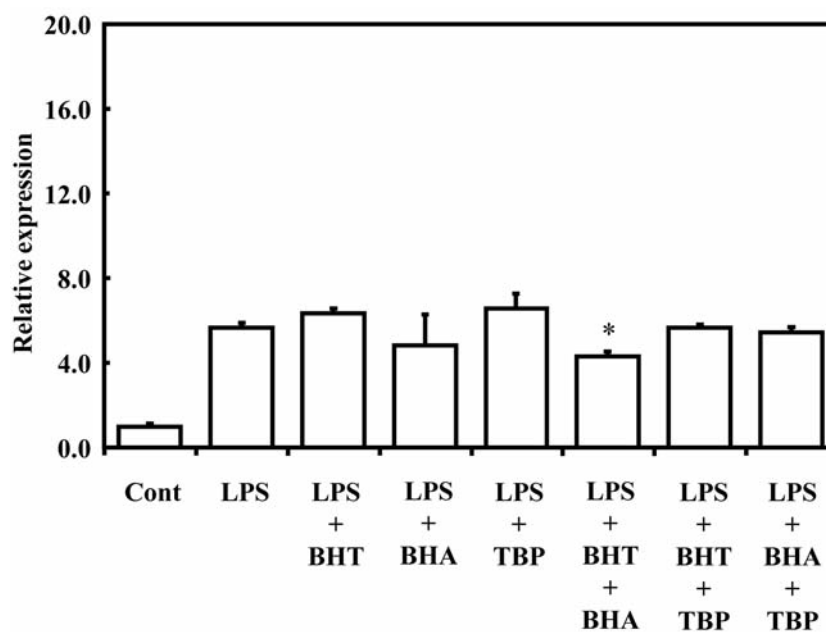


Figure 3. Effect of 2,6-di-tert-butyl-4-methylphenol (BHT), 2-tert-butyl-4-methoxyphenol (BHA) and 2,4,6-tri-tert-butylphenol (TBP) on lipopolysaccharide (LPS)-stimulated expression of the tumor necrosis factor alpha (Tnfa) gene in RAW264.7 cells. The cells were pretreated for 30 min with these BHT-related compounds alone at 10 μ M, or in three combinations at the same concentration. They were then incubated for 3 h with or without LPS at 100 ng/ml, and their total RNAs were then prepared. Each cDNA was synthesized, and the expression levels of Tnfa mRNA were determined by real-time polymerase chain reaction and standardized against the expression of 18S rRNA. The results are presented as means \pm SE of three independent experiments. SE<15%. Significantly different at * p <0.05 vs. LPS.

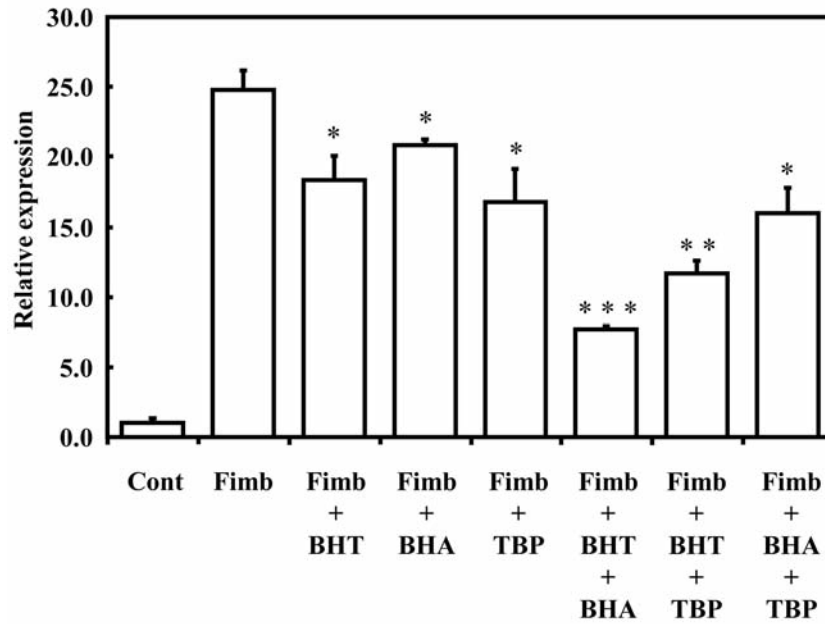


Figure 4. Effect of 2,6-di-tert-butyl-4-methylphenol (BHT), 2-tert-butyl-4-methoxyphenol (BHA) and 2,4,6-tri-tert-butylphenol (TBP) on *Porphyomonas gingivalis* fimbria-stimulated expression of the cyclooxygenase-2 (Cox2) gene in RAW264.7 cells. The cells were pretreated for 30 min with these BHT-related compounds alone at 10 μ M, or in three combinations at the same concentration. They were then incubated for 3 h with or without the fimbriae at 4 μ g/ml, and their total RNAs were then prepared. Each cDNA was synthesized, and the expression levels of Cox2 mRNA were determined by real-time polymerase chain reaction and standardized against the expression of 18S rRNA. The results are presented as means \pm SE of three independent experiments. SE<15%. Significantly different at * p <0.05, ** p <0.01 and *** p <0.005 vs. fimbriae.

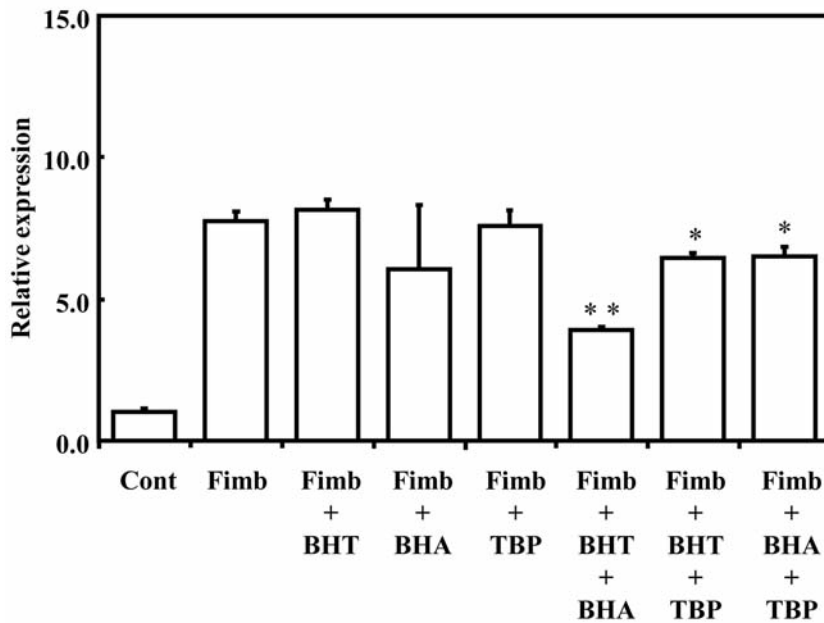


Figure 5. Effect of 2,6-di-tert-butyl-4-methylphenol (BHT), 2-tert-butyl-4-methoxyphenol (BHA) and 2,4,6-tri-tert-butylphenol (TBP) on *Porphyomonas gingivalis* fimbria-stimulated expression of the tumor necrosis factor alpha (Tnfa) gene in RAW264.7 cells. The cells were pretreated for 30 min with these BHT-related compounds alone at 10 μ M, or in three combinations at the same concentration. They were then incubated for 3 h with or without the fimbriae at 4 μ g/ml, and their total RNAs were then prepared. Each cDNA was synthesized, and the levels of expression of Tnfa mRNA were determined by real-time polymerase chain reaction and standardized against the expression of 18S rRNA. The results are presented as means \pm SE of three independent experiments. SE<15%. Significantly different at * p <0.05 and ** p <0.01 vs. fimbriae.

