Inhibitory Effects of Tocopherols on Expression of the Cyclooxygenase-2 Gene in RAW264.7 Cells Stimulated by Lipopolysaccharide, Tumor Necrosis Factor-α or Porphyromonas gingivalis Fimbriae

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Abstract. Background: Tocopherols, which include α-, β-, γ-, and δ-tocopherol, protect cells against harmful free radicals and play an important role in preventing many human diseases such as cancer, inflammatory disorders, and ageing itself. However, the causal relationships between periodontal or oral chronic diseases and tocopherols have not been sufficiently studied. The present study investigated the inhibitory effects of these compounds on the expression of cyclooxygenase-2 (COX2) mRNA in RAW264.7 cells stimulated with lipopolysaccharide (LPS), tumor necrosis factor-α (TNFα) or fimbriae of Porphyromonas gingivalis (Pg), an oral anaerobe. Materials and Methods: The cytotoxicity (EC50) of tocopherols toward RAW cells was determined using a cell counting kit (CCK-8). The regulatory effect of these compounds on the expression of COX2 mRNA stimulated with LPS, TNFα or Pg fimbriae was investigated using real-time polymerase chain reaction (PCR). Results: Each tocopherol had similarly low cytotoxicity. COX2 gene expression in RAW cells after exposure to the three different macrophage activators was inhibited by the tocopherols (p<0.01). Compared to α-tocopherol, β-, γ- and δ-tocopherol exhibited greater inhibitory effects (p<0.05). Conclusion: Tocopherol exhibit anti-inflammatory activity, and β-, γ- and δ-tocopherol have particularly more potent anti-inflammatory activity than α-tocopherol. Tocopherols may have potential utility for prevention of periodontal and chronic oral diseases.

Tocopherols exist in four isomers, designated α-, β-, γ-, and δ-tocopherol (Figure 1), which differ according to the number and position of the methyl group on the chroman ring, and also in their activity. Tocopherols have three different domains: the functional domain, responsible for their antioxidant activity due to the phenolic O-H group; the signaling domain, which is a regulator of protein kinase C; and the hydrophobic domain, bearing a saturated phytyl side chain, which is a component of biological membranes and lipoproteins. α-Tocopherol (vitamin E) is the most biologically active form, and is an important component of cell membranes (1).

A number of studies have investigated the relationship between vitamin E, mostly in the form of tocopherols, and periodontal diseases. It has been shown that deficiency of vitamin E does not exacerbate destruction of the periodontium in the presence of periodontitis in rats (2), and that patients with periodontal disease have no significant reduction of vitamin E levels (3). In contrast, some positive effects on rat bone loss induced by stress have been observed after administration of vitamin E, and these effects were exerted only when the stress was introduced acutely (4). Recently, it has been suggested that low serum levels of ascorbic acid and α-tocopherol may be a risk factor for periodontal disease in elderly Japanese individuals (5). However, the causal link between periodontal disease and tocopherols has not been sufficiently clarified.

Cyclooxygenases (COX1 and COX2) catalyze the conversion of arachidonic acid to prostaglandin H2 (PGH2), the common precursor of prostaglandins and thromboxanes, which are crucial lipid mediators for regulation of many physiological and pathological responses (6). COX1 is constitutively expressed in many tissues and cells, including platelets, where thromboxanes are generated by this enzyme to promote platelet aggregation. In contrast, COX2 is often induced under acute/chronic inflammatory conditions and is mainly responsible for the
generation of pro-inflammatory eicosanoids including prostaglandin E2 (PGE2) (7). Chronic inflammation has been identified as a significant factor in the development of many human diseases, such as cancer, and COX inhibitors such as non-steroidal anti-inflammatory drugs (NSAIDs), are effective chemopreventive agents for the treatment of many chronic human diseases (8). Bacterial lipopolysaccharide (LPS) acts on macrophages to promote the secretion of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β (9). COX2 is expressed at very low levels and is strongly induced by LPS and activated oncogenes (9).

