Basic nutritional investigation

Low and high levels of α-tocopherol exert opposite effects on IL-2 possibly through the modulation of PPAR-γ, IκBα, and apoptotic pathway in activated splenocytes

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Abstract

Objective: We previously demonstrated that a high dose of α-tocopheryl succinate inhibits interleukin-2 (IL-2) mRNA and production in autoimmune-prone MRL/lpr mice. In the present study, we investigated the regulation of α-tocopherol (αTOC) on IL-2 gene expression by examining the mRNA of IL-2, inhibitor κBα (IκBα), and peroxisome proliferator-activated receptor-γ (PPARγ).

Methods: Messenger RNA expression in active splenocytes of BALB/c mice was investigated with reverse transcriptase polymerase chain reaction.

Results: Levels of IL-2 mRNA in phorbol 12-myristate 13-acetate/ionomycin activated splenocytes and cytokine in T–helper-1 cells were increased by 50 M of αTOC but decreased by 1 mM of αTOC. In addition, the IκBα gene expression significantly increased by the high dose (≥500 μM) of αTOC, suggesting an inhibition on nuclear factor-κB pathway for activation of IL-2 expression. PPARγ mRNA level in activated splenocytes was upregulated by 1 mM of αTOC. PPARγ mRNA level in unstimulated splenocytes was upregulated by αTOC in a dose-dependent manner, suggesting that αTOC might enhance the PPARγ signaling pathway. High-dose αTOC induced apoptosis of splenocytes and inhibited phytohemagglutinin-stimulated T-cell proliferation. Conversely, the proliferative response of splenocytes was enhanced by 5 μM of αTOC. Low-dose (50 μM) αTOC increased IL-2 expression, which may have been due to the absence of downregulation of PPARγ and IκBα on the IL-2 gene.

Conclusion: The results indicated that low and high doses of αTOC exert opposite effects on IL-2, possibly through modulation of PPARγ, IκBα, and apoptosis pathways. The present findings support our previous observation of opposite effects of low- and high-dose vitamin E on survival of MRL/lpr mice. © 2006 Elsevier Inc. All rights reserved.

Keywords: α-Tocopherol; Lymphocyte; Interleukin-2; IκBα; Peroxisome proliferator-activated receptor-γ

Introduction

Vitamin E is the most important chain-breaking antioxidant that is well known for its function in maintaining the structural integrity of cells. In addition, vitamin E is recognized to play an essential role in sustaining an appropriate immune response, regulation of cytokine balance, T-cell differentiation, and proliferation, and reduction of inflammation [1,2]. α-Tocopherol (αTOC) may exert its antioxidant-independent function through mechanisms involving modulation of cellular signaling, transcriptional regulation, and induction of apoptosis [3]. Genes that could be regulated by tocopherols include those that are implicated in the uptake and degradation of αTOC, lipid uptake and atherosclerosis, inflammation and cell adhesion, cell signaling and cell cycle regulation [4]. Therefore, the mechanisms by which αTOC modulates cellular immune functions could also be through the
regulation of gene expression of splenocytes, but this is still not well characterized.

Vitamin E has been shown to ameliorate impaired interleukin-2 (IL-2) secretion and cell proliferation of splenocytes caused by aging in human and animal studies [5,6]. We previously reported that the IL-2 mRNA of splenocytes from autoimmune-prone MRL/lpr mice was activated by low doses of vitamin E. Conversely, high doses of vitamin E significantly decreased IL-2 mRNA expression and cytokine production in MRL/lpr mice [7]. The unexpected opposite effects of vitamin E on IL-2 secretion awaited to be clarified.

