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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 Poryphyromonas gingivalis (Pg), an oral anaerobe. Materials and Methods: The cytotoxicity (EC_{50}) 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: Tocopherols 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.*

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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 H_2 (PGH_2), 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

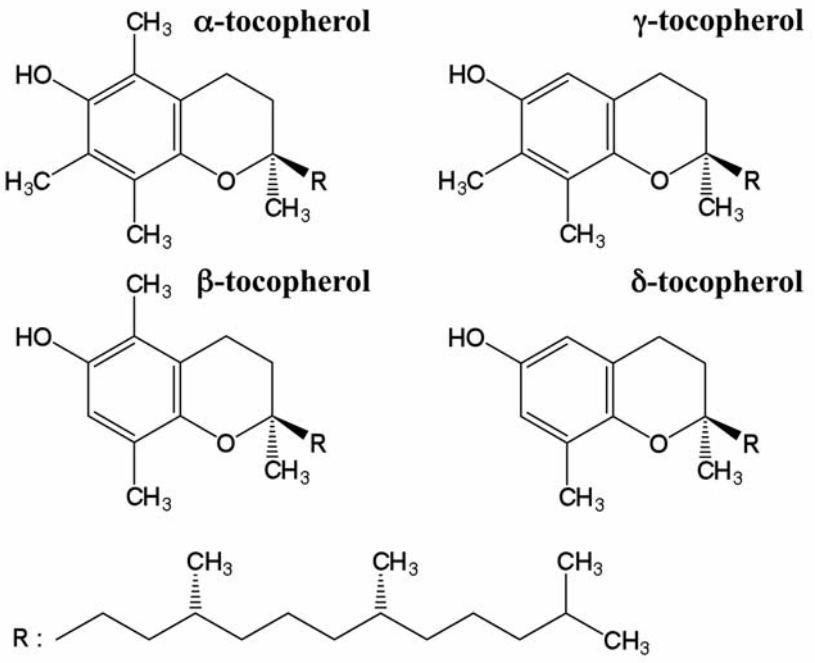

Figure 1. *The chemical structures of α-, β-, γ-, and δ-tocopherol.*

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 *Poryphyromonas 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, and found that the *ortho* dimer of butylated hydroxyanisole (BHA) inhibited COX2 expression and NFκB activation (14, 15). Furthermore, we recently reported that the phenolic compounds magnolol and honokiol inhibited *COX2* gene expression and NF-κB activation in 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% CO_2 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,

Japan) (17). In brief, RAW264.7 cells (3×10^4 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 (CC_{50}) 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 standad 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

EC_{50}. The results are shown in Figure 2. The EC_{50} (mM) declined in the order α- tocopherol (αT) (4.625)>βT (4.500)>γT (4.250)>δT (4.125) (Figure 2). The differences in cytotoxicity among the tocopherols were not significant. Tocopherols exhibited markedly low cytotoxicity relative to the EC_{50} 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

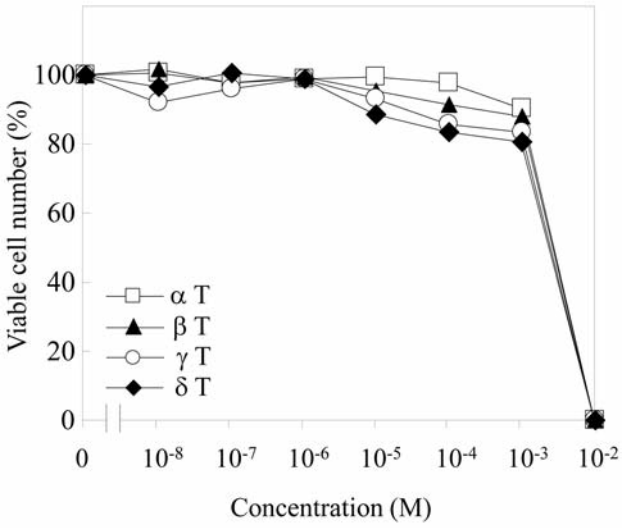

Figure 2. *The cytotoxicity of α-, β-, γ-, and δ-tocopherol (T) towards RAW264.7 cells. The procedures employed are described in Materials and Methods. The results are represented as the means of three independent experiments.*

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.

