Introduction

Inflammation is a major contributor to the development of degenerative diseases, including cancer, cardiovascular diseases, and neurodegenerative disorders (1, 2). It is a complex process mediated by activation of various immune cells, including leukocytes, macrophages, mast cells, and platelets (3). Among participating cells, macrophages play a pivotal role in mediating inflammatory responses by organizing the production of inflammatory mediators, such as nitric oxide (NO) and prostaglandin (PG) E₂, and pro-inflammatory cytokines, such as interleukin-1β and tumor necrosis factor-α (4, 5).

NO is produced during the conversion of L-arginine to L-citrulline, a reaction catalyzed by three isoforms of nitric oxide synthase (NOS), one of which is inducible NOS (iNOS) (6). The production of PGE₂ begins with the liberation of arachidonic acid (AA) from membrane phospholipids by phospholipase A₂. Subsequently, cyclooxygenase (COX) enzymes convert AA to PGH₂, which is converted to various prostaglandins, including PGE₂. Of the two isoforms of COX, COX-2 is often induced under inflammatory conditions (7). Therefore, iNOS and COX-2 are mainly responsible for the increased levels of NO and PGE₂ during the inflammation response.

Antioxidant vitamins, which protect against oxidants produced during inflammation, are believed to be important in public health and disease prevention primarily by virtue of their inhibition of inflammatory responses (8). Alpha-tocopherol (αTOL) is the most potent compound of the antioxidant vitamin E family and is thus the pre-
dominant form of vitamin E in vitamin supplements (9). αTOL has drawn the most attention, and extensive studies have shown that it exhibits antithrombotic, anticoagulant, neuroprotective, antiproliferative, immunomodulatory, and antiviral effects, which may or may not be associated with its antioxidant activity (10, 11). αTOL has been shown to suppress oxidized low-density lipoprotein-induced phospholipase A2 activity in rat mesangial cells (12) and attenuate lipopolysaccharide (LPS)-induced COX-2 transcription and synthesis in microglial cells, leading to the suppression of PGE2 production (13). In macrophages and aged mice, αTOL has been reported to decrease COX activity without affecting COX mRNA or protein expression levels (14, 15). αTOL also inhibits LPS-stimulated iNOS expression in macrophages (16). Taken together, these findings suggest that supplementation with αTOL may be beneficial in suppressing NO- and/or PGE2-mediated inflammatory responses, but the mechanism underlying αTOL effects may be diverse and cell-type dependent.

In addition to αTOL, other isoforms of vitamin E differing in methylation patterns of the chromanol ring (α-, β-, γ-, δTOL) are present in nature. Also, synthetic analogs of αTOL, such as α-tocopheryl acetate (αTOA) and α-tocopheryl succinate (αTOS), which exhibit increased stability compared to αTOL, are commercially available. Recent studies, including those from our laboratory (17), indicate that other forms of vitamin E may exhibit more potent anti-inflammatory activities than αTOL. For example, γTOL inhibits PGE2 production and COX activity in epithelial cells and LPS-activated macrophages at a concentration at which αTOL is not effective, although its antioxidant activity is similar to that of αTOL (14). Moreover, γTOL is more effective in decreasing iNOS expression and NO production in macrophages than αTOL (14). We also documented that αTOS, a succinyl analog of αTOL, more effectively suppresses COX activity and PGE2 production in lung epithelial cells, despite its inferior antioxidant activity compared to αTOL (17). These data indicate that other natural or synthetic isoforms of TOL may have more potent anti-inflammatory activities than αTOL. To perform a comparative analysis of the potential anti-inflammatory activities of these TOL analogs as part of a search for agents that are more effective in preventing and treating human diseases associated with inflammation, in the present study, we examined the effects of αTOL, αTOS, and γTOL on the production of NO and PGE2 in LPS-stimulated RAW264.7 macrophages.

Materials and Methods

Chemicals and reagents
LPS from an Escherichia coli source, αTOS, αTOL, γTOL, and sulforaphane were purchased from Sigma-Aldrich (St. Louis, MO, USA). AA, celecoxib, 1-N’-monomethyl arginine (NMMA), and antibodies to murine COX-1, COX-2, and iNOS were obtained from Cayman Chemical (Ann Arbor, MI, USA). Antibodies to β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other chemicals were of reagent grade and used without further purification. αTOL analogs were dissolved in DMSO at 50 mM and then diluted with culture media. The final concentration of DMSO in all samples did not exceed 0.1%. All other chemicals were of reagent grade and used without further purification.

Cell culture
RAW264.7 mouse macrophages were obtained from the Korean Cell Line Bank (Seoul, Korea). Cells were grown in a humidified atmosphere containing 5% CO2 and 95% air at 37°C and maintained in RPMI-1640 medium (Gibco, Grand Island, NY, USA) containing 10% heat-inactivated fetal bovine serum (FBS) and 100 units/ml penicillin.

Six- to eight-week-old male Balb/c mice were obtained from Orient (Seoul, Korea) and housed and cared for according to the standards of the Sejong University for animal care and were used under a protocol approved by the Sejong University. Peritoneal macrophages were obtained from mice by a lavage of the peritoneal cavity with 5 ml of RPMI medium. The cells were then centrifuged, washed twice, resuspended in RPMI-1640 containing 10% heat-inactivated FBS, and then seeded in 6-well tissue culture plates at densities of 1 × 10⁶ cells/well. The macrophages were allowed to adhere for 2 – 3 h at 37°C in a 5% CO₂ humidified atmosphere and the non-adherent cells were removed by washing the plates twice with prewarmed medium. More than 95% of the cell preparations were viable and contained > 95% macrophages.

Assay for nitrite release
Cells (5 × 10⁴ cells/ml) plated in 24-well tissue culture plates in RPMI-1640 medium containing 5% FBS were treated with various reagents. After incubation, the supernatant conditioned medium was harvested and then assayed for the concentration of NO using a NO detection kit (Intronbio, Sungnam, Korea) according to the manufacturer’s instructions. Briefly, NO concentration in the cell cultures was measured as its stable oxidative metabolite, nitrite. After incubation, the nitrite concen-
Measurement of PGE2 production and COX activity

After incubation of cells with the appropriate reagent, the supernatant conditioned medium was harvested and then assayed for PGE2 levels using a specific ELISA kit according to the manufacturer’s instructions (Cayman Chemical). Medium alone without cells was incubated under the same conditions and used as blank control for the ELISA. Levels of PGE2 were normalized to the number of cells.

In separate experiments designed to determine the activity of the COX enzyme, COX activity was quantified by providing cells with exogenous AA, the substrate for COX, and measuring its conversion to PGE2. Briefly, cells were treated, after which cells were washed with phosphate-buffered saline (PBS, pH 7.4), and fresh medium containing AA was added for 20 – 30 min at 37°C; then the medium was collected and subjected to the PGE2 enzyme immuno-assay.

Immunoblotting

Treated cells were scraped from the culture, washed with PBS, and incubated for 15 – 30 min on ice in lysis buffer [containing 150 mM NaCl, 10mM Tris-Cl, 0.3% NP-40, 0.2 mM Na3VO4, 0.2% Triton X-100, and protease inhibitors cocktail (pH 7.4) (Roche, Seoul, Korea)]. After centrifugation for 20 min at 13,000 × g, supernatants were collected and protein concentration in each was measured using the Bradford method. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a nitrocellulose membrane. Membranes were blocked with 5% nonfat dry milk and probed with specific primary antibodies, followed by incubation with appropriate peroxidase-conjugated secondary antibodies. Blots were developed with ECL Plus reagent (Amersham, Arlington Heights, IL, USA) according to the manufacturer’s protocol.

RT-PCR

Following treatment, total RNA was isolated by using the Trizol Reagent (Gibco) according to the manufacturer’s instructions. Single-stranded oligo (dT)-primed cDNA from 3 μg of total RNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). The total RNA (3 μg) was incubated with oligo-dT15 primers for 5 min at 70°C and mixed with a 5 × first-strand buffer, 10 mM of dNTP, and 0.1 M dithiothreitol. It was incubated for 5 min at 37°C and a further incubated for 60 min following addition of M-MLV RT (2U). The reactions were terminated by heating at 70°C for 10 min and the total RNA was depleted with RNase. PCR was performed with the incubation mixture [2 μg cDNA, 4 μM 5′ and 3′ primers, a 10 × buffer, 250 μM of dNTP, 25 mM of MgCl2, and 1 unit of Tag polymerase (Promega)] under the following incubation conditions: a 60-s denaturation time at 94°C, an annealing time of 45 s between 55°C and 60°C, an extension time of 60 s at 72°C, and a final extension for 7 min at 72°C at the end of 35 cycles. PCR primers used are follows: COX-2 (sense primer) 5′-CCGTGGTGAAAT GTATGAGCA-3′ and (antisense primer) 5′-CCTCGCT TCTGATCTGTCTT-3′, GAPDH (sense primer) 5′-GTC AACGGATTGGTTCGTATT-3′ and (anti sense primer) 5′-AGTCTTCTGGGCGAGTG-3′.

Statistical analyses

Statistically significant differences between values obtained under different experimental conditions were determined using two-tailed unpaired Student’s t-tests or one-way analysis of variance (ANOVA), followed by Tukey post hoc tests. P ≤ 0.05 was considered statistically significant.

Results

LPS-induced NO and PGE2 production

To analyze the anti-inflammatory effects of TOL analogs, we used RAW264.7 cells, which have been extensively used as a cell model for evaluating potential inhibitors of the inflammatory response. We first studied time-dependent changes in LPS-induced NO and PGE2 production in RAW264.7 cells. Neither iNOS nor COX-2 protein was detected in unstimulated RAW264.7 cells, whereas addition of LPS rapidly induced the expression of both (Fig. 1: A and B, inset): iNOS and COX-2 expression were detected within 8 h of adding LPS and were maintained or increased up to 24 h. The release of NO and PGE2 into the supernatant gradually increased up to 24 h, consistent with the iNOS and COX-2 expression profiles (Fig. 1: A and B). In subsequent experiments, we therefore tested the efficacy of TOL analogs using RAW264.7 cells incubated with LPS for at least 8 h.

Effects of TOL analogs on LPS-induced NO release

We next analyzed the effect of TOL analogs on the production of NO. All three TOL analogs only slightly suppressed NO release into the culture medium at the highest concentration used; at 20 μM, αTOL, γTOL, and αTOS decreased NO release by 4%, 11%, and 16%, respectively (Fig. 2). The decrease induced by any of the TOL analogs was not statistically significant (P > 0.05). Increasing the concentration of each analog up to 70 μM...
was not effective in further decreasing NO release (data not shown). In addition, none of the three TOL analogs significantly changed LPS-stimulated iNOS expression (data not shown). Therefore, it seems unlikely that αTOS suppresses LPS-stimulated NO production by inhibiting the iNOS induction process.

Effects of TOL analogs on LPS-induced PGE2 production

Preincubation of RAW264.7 cells with TOL analogs for 10 h prior to incubation with LPS altered the extent of LPS-induced PGE2 production differently depending on the TOL analog used. At concentrations of 10, 20, and 30 μM, αTOS inhibited LPS-induced PGE2 production by 11.9%, 49.1%, and 70.1%, respectively, indicating that the αTOS effect is concentration-dependent (Fig. 3A). In contrast, the PGE2-suppressive effects of αTOL and γTOL pretreatment were much less pronounced. A comparison of the suppressive effect among the three analogs at the same concentration (30 μM) showed that αTOS, αTOL, and γTOL inhibited LPS-induced PGE2 production by 70.1%, 26.8%, and 27.9%, respectively. Increasing the concentration of αTOL or γTOL to as high as 70 μM was not effective in further decreasing PGE2 production (data not shown). Taken together, these data indicate that αTOS more potently inhibits LPS-induced PGE2 production in RAW264.7 cells than αTOL or γTOL.

To investigate whether the suppressive effect of αTOS on PGE2 suppression is also observed in other macrophages, we obtained peritoneal macrophages from BALB/c mice. As in RAW264.7 cells, pretreatment with αTOS exerted a concentration-dependent suppression of LPS-stimulated PGE2 production in cultured peritoneal macrophages. At concentrations of 10, 20 and 30 μM, αTOS inhibited LPS-induced PGE2 production by 15%, 53%, and 63%, respectively, whereas αTOL at concentrations up to 70 μM did not (Fig. 3B). The relative non-effectiveness of αTOL in peritoneal macrophages compared to RAW264.7 cells may be due to the differences in the intracellular αTOL availability caused by different cellular uptake, metabolism, and localization of αTOL in both cells. Our data confirm that the suppressive effect of αTOS on LPS-induced PGE2 production is not limited to RAW264.7 cells and demonstrate that the efficacy of αTOS against PGE2 production in peritoneal macrophages, as in RAW264.7 cells, is much greater.

Fig. 1. LPS-induced NO (A) and PGE2 (B) production in a time-dependent manner. After RAW264.7 cells were incubated with LPS (2.5 μg/ml) for the indicated times, the supernatant-conditioned medium was collected and the NO and PGE2 concentration was determined as described in the text. Results are from duplicate assays in each of at least two independent experiments (mean ± S.D.). An asterisk denotes significantly higher level than that of the time-0 group (P < 0.05). Insets: immunoblots were performed to determine the expression level of iNOS (A) and COX-2 (B) induced by LPS.

Fig. 2. TOL analogs only slightly suppressed NO release in LPS-stimulated RAW264.7 cells. RAW264.7 cells pretreated with indicated concentrations of αTOS, αTOL, or γTOL for 10 h were incubated with LPS (2.5 μg/ml) for an additional 16 h. Supernatants were collected and the NO concentration was determined. Cells pretreated with the NOS inhibitor NMMA (1 mM) prior to LPS stimulation were included as a positive control. Results are from duplicate assays in each of at least two independent experiments (mean ± S.D.). An asterisk denotes significantly lower level than that of the LPS-treated group (P < 0.05 by ANOVA).
Superior Anti-inflammatory Effect of αTOS than that of αTOL or γTOL.

Effects of TOL analogs on LPS-induced COX-2 expression and activity

To investigate the mechanism underlying the αTOS-mediated inhibition of LPS-induced PGE$_2$ production, we measured changes in COX-2 mRNA and protein levels. Pretreatment of RAW264.7 cells for 10 h with αTOS did not affect the up-regulation of COX-2 mRNA (Fig. 4A) or protein (Fig. 4B) induced by subsequent treatment with LPS. These data suggest that αTOS does not interfere with the process leading to COX-2 up-regulation.

In our earlier study, we suggested inhibition of COX activity as one mechanism by which αTOS blocks the production of prostaglandins in lung epithelial cells (17). To investigate whether αTOS blocks LPS-induced PGE$_2$ production in macrophages in a similar manner, we determined αTOS effects on COX activity in LPS-stimulated RAW264.7 cells using a post-exposure paradigm. Post-incubation with αTOS resulted in a concentration-dependent decrease in COX activity: at 10, 20, and 30 μM, αTOS inhibited LPS-induced COX activity by 31%, 45%, and 52%, respectively (Fig. 5A). These data suggest that αTOS inhibits COX activity, consistent with its anti-inflammatory properties.

Fig. 3. αTOS more potently inhibited PGE$_2$ production in LPS-stimulated macrophages. RAW264.7 (A) and peritoneal macrophages (B) were pretreated with the indicated concentrations of αTOS, αTOL, or γTOL for 10 h and subsequently incubated with LPS for an additional 8 h. The PGE$_2$ concentration from the supernatants was determined by an ELISA. Cells pretreated with the COX inhibitor celecoxib (10 nM) were included as a positive control. Results are from duplicate assays in each of two independent experiments (mean ± S.D.). An asterisk denotes significantly lower level than that in the LPS-treated group and a sharp mark denotes significantly lower level than that of the LPS plus αTOL– or γTOL-treated (same dose) group (P < 0.05 by ANOVA).