IL-2 plays a key role in T-cell activation and proliferation, and then T-cell activation leads to cytokine production, thereby promoting the adaptive immune response [8]. IL-2 gene expression is tightly regulated at the transcriptional level. Its promoter contains binding sites for classic transcription factors such as activator protein-1 (AP-1), octamer binding protein (Oct), nuclear factor of activated T cells (NFAT), and nuclear factor-κB (NF-κB) [9]. NF-κB has been recognized as one of the most important transcription factors for coordinating the expression of a wide range of genes that control immune responses [10]. In vivo footprinting of the IL-2 promoter indicated the NF-κB site was bound by a nuclear factor in stimulated T cells [11]. Failure of NF-κB activation results in a deficiency in IL-2 production, suggesting that NF-κB activity is essential for lymphocytic survival and activation [10]. In unstimulated cells, NF-κB is found in the cytoplasm bound to an inhibitory protein, inhibitor κB (IκB). Upon stimulation, IκB is phosphorylated, ubiquitinated, and degraded. This process, which is a key step in the regulation of the NF-κB pathway, liberates the NF-κB proteins from IκB and leads to their nuclear translocation to result in activation of gene expression [12].

The reactive oxygen species has been proposed to activate the NF-κB pathway and thus induce genes encoding for proinflammatory mediators. Therefore, inhibition of the liberation of the NF-κB proteins by antioxidants might explain the anti-inflammatory properties of vitamin E, which are due in part to its ability to downregulate NF-κB [13]. A recent study has demonstrated that αTOC inhibits IκBα degradation and then prevents nuclear translocation of NF-κB in lipopolysaccharide-activated neutrophils [14]. Therefore, αTOC may also inhibit NF-κB nuclear translocation mainly through preventing the release of NF-κB by its inhibitor IκB.

In addition, NF-κB activity was found to be inhibited by peroxisome proliferator-activated receptor-γ (PPARγ) ligands [15]. PPARγ is a ligand-activated nuclear receptor that has an essential role in adipocyte differentiation and lipid metabolism [16]. PPARγ is also expressed in fibroblasts, breast cells, spleen, monocytes/macrophages and human bone marrow precursors [17]. PPARγ is a negative regulator of macrophage activation and expression of inflammatory cytokines by inhibiting transcriptional induction of genes such as tumor necrosis factor-α, IL-6, and inducible nitric oxide synthase [18,19]. Ligands for PPARγ have been shown to be anti-inflammatory in models of inflammatory bowel disease [20], collagen-induced arthritis [21], and experimental autoimmune encephalomyelitis [22].

In addition to its anti-inflammatory role, PPARγ has been shown to inhibit IL-2 secretion and proliferation of activated T lymphocytes through suppression of the DNA-binding activities of transcription factors NFAT, AP-1, and NF-κB [16,23]. PPAR activators inhibit IL-2 mRNA and protein expression [24]. Moreover, PPARγ activation has antiproliferative, prodifferentiation, and proapoptotic effects on cancer cells by regulating downstream genes expression [25].

In our previous study, IL-2 secretion and mRNA expression were decreased in phytohemagglutinin (PHA)-stimulated splenocytes cultured in medium supplemented with high doses of α-tocopheryl succinate [7]. High doses of αTOC were also shown to induce PPARγ expression in a dose-dependent manner in hepatocytes [26]. αTOC and γTOC can upregulate the expression of PPARγ mRNA and protein in SW480 human colon cancer cell lines [27]. Therefore, the purpose of this present study was to observe whether inhibition of IL-2 secretion and mRNA expression in activated splenocytes are related to activation of the PPARγ signaling pathway by high doses of αTOC. In the present study, we demonstrate that inhibition of IL-2 mRNA level, IL-2 production, and splenocytic proliferation by a high dose of αTOC is associated with the increase of PPARγ and 1κBα mRNA expression and the induction of apoptosis in splenocytes.

Materials and methods

Experimental mice

Female BALB/c mice and DO11.10 T-cell receptor transgenic mice specific for ovalbumin BALB/c mice were purchased from the Laboratory Animal Center at the National Taiwan University College of Medicine (Taipei, Taiwan) and maintained at the Institute of Microbiology and Biochemistry at the National Taiwan University. The mice used were between the ages 8 and 12 wk. Animal care and handling conformed to the National Institute of Health’s Guide for the Care and Use of Laboratory Animals [28].

