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ORIGINAL ARTICLE

Tocotrienols Suppress Proinflammatory Markers and Cyclooxygenase-2 Expression in RAW264.7 Macrophages

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Abstract Tocotrienols are powerful chain breaking antioxidant. Moreover, they are now known to exhibit various non-antioxidant properties such as anti-cancer, neuroprotective and hypocholesterolemic functions. This study was undertaken to investigate the anti-inflammatory effects of tocotrienol-rich fraction (TRF) and individual tocotrienol isoforms namely δ-, γ-, and α-tocotrienol on lipopolysaccharide-stimulated RAW264.7 macrophages. The widely studied vitamin E form, α-tocopherol, was used as comparison. Stimulation of RAW264.7 with lipopolysaccharide induced the release of various inflammatory markers. 10 μg/ml of TRF and all tocotrienol isoforms significantly inhibited the production of interleukin-6 and nitric oxide. However, only α-tocotrienol demonstrated a significant effect in lowering tumor necrosis factor-α production. Besides, TRF and all tocotrienol isoforms except γ-tocotrienol reduced prostaglandin E_2 release. It was accompanied by the down-regulation of cyclooxygenase-2 gene expression by all vitamin E forms except α-tocopherol. Collectively, the data suggested that tocotrienols are better anti-inflammatory agents than α-tocopherol and the most effective form is δ-tocotrienol.

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Abbreviations

COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
IL-6	Interleukin-6
LPS	Lipopolysaccharide
NO	Nitric oxide
PGE_2	Prostaglandin E_2
TNF- α	Tumor necrosis factor
TRF	Tocotrienol-rich fraction
αT	Alpha-tocopherol
αT_3	Alpha-tocotrienol
γT_3	Gamma-tocotrienol
δT_3	Delta-tocotrienol

Introduction

Inflammation is a complex response that protects the body from various harmful agents such as microbes and toxins [1]. Nevertheless, it is a known fact that unregulated inflammation is associated with many chronic diseases, contributing to the increase in worldwide morbidity and mortality rates. The relationship between cancer and inflammation was hypothesized in 1863 by Rudolf Virchow that cancer originates from sites of chronic inflammation where the inflammatory event serves as the cofactor in carcinogenesis [2]. The imbalance in inflammatory mediators during chronic inflammation causes damage to the cartilage and bone, leading to the debilitating rheumatoid arthritis [3]. Besides that, inflammation has also

been found to participate in all phases of the atherosclerotic process, from the initial atherogenesis through lesions progression and finally, the subsequent thrombotic complications [4]. In other words, inflammation plays a great role in the underlying mechanisms of almost all pathological states and diseases.

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most widely prescribed drugs in inflammation therapy. Alleviation of pain and arrestment of the tissue-damaging process serve as the therapeutic strategies in patients with inflammation [5]. However, the short-coming of these drugs is their association with a wide range of adverse effects on the gastrointestinal tract, hematological, and renal functions [5, 6]. A study by Tomisato and colleagues shows that NSAIDs have the possibility of inducing gastric lesions through direct cytotoxic effects on the gastric mucosa [7]. Moreover, it has been put forward that a causal association exists between bowel perforation or hemorrhage and NSAIDs ingestion [8]. Hence, any alternatives to address the shortcomings of NSAIDs would be hailed.

Palm vitamin E comprising 30% tocopherols and 70% tocotrienols has been extensively studied for its nutritional properties and health benefits [9]. Both tocopherols and tocotrienols have different biological properties and are further separated into four isoforms, α-, β-, δ- and γ-, respectively. It is widely known that vitamin E possesses potent antioxidant properties as well as other beneficial values. It confers protection against peroxynitrite-induced lipid oxidation in vivo and prevents cholesterol-induced atherosclerotic lesions in rabbits [10, 11]. Besides, tocotrienols are shown to inhibit the growth of a human breast cancer cell line, ZR-75-1 [12]. Reactive oxygen metabolites are involved in the mediation of tissue injuries as well as amplifying the existing inflammatory response [13]. Therefore, it is possible that the use of antioxidants may have direct or indirect effects on inflammation. Moreover, some vitamin E forms have been shown to possess anti-inflammatory effects in vivo and in vitro [14, 15].

Materials and Methods

Chemicals and Reagents

Tocotrienol-rich fraction (TRF) was obtained from Golden Hope Plantations (Selangor, Malaysia). α-Tocopherol (αT) was purchased from Aldrich Chemical Company Inc. (Milwaukee, USA) whereas δ-, γ-, and α-tocotrienol (T_3) were acquired from Eisai Food & Chemical Co. Ltd. (Tokyo, Japan). All media and reagents for tissue culture were purchased from GIBCO BRL (Paisley, Scotland). A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT) kit for cell viability assay was purchased from Chemicon (Temecula, CA, USA). Lipopolysaccharide (LPS) from a *Escherichia coli* source was obtained from Sigma (St Louis, Missouri, USA). ELISA kit for tumor necrosis factor (TNF)-α and interleukin (IL)-6 was from eBioscience (San Diego, CA, USA) and the Amersham Prostaglandin E_2 Biotrak Enzyme immunoassay (EIA) System was from GE Healthcare Ltd. (Buckinghamshire, UK). Griess reagent for the determination of nitrite was purchased from Molecular Probes (Eugene, Oregon, USA). Meanwhile, TRI reagent was from the Molecular Research Center, Inc (Cincinnati, OH, USA) and reagents for polymerase chain reaction (PCR) applications were all from Invitrogen Life Technologies (Carlsbad, CA, USA). Protein assay dye reagent concentrate for the Bradford assay was from Bio-Rad (Hercules, CA, USA).