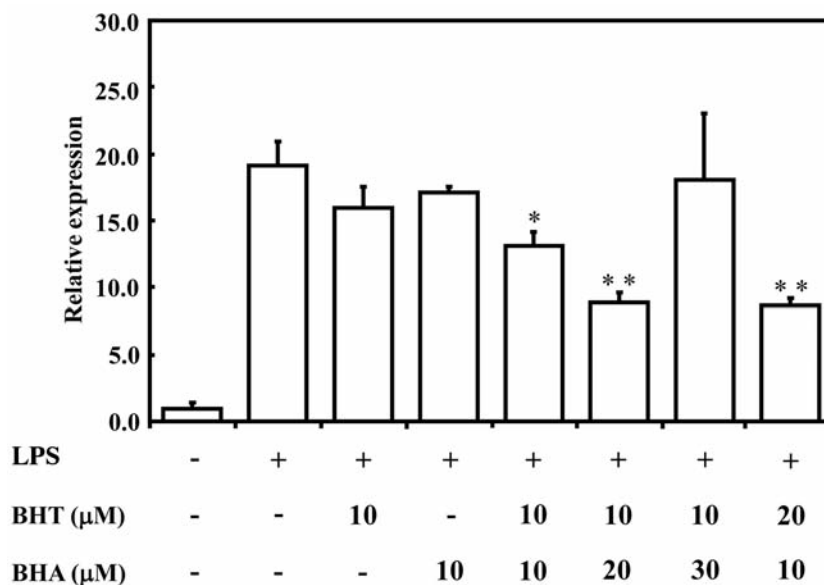


Figure 6. Synergistic inhibitory effect of 2,6-di-tert-butyl-4-methylphenol (BHT) and 2-tert-butyl-4-methoxyphenol (BHA) on lipopolysaccharide (LPS)-stimulated expression of the cyclooxygenase-2 (Cox2) gene in RAW264.7 cells. The cells were pretreated for 30 min with the indicated doses of these BHT-related compounds. They were then incubated for 3 h with or without LPS at 100 ng/ml, and their total RNAs were then prepared. Each cDNA was synthesized, and the levels of expression of Cox2 mRNA were determined by real-time polymerase chain reaction and standardized against the expression of 18S rRNA. The results are presented as means±SE of three independent experiments. SE<15%. Significantly different at * $p<0.05$ and ** $p<0.01$ vs. LPS.

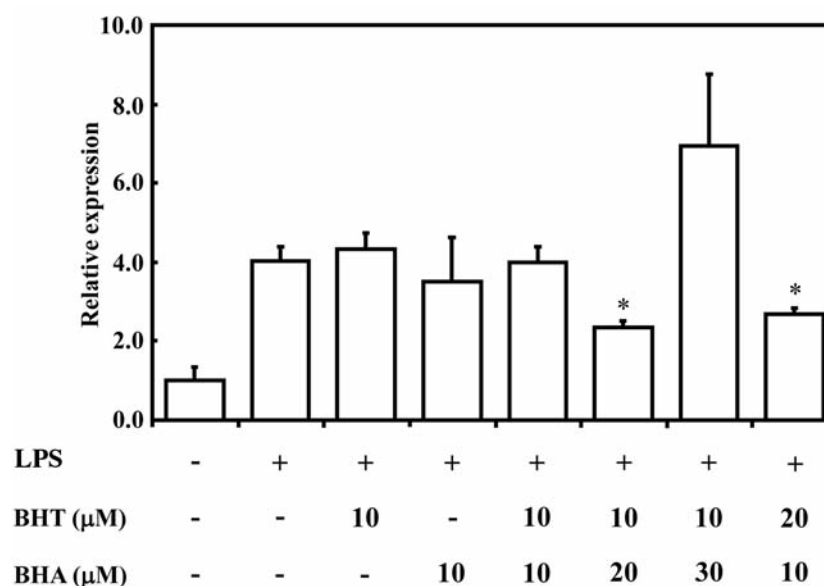


Figure 7. Synergistic inhibitory effect of 2,6-di-tert-butyl-4-methylphenol (BHT) and 2-tert-butyl-4-methoxyphenol (BHA) on lipopolysaccharide (LPS)-stimulated expression of the tumor necrosis factor alpha (Tnfa) gene in RAW264.7 cells. The cells were pretreated for 30 min with the indicated doses of these BHT-related compounds. They were then incubated for 3 h with or without LPS at 100 ng/ml, and their total RNAs were then prepared. Each cDNA was synthesized, and the levels of expression of Tnfa mRNA were determined by real-time polymerase chain reaction and standardized against the expression of 18S rRNA. The results are presented as means±SE of three independent experiments. SE<15%. Significantly different at * $p<0.05$ vs. LPS.