Cell culture and treatment

The spleens were removed and splenocytes were isolated by lysing erythrocytes and washed before use. Splenocytes at a concentration of 5 × 10⁶ cells/mL were placed in RPMI-1640 medium supplemented with 10% mouse serum replacement (TCM, Celox Corp., Hopkins, MN, USA). Cultures were maintained in a humidified atmosphere with 5% CO₂ at 37°C. A stock solution of dl-αTOC (Sigma, St.
Louis, MO, USA) at 500 mM was prepared in ethanol and stored at −20°C. To supplement cells in culture with αTOC, the stock solution was first mixed with fetal bovine serum at a dilution of 1:9 and then incubated at 37°C for 15 min during which time it was vortexed briefly every 5 min [29]. The αTOC/fetal bovine serum solution was then diluted to appropriate concentrations in complete RPMI-1640 medium. The volume of ethanol added to the culture medium was less than 0.2% (v/v). For IL-2 mRNA analysis, cells were pretreated with various doses of αTOC for 24 h and stimulated with 50 ng/mL of phorbol 12-myristate 13-acetate (PMA; Sigma) and 500 ng/mL of ionomycin (Sigma) for 17 h in the presence of αTOC. For PPARγ and IxBα gene expressions, cells were also pretreated with αTOC for 24 h and stimulated with PMA plus ionomycin for 6 h in the presence of αTOC.  

Generation of T–helper-1 cells and determination of IL-2 secretion by enzyme-linked immunosorbent assay

CD4+ T cells (0.5 × 10⁶ cells/mL) were isolated from spleens of DO11.10 T-cell receptor transgenic mice by using a murine cell enrichment kit (>95% CD4+ purity; StemCell Technologies, Vancouver, British Columbia, Canada) and cultured in the presence of irradiated antigen-presenting cells (2 × 10⁶ cells/mL) with 1 µg/mL of ovalbumin peptide, anti–IL-12 antibody (1 ng/mL), and anti–IL-4 antibody (2 µg/mL) for 10 d to generate a homogeneous population of T–helper-1 cells. Then cells were stimulated with ovalbumin peptide in the presence of αTOC for 48 h. The collected supernatants were assayed for IL-2 by sandwich enzyme-linked immunosorbent assay as previously described [7]. Briefly, the anti–IL-2 capture antibody (PharMingen, San Diego, CA, USA) was coated on plates. After incubation, plates were washed, blocked, incubated at 25°C for 2 h, and washed again and then the supernatant was added and incubated for 2 h. After washing, biotin-conjugated anti–IL-2 antibody (PharMingen) was added. After washing, horseradish peroxidase-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA, USA) was added for 30 min. After washing, plates were incubated with 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (Sigma) and measured for absorbance at 415 nm. Data were calculated according to the cytokine standard curve (PharMingen).

Reverse transcriptase polymerase chain reaction: Semiquantitation of mRNA

Splenocytes were treated with various doses of αTOC for 24 h and then stimulated by PMA plus ionomycin in the absence or presence of αTOC for 17 h. Semiquantitative reverse transcription polymerase chain reaction (PCR) was performed by using a set of primers specific for IL-2, IxBα, or PPARγ. The relative density of the mRNA was expressed by using β-actin as an internal control. Total cellular RNA from the spleen was extracted by using an RNeasy Mini kit (Qiagen, GmbH, Hilden, Germany). The RNA was reverse transcribed to cDNA and then products were amplified through reverse transcriptase PCR by using a ProSTAR high-fidelity reverse transcriptase PCR system kit (Stratagene, La Jolla, CA, USA). The primers had the following sequences for 5' and 3' primers, respectively: IL-2, 5'-TTCAGGCTCAGCTCAGGTAATCGTGTAGTGGTTA-3' and 5'-GACAGAGGATCTCCGTGATCTGCACTCC-3'; IxBα, 5'-AAGAGGGAGC-GGTGGGTGAGC-3' and 5'-CAGCCGAGTGGAGG-3'; PPARγ, 5'-ACCCTCCCATCCTTTGAC-3' and 5'-AGGCTTTTGGAGAATCC-3'; and β-actin, 5'-GTGGCCCGCTCTAGGCAACCG-3' and 5'-CTCCTTGTGACACAT-3'. After amplification, the PCR products were visualized after electrophoresis in 1.2% agarose gels by staining with ethidium bromide. The PCR products for IL-2, IxBα, or PPARγ mRNAs, using β-actin as an internal control, after TOC treatment compared with those without TOC treatment.