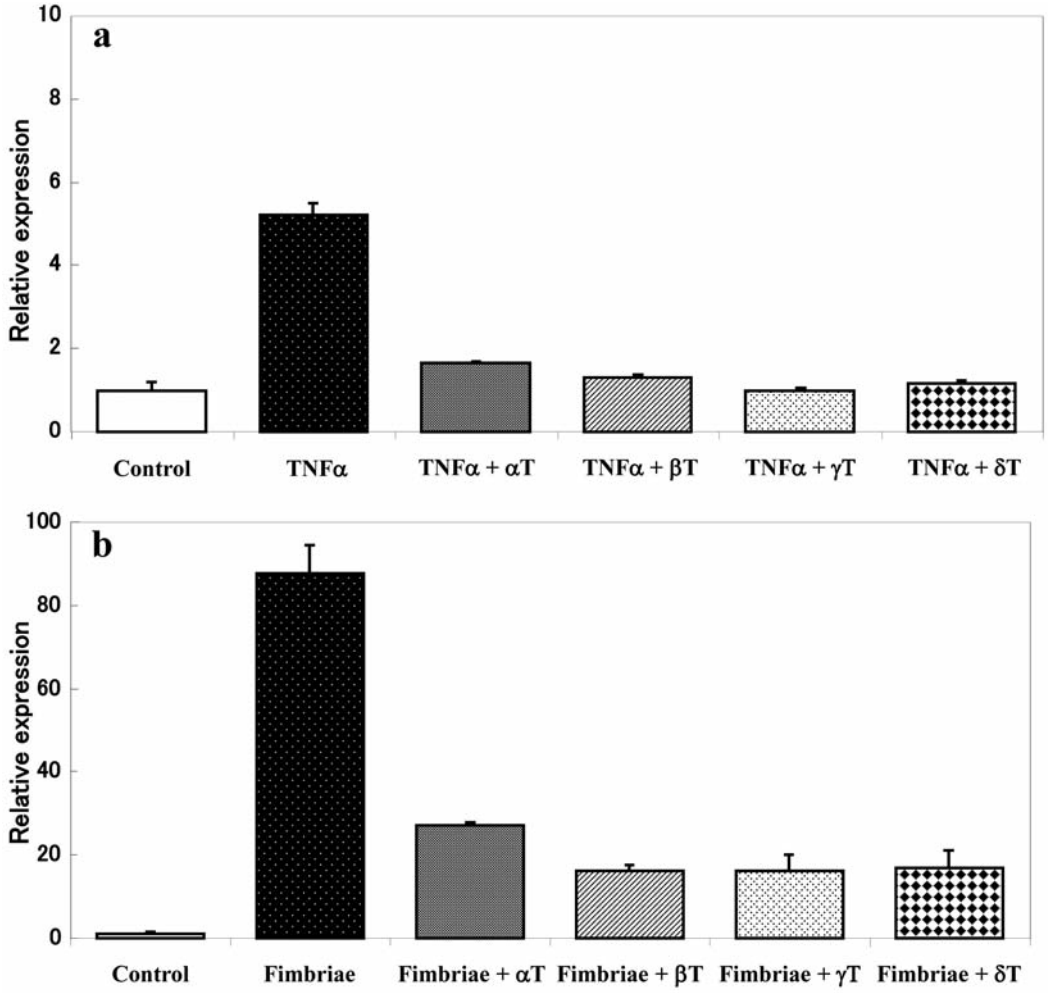

Figure 3. *Regulatory effect of tocopherols on Escherichia coli lipopolysaccharide (LPS)-stimulated cyclooxygenase 2 (COX2) gene expression. The cells were pre-treated for 30 min with 500 μM (a), or 100 μM (b) α-, β-, γ-, and δ-tocopherol (T), respectively. They were then incubated for 3 h with or without LPS at 1 μg/ml, 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 both panels a and b, LPS vs. LPS + αT, βT, γT or δT, $p<0.01$; LPS + αT vs. LPS + βT, γT or δT, $p<0.05$.*

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. 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 PGE_2 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