Cell Culture

RAW264.7 macrophages were purchased from American Type Culture Collection (ATCC) (Manassas, Virginia, USA) and grown in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin and 1% L-glutamine in a humidified atmosphere of 5% CO_2 at 37 °C. For treatment, cells were cultured in RPMI medium without phenol red, supplemented with 5% FBS, 1% penicillin-streptomycin and 1% L-glutamine. 1×10^5 cells/well were plated in 96-well plates and incubated overnight. The next day, treatments and stimulant were given accordingly and the cells were incubated for 24 h. Cell supernatant was collected for the measurement of cytokines (IL-6 and TNF-α), nitric oxide (NO) and prostaglandin E_2 (PGE_2).

Cell Viability Assay

RAW264.7 macrophages were cultured and left overnight in 96-well plates at a density of 1×10^5 cells/well. The next day, treatment with different concentrations of TRF, αT, and tocotrienol isoforms (δ-, γ-, and αT_3) in the presence of 10 ng/ml LPS were given. The cells were incubated for 24 h. MTT solution was added into the wells containing fresh RPMI medium and the cultures were returned to the incubator. Four hours later, the blue formazan crystals that had formed were dissolved in isopropanol–HCl and the optical density was measured at 570 nm using a microplate reader.

Cytokine Assays

1×10^5 cells/well of RAW264.7 macrophages were incubated overnight in 96-well plates. Next, 10 ng/ml of

LPS was added for stimulation, together with 10 μg/ml of vitamin E supplementation and the cells were incubated for 24 h. The levels of IL-6 and TNF-α in culture supernatants were determined using commercially available ELISA kits (eBioscience). Assays were carried out according to the manufacturer's instructions. Plates were read at 540 nm using a microplate reader.

Griess Assay

Determination of NO production was performed using a Griess reagent kit (Molecular Probes) which measures the levels of nitrite formed from the spontaneous oxidation of NO. Briefly, 1×10^5 cells/well of RAW264.7 were cultured overnight in DMEM in 96-well plates, and then subjected to their respective treatments for another 24 h in the presence of 10 ng/ml of LPS. To measure nitrite, supernatants were incubated for 30 min with an equal volume of *N*-(1-naphthyl)ethylenediamine dihydrochloride and sulfanilic acid against a nitrite standard. Absorbance was measured using a microplate reader at 548 nm.

Prostaglandin E_2 Enzyme Immunoassay

Measurement of PGE_2 in the cell culture supernatant of treated RAW264.7 was carried out using the Amersham Prostaglandin E_2 Biotrak Enzyme immunoassay (EIA) System according to the manufacturer's instructions. Optical density was read at 450 nm using a microplate reader.

RNA Extraction and Reverse Transcriptase-PCR

RAW264.7 macrophages were maintained in DMEM in culture flasks. Upon confluency, treatments were given in the presence of 10 ng/ml LPS and incubated for 24 h in 5% CO_2. Total RNA was extracted using TRI reagent (Molecular Research Center, Inc.) according to manufacturer's instructions. Next, the RNA was reversed transcribed into cDNA using reverse transcriptase and the resultant cDNA was subjected to PCR using gene-specific primers. The primers for β-actin was designed using Primer 3 software whereas the sequences for *COX-1* and *COX-2* have previously been reported [16, 17]. All the primers were purchased from Invitrogen (Carlsbad, CA, USA). The sequences and the product sizes are as follows: β-actin (forward: 5′-GTG GGGCGCCCCAGGCACCA-3′, reverse: 5′-CTCCTTAAT GTCACGCACGATTTC-3′) 540 bps, *COX-1* (forward: 5′-CTTTGCACAACACTTCACCCACC-3′, reverse: 5′-AG CAACCCAAACACCTCCTGG-3′) 285 bps, and *COX-2* (forward: 5′-GCATTCTTTGCCCAGCACTT-3′, reverse: 5′-AGACCAGGCACCAGACCAAAGA-3′) 299 bps. The amplified products were resolved on a 1.5% agarose gel containing ethidium bromide and visualized using an imager. The bands were quantified using ImageJ software and results were expressed as the relative fold change after normalization against β-actin [18].

Real-Time RT-PCR

Total RNA was subjected to DNase treatment. Gene expression of *COX-2* was examined using SuperScript III Platinum SYBR Green kit (Invitrogen) which employs the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, CA, USA). β-Actin was used as the endogenous control. Primers for β-actin and *COX-2* were purchased from Invitrogen (Carlsbad, CA, USA) and Bioneer (Korea), respectively. The primer sequences are: β-actin (forward: 5′-AGAAGGATTCCTATGTGGGGG-3′, reverse: 5′-CAT GTCGTCCCAGTTGGTGAC-3′) and *COX-2* (forward: 5′-CATACTCAAGCAGGAGCATCC-3′, reverse: 5′-ACC GCTCAGGTGTTGCACGTAGTC-3′). The reactions were assayed as triplicates and performed in a final volume of 50 μl. Melting curve analysis was also done. The mRNA levels for *COX-2* were normalized against β-actin and expressed as fold change relative to control. The quantification was done using the delta-delta C_t method.

Western Blot

Total proteins were extracted from treated-RAW264.7 for Western blot analysis. Briefly, cells were trypsinized, washed with ice-cold PBS and resuspended in lysis buffer (10 mM Tris–HCl pH 7.4, 1.92 mM $MgCl_2$, 1 mM EDTA pH 8.0, 50 mM NaCl, 6 mM β-mercaptoethanol and 2% Triton X-100) containing 1% protease inhibitor cocktail (Calbiochem, San Diego, CA, USA) and incubated for 5 min on ice. Afterwards, the cells were passed through a 21G needle syringe twice, and sonicated for 5 min. The lysates were then centrifuged at 1,000*g* for 10 min and the supernatants were collected. Determination of protein concentration was done using the Bradford method. A 30-μg amount of the protein lysates were resolved on 10% SDS-PAGE gels and electroblotted onto nitrocellulose membranes using a semi-dry transfer system (Bio-Rad, CA, USA). The membranes were then incubated overnight at 4 °C with 5% blocking agent (Amersham, UK) in wash buffer (0.1% (v/v) Tween 20 in PBS). After the blocking step, membranes were probed with rabbit polyclonal COX-2 antibody (Santa Cruz, USA) for 1 h at room temperature. Subsequently, the membranes were incubated for another hour at room temperature with anti-rabbit secondary antibody (Amersham, UK). Detection was performed using an ECL detection kit from Amersham (UK), according to the manufacturer's instructions. Following that, the membranes

were stripped off from the probed antibodies, blocked, and re-incubated with mouse monoclonal actin (Sigma, USA) followed by horseradish peroxidase-labeled anti-mouse secondary antibody (Santa Cruz, USA). Bands on autoradiography films were quantified using ImageJ software and normalized against actin [18].

Statistical Analysis

All results were expressed as means ± SD and analyzed using one way ANOVA. Dunnett's multiple comparisons test was applied to determine the significant differences between treatment groups. Results were accepted to be significant at $P < 0.05$.

Results

Effects of TRF, αT, and Individual Tocotrienol Isoforms (δ-, γ-, and αT_3) on the Viability of LPS-Stimulated RAW264.7

An MTT assay was performed to determine the viability of RAW264.7 following treatment with different vitamin E forms. Figure 1 shows the percent cell viability of LPS-stimulated RAW264.7 after 24 h incubation with different concentrations of TRF, αT and tocotrienol isoforms. It demonstrated that 10 ng/ml of LPS, the concentration used for RAW264.7 stimulation throughout the study, did not cause any change in RAW264.7 viability. No cytotoxicity was observed in all treatment groups for up to 15 μg/ml except for δ- and γT_3. A significant decrease in the percentage cell viability was observed in these two groups at 15 μg/ml.

TRF, αT and Tocotrienol Isoforms (δ-, γ-, and αT_3) Have Different Effects on Proinflammatory Cytokines (IL-6 and TNF-α)

IL-6 and TNF-α are two important proinflammatory cytokines that are released in various inflammatory conditions. The presence of 10 ng/ml of LPS stimulated the release of these cytokines in RAW264.7 (Fig. 2a, b). TRF and all tocotrienol isoforms (δ-, γ-, and αT_3) at 10 μg/ml significantly reduced the release of IL-6 in LPS-stimulated RAW264.7 when compared to the untreated LPS-stimulated group (Fig. 2a). On the other hand, αT has no effect on this context. Of all the treatments, δT_3 showed the best inhibitory effect, being able to reduce the production of IL-6 by more than 50%, followed by γT_3, αT_3 and TRF. In contrast, co-treatment of vitamin E forms and LPS has different effects on the production of TNF-α in RAW264.7 (Fig. 2b). TNF-α levels were increased in TRF, αT and δT_3-treated groups, remained unaltered in a γT_3-treated group, but underwent a significant decrease in αT_3-treated RAW264.7. This decrease was equivalent to a 12% inhibition when compared to an untreated LPS-stimulated group. The significant increase in TNF-α production was

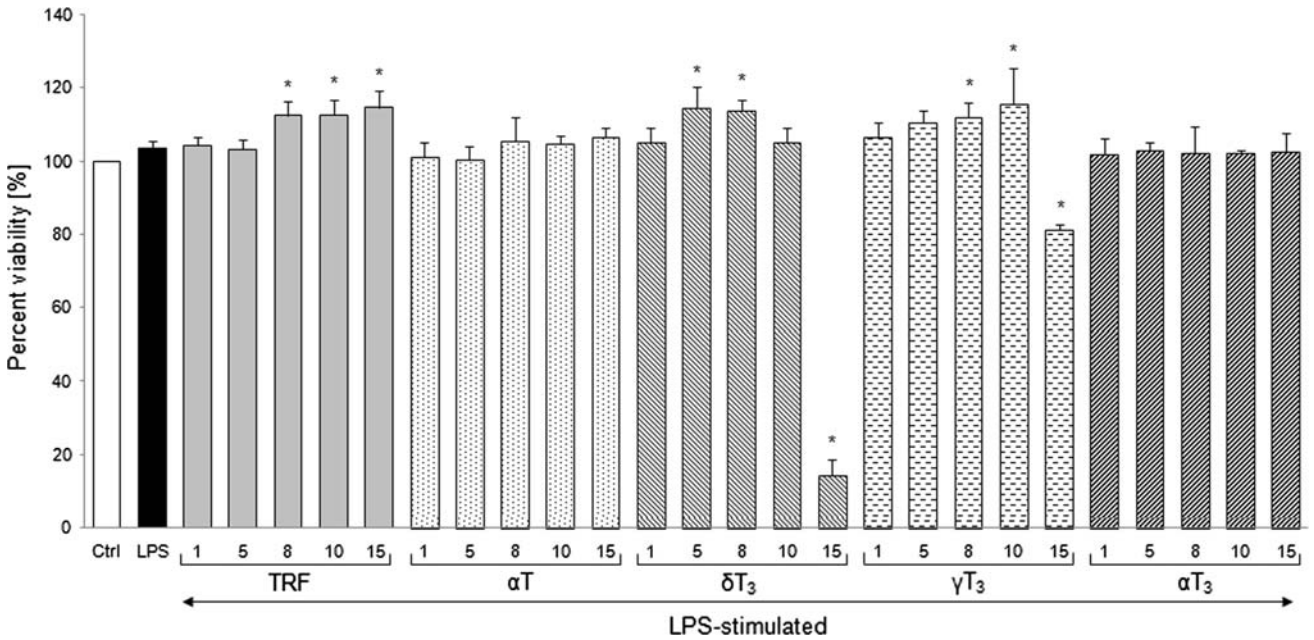

Fig. 1 Percentage cell viability of LPS-stimulated RAW264.7 following 24 h incubation with TRF, αT, and tocotrienol isoforms (δ-, γ-, and αT_3) as assessed using the MTT assay. Data are expressed as means ± SD of triplicate wells. *$P < 0.05$ compared with control group