Apoptosis assay by propidium iodide staining

Propidium iodide (PI; Sigma) is a fluorescent probe that was stained for nucleic acid to evaluate intracellular DNA contents using flow cytometry. Cells that displayed a smaller cell and a high permeability to PI were considered dead cells [30]. After treatment, cells were harvested and then washed twice. Cells were added to 200 µL of phosphate buffered saline and fixed in ice-cold 100% ethanol at 4°C overnight, washed once, and resuspended in 100 µg/mL of ribonuclease A (Calbiochem, La Jolla, CA, USA) at 37°C for 30 min. Then cells were stained with 10 µg/mL of PI for 20 min. Cellular DNA in 10,000 cells was analyzed in a FACSscan flow cytometer (Becton Dickinson, Mountain View, CA, USA). Data are expressed as percentages of total cells.

Proliferation assays

Splenocytes were plated in 96-well plates at a concentration of 1 × 10⁶ cells/mL in complete RPMI-1640 medium supplemented with various doses of αTOC in the absence or presence of 5 µg/mL of PHA (Sigma). Cells were incubated at 37°C for 72 h and then labeled with 1 µCi of [3H] thymidine that was added to each well for the final 24 h. Cells were harvested and radioactivity was measured with a β-counter (Beckman LS 5000 CE, Beckman, Los Angeles, CA, USA). The data were expressed as stimulation index: (samplecpm−blankcpm)/(controlcpm−blankcpm), where cpm is the counts/min.
Statistical analysis

Data were presented as mean ± standard error of the mean for each group. Student’s t test was used to assess differences between the control and treatment groups. This statistical analysis was conducted with SAS/STAT 8 (SAS Institute, Cary, NC, USA). The difference was considered statistically significant at $P < 0.05$.

Results

To investigate the effects of various doses of αTOC on IL-2 production, IL-2 gene expression and cytokine secretion in mitogen-stimulated splenocytes were treated with or without αTOC (Fig. 1). Data are expressed as fold increase of mRNA after αTOC treatment versus without αTOC treatment in Fig. 1A. The level of IL-2 mRNA increased significantly to 63% ($P = 0.0254$) when treated with 50 μM of αTOC. Conversely, IL-2 mRNA decreased by about 23% when treated with 1 mM of αTOC. To determine the effect of αTOC on IL-2 secretion, CD4$^+$ T cells specific for ovalbumin from DO11.10 T-cell receptor transgenic mice were used to obtain a homogeneous population of T-helper-1 cells. IL-2 secretion was significantly increased ($P = 0.0194$) when treated with 50 μM of αTOC but decreased by about 52% ($P = 0.1116$) when treated with 1 mM of αTOC (Fig. 1B). IL-2 secretions also showed the same tendency under various concentrations of αTOC treatment.

Spontaneous IL-2 production by splenocytes without mitogen stimulation also decreased significantly with addition of αTOC at a concentration higher than 500 μM (data not shown). Therefore, the opposite effect of low and high doses of αTOC on IL-2 production may be due to the regulation of gene expression.

IκBα, a NF-κB inhibitory subunit, was reported to be higher, whereas NF-κB DNA-binding was decreased [31]. To determine whether inhibition of IL-2 production might be due to decreased NF-κB DNA-binding, mRNA expres-
Fig. 3. Effect of αTOC on PPARγ mRNA expression by stimulated splenocytes. Cells were pretreated with various doses of αTOC for 24 h and treated with 50 µg/ml of PMA plus 500 µg/L of ionomycin for 6 h in the presence of αTOC and then analyzed by semiquantitative reverse transcriptase polymerase chain reaction. Total RNA was extracted and polymerase chain reaction products were visualized after electrophoresis. Values are densitometric quantifications of each band represented as fold increases in relative density of PPARγ mRNA. Data are representative of three independent experiments. Values are means ± standard error of the mean (n = 3). *Different from the control sample (PMA/ionomycin only) as analyzed by Student’s t test (P < 0.05). αTOC, α-tocopherol; PMA, phorbol 12-myristate 13-acetate; PPARγ, peroxisome proliferator-activated receptor-γ.