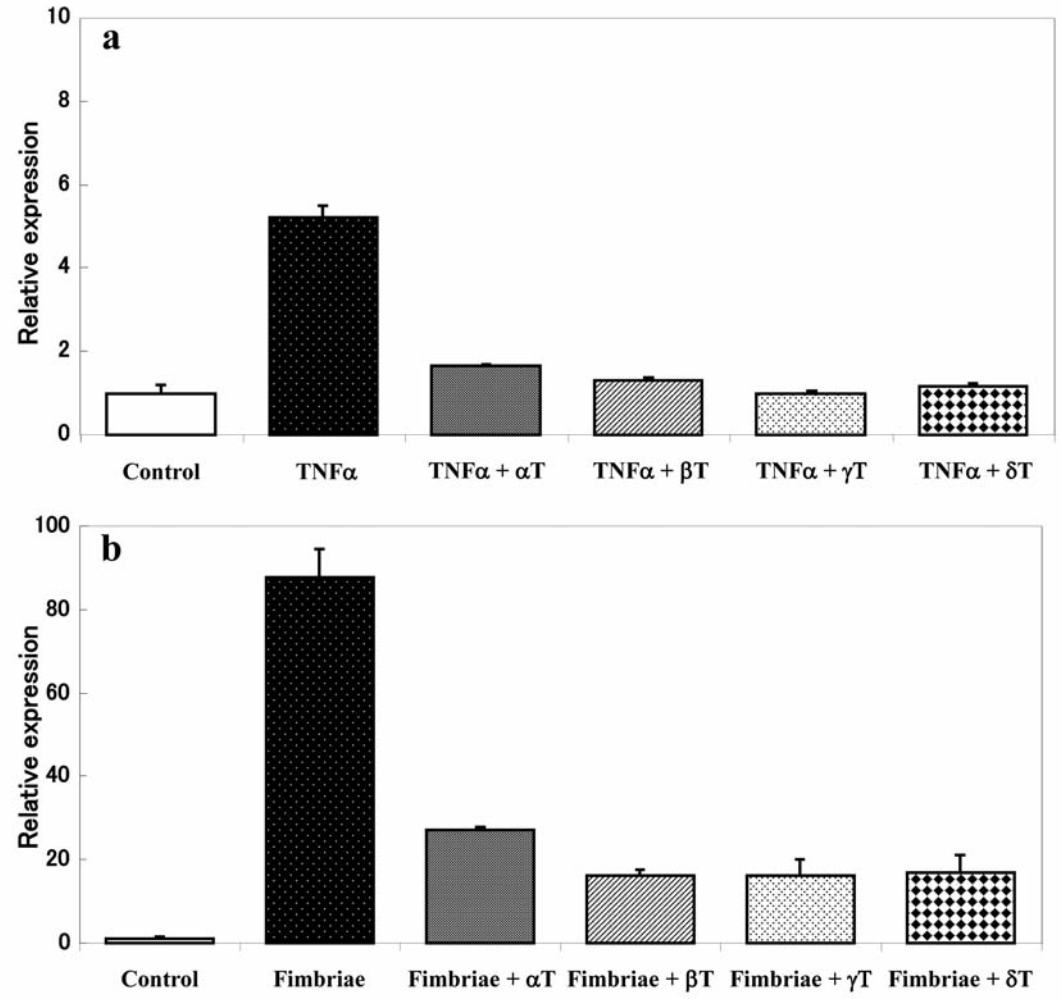

Figure 4. *Regulatory effect of α-, β-, γ-, and δ-tocopherols (T) on tumor necrosis factor-α (TNFα) and Poryphyromonas gingivalis (Pg) fimbriae-stimulated expression of the cyclooxygenase (COX)-2 gene in RAW264.7 cells. The cells were pre-treated for 30 min with 500 μM αT, βT, γT, and δT, respectively. 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.*

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 (k_{inh}) (Table I). COX activity requires the presence of oxidant hydroperoxide, including PGE_2 (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 k_{inh} 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

Table I. *Values of inhibition rate constants (k_{inh}) using induction period methods of tocopherols.*

	Methyl methacrylate[a]				Linolic acid in 0.5M SDS[b]	Stylene in chlorobenzene[c]
Tocopherol	n*	(Rp_{inh}/Rp_{con})#	(k_{inh}/k_p)+	$k_{inh} \times 10^{-4}$ $M^{-1}s^{-1}$	$k_{inh} \times 10^{-4}$ M-1s^{-1}	$k_{inh} \times 10^{-4}$ $M^{-1}s^{-1}$
α	0.33	0.83	47.24	3.77	3.7	320
β	1.03	0.85	14.92	1.79	2.2	130
γ	1.36	0.93	10.22	0.81	2.2	140
δ	1.93	0.9	7.46	0.59	0.89	44

