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γ-Tocotrienol inhibits lipopolysaccharide-induced interlukin-6 and granulocyte colony-stimulating factor by suppressing C/EBPβ and NF-κB in macrophages☆

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Abstract

Cytokines generated from macrophages contribute to pathogenesis of inflammation-associated diseases. Here we show that γ-tocotrienol (γ-TE), a natural vitamin E form, inhibits lipopolysaccharide (LPS)-induced interleukin (IL)-6 production without affecting tumor necrosis factor α (TNF-α), IL-10 or cyclooxygenase-2 (COX-2) up-regulation in murine RAW264.7 macrophages. Mechanistic studies indicate that nuclear factor κB (NF-κB), but not c-Jun NH(2)-terminal protein kinase, p38 or extracellular signal-regulated kinase mitogen-activated protein kinases (MAPKs), is important to IL-6 production and that γ-TE treatment blocks NF-κB activation. In contrast, COX-2 appears to be regulated by p38 MAPK in RAW cells, but γ-TE has no effect on LPS-stimulated p38 phosphorylation. Despite necessary for IL-6, NF-κB activation by TNF-α or other cytokines is not sufficient for IL-6 induction with exception of LPS. CCAAT/enhancer-binding protein (C/EBP) β appears to be involved in IL-6 formation because LPS induces C/EBPβ up-regulation, which parallels IL-6 production, and knockdown of C/EBPβ with small interfering RNA results in diminished IL-6. LPS but not individual cytokines is capable of stimulating C/EBPβ and IL-6 in macrophages. Consistent with its dampening effect on IL-6, γ-TE blunts LPS-induced up-regulation of C/EBPβ without affecting C/EBPδ. γ-TE also decreases LPS-stimulated granulocyte colony-stimulating factor (G-CSF), a C/EBPβ target gene. Compared with RAW264.7 cells, γ-TE shows similar or stronger inhibitory effects on LPS-triggered activation of NF-κB, C/EPBβ and C/EBPδ and more potently suppresses IL-6 and G-CSF in bone marrow-derived macrophages. Our study demonstrates that γ-TE has antiinflammatory activities by inhibition of NF-κB and C/EBPs activation in macrophages.

Keywords: Vitamin E; Tocopherol; Tocotrienol; Inflammation; IL-6; C/EBPβ

1. Introduction

Macrophages play important roles in promoting inflammation and inflammation-associated diseases including cancer [1–3]. During inflammation, in response to endotoxin and cytokines, macrophages release excessive amount of proinflammatory mediators including reactive oxygen species, eicosanoids and cytokines such as interleukin (IL)-6. IL-6 has been recognized as a key proinflammatory cytokine that contributes to arthritis, cancer and obesity-related promotion of carcinogenesis [3,4]. Targeting IL-6 by anti-IL-6 has been used clinically to treat anti-tumor necrosis factor α (TNF-α) nonresponsive arthritis [5,6]. Therefore, modulation of IL-6 formation appears to be an attractive strategy for regulating excessive immune response and attenuating inflammation-associated damage.

Vitamin E is a group of lipophilic antioxidants, which include α-, β-, γ- and δ-tocopherol and α-, β-, γ- and δ-tocotrienol. All members of vitamin E family contain a chromanol ring linked with a phytyl side chain, in which tocopherols are saturated and tocotrienols have three double bonds [7,8]. In addition to antioxidant activities, specific forms of vitamin E have been shown to have antiinflammatory properties [9]. We have demonstrated that γ-tocopherol (γ-T), δ-tocopherol (δ-T) and γ-tocotrienol (γ-TE) as well as their long-chain metabolites suppress cyclooxygenase- and 5-lipoxygenase-catalyzed proinflammatory eicosanoids [10–13]. Consistently, γ-T supplementation decreases proinflammatory eicosanoids and suppresses eosinophilia in an acute inflammatory and an allergic airway inflammation model [12,14].

Besides inhibitory effects on eicosanoids, specific vitamin E forms have been reported to modulate cytokine formation. For instance, γ-TE appears to be stronger than its tocopherol counterparts in inhibition of IL-13-stimulated eotaxin-3 in human lung epithelial cells by up-regulation of prostate apoptosis response 4, which subsequently blocks atypical protein kinase C (PKC)-mediated activaton of signal transducer and activator of transcription 6 [15,16]. It has recently been reported that γ-TE inhibits lipopolysaccharide (LPS)-stimulated IL-6 in murine RAW276.4 macrophages [17]. However, the mechanisms underlying this inhibitory effect have not been identified. In the present study, we investigated the effects and mechanisms of various forms of vitamin E, including α-tocopherol (α-T), γ-T, δ-T and γ-TE, on LPS-stimulated cytokine formation in murine RAW264.7 macrophages. We also extended our study to primary bone marrow-derived macrophages (BMDMs) for verifying the observed activities and mechanisms.

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2. Materials and methods

2.1. Materials

α-T (99%), γ-T (97–99%) and δ-T (97%) were purchased from Sigma (St. Louis, MO, USA). γ-TE (>97%) was a gift from BASF (Germany). Bacterial LPS (B *Escherichia coli* 055:B5), recombinant mouse TNF-α, IL-1α, IL-1β, type II interferon (IFN-γ) and macrophage colony-stimulating factor (M-CSF) were from Sigma. Primary antibodies against phosphor-inhibitor of nuclear factor κB (NF-κB) α (IκBα) (sc-8404), IκBα (sc-371), CCