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Preventive effect of *bis*-eugenol, a eugenol *ortho* dimer, on lipopolysaccharide-stimulated nuclear factor kappa B activation and inflammatory cytokine expression in macrophages

Yukio Murakami[a], Masao Shoji[a], Shigemasa Hanazawa[b], Shoji Tanaka[a], Seiichiro Fujisawa[a,*]

[a]*Department of Oral Diagnosis, Meikai University School of Dentistry, 1-1 Keyakidai, Sakado City, Saitama 350-0283, Japan*
[b]*Division of Oral Infectious Diseases and Immunology, Faculty of Dental Science, Kyushu University, Maidashi, Higasi-ku, Fukuoka City, Fukuoka 812-8582, Japan*

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Abstract

Eugenol exhibits antioxidant and anti-inflammatory activities, but at higher concentrations acts as an oxidant and potent allergen. It was earlier shown that *bis*-eugenol synthesized by the oxidation of eugenol was less cytotoxic and more highly antioxidative than eugenol. But its anti-inflammatory mechanism remains yet unclear. Since nuclear factor-κB (NF-κB) is a key transcriptional factor in the expression of inflammatory cytokines, we examined whether eugenol and *bis*-eugenol are inhibitors of NF-κB activation. We observed that *bis*-eugenol, but not eugenol, clearly inhibited the degradation of inhibitory κB-α in RAW264.7 murine macrophages stimulated with lipopolysaccharide and, consequently, the transcriptional activity of the stimulated NF-κB in the cells. In addition, *bis*-eugenol actually inhibited LPS-stimulated expression of inflammatory cytokines at both gene and protein levels. These findings suggest that *bis*-eugenol acts as a potent inhibitor of NF-κB.

Keywords: Eugenol; *bis*-Eugenol; NF-κB; LPS; Inflammatory cytokine; Macrophage

1. Introduction

Eugenol (4-allyl-2-methoxyphenol), a component of clover oil, is commonly used as a flavoring agent in cosmetics and food products, and particularly, as a dental material such as filling cements and pulp capping agents that are made by chelation of eugenol with zinc oxide. Several previous studies demonstrated that eugenol has the beneficial properties of having antioxidant and anti-inflammatory activities, which stem from the inhibition of prostaglandin synthesis, neutrophil chemotaxis, and pyretic activity [1–4]. In addition, eugenol has an antigenotoxic and anticarcinogenic potential [5,6]. However, at higher concentrations eugenol has adverse effects of causing inflammatory and allergic reactions such as allergic contact dermatitis [7,8], possibly due to the formation of phenoxyl radicals and, subsequently, of quinone methide intermediates via its pro-oxidant activity. Thus, to lessen the potent oxidative activity of eugenol, we recently synthesized various dimers of eugenol-related compounds and investigated their antioxidant activities and cytotoxicity. In particular, the eugenol dimer *bis*-eugenol (3,3′-dimethoxy-5,5′-di-2-propenyl-1,1′-biphenyl-2,2′-diol) was less cytotoxic and had a significantly higher antioxidant activity than eugenol [9,10], suggesting that *bis*-eugenol might act as a potent inhibitor of inflammatory and allergic responses. However, the mechanism of *bis*-eugenol inhibition of inflammatory responses has still not been clarified in detail.

NF-κB is an important well-known transcriptional factor that regulates inflammatory responses and the expression of inflammatory cytokines [11–14]. Thus, to elucidate the anti-inflammatory mechanism of eugenol and *bis*-eugenol, it is of interest to investigate whether these compounds act

* Corresponding author. Tel.: +81-49-285-5511; fax: +81-49-287-6657.
E-mail address: fujisawa@dent.meikai.ac.jp (S. Fujisawa).

Abbreviations: NF-κB, nuclear factor kappa B; IκB, inhibitory kappa B; IL-1, interleukin-1; TNF-α, tumor necrosis factor-alpha; LPS, lipopolysaccharide.

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as an inhibitor of NF-κB. Since LPS is a potent stimulator of NF-κB in macrophages [15], in the present study, we focused on whether eugenol and *bis*-eugenol could act as an inhibitor of LPS-stimulated NF-κB.