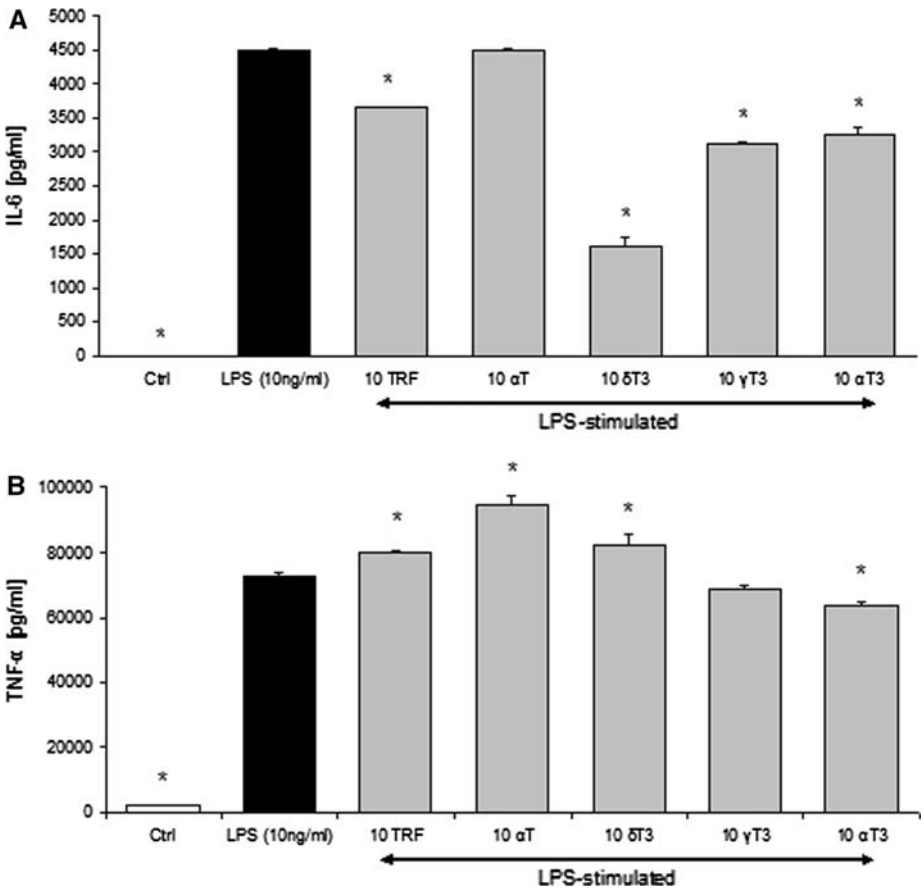

Fig. 2 The changes in (**a**) IL-6 and (**b**) TNF-α levels in LPS-stimulated RAW264.7 after 24 h treatment with various vitamin E forms. Supernatants were collected and assayed by ELISA. Data are presented as means ± SD. $^*P < 0.05$ compared with LPS group

highest in an αT-treated group, followed by δT_3- and TRF-treated groups. Data suggested a possible proinflammatory effect of these vitamin E forms in LPS-stimulated RAW264.7.

All Vitamin E Forms Inhibited the Production of NO in LPS-Stimulated RAW264.7

The addition of 10 ng/ml LPS to RAW264.7 macrophages stimulated the production of NO from a baseline 0.2 μM in the control to 37 μM in the LPS group (Fig. 3). Treatment with 10 μg/ml of all vitamin E forms showed a positive effect in inhibiting NO production. The best effect was observed in the δT_3-treated group in which the level of NO was limited to 26 μM which corresponds to a 31% inhibition when compared to the LPS-stimulated group. It was followed by γT_3 which showed a 19% inhibition at 30 μM, compared to the LPS group. Compared to δ- and γT_3, other treatments also showed significant inhibitory effects in NO production although not as strongly. The levels of NO were detected at 33, 34, and 35 μM in αT_3, TRF, and αT-treated groups, respectively. It is significant that αT treatment demonstrated the least inhibitory effect of only 5% when compared to the other vitamin E forms.

Tocotrienols Inhibited the Production of PGE_2 in LPS-Stimulated RAW264.7

Following LPS stimulation, the level of PGE_2 in the culture supernatant increased to approximately 1,000 pg/ml (Fig. 4). Treatment of LPS-stimulated RAW264.7 with TRF and tocotrienols, except γT_3, significantly inhibited the level of PGE_2 when compared with the LPS group. The greatest inhibition was exerted by δT_3, which showed approximately 55% reduction, followed by TRF and αT_3. No change in the level of PGE_2 was seen in the γT_3-treated group. It was interesting that the PGE_2 level was significantly elevated in the αT-treated group when compared to the LPS group.