We previously reported that phytophenols and related NSAID-like natural phenolic compounds inhibited LPS-induced COX2 gene expression in RAW264.7 macrophage-like cells, and that dehydrodi-isoeugenol, an isoeugenol dimer, preferentially inhibited LPS-induced nuclear factor kappa B (NFκB) activation and COX2 gene expression, and also inhibited LPS-induced phosphorylation-dependent proteolysis of the inhibitor κBα and transcriptional activity of NFκB (10). Fimbriae of Porphyromonas gingivalis (Pg), in addition to LPS, are also involved in the pathogenesis of periodontal disease (11-13). We previously investigated the effect of synthetic phenolic compounds on COX2 expression and NF-κB activation in RAW264.7 cells stimulated with Pg fimbriae-stimulated RAW264.7 cells (16).

In the present study, to clarify the causal link between periodontal disease and tocopherols, we investigated whether α-, β-, γ-, and δ-tocopherol inhibited COX2 gene expression in RAW264.7 cells stimulated with LPS, TNFα, or Pg fimbriae.

**Materials and Methods**

**Materials.** Tocopherols (α-, β-, γ-, and δ-tocopherol) were purchased from Merck Millipore, Inc. (Billerica, MA, USA). Solutions of each molecule were made by dissolving them in dimethyl sulfoxide, and they were then diluted to the indicated concentrations using serum-free RPMI-1640 (Invitrogen Co., Carlsbad, CA, USA) as test samples. RPMI-1640 was purchased from Invitrogen Corp. Fetal bovine serum (FBS) was from HyClone (Logan, UT, USA). Recombinant mouse TNF-α was obtained from R&D Systems, Inc. (Minneapolis, MN, USA). *Escherichia coli* O111 B4-derived LPS was 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 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. They were then washed again and treated with the test samples.

**Cytotoxicity.** The relative number of viable cells was determined using a Cell Counting Kit-8 (CCK-8) (Dojindo Co., Kumamoto,
In brief, RAW264.7 cells (3x10^4 per well) were cultured in NUNC 96-well plates (flat-well-type microculture plates) for 48 h, after which the cells were incubated with test samples for 24 h. CCK-8 solution was added to each well and then the absorbance was measured at 450 nm with a microplate reader (Biochromatic, Helsinki, Finland). The 50% cytotoxic concentration (CC50) was determined from the dose–response curves. Data are expressed as means of three independent experiments. Statistical analyses were performed using Student’s t test.

Preparation of Pg fimbriae. *P. gingivalis* ATCC33277 fimbriae were prepared and purified from cell washings by the method of Yoshimura et al. (11). As documented previously, purified fimbria-induced biological activities were not attributable to LPS contaminants in the preparation (12, 13). Viability of the cells after exposure to the fimbriae at the concentrations used was over 90% by CCK-8. The protein content of the fimbriae was measured by the method of Smith et al. (18).

Preparation of total RNA and real-time polymerase chain reaction (PCR). The preparation of total RNA and the procedure for real-time PCR have been described previously (16). In brief, the cells in NUNC 96-flat-well-type microculture plates (10^5 cells per well) were treated with the test samples. The 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 μg) of each sample by random priming using a High Capacity RNA-to-cDNA Kit (Life Technologies Japan, Tokyo, Japan). Reaction mixtures without the RT were also used as a negative control. An aliquot of each cDNA synthesis reaction mixture was diluted and used for real-time PCR quantification. An equal-volume aliquot of each cDNA was mixed, serially diluted, and used as a standard. TaqMan probes/primers for COX2 and 18s rRNA and the PCR enzyme mix for real-time PCR were purchased from Life Technologies Japan. Real-time PCR quantification 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 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 18S rRNA.

Results

EC50. The results are shown in Figure 2. The EC50 (mM) declined in the order α-tocopherol (αT) (4.625)>βT (4.500)>γT (4.250)>δΤ (4.125) (Figure 2). The differences in cytotoxicity among the tocopherols were not significant. Tocopherols exhibited markedly low cytotoxicity relative to the EC50 of about 0.5 mM for phenolic compounds such as BHA and eugenol towards RAW264.7 cells (14-16). Tocopherols were not toxic to RAW264.7 cells at concentrations up to 1 mM.