Our findings reported herein suggest that the anti-inflammatory action of *bis*-eugenol is mediated via the inhibition of NF-κB in RAW264.7 cells.

2. Materials and methods

2.1. Reagents

Eugenol was purchased from Tokyo Kasei Co. *bis*-Eugenol was synthesized from eugenol monomers by the *ortho* coupling reaction described previously [10]. The chemical structures of eugenol and *bis*-eugenol are shown in Fig. 1. Megaprime DNA labeling system, 5′-end labeling system, 5′-[α-^{32}P]-dCTP, and [γ-^{32}P]-ATP were purchased from Amersham Pharmacia Biotech. The phospho-specific anti-IκB-α antibody (Ser 32) and anti-IκB-α, both rabbit polyclonal antibodies, and horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG were purchased from New England Biolabs, Inc. RPMI 1640, Opti-MEM, and Lipofectin were obtained from Invitrogen Corp. FBS was from HyClone; and *Escherichia coli* O111 B4-derived LPS, from List Biological Laboratories Inc.

2.2. Cell culture

Cells of the murine macrophage cell line RAW264.7, obtained from Riken Cell Bank, were used. They were cultured to the subconfluent state in RPMI 1640 medium supplemented with 10% FBS at 37° and 5% CO_2 in air, washed, and then incubated overnight in serum-free RPMI 1640. They were then washed further and treated with test samples.

2.3. Gel mobility shift assay

Cells in 15-cm diameter dishes (10^7 cells) were treated with test samples. Their nuclei were isolated, and the extracts were prepared for the gel mobility shift assay described previously [16]. Briefly, the binding reactions were performed for 20 min at room temperature with 10 μg of the nuclear proteins in 2 mM Tris (pH 7.5) containing 8 mM NaCl, 0.2 mM EDTA, 0.8% (v/v) glycerol, 0.2 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mg of poly(dI-dC), and 20,000 cpm of a ^{32}P-labeled NF-κB oligonucleotide in a final volume of 20 μL. The double-stranded oligonucleotide containing a tandem repeat of the consensus sequence for the binding site -GGGGACTTTCC- for NF-κB was end-labeled by the T4 polynucleotide kinase-[γ-^{32}P]-ATP method. DNA–protein complexes were electrophoresed on native 5% polyacrylamide gel in 0.25× TBE buffer (22 mM Tris (pH 8.0), 22 mM boric acid, 0.6 mM EDTA). The gel was dried, and then exposed to Kodak X-ray film at −70°.

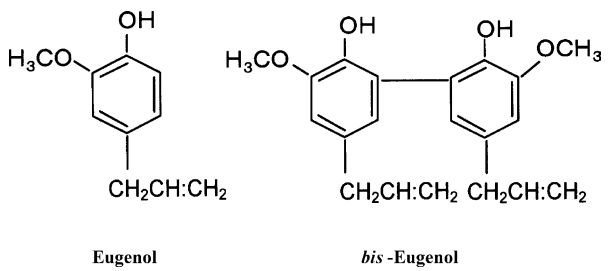

Fig. 1. Chemical structures of eugenol and *bis*-eugenol.

2.4. Western blot analysis for IκB-α

Cells in 5-cm diameter dishes (10^6 cells) were treated with test samples. Then the cells were solubilized with lysis buffer (20 mM Tris (pH 7.9), 1% sodium deoxycholate, 1% (v/v) Nonidet P-40, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 20 mM EDTA, 10 μg/mL aprotinin, 1 mM PMSF) as described previously [17]. The protein concentrations were measured by the method of Smith *et al.* [18]. Next, the sample (10 μg of protein) was subjected to SDS–PAGE on a 12.5% polyacrylamide gel, and the separated proteins were transferred to a polyvinylidene difluoride membrane (Millipore Co.). Then, the blots were blocked with 5% skim milk, washed, and treated with primary antibody against IκB-α. Proteins were detected with a Phototope-HRP Western blot detection kit (New England Biolabs), and the blots were exposed to Kodak X-ray film.