Effects of TRF, αT and Tocotrienol Isoforms (δ-, γ-, and αT_3) on *COX-1* and *COX-2* Gene Expression in LPS-Stimulated RAW264.7

COX-1 and COX-2 are two isoforms of the COX enzyme which regulate the synthesis of various prostanoids via the arachidonic acid pathway [19]. COX-1, being the constitutive isoform is important in the regulation of homeostasis whereas COX-2 is the inducible isoform that plays

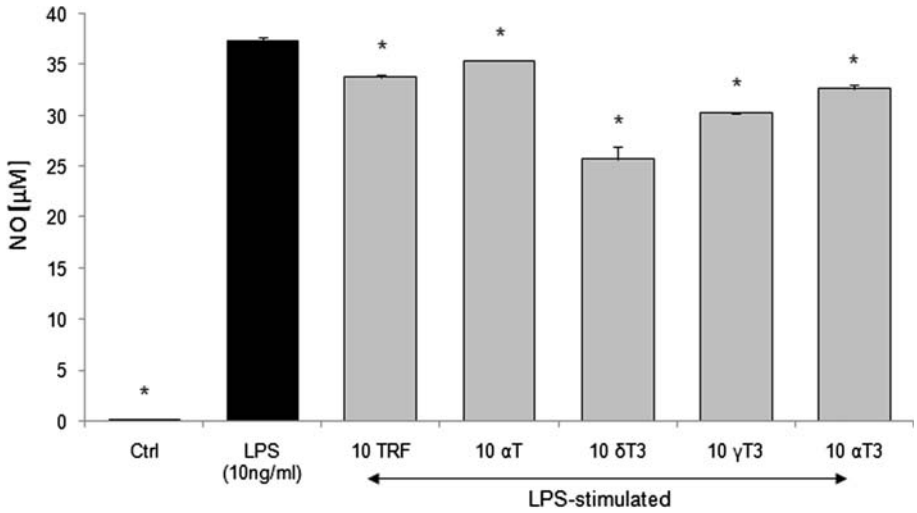

Fig. 3 The production of NO in LPS-stimulated RAW264.7 following treatment with different vitamin E forms. Griess assay that measures nitrite formation was used as an indirect method to determine the levels of NO accumulation in culture supernatants. The data are expressed as means ± SD of triplicate wells. $*P < 0.05$ compared with LPS group

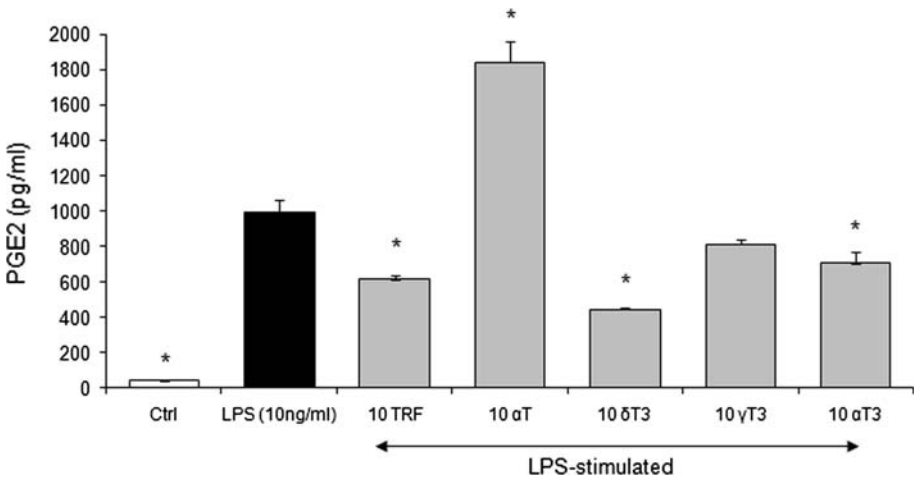

Fig. 4 The changes in PGE_2 release following treatment of LPS-stimulated RAW264.7 with different vitamin E forms. The PGE_2 levels in cell supernatants were measured using an enzyme immunoassay kit. The data are expressed as means ± SD of triplicate wells. $*P < 0.05$ compared with LPS group

important roles in the pathophysiological aspects of inflammation (20). The expression of COX-2 can be initiated by a variety of stimuli and LPS is known to be a potent inducer for these enzymes [21, 22]. In order to elucidate the effects of different vitamin E forms on *COX-1* and *COX-2* gene expressions in LPS-stimulated RAW264.7, RT-PCR was performed using gene-specific primers. Data showed that gene expression of *COX-1* remained unaltered in all groups (Fig. 5b). This observation agrees with the fact that COX-1 remains a constitutive isoform of COX and being expressed equally in all samples. Meanwhile, 10 ng/ml of LPS up-regulated the expression of *COX-2* to a significant 6.4-fold relative to the control. Among the treated groups, δT_3 showed the best inhibition in *COX-2* expression. δT_3, at 10 μg/ml down-regulated the expression of *COX-2* for 1.5-fold when compared with the LPS group but the change was not statistically significant.

TRF and Individual Tocotrienol Isoforms, but Not αT Down-Regulated the Expression of *COX-2* as Determined Using Semi-Quantitative Real-Time PCR

To ascertain the *COX-2* expression results obtained using the conventional RT-PCR method, semi-quantitative Real-Time PCR was applied. Real-Time PCR is a more sensitive method for direct detection of PCR product during the exponential phase of the reaction [23]. Data obtained showed a 145-fold increase in *COX-2* expression when compared to control (Fig. 6). Supplementation of LPS-stimulated RAW264.7 with all the vitamin E forms at 10 μg/ml effectively reduced the expression of *COX-2*. However, the down-regulation was only statistically significant for TRF and other tocotrienol isoforms, but not αT. The most apparent change was seen in the δT_3-treated group where the expression of *COX-2* was reduced 29-fold