Fig. 4. Effect of αTOC on PPARγ mRNA expression in splenocytes. Cells were treated with various doses of αTOC or 20 µmol/L of ciglitazone for 48 h and then analyzed by semiquantitative reverse transcriptase polymerase chain reaction. Total RNA was extracted and polymerase chain reaction products were visualized after electrophoresis. Values are densitometric quantifications of each band represented as fold increases in relative density of PPARγ mRNA. Data are representative of three independent experiments. Values are means ± standard error of the mean (n = 3). *Different from control sample (0 µmol/L of αTOC) as analyzed by Student’s t test. αTOC, α-tocopherol; PPARγ, peroxisome proliferator-activated receptor-γ.

Fig. 5. Effect of αTOC on the PI percentage in splenocytes. Cells were incubated with various concentrations of αTOC for 24 h, and apoptosis was determined by PI staining. Data are representative of three independent experiments. Values are means ± standard error of the mean (n = 3). *Different from control sample (0 µmol/L of αTOC) as analyzed by Student’s t test (P < 0.05). αTOC, α-tocopherol; PI, propidium iodide.
apoptosis of splenocytes, PI staining, which was stained for nucleic acid to evaluate intracellular DNA content, was determined. As shown in Fig. 5, after 24 h of incubation, apoptosis occurred spontaneously in about 10% of cells. When cells were treated with low doses of \( \alpha \)TOC, slight protection was seen at 5 \( \mu \)M of \( \alpha \)TOC. However, splenocytes treated with higher concentrations of \( \alpha \)TOC, underwent dose-dependent apoptosis, which reached 34% at a dose of 1 mM. Apoptotic cells were significantly higher, about 259% \( (P = 0.0041) \), and cell viability was significantly lower (data not shown) with treatment with 1 mM of \( \alpha \)TOC compared with control cells.

Spleen cells were stimulated with PHA in the presence of \( \alpha \)TOC, and the proliferative response is expressed as stimulation index, as shown in Fig. 6. Cell proliferation of PHA-activated splenocytes was significantly increased by the addition of low doses of \( \alpha \)TOC (5 \( \mu \)M, \( P = 0.0121 \)) but significantly decreased by high doses of \( \alpha \)TOC \((\approx 500 \mu \text{M}, \ P < 0.005) \). This suggested that there was also an opposite effect of opposite effect of low and high doses of \( \alpha \)TOC on spleen cell proliferation under stimulation. High doses of \( \alpha \)TOC might be negative regulators of T-cell activation.

**Discussion**

Vitamin E is necessary for the maintenance of an appropriate immune response and optimal immune function [1,2]. However, vitamin E may affect lymphocyte function through mechanisms other than its antioxidant effects. For example, the effects of \( \alpha \)TOC on cellular signaling may be due to inhibition of protein kinase-C [13]. The anticarcinogenic effects of \( \alpha \)TOC may be explained by its modulation of immune function and induction of apoptosis [13,32]. \( \alpha \)-Tocopheryl succinate has been shown to be the most effective form among the vitamin E analogues to induce apoptosis [33]. Therefore, \( \alpha \)TOC was used in this study to avoid strong apoptotic effects.

Antioxidant and modulation effects on gene expression of \( \alpha \)TOC have been widely accepted to explain its protective effects with regard to cardiovascular disease and cancer. Moreover, an autoimmune disease, systemic lupus erythematosus, has been suggested to be associated with impaired antioxidant status in patients who have systemic lupus erythematosus [34]. Vitamin E supplementation to autoimmune-prone NZB/W F1 mice under oxidative stress decreased autoantibody production and inflammation and thus prolonged the life span [35]. One study showed that low-dose (0.4 mg/d) vitamin E supplementation eliminated autoimmune deterioration in autoimmune-prone MRL/lpr mice when compared with a vitamin E–deficient group [36]. The beneficial effect of low-dose vitamin E on MRL/lpr mice was also observed in our previous study. However, high-dose vitamin E supplementation to MRL/lpr mice significantly decreased IL-2 secretion, increased autoantibody, and shortened life span. The opposite effects of low- and high-dose supplementation of vitamin E on survival of MRL/lpr mice were first demonstrated by Hsieh and Lin [7]. Therefore, the possibility of different effects on IL-2 expression through pathways of NF-\( \kappa \)B or PPAR\( \gamma \) was proposed.