2.5. cDNA hybridization probe

Plasmids containing mouse IL-1β and TNF-α cDNAs were provided by T. Hamilton, and those containing mouse neutrophil chemoattractant KC cDNA, by C.D. Stiles. A plasmid with β-actin cDNA was obtained from the Japanese Cancer Research Bank. The methods used for plasmid preparation were described previously [19].

2.6. Northern blot analysis

Cells in 5-cm diameter dishes (10^6 cells) were treated with test samples. Total cellular RNA was extracted by the AGPC procedure [20]. As described previously [16], the RNA was subjected to 1% agarose electrophoresis and blotted onto nylon membranes (MSI Magnagraph). The membranes were hybridized with each cDNA probe labeled with 5′-[α-^{32}P]-dCTP by use of a megaprime DNA labeling system. After hybridization, the membranes were washed, dried, and exposed overnight to Kodak X-ray film at −70°. β-Actin was used as internal standard for quantification of total RNA in each lane of the gel.

2.7. Plasmid construction and transient expression assay

The plasmid pNF-κB-Luc (Clontech) was constructed by inserting a synthetic oligonucleotide containing four tandem copies of the NF-κB consensus sequence into the corresponding sites of pTAL-Luc (Clontech), which contains the HSV thymidine kinase (HSV-TK) promoter enhancer region located upstream of the firefly luciferase gene. Also used was pRL-TK, which contains the HSV-TK promoter located region upstream of *Renilla* luciferase (Promega). Transient expression was assayed as described previously [21]. Briefly, cells in 5-cm diameter dishes (10^6 cells) were incubated for 1 hr with serum-free Opti-MEM. Then they were transfected with the reporter plasmid at 2 μg and pRL-TK at 0.2 μg by using Lipofectin (Invitrogen). After incubation for 24 hr, the transfected cells were treated with test samples in serum-free RPMI 1640. Then, the cellular extracts were prepared with reporter passive lysis buffer (Promega) and examined for firefly luciferase activity after determination of *Renilla* luciferase activity (pRL-TK). The latter was used as an internal control to normalize for variations in transfection efficiency. The results were expressed as a percentage of the maximum.

2.8. Measurement of IL-1β and TNF-α

Cells in 24-well microculture plates were treated with test samples, and then their cell culture supernatants were harvested. IL-1β and TNF-α protein in the culture supernatants were measured with enzyme-linked immunosorbent assay (ELISA) kits utilizing anti-mouse IL-1β and TNF-α antibody (BioSource International, Inc.), as described previously [22].

3. Results and discussion

As demonstrated by many previous studies [11,13–15,17], NF-κB is an important transcriptional factor for the expression of various inflammatory cytokines. Since it is well known that the activation of this transcriptional factor is initiated by phosphorylation-dependent degradation of IκB, an inhibitor of NF-κB [12,14], we focused on the inhibitory effects of *bis*-eugenol and eugenol on NF-κB, as a possible explanation for the anti-inflammatory action of these compounds. First we examined using a gel mobility shift assay whether *bis*-eugenol or eugenol was capable of inhibiting LPS-stimulated transcriptional activity of NF-κB in RAW cells. As shown in Fig. 2, pretreatment of LPS-stimulated cells with *bis*-eugenol clearly inhibited NF-κB binding to its consensus sequence in the gel mobility shift assay, whereas such an inhibitory effect was not observed with eugenol-pretreated cells. These findings suggested that the NF-κB inhibition by *bis*-eugenol may be associated with suppression of LPS-stimulated IκB degradation. Therefore, by using the Western blot assay, we explored the inhibitory effect of *bis*-eugenol on LPS-stimulated degradation of IκB-α in RAW cells. Figure 3A and B show that LPS dramatically stimulated IκB-α degradation by 30 min after the start of treatment and that this degradation was clearly inhibited by the *bis*-eugenol treatment. We further investigated whether *bis*-eugenol could inhibit LPS-stimulated phosphorylation of IκB-α in the cells by conducting a Western blot assay with phospho-specific anti-IκB-α antibody. As expected, *bis*-eugenol, but not eugenol, clearly inhibited LPS-stimulated phosphorylation of IκB-α by 15 min after the start of treatment (data not shown). These findings strongly suggest that *bis*-eugenol inhibited the LPS-stimulated transcriptional activity of NF-κB by suppressing phosphorylation-dependent proteolysis of IκB-α in the cells. To demonstrate further the effect of *bis*-eugenol on NF-κB, we conducted an NF-κB-promoted luciferase assay. As expected, we observed that *bis*-eugenol actually was able to inhibit the LPS-stimulated transcriptional activity of NF-κB in the cells (Fig. 4). However, this inhibitory effect was not observed with eugenol.