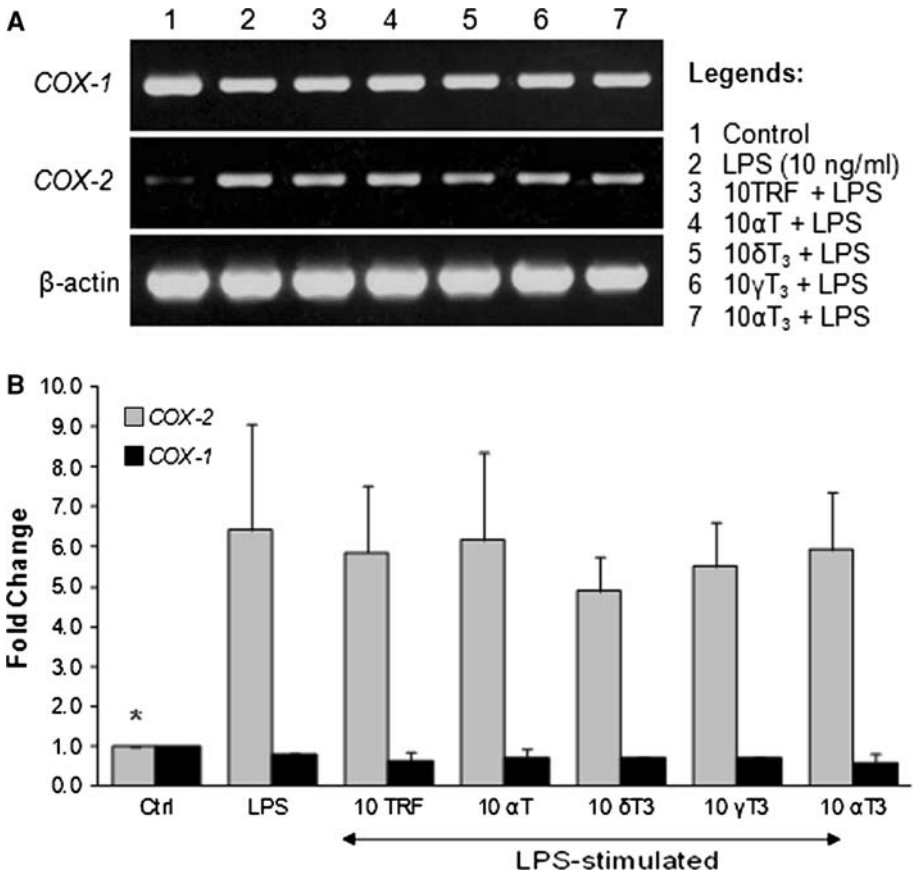

Fig. 5 Gene expressions of *COX-1* and *COX-2* in LPS-stimulated RAW264.7. Total RNA was extracted from the treated macrophages and RT-PCR was carried out to study the gene expressions of *COX-1* and *COX-2*. (**a**) PCR bands as visualized from ethidium bromide-stained agarose gel. The images are representatives of three independent experiments. (**b**) Gene expressions are expressed as relative fold change following normalization against β-actin. Data are shown as means $\pm$ SD of three replicates. $^*P < 0.05$ compared with LPS group

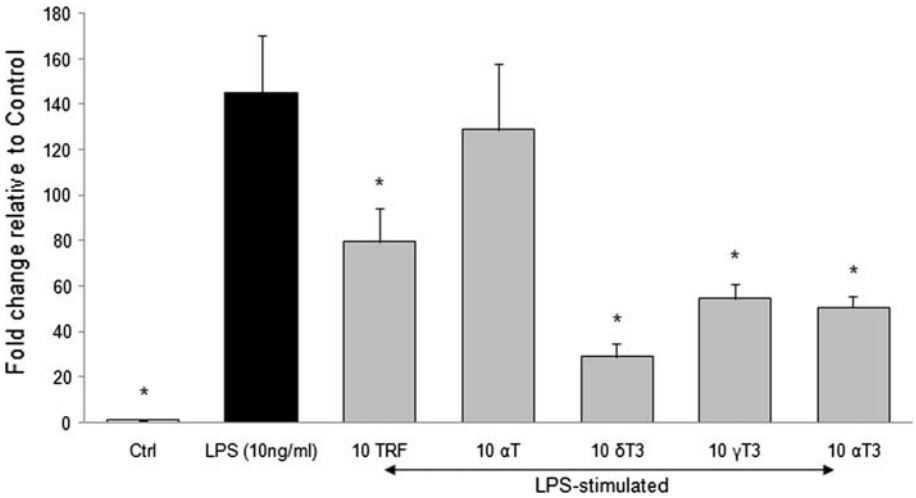

Fig. 6 Relative *COX-2* mRNA expression in LPS-stimulated RAW264.7 after 24 h treatment with TRF, αT and tocotrienol isoforms (δ-, γ-, and αT_3) as determined by Real-Time PCR. Total RNA extracted using the TRIzol method was DNase-treated and subjected to Real-Time PCR to determine the expression of *COX-2*. Data represent expressional fold change relative to control after normalization against β-actin. Data are expressed as means $\pm$ SD of triplicate wells. $^*P < 0.05$ compared with LPS group

relative to the control, which was equivalent to a 116-fold difference from the LPS group. It was followed by αT_3, γT_3, TRF and αT with *COX-2* expression levels at 51-, 55-, 79- and 129-fold relative to the control, respectively. In terms of differences from the LPS group, it corresponded to 94-, 90-, 66-, and 16-fold, respectively. Using this more sensitive approach to determine the expression of the *COX-2* gene, it showed that the most effective *COX-2* inhibitor was δT_3 while αT has no effect.