Inhibition of *E. coli* LPS-induced COX2 gene expression by tocopherols. The results are shown in Figure 3. Tocopherols significantly inhibited the expression of the COX2 gene in RAW264.7 cells induced by LPS (p<0.01). A significant difference in the inhibition of COX2 gene expression was evident between α-tocopherol and β-, γ- or δ-tocopherol at both 500 μM and 100 μM (p<0.05), but there were no significant differences between β-, γ- and δ-tocopherol. Tocopherols also exhibited an anti-inflammatory activity. The present findings were in good agreement with others reported previously (19-22).

Inhibition of TNFα- and Pg fimbriae-induced COX2 gene expression by tocopherols. TNFα is well-known as a cytokine with particular relevance to periodontal diseases. This cytokine is very active in the mouth, and therefore related to destructive inflammation. Pg is also a likely major pathogen in adult periodontitis (12, 13). In particular, fimbriae may play a crucial role in facilitating the initial interaction between bacteria and the human host, thus triggering periodontitis. We investigated the inhibitory effects of tocopherols on COX2 gene expression in RAW264.7 cells stimulated with TNFα or Pg fimbriae, and the results are shown in Figures 4a and b, respectively. COX2 gene expression in RAW cells stimulated with TNFα as well as with Pg fimbriae, was significantly inhibited by tocopherols (p<0.01). For both macrophage activators, a significant difference in the inhibition of COX2 gene expression was also observed between α-tocopherol and β-, γ- or δ-tocopherol (p<0.05), but there were no significant differences among β-, γ- and δ-tocopherol.
Discussion

In this study, we investigated whether LPS-, TNF-α- or Pg fimbriae-stimulated COX2 mRNA expression in RAW 264.7 cells was inhibited by α-, β-, γ- and δ-tocopherol (T), respectively. Such inhibition was indeed observed, but the inhibitory activity of α-tocopherol was lower than those of the other tocopherols. It has been shown that α-tocopherol attenuates COX2 transcription and COX2 activity; in murine macrophages, α-tocopherol significantly reduces LPS-stimulated COX2 mRNA expression, COX2 protein synthesis and PGE2 release (19). α-Tocopherol has also been reported to diminish the expression of microglial COX2, an enzyme involved in various neurodegenerative diseases (20). Furthermore, vitamin E has been shown to inhibit COX2-catalyzed PGE2

in IL1β-stimulated A549 cells without affecting COX2 expression, with a relative order of potency of δ->γ->α- and β-tocopherol (21). In addition, δ- and γ-tocopherols more efficiently inhibited colon carcinogenesis in azoxymethane-treated F344 rats than α-tocopherol (22); there is a possible link between chronic human diseases and the effects of tocopherols on COX2 expression.

The present study showed that α-tocopherol had the least potent anti-inflammatory effect. Although α-tocopherol is the predominant form of vitamin E in tissues, other tocopherols appear to have superior bioactivities that are important for disease prevention and therapy (23). The reasons for the differences in bioactivity between α-tocopherol and other tocopherols are not well-understood, but may be partly attributable to their distinct metabolism; α-tocopherol is well...
retained in tissues because it is preferentially protected by degradation of α-tocopherol transfer protein (23, 24). However, it is not yet clear why δ- and γ-tocopherols exhibit more potent anti-inflammatory activity than α-tocopherol.