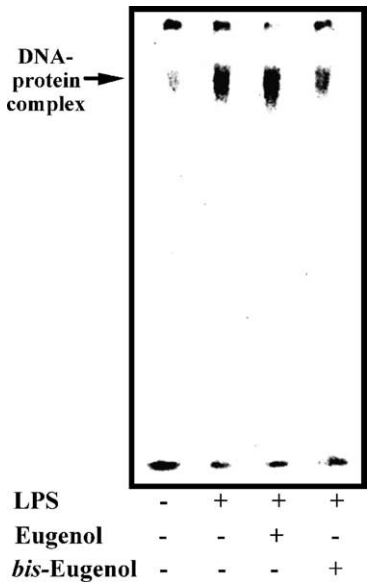

Fig. 2. *bis*-Eugenol inhibits LPS-stimulated NF-κB binding in RAW264.7 cells. The cells were pretreated or not for 30 min with eugenol or *bis*-eugenol at 500 μM and then treated or not with LPS at 100 ng/mL for 45 min. Then the nuclear proteins were prepared for a gel mobility shift assay, which was performed with ^{32}P-labeled oligonucleotide containing the NF-κB consensus sequence in the presence of the nuclear proteins. An identical experiment independently performed gave similar results.

In addition, it is very important to investigate whether *bis*-eugenol actually inhibits LPS-stimulated expression of inflammatory cytokines in RAW cells. Therefore, using the Northern blot assay, we examined the effect of *bis*-eugenol on the expression of IL-1β, TNF-α, and KC genes in LPS-stimulated cells. Figure 5A shows that LPS-induced expression of these genes in the cells was strongly inhibited by *bis*-eugenol. A similar inhibitory effect of *bis*-eugenol

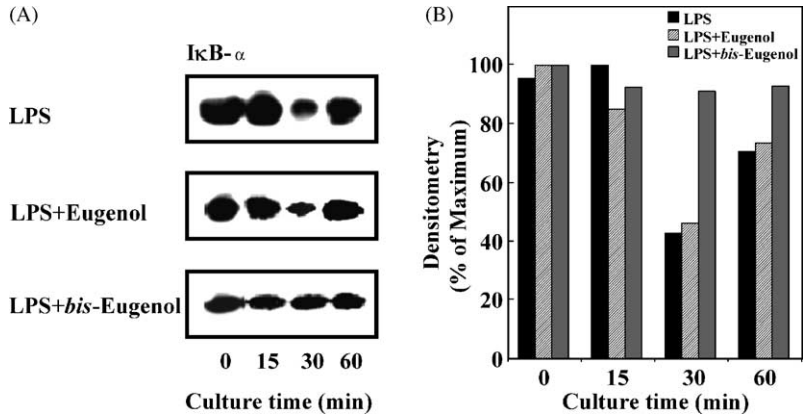

Fig. 3. *bis*-Eugenol inhibits LPS-stimulated IκB-α degradation in RAW264.7 cells. (A) The cells were pretreated or not for 30 min with eugenol or *bis*-eugenol at 500 μM and then treated or not for the indicated times with LPS at 100 ng/mL. Thereafter, their cell lysates were prepared. Equal amounts of cell lysates were analyzed after SDS–PAGE with anti-IκB-α antibody. An identical experiment independently performed gave similar results. (B) Quantification of expression of IκB-α in (A) was done by densitometry, and the data are expressed as a percentage of the maximum.