Effects of TRF, αT and Tocotrienol Isoforms (δ-, γ-, and αT_3) on COX-2 Protein Expression

Next, the effects of vitamin E forms on protein expression of COX-2 were evaluated using Western blot analysis. Stimulation of RAW264.7 with 10 ng/ml of LPS significantly up-regulated the expression of COX-2 protein to 5.9-fold relative to control (Fig. 7b). TRF, at the concentration tested showed the best effect in down-regulating the

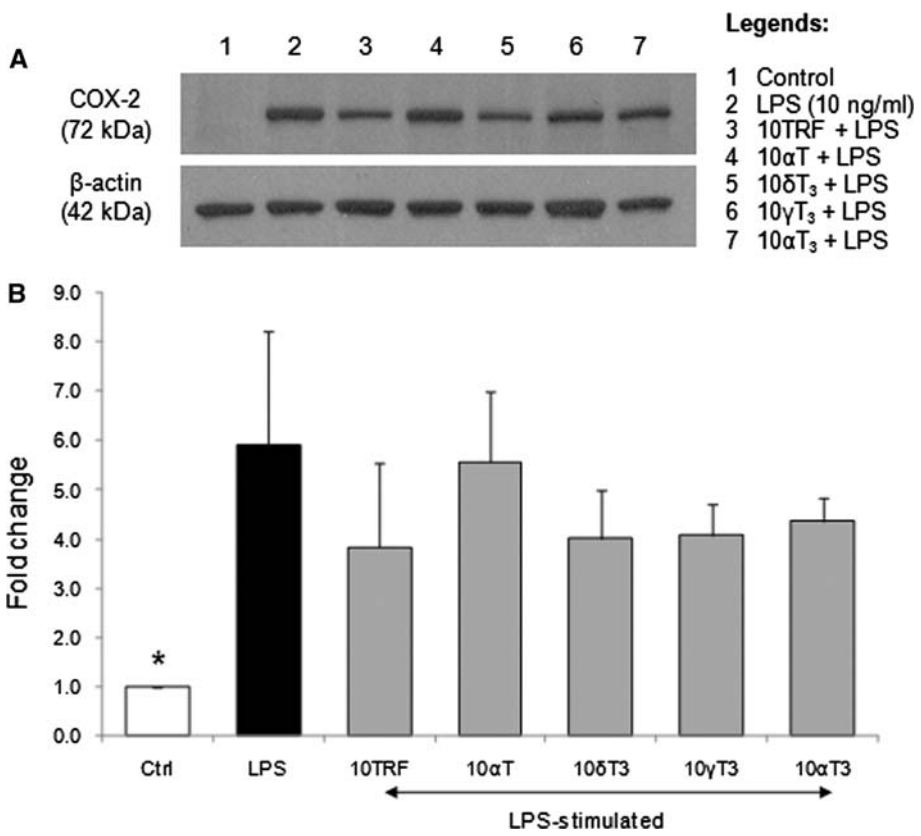

Fig. 7 Protein expression of COX-2 in LPS-stimulated RAW264.7. Cell lysates from treated macrophages were obtained and analyzed for COX-2 protein expression by Western blot. (**a**) COX-2 and β-actin bands on autoradiography films following Western blotting. Images are representatives of three separate experiments. (**b**) Following densitometry analysis, data are expressed as the number of times the change is relative to the control group after normalization against β-actin. *$P < 0.05$ compared with the LPS group

expression of COX-2 protein. In the TRF group, COX-2 protein expression level was 3.8-fold relative to the control and this corresponded to a 2.1-fold difference from the LPS group. However, this down-regulation shown by TRF was not statistically significant. Other treated groups (δ-, γ-, and αT_3) also demonstrated a slight decrease in the number of times different compared to the LPS group but again, the changes were not significant. No obvious effect was seen in the αT-treated group.

Discussion

Recent studies have shown that tocotrienols possess powerful neuroprotective, anti-cancer, as well as hypocholesterolemic properties that are not seen in tocopherols [24]. The objective of the study was to investigate the anti-inflammatory effects of TRF and individual tocotrienol isoforms (δ-, γ-, and αT_3), using αT as comparison. RAW264.7, a transformed murine macrophages cell line was used as the in vitro model for the study. The cells were incubated for 24 h with 10 μg/ml of one of the vitamin E forms together with 10 ng/ml of LPS as stimulation. The concentration of vitamin E form used, 10 μg/ml, was selected based on the cell viability assay as the highest non-toxic concentration for all tested vitamin E forms. The levels of IL-6 in the culture supernatant were found to be reduced in all treated groups except αT. The decrease was not due to the cytotoxic effects of the treatments because as shown by the MTT assay, cells were still viable at 10 μg/ml for each treatment. On the contrary, treatment with vitamin E forms not only failed to reduce TNF-α levels, its production was increased in αT, δT_3 and TRF-treated groups. When compared with the LPS group, only αT_3 was able to inhibit TNF-α significantly whereas its production was unaffected by γT_3. NO, as estimated using Griess assay for the measurement of nitrite, was significantly reduced by all the vitamin E forms when compared with the LPS group. All the treatments, with the exception of αT and γT_3 also inhibited the release of PGE_2. In addition, gene expression of *COX-2* was found to be down-regulated in all treated group except αT. Of all the treatments, δT_3 exhibited the best effect, followed by αT_3, γT_3, and finally, TRF. No significant effect was observed in all the treated groups on the expression of COX-2 protein.

IL-6 and TNF-α are among the main cytokines produced by activated macrophages which are involved in both acute and chronic inflammation [25]. Many studies have used these two cytokines as important markers of inflammation [26, 27]. The up-regulation of IL-6 and TNF-α is seen in many disorders such as rheumatoid arthritis, multiple myeloma and chronic obstructive pulmonary disease [28–30]. Our findings on IL-6 release showed that all treatments except αT were able to reduce the production of

this cytokine significantly. This result contradicts a previous finding which showed a significant decrease in IL-6 level when 20 μM of αT was supplemented [27]. The same report also demonstrated a reduction in TNF-α production at the same concentration of αT. This is in opposition to what we obtained where all the vitamin E forms except αT_3 failed to demonstrate a significant reduction in TNF-α production. The discrepancies in the results may be probably due to the use of different approaches in the experimental protocols as the effects caused by a particular mediator depend on the nature of the toxicant, target and quantities of the mediator produced [31].