[a]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 mole of inhibitory moiety unit; #The ratio of the intial 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 (kinh) to that of propagation (k_p). k_p=797 $M^{-1}s^{-1}$. [b]Pryor *et al*. (26) in linoleic acid in aqueous sodium dodecyl sulphate micelle solution (SDS) initiated by 2,2 azobis (2-aminodinopropane) dihydrochloride, k_p=37 $M^{-1}s^{-1}$. [c]Burton and Ingold (1) in chlorobenzene-styrene solution initiated by azobis (isobutyronitrile). k_p=41 $M^{-1}s^{-1}$.

styrene – a polymeric peroxyl radical – as the auto-oxidizable substrate, whereas the latter employed linoleic acid – a monomeric unsaturated fatty acid peroxyl radical – in an aqueous sodium dodecyl sulfate (SDS) micelle system. The k_{inh} was shown to decline in the order α->β- ≃ γ-> δ-tocopherol. In contrast, our experiment was carried out in methyl methacrylate (MMA) to produce a poly MMA radical initiated by the thermal decomposition of benzoyl peroxide (BPO) (*i.e.* the $PhCOO^{\bullet}$ radical) under nearly anaerobic conditions, showed that the rank order of k_{inh} in three experiments declined in the order α->β->γ-> δ-tocopherol (27). Although the experimental methodologies differed among the studies, all of the tocopherols were shown to be good chain-breaking antioxidants *in vitro*, and α-tocopherol had the larger k_{inh}. However, the k_{inh} value may not be related to anti-inflammatory activity, and this may explain why the inhibitory activity of α-tocopherol against COX2 gene expression was lower than that of δ- and γ-tocopherols.

The causal link between the antioxidant activity of tocopherols and their effect on COX2 expression is somewhat varied. The anti-inflammatory activity of tocopherols is not mediated by their antioxidant activity alone. As shown in Figure 1, α-tocopherol is a hindered phenol, whereas β-, γ- and δ-tocopherols are less hindered phenols. Tocohperol-induced regulation of COX2 protein and gene expression may be related to the molecular structure of tocopherols, *i.e.* a steric effect. α-Tocopherol, a hindered phenol may prevent much more a loss of α-tocopherol itself from inflicting damage on attacking peroxyl free radicals, ROO. and other free radicals, compared to that of other tocopherols.

Additionally to their antioxidant role, tocopherols affect gene expression and regulation, apoptosis, and signal transduction (28, 29). Naturally-occurring isomers of tocopherols have an R,R.R-configuration, and there is agreement that RRR-α-tocopherol is the most bioactive compound. Azzi's group (30) have described the non-antioxidant cell signaling function of α-tocopherol, and demonstrated that vitamin E inhibits protein kinase C (PKC) and 5-lipoxygenase and activates protein phosphatase 2A and diacylglycerol kinase, a pertinent cancer target. Rimbach *et al*. have studied the gene-regulatory activity of α-tocopherol using gene chip technology, and demonstrated that genes encoding proteins involved in peripheral α-tocopherol transport and degradation are significantly affected by the apolipoprotein E (apoE) genotype (31). α-Tocopherol also regulates signal transduction cascades not only at the mRNA level but also the miRNA level, since miRNA-122a (involved in lipid metabolism) and miRNA-125b (involved in inflammation) are down-regulated by α-tocopherol. A role of miRNA-125b in inflammation has been supported by a study that identified TNFα as a direct target of miRNA-125b. miRNA-125b is down-regulated in LPS-stimulated RAW 264.7 cells and a decrease of miRNA-125b is resulted in increased TNFα production; the down-regulation in the response of LPS may be required for proper TNFα production in RAW 264.7 cells (32). The reduced miRNA-125b levels observed in vitamin E-deficient rats may be associated with an enhanced inflammatory and allergy response due to oxidative stress (33, 34). It may be possible to identify the mechanism responsible for the biological activity of tocopherols based on their gene-regulatory activity. We previously showed that Pg fimbriae strongly induced expression of the TNFα (35) and IL1 (12) genes in macrophages, indicating a possible link between the production of cytokines and Pg fimbriae. In the present study, Pg fimbriae (or TNFα)-stimulated *COX2* gene expression in RAW264.7 cells was inhibited by tocopherols, suggesting that there may be a relationship between the anti-inflammatory activity of tocopherols and chronic inflammatory periodontal disease. Tocopherols may, therefore, be applicable for chemoprevention of periodontal disease. Chronic inflammation of oral tissues is also a well-recognized risk

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

1 Burton GW and Ingold KU: Autoxidation of biological molecules. 1. The antioxidant activity of vitamin E and related chain-breaking phenolic antioxidant *in vitro*. J Am chem Soc *110*: 2224-2229, 1988.