was also observed in the case of LPS-induced expression of cyclooxygenase-2 and monocyte chemoattractant protein-1 genes in the cells (data not shown). However, eugenol did not exhibit such inhibition. These findings indicate that *bis*-eugenol, in addition to blocking at the transcriptional level, also may be capable of inhibiting the production of inflammatory cytokines by LPS-stimulated cells. Therefore, we finally examined the inhibitory effect of *bis*-eugenol on LPS-stimulated production of IL-1β and TNF-α by the cells. As expected, *bis*-eugenol dramatically inhibited LPS-stimulated production of IL-1β and TNF-α by the cells (Fig. 5B and C).

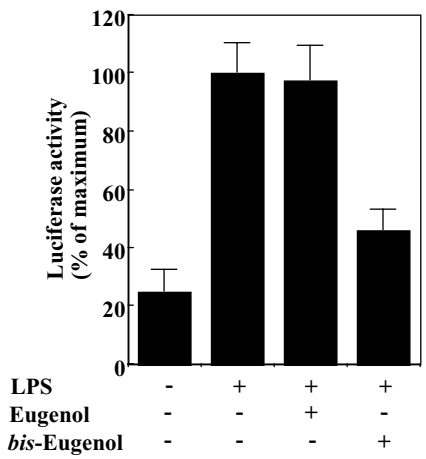

Fig. 4. *bis*-Eugenol inhibits LPS-stimulated activation of NF-κB in RAW264.7 cells. The cells were cotransfected with pRL-TK and reporter plasmid (pNFκB-Luc) and then were washed three times. Next, the transfected cells were pretreated or not for 30 min with eugenol or *bis*-eugenol at 500 μM in serum-free RPMI 1640 and then treated or not with LPS at 100 ng/mL. The cellular extracts were prepared 12 hr later and subsequently subjected to the luciferase assay. The pRL-TK plasmid was used as an internal control to normalize for variations in transfection efficiency. The luciferase activity is expressed as a percentage of the maximum obtained with LPS alone. An identical experiment independently performed gave similar results.

We propose here suppression of NF-κB transcriptional activity as a possible mechanism by which *bis*-eugenol inhibits LPS-stimulated inflammatory cytokine expression. Since several studies have shown an anti-inflammatory effect of eugenol on experimental inflammation [1–4], these protective effects of eugenol might be associated with its antioxidative activity. However, eugenol produces phenoxyl radicals and consequently, has a catalytic action. Eugenol undergoes quinone methide formation and dimer formation under some circumstances [7,23]. The phenoxyl radical-generating activity of eugenol was previously reported to be associated with the dissociation energy (ΔH) for the hydrogen abstraction from its phenolic OH group; and the ΔH of eugenol was found to be significantly smaller than that of *bis*-eugenol, suggesting that eugenol can more efficiently produce phenoxyl radicals [10]. NF-κB is also stimulated by intracellularly generated reactive oxygen species [24], and so phenoxyl radicals (oxygen-centered radical) of eugenol may also be able to stimulate NF-κB. In contrast, *bis*-eugenol with a lower radical production potential inhibited LPS-stimulated NF-κB activation.

We demonstrated previously that LPS and/or fimbriae of periodontopathic bacteria significantly stimulated inflammatory cytokine production via NF-κB [17,25]. As NF-κB is a key transcriptional factor of osteoclast activation [26], the inhibitory effect of *bis*-eugenol on the NF-κB activation may prevent the bacterium-stimulated alveolar bone resorption related to adult periodontal diseases. At present, we still do not know the precise mechanism by which *bis*-eugenol inhibits LPS-stimulated NF-κB, although the suppression of phosphorylation-dependent IκB-α degradation appears to be a part of it. In a further study, we plan to explore the molecular mechanism in greater detail.

In conclusion, we have proposed here a possible mechanism for the anti-inflammatory action of *bis*-eugenol, and suggest that *bis*-eugenol might be useful for the chemoprevention of oral diseases due to its inhibitory activity toward NF-κB and/or inflammatory responses.