NO is an important mediator that is involved in the regulation of homeostatic conditions and immune responses. Synthesis of NO requires the amino acid L-arginine as substrate and the process is catalyzed by a group of enzymes termed the nitric oxide synthases (NOS) [32, 33]. NOS co-exist in three isoforms, each with its own biological functions. The constitutive form of NOS, the cNOS, is important in maintaining homeostasis and it is further divided into the neuronal NOS (nNOS or type 1 NOS) and endothelial NOS (eNOS or type 3 NOS). The third isoform, the inducible NOS (iNOS or type 2 NOS) is the form that is involved in a wide range of pathophysiological events. Our findings showed that all vitamin E forms at 10 μg/ml significantly reduced the generation of NO as assayed using Griess reagent that measures nitrite, the breakdown product of NO. This may be attributed to the antioxidant properties of vitamin E. NO is an effector molecule of innate immunity that forms the pro-oxidant and toxic peroxynitrite ($ONOO^-$) in the presence of superoxide (O_2^-) [34]. In addition, the data also agree with those reported by Berharka and colleagues stating that supplementation of vitamin E reduces the production of inducible NO in peritoneal macrophages derived from aged mice [35]. Study of *iNOS* gene expression was also carried out but none of the treatments showed a significant effect (results not shown). This suggested that vitamin E forms can only inhibit iNOS activity as demonstrated by the reduction in NO synthesis, and not at the gene level.

Being one of the most studied prostaglandins, PGE_2 is produced by fibroblasts, macrophages, and some types of malignant cells and its over-production is implicated in various pathophysiological and diseased states [36, 37]. Similar to NOS, the COX enzymes can be divided into the constitutive and inducible isoforms. COX-1, being the constitutive isoform plays important roles in the regulation of various physiological functions such as gastric cytoprotection, and the maintenance of kidney and platelet functions [20, 38]. On the other hand, COX-2 is the inducible isoform of COX, being expressed by immune cells and engages in the pathological aspects of inflammation [38]. The present study revealed that treatment of LPS-stimulated RAW264.7 with TRF, δ-, and αT_3 suppressed the release of PGE_2 into the culture supernatant. On the other hand, study of gene expression demonstrated that treatment with 10 μg/ml of TRF and tocotrienol isoforms (δ-, γ-, and αT_3) down-regulated the expression of *COX-2*. These levels of *COX-2* expression were in accordance with the amount of PGE_2 measured in the cell supernatants. The expression level of *COX-1*, the constitutive isoform which is responsible for various housekeeping roles, remained unaffected in all groups. This shows that the changes of PGE_2 , as shown in Fig. 4, have nothing to do with COX-1 activity. The down-regulation of the *COX-2* gene suggested that vitamin E forms show an effect at the gene transcriptional level, subsequently affecting the levels of PGE_2 released. Although some marginal down-regulation was observed in the present study, treatment with all vitamin E forms seemed to have no significant effect on the protein translational level. Previous findings have shown that PGE_2 inhibition is accompanied by the down-regulation of the COX-2 gene and protein expression [39]. However, other findings also demonstrated that it does not necessarily follow in that manner as COX-2 enzymatic activity could be altered without any effects being shown at the transcription and translation levels [40, 41]. This suggests that each tocotrienol forms could modulate PGE_2 production through different mechanisms.

Tocotrienols, the vitamin E form which has previously been neglected, are now starting to gain attention due to their newly found properties. Besides their novel antioxidant functions, researchers have shown great interest in the non-antioxidant properties of this vitamin E form [42]. On top of that, studies are beginning to strengthen the notion that tocotrienols are superior to tocopherols in many aspects. TRF and individual tocotrienols, in particular δ- and γT_3, are potent anti-proliferative agents, being able to induce apoptosis and cell cycle arrest in PC-3, a human prostate cancer cell line [43]. αT and αT_3 show no effect in that context. Besides, αT_3 is shown to provide the best neuroprotective effects in rat striatal cultures while αT failed to do so [44]. In the inflammation context, palm TRF has been shown to suppress the transcription of proinflammatory cytokines, and blocking the expression of COX-2 and iNOS in THP-1, a human monocytic cell line [45]. γT_3 was found to inhibit the production of PGE_2 in human lung epithelial A549 cells activated by interleukin-1β while no effect was seen in αT [46]. Meanwhile, another study has demonstrated that γT_3, but not tocopherol, is a potent inhibitor of nuclear factor-κB (NF-κB) activation that leads to the down-regulation of various gene products including COX-2, as well as potentiating apoptosis [47]. NF-κB is a principal transcription factor that modulates the expression of various cytokines, growth factors and

proinflammatory enzymes [48]. The activation of NF-κB pathway is involved in the pathogenesis of many diseases such as those related to inflammation, enhanced cellular proliferation, infections and genetic diseases [49]. The expression of many proinflammatory genes including *COX-2* and *iNOS* is regulated by the activation of NF-κB [50]. Therefore it is plausible that the inhibitory effects of vitamin E forms on *COX-2* expression in this study were due to the blockade of the NF-κB pathway. Suppression of the NF-κB pathway will eventually lead to a lower expression of proinflammatory enzymes and/or cytokines.

In conclusion, the results of this study showed that TRF and tocotrienol isoforms (δ-, γ-, and αT_3) possess anti-inflammatory properties with each displaying different levels of potency. It should be remembered that different effects might be observed if other cell types or different stimulation and incubation methods were used. Nevertheless, in this model of LPS-stimulated RAW264.7 macrophages, δT_3 was demonstrated to be the most effective anti-inflammatory agent among all the vitamin E forms tested, being able to suppress the production of IL-6, NO, PGE_2, and at the same time inhibiting the expression *COX-2*. αT showed the least effect in alleviating an inflammatory response. It was suggested that individual isoforms of tocotrienol might have distinct and different effects, depending on the cell-type and on which parameters are being studied.

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