Therefore, in a mixture of BHT and TBP, phenoxy radicals derived from each antioxidant may be produced simultaneously in the presence of ROS and, consequently,

BHT radicals possibly interact with TBP radicals, and *vice versa*, subsequently reacting with ROO \cdot radical and a chain terminator.

In the present study, BHA, BHT and TBP alone showed a slight but significant inhibitory effect on the expression of *Cox2* mRNA stimulated with *P. gingivalis* fimbriae (Figure 3), whereas they exerted no significant inhibitory effect on the expression of *Tnfa* mRNA in RAW264.7 cells stimulated with LPS (Figure 2). At a molar ratio of 1:1, the BHT/BHA combination exerted anti-inflammatory activity in the presence of both types of stimulation. By contrast, the BHT/TBP and BHA/TBP combinations did not exert any activity in the presence of LPS, whereas they exerted relatively weak activity in the presence of *P. gingivalis* fimbriae. These findings indicate that there is a possible difference in the inhibitory effects of phenolic antioxidants under stimulation by LPS and *P. gingivalis* fimbriae. LPS quickly stimulated ROS in macrophages and also stimulated macrophages to release the inflammatory cytokines, IL-1 β and *Tnfa*. ROS production in RAW264.7 cells stimulated with LPS may be considerably greater than when stimulated by *P. gingivalis* fimbriae. The lack of anti-inflammatory activity of BHT, BHA and TBP obtained using LPS stimulation may be due to their declining phenol function because these antioxidants scavenged a large amount of ROS derived from LPS. Whereas the weak anti-inflammatory activity for BHT, BHA and TBP obtained using the *P. gingivalis* fimbriae stimulatory system suggests that their antioxidant activities are operative in that system. By contrast, for the BHT/BHA combination, BHT acts as the synergist, the regenerator of BHA from phenoxy radical of BHA (28). The BHT/BHA combination at a molar ratio of 0.5-2 exerted potent anti-inflammatory activity in RAW264.7 cells stimulated with LPS, possibly being associated with its high ROS-scavenging capacity. ROS, a universal second messenger, are important chemical mediators that regulate the transduction of signal protein activity *via* redox chemistry. The OH \cdot radicals of ROS would not act as a messenger because they have an extremely high reactivity and short life, whereas antioxidant radicals such as phenoxy radicals arising from BHA and BHT would be stable. In human and murine macrophages, COX2 expression is induced by LPS (29) and ROS, and this mediates nicotinamide adenine dinucleotide phosphate oxidase in monocyte differentiation (30). At a molar ratio of 1:1, the BHT/BHA combination showed greater anti-inflammatory activity than both the BHT/TBP and BHA/TBP combinations. This may be related to BHT being able to regenerate BHA.

On the other hand, many reports have indicated that BHA and BHT have adverse effects. Thomson *et al.* reported that BHA enhances BHT-induced lung toxicity in mice (31). BHA may facilitate the activation of BHT in the lung as a result of both increased hydrogen peroxide formation and subsequent peroxidase-dependent formation of BHT-QM through direct interaction of BHA with BHT (32).

Festjens *et al.* reported that BHA has a clearly more marked antinecrotic effect than BHT on L929sAhFas cells, and that this effect of BHA reflects its preferential ROS-scavenging activity and ability to inhibit complex I [nicotinamide adenine dinucleotide hydrate (NADH) quinone oxidoreductase] and lipoxygenases (33). The discrepancy in ROS-scavenging and anti-necrotic activities between BHA and BHT might be attributable to the fact that BHA is more lipophilic and less sterically hindered than BHT (33); phenoxy radicals of BHA may tend to be produced at cellular membranes *via* oxidative stress due to this lipophilicity. We have reported that BHA preferentially undergoes dimerization due to a radical-radical coupling reaction and that the resulting dimer, *bis*-BHA, possesses potent anti-inflammatory activity (15, 16, 19). By contrast, Oikawa *et al.* investigated DNA damage and apoptosis induced by BHT metabolites and found that BHT-OOH and BHT-Q induced DNA strand breaks in cultured cells, whereas BHT-CHO did not (34). BHT-QM, an electrophile, mediates tumor promotion by BHT-OOH (5).