Fig. 4. αTOS did not affect LPS-induced COX-2 mRNA (A) and protein (B) up-regulation in RAW264.7 cells. Cells were stimulated by LPS for 8 h with or without 10-h pretreatment with αTOS or αTOL before analysis. A: Expression of COX-2 mRNA was determined by semi-quantitative RT-PCR, with GAPDH mRNA serving as a loading control. B: Immunoblot analysis of COX-2 protein expression in cells. Immunoblotting with an antibody to COX-1 was used to control for equal loading of protein per lane (bottom panel).
removed and replaced with a fresh serum-free media containing 1 μM AA for 20 min. PGE2 in the medium was measured. Cells pretreated 30 min. PGE2 in the medium was measured. COX activity (%) was indicated concentrations of AA was added and incubated for another 8 h. After removing the supernatant, fresh media containing the TOS-induced decreases in COX activity. RAW264.7 cells were incubated with LPS for 4 h and then with 20 μM TOS for an additional 8 h. After removing the supernatant, fresh media containing the indicated concentrations of AA was added and incubated for another 30 min. PGE2 in the medium was measured. COX activity (%) was expressed as the ratio of PGE2 produced in cells treated with LPS/αTOS to that in cells treated with LPS/vehicle at the designated AA concentrations. The results shown are the average of two different experiments assayed in duplicate.

Fig. 5. αTOS inhibited COX activity in LPS-stimulated RAW264.7 cells. A: Cells were pretreated with LPS. After 4-h incubation, cells were washed and incubated with fresh medium containing vehicle or the indicated concentration of αTOS for 8 h. Medium was then removed and replaced with a fresh serum-free media containing 1 μM AA for 20 min. PGE2 in the medium was measured. Cells pretreated with the COX inhibitor celecoxib (10 nM) were included as a positive control. An asterisk denotes significantly lower level than that of the LPS-treated group (P < 0.05). B: Effect of AA concentration on the αTOS-induced decreases in COX activity. RAW264.7 cells were incubated with LPS for 4 h and then with 20 μM αTOS for an additional 8 h. After removing the supernatant, fresh media containing the indicated concentrations of AA was added and incubated for another 30 min. PGE2 in the medium was measured. COX activity (%) was expressed as the ratio of PGE2 produced in cells treated with LPS/αTOS to that in cells treated with LPS/vehicle at the designated AA concentrations. The results shown are the average of two different experiments assayed in duplicate.

suggest that αTOS acts as a COX inhibitor in intact RAW264.7 cells without affecting COX expression. Consistent with our earlier study with lung epithelial cells, the ability of αTOS to inhibit COX activity decreased with increasing AA concentration (up to 2 μM) in the assay medium (Fig. 5B). This suggests that αTOS may inhibit the production of PGE2 by acting as a competitive inhibitor of AA in macrophages, as we previously observed in lung epithelial cells.

Effect of combined αTOS/sulforaphane treatment on PGE2 production in macrophages

Sulforaphane, a natural isothiocyanate present in cruciferous vegetables, is known to possess potent anti-inflammatory properties. Sulforaphane inhibits the LPS-stimulated induction of COX-2 in peritoneal macrophages (18). With this in mind, we anticipated that combined treatment with αTOS, a COX inhibitor, and sulforaphane would exert a cooperative inhibitory effect on PGE2 production that would be more effective than either agent alone. To test this possibility, we first analyzed the concentration of sulforaphane required to block LPS-induced COX-2 expression under our experimental conditions. Immunoblotting analysis demonstrated that 10 – 20 μM sulforaphane suppressed LPS-stimulated COX-2 protein expression in a concentration-dependent manner; at 20 μM, sulforaphane completely blocked LPS-stimulated COX-2 expression without affecting COX-1 expression (Fig. 6A).