The variation of COX2-inhibitory activity among the tocopherols may be explained by differences in their antioxidant activity and inhibition rate constant (kinh) (Table I). COX activity requires the presence of oxidant hydroperoxide, including PGE2 (25). Tocopherols scavenge free radicals such as reactive oxygen species, including lipid peroxyl radicals formed during oxidation of polyunsaturated fatty acids (PUFAs) that contain more than one double bond in their backbone. Tocopherols inhibit peroxidation of PUFAs in biological systems and neutralize them; tocopherols are able to attenuate COX activity by scavenging the harmful free radicals necessary for COX activation. Therefore, it is important to determine the rates at which tocopherols react with peroxyl radicals to stop PUFA auto-oxidation. Previous studies have quantitatively investigated the stoichiometry of the radical-scavenging activity of tocopherols in vitro, and the reported kinh values of tocopherols, together with those determined in our previous study, are summarized in Table I. The studies conducted by Burton and Ingold (1) and Pyror et al. (26) were carried out under aerobic conditions using the induction period method with azo initiators (LOO. radical), and the former employed

Figure 4. Regulatory effect of α-, β-, γ-, and δ-tocopherols (T) on tumor necrosis factor-α (TNFα) and Porphyromonas gingivalis (Pg) fimbriae-stimulated expression of the cyclooxygenase (COX)-2 gene in RAW264.7 cells. The cells were pre-treated for 30 min with 500 μM αT, βT, γT, and δT, respectively. They were then incubated for 5 h for with or without TNFα 100 ng/ml (a), or for 3 h with or without fimbriae at 4 μg/ml (b), and their total RNAs were then prepared. Each cDNA was synthesized, and the expression level of COX2 mRNA was determined by real-time PCR and standardized against the expression of 18s rRNA. The results are presented as means±SE of three independent experiments. SE<15%. For (a), TNFα vs. TNFα + αT, βT, γT or δT, p<0.01; TNFα + αT vs. TNFα + βT, γT or δT, p<0.05. For (b), Pg fimbriae vs. Pg fimbriae + αT, βT, γT or δT, p<0.01; Pg fimbriae + αT vs. Pg fimbriae + βT, γT or δT, p<0.05.
In the polymerization of MMA initiated by thermal decomposition of benzoyl peroxide (BPO), the number of moles of PhCOO• (BPO radical) trapped by the tocopherol is calculated with respect to 1 mol of inhibitory moiety unit. The ratio of the initial rate of polymerization in the presence of inhibitor (Rp inh) to that in the absence of inhibitor (Rp con): The ratio of rate constant of inhibition (k inh) to that of propagation (k p). k p=797 M–1s–1. Pryor et al. (26) in linoleic acid in aqueous sodium dodecyl sulphate micelle solution (SDS) initiated by 2,2 azobis (2-aminodimopropane) dihydrochloride, k p=37 M–1s–1. Burton and Ingold (1) in chlorobenzene-styrene solution initiated by azobis (isobutynitrile). k p=41 M–1s–1.

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<th>Tocopherol</th>
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α-Fujisawa and Kadoma (27) in the polymerization of MMA initiated by thermal decomposition of benzoyl peroxide (BPO). *Stoichiometric factor, the number of moles of PhCOO• (BPO radical) trapped by the tocopherol is calculated with respect to 1 mol of inhibitory moiety unit. †The ratio of the initial rate of polymerization in the presence of inhibitor (Rp inh) to that in the absence of inhibitor (Rp con); ‡The ratio of rate constant of inhibition (k inh) to that of propagation (k p). k p=797 M–1s–1. β-Pryor et al. (26) in linoleic acid in aqueous sodium dodecyl sulphate micelle solution (SDS) initiated by 2,2 azobis (2-aminodimopropane) dihydrochloride, k p=37 M–1s–1. γ-Burton and Ingold (1) in chlorobenzene-styrene solution initiated by azobis (isobutynitrile). k p=41 M–1s–1.
factor for oral squamous cell carcinoma. Cytokines, reactive oxygen species and mediators of the inflammatory pathway (NF-κB and COX2) are well-known for causing loss of cancer suppressor function and for stimulating oncogene expression. In the present study, tocopherols with antioxidant activity inhibited the production of cytokines and showed an inhibitory effect on COX2 expression, suggesting that these compounds may be useful in reducing the risk of oral cancer. However, further studies are necessary for clarifying the relationships between oral diseases and tocopherols.

References