To clarify the mechanism of radical-mediated toxicity, we previously investigated the radical-scavenging activity of BHT metabolites using free radicals derived from azobisisobutyronitrile at 70°C and found that the *n* values for BHT-Q and both BHT-CHO and BHT-OOH are approximately 0.003, 0.08 and 0.08, respectively. BHT-Q had the highest inhibition rate constant ($k_{inh}=3.5\times 10^4\text{ M}^{-1}\text{ s}^{-1}$), followed by BHT-CHO ($k_{inh}=1.3\times 10^4\text{ M}^{-1}\text{ s}^{-1}$) and BHT-OOH ($k_{inh}=1.9\times 10^4\text{ M}^{-1}\text{ s}^{-1}$) (35). It was noteworthy that the *n* and k_{inh} values for BHT were 1.2 and $0.2\times 10^4\text{ M}^{-1}\text{ s}^{-1}$, respectively. The *n* value of about 1 for the antioxidant activity of BHT suggests that oxidized BHT undergoes dimerization due to the *para-para* radical coupling reaction. Oxidation of BHT produces the phenol-type BHT dimer (*bis*-BHT), which can be further oxidized to the quinone-type dimer (STQ) (36). Moreover, although BHT metabolites (or their intermediates) possess a small *n* value, these compounds scavenge free radicals, maintaining a large k_{inh} value (35). Furthermore, we investigated the relationship between the cytotoxicity and k_{inh} value for 2- and 2,6-di-*tert*-butyl phenols and demonstrated that there is a significant linear relationship between the two parameters: as the k_{inh} value increases, cytotoxicity is enhanced. These findings suggested that complex phenols with *tert*-butyl substituents are governed by a radical-mediated toxicity mechanism (37).

The amounts of ROS and electrophiles produced can be metabolically controlled in biological systems, and small quantities of these species induce expression of cell survival genes. By contrast, under certain pathological conditions, electrophiles and ROS are produced in excess, and consequently accelerate cell damage. It is well-known that phenolic compounds are metabolized by peroxidase to form pro-oxidant phenoxy radicals, which are sufficiently reactive

to co-oxidize glutathione or NADH, accompanied by extensive oxygen uptake and ROS formation. Therefore, a combination of BHA and BHT would be expected to suppress infection under certain conditions.

The radical-scavenging activity and anti-inflammatory activity of phenolic compounds are inseparably connected. In this study, the intermediates derived from the BHT/BHA combination at an appropriate molar ratio exhibited anti-inflammatory activity, whereas BHT with a large excess of BHA did not. Some BHT intermediates derived from complex phenols are electrophilic Michael acceptors that are stabilized by conjugation. These acceptors can interact with proteins and thiols with an SH group (nucleophiles) in biological systems, resulting in detoxification.

Quantum chemical calculation might provide a closer insight into the molecular mechanisms of radical-scavenging and anti-inflammatory activity of these phenolic compounds. Molecules with a relatively small highest occupied molecular orbital / lowest unoccupied molecular orbital gap value are generally reactive, while those with a relatively large value are generally not (38). As shown in Table I, BHA has a small gap value and, therefore, is more reactive than BHT, and TBP. In addition, the IP is the most important energetic factor for evaluation of scavenging ability. A relatively high IP decreases the rate of electron transfer between a phenolic antioxidant and oxygen, and therefore phenolic compounds with a higher IP have lower pro-oxidative potency, and *vice versa* (36). BHA has potentially higher pro-oxidant activity than BHT or TBP.

Although synthetic antioxidants such as BHA and BHT exert beneficial effects, they are suspected to have potentially harmful effects on human health (39). Further studies are necessary to clarify the mechanism of anti-inflammatory activity and anti-proliferative activity exerted by various combinations of such complex phenols.

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