To test the effectiveness of the αTOS/sulforaphane combination in suppressing PGE2 production, we selected concentrations of αTOS and sulforaphane that were lower than those required to exert a maximal suppression effect when used alone. In RAW264.7 cells preincubated with αTOS/sulforaphane for 30 min and then treated with LPS for 6 h, 20 μM αTOS did not further enhance the sulforaphane-mediated blockade of LPS-stimulated COX-2 expression at any sulforaphane concentration (data not shown). However, 20 μM αTOS, in combination with 5 or 10 μM sulforaphane, more efficiently reduced LPS-stimulated PGE2 production: 5 μM sulforaphane and 20 μM αTOS each inhibited PGE2 production by only 58.0% and 53.9%, respectively, whereas combined treatment with these same concentrations of αTOS and sulforaphane suppressed PGE2 production by 85.8% (Fig. 6B). In contrast, 70 μM αTOL, in combination with 5 μM sulforaphane, did not enhance the sulforaphane-mediated blockade of LPS-stimulated PGE2 production. Our data suggest that the αTOS/sulforaphane combination may be an effective anti-inflammatory regimen.

Discussion

Although αTOL has been considered the most bioactive compound of the vitamin E family, mainly owing to its potent antioxidant activity, recent findings by our group and others have shown that certain types of TOL analogs, including αTOS and γTOL, inhibit PGE2 production more effectively than αTOL. In these studies, αTOS and γTOL appeared to suppress PGE2 production through regulation of COX activity independent of their associated antioxidant activities (14, 17). Because the
Sulforaphane was added. At 6-h postincubation with LPS, the PGE2 production was significantly lower level than that in groups treated with αTOL alone and sulforaphane inhibition of COX-2 protein induction. Our study further demonstrates that the effect of αTOS on PGE2 production in LPS-stimulated macrophages was primarily due to its inhibition of COX activity, as was also observed in our earlier study on lung epithelial cells. This finding is supported by our observations that 1) αTOS inhibited LPS-stimulated PGE2 synthesis without altering expression of COX-1 or COX-2; 2) post-incubation with αTOS inhibited COX activity in macrophages prestimulated with LPS; and 3) the inhibition of COX activity by αTOS decreased with increasing concentrations of the COX substrate AA. Therefore, pharmacologically relevant concentrations (20) of αTOS inhibited COX activity and PGE2 synthesis in macrophages.

Considerable recent attention has focused on the use of combinations of preventive and therapeutic agents, a strategy predicated on the idea that combination treatments may enhance the efficacy of individual constituents, thereby lowering the required doses of each agent and reducing the potential for toxicity that could occur at higher doses. Here, we showed that treatment of macrophages with the combination of αTOS and sulforaphane produced almost complete suppression of LPS-stimulated PGE2 production, whereas the same concentration of each agent alone modestly blocked PGE2 production. A plausible explanation for this complete suppression is a cooperative effect of αTOS inhibition of COX activity and sulforaphane inhibition of COX-2 protein induction. The fact that the doses of each agent required to exert maximal effect when used in combination (20 μM αTOS and 5 μM sulforaphane) were much lower than those required when each agent was used alone suggests that combined administration of αTOS and sulforaphane may provide an effective means for inhibiting PGE2-mediated responses and thereby inhibiting the initiation and progression of inflammation. Designing supplements that combine αTOS with other preventive vitamins/agents may contribute to better strategies against inflammatory diseases related to inflammation.

Our data indicate that the extent of NO inhibition by all three TOL analogs was very limited compared to PGE2 inhibition. Our data is somewhat inconsistent with that of Jiang et al. who reported a significant inhibitory effect of γTOL on NO in RAW264.6 cells (14). This discrepancy may reflect differences in experimental conditions. For example, we used 2.5 μg/ml LPS in our current study, whereas Jiang et al. used 0.5 μg/ml LPS. In addition, our cell culture media contained 10% FBS, whereas Jiang et al. performed their experiments using media containing 0.5% serum. Binding of TOL to serum proteins present in culture media may retard TOL uptake by cells.

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diseases and cancer.

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