2 Parrish JH Jr., DeMarco TJ and Bissada NF: Vitamin E and periodontitis in the rat. Oral Surg Oral Med Oral Pathol *44*: 210-218, 1977.

3 Slade EW Jr., Bartuska D, Rose LF and Cohen DW: Vitamin E and periodontal disease. J Periodontol *47*: 352-354, 1977.

4 Cohen ME and Meyer DM: Effect of dietary vitamin E supplementation and rotational stress on alveolar bone loss in rice rats. Arch Oral Biol *38*: 601-606, 1993.

5 Iwasaki M, Manz MC, Taylor GW, Yoshihara A and Miyazaki H: Relations of serum ascorbic acid and α-tocopherol to periodontal disease. J Dent Res *91*: 167-172, 2012.

6 Dubois RN, Abramson SB, Crofford L, Gupta RA, Simon LS, Van De Putte LB and Lipsky PE: Cyclooxygenase in biology and disease. FASEB J *12*: 1063-1073, 1998.

7 Vane JR and Botting RM: Anti-inflammatory drugs and their mechanism of action. Inflamm Res *47(Suppl 2)*: S78-87, 1998.

8 Gupta RA and Dubois RN: Colorectal cancer prevention and treatment by inhibition of cyclooxygenase-2. Nat Rev Cancer *1*: 11-21, 2001.

9 Eliopoulos AG, Dumitru CD, Wang CC, Cho J and Tsichlis PN: Induction of COX2 by LPS in macrophages is regulated by Tpl2-dependent CREB activation signals. EMBO J *21*: 4831-4840, 2002.

10 Murakami Y, Shoji M, Hirata A, Tanaka S, Yokoe I and Fujisawa S: Dehydrodiisoeugenol, an isoeugenol dimer, inhibits lipopolysaccharide-stimulated nuclear factor kappa B activation and cyclooxygenase-2 expression in macrophages. Arch Biochem Biophys *434*: 326-332, 2005.

11 Yoshimura F, Takahashi K, Nodasaka Y and Suzuki T: Purification and characterization of a novel type of fimbriae from the oral anaerobe *Bacteroides gingivalis*. J Bacteriol *160*: 949-954, 1984.

12 Hanazawa S, Murakami Y, Hirose K, Amano S, Ohmori Y, Higuchi H and Kitano S: *Bacteroides* (*Porphyromonas*) *gingivalis* fimbriae activating mouse peritoneal macrophages and gene expression and production of interleukin-1. Infect Immun *59*: 1972-1977, 1991.

13 Hanazawa S, Kawata Y, Murakami Y, Naganuma K, Amano S, Miyata Y and Kitano S: *Porphyromonas gingivalis* fimbria-stimulated bone resorption *in vitro* is inhibited by a tyrosine kinase inhibitor. Infect Immun *63*: 2374-2377, 1995.

14 Murakami Y, Shoji M, Hirata A, Tanaka S, Hanazawa S, Yokoe I and Fujisawa S: An ortho dimer of butylated hydroxyanisole inhibits nuclear factor kappa B activation and gene expression of inflammatory cytokines in macrophages stimulated by *Porphyromonas gingivalis* fimbriae. Arch Biochem Biophys *449*: 171-177, 2006.

15 Murakami Y, Shoji M, Ogiwara T, Tanaka S, Yokoe I and Fujisawa S: Preventive effect of ortho dimer of butylated hydroxyanisole on activator protein-1 activation and cyclooxygenase-2 expression in macrophages stimulated by fimbriae of *Porphyromonas gingivalis*, an oral anaerobe. Anticancer Res *26*: 2915-2920, 2006.

16 Murakami Y, Kawata A, Seki Y, Koh T, Yuhara K, Maruyama T, Machino M, Ito S, Kadoma Y and Fujisawa S: Comparative inhibitory effects of magnolol, honokiol, eugenol and *bis*-eugenol on cyclooxygenase-2 expression and nuclear factor kappa B activation in RAW264.7 macrophage-like cells stimulated with fimbriae of *Porphyromonas gingivalis*. In Vivo *26*: 941-950, 2012.

17 Ishiyama M, Miyazono Y, Sasamoto K, Ohkura Y and Ueno K: A highly water-soluble disulfonated tetrazolium salt as a chromogenic indicator for NADH as well as cell viability. Talanta *44*: 1299-1305, 1997.

18 Smith PM, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ and Klenk DC: Measurement of protein using bicinchoninic acid. Anal Biochem *150*: 76-85, 1985.

19 Abate A, Yang G, Dennery PA, Oberle S and Schröder H: Synergistic inhibition of cyclooxygenase-2 expression by vitamin E and aspirin. Free Radic Biol Med *29*: 1135-1142, 2000.

20 Egger T, Schuligoi R, Wintersperger A, Amann R, Malle E and Sattler W: Vitamin E (α-tocopherol) attenuates cyclooxygenase 2 transcription and synthesis in immortalized murine BV-2 microglia. Biochem J *370*: 459-467, 2003.

21 Jiang Q, Yin X, Lill MA, Danielson ML, Freiser H and Huang J: Long-chain carboxychromanols, metabolites of vitamin E, are potent inhibitors of cyclooxygenases. Proc Natl Acad Sci USA *105*: 20464-20469, 2008.

22 Guan F, Li G, Liu AB, Lee MJ, Yang Z, Chen YK, Lin Y, Shih W and Yang CS: δ- and γ-tocopherols, but not α-tocopherol, inhibit colon carcinogenesis in azoxymethane-treated F344 rats. Cancer Prev Res *5*: 644-654, 2012.

23 Jiang Q, Christen S, Shigenaga MK and Ames BN: γ-Tocopherol, the major form of vitamin E in the US diet, deserves more attention. Am J Clin Nutr *74*: 714-722, 2001.

24 Brigelius-Flohé R and Traber MG. Vitamin E: function and metabolism. FASEB J *13*: 1145-1155, 1999.

25 Lu Y and Wahl LM: Oxidative stress augments the production of matrix metalloproteinase-1, cyclooxygenase-2, and prostaglandin E2 through enhancement of NFκB activity in lipopolysaccharide-activated human primary monocytes. J Immunol *175*: 5423-5429, 2005.

26 Pryor WA, Strickland T and Church, DF: Comparison of the efficiency of several natural and synthetic antioxidants in aqueous sodium dodecyl sulpfate micelle solutions. J Am Chem Soc *110*: 2224-2229. 1988.

27 Fujisawa S and Kadoma Y: Kinetic study of the radical-scavenging activity of vitamin E and ubiquinone. In Vivo *19*: 1005-1012, 2005.

28 Zingg JM and Azzi A: Non-antioxidant activities of vitamin E. Curr Med Chem *11*: 1113-1133, 2004.

29 Azzi A, Gysin R, Kempná P, Munteanu A, Negis Y, Villacorta L, Visarius T and Zingg JM: Vitamin E mediates cell signaling and regulation of gene expression. Ann NY Acad Sci *1031*: 86-95, 2004.

30 Azzi A, Breyer I, Feher M, Pastori M, Ricciarelli R, Spycher S, Staffieri M, Stocker A, Zimmer S and Zingg JM: Specific cellular responses to alpha-tocopherol. J Nutr *30*: 1649-1652, 2000.

31 Rimbach G, Moehring J, Huebbe P and Lodge JK: Gene-regulatory activity of alpha-tocopherol. Molecules *15*: 1746-1761, 2010.

32 Tili E, Michaille JJ, Cimino A, Costinean S, Dumitru CD, Adair B, Fabbri M, Alder H, Liu CG, Calin GA and Croce CM: Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-α stimulation and their possible roles in regulating the response to endotoxin shock. J Immunol *179*: 5082-5089, 2007.

33 Lim Y, Vasu VT, Valacchi G, Leonard S, Aung HH, Schock BC, Kenyon NJ, Li CS, Traber MG and Cross CE: Severe vitamin E deficiency modulates airway allergic inflammatory responses in the murine asthma model. Free Radic Res *42*: 387-396, 2008.

34 Yamaoka S, Kim HS, Ogihara T, Oue S, Takitani K, Yoshida Y and Tamai H: Severe Vitamin E deficiency exacerbates acute hyperoxic lung injury associated with increased oxidative stress and inflammation. Free Radic Res *42*: 602-612, 2008.

35 Murakami Y, Hanazawa S, Nishida K, Iwasaka H and Kitano S: N-acetyl-D-galactosamine inhibits TNF-α gene expression induced in mouse peritonal macrophages by fimbriae of *Porphyromonas* (*Bacteroides*) *gingivalis*, an oral anaerobe. Biochem Biophys Res Commun *192*: 826-832, 1993.

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