In the present in vitro study, mitogen-stimulated splenocytes had significantly increased IL-2 gene expression and higher cytokine secretion in the presence of low-dose \( \alpha \)TOC. Conversely, IL-2 gene expression and cytokine secretion decreased when \( \alpha \)TOC concentrations were higher than 1 mM. Therefore, the opposite effect of low- and high-dose supplementation of vitamin E on IL-2 gene expression and cytokine secretion was demonstrated again, despite the fact that two different forms of vitamin E were used and splenocytes were isolated from different species of mice in these two studies.

The IL-2 promoter can be inactivated by insufficient binding of AP-1/NFAT in \( lpr/lpr \) CD4 \( ^{+} \)CD8 \( ^{-} \) T cells and mutation of any single transcription site or by cyclic adenosine monophosphate inhibition of a single NF-\( \kappa \)B site [37]. Because activation of NF-\( \kappa \)B is required for IL-2 expression in activated lymphocytes, mitogen-induced nuclear translocation of NF-\( \kappa \)B requires degradation of its inhibitor I\( \kappa \)B. Moreover, the newly synthesized I\( \kappa \)B\( \alpha \) protein enters the nucleus and dissociates NF-\( \kappa \)B from promoter elements to repress NF-\( \kappa \)B function [38]. Therefore, NF-\( \kappa \)B activation was indirectly investigated by analysis of its inhibitor I\( \kappa \)B expression in the present study.

\( \alpha \)TOC has been shown to decrease I\( \kappa \)B\( \alpha \) degradation and inhibit stimuli-induced NF-\( \kappa \)B translocation in hepatocytes and neutrophils [14,31]. Our results showed decreased I\( \kappa \)B\( \alpha \) expression after PMA/ionomycin activation, suggesting that inhibition of I\( \kappa \)B\( \alpha \) gene expression may be one way to maintain NF-\( \kappa \)B activation in lymphocytes after mitogen stimulation. When splenocytes were activated in the presence of high-dose \( \alpha \)TOC, I\( \kappa \)B\( \alpha \)
gene expression was significantly increased, implying the NF-κB activation might be suppressed and thus decreasing IL-2 expression. Kiemer et al. [39] also reported that atrial natriuretic peptide specifically suppressed tumor necrosis factor-α–induced activation of NF-κB by induction of IkBα gene expression. Kovács et al. [40] recently demonstrated that dissociation of NF-κB from κB promoter elements may be caused by rapid transcription of IkBα genes, leading to a largely increased IkBα protein. Therefore, an increase in the level of IkBα by a high dose of αTOC may prolong NF-κB inactivation and thus affect IL-2 expression in activated lymphocytes.

There have been studies showing that αTOC can also upregulate expression of PPARγ mRNA in hepatocytes and SW480 human colon cancer cell lines [26,27]. The PPAR family has been the focus of much interest for its possible role in the regulation of immune responses and inflammatory cytokines by ligands involving aberrant inflammatory or proliferation [19,24]. PPAR also has been demonstrated to inhibit proliferation of mitogen-stimulated T cells. The decreased cell viability was a result of apoptosis of T cells, which occurred when cells were treated with PPARγ agonists [43]. A high dose of αTOC (1 mM) was found to induce apoptosis of unstimulated splenocytes in the present study, suggesting that an association between αTOC and PPARγ on T-cell apoptosis. The proliferative responses of PHA-stimulated splenocytes were also suppressed by αTOC at a high concentration (≥500 μM), suggesting that high doses of αTOC alters T-lymphocyte function. The maintenance of appropriate T-lymphocyte populations is important in immune regulation and autoimmune development [7,44]. The present findings support our previous observation that there are opposite effects of low- and high-dose supplementation of vitamin E on survival of autoimmune-prone MRL/lpr mice.

In the present study we have reported evidence that a low dose of αTOC increases IL-2 gene expression and that a high dose has the opposite effect that is associated with the upregulated PPARγ pathway, thereby increasing IkBα expression, inducing apoptosis, and suppressing proliferative responses of splenocytes.

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