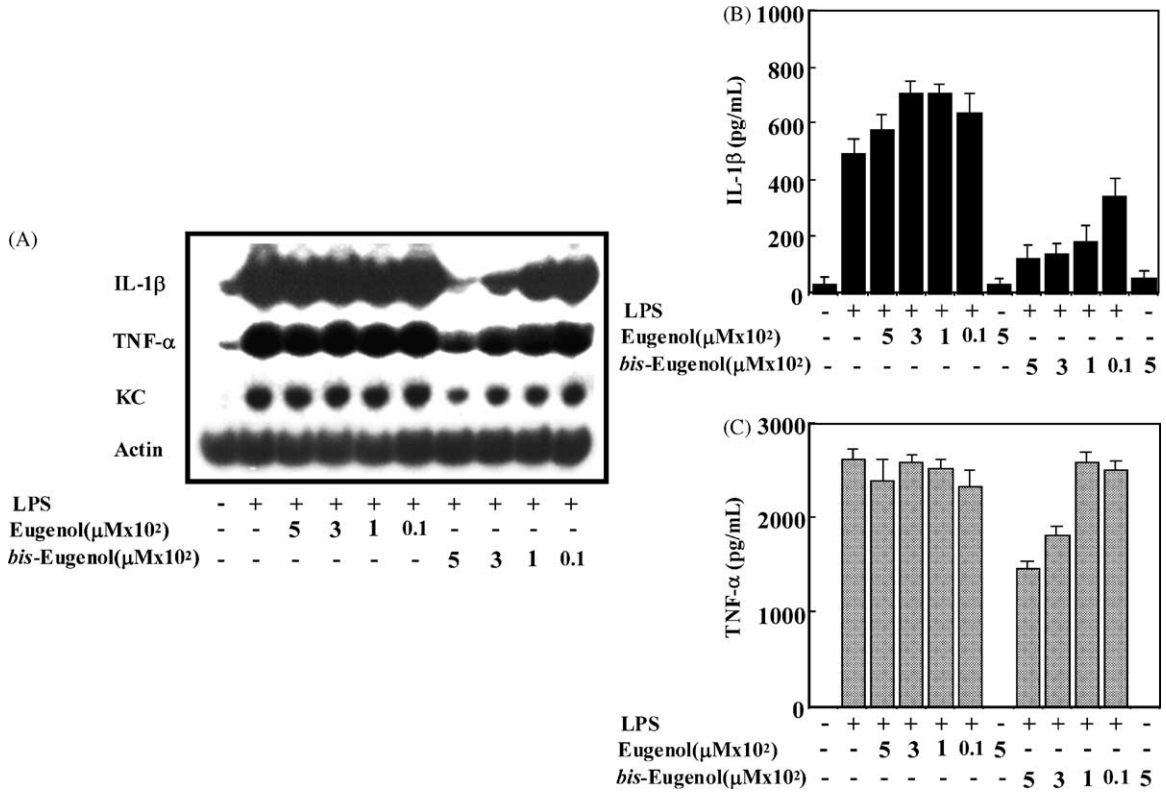

Fig. 5. *bis*-Eugenol inhibits LPS-induced expression of inflammatory cytokine mRNA and protein levels in RAW264.7 cells. (A) The cells were pretreated or not for 30 min with the indicated dosage of eugenol or *bis*-eugenol and then treated or not with LPS at 100 ng/mL. Thereafter, their total RNA was prepared at 1 hr after the LPS addition. Northern blot analysis was performed with IL-1β, TNF-α, KC, and β-actin cDNAs used as probes. An identical experiment independently performed gave similar results. (B and C) The cells were pretreated or not for 30 min with the indicated dosages of eugenol or *bis*-eugenol and then treated or not with LPS at 100 ng/mL. Thereafter, their culture supernatant was collected at 3 hr after the LPS addition. Levels of IL-1β (B) and TNF-α (C) protein were measured by ELISA. The results are expressed as the mean ± SD of three different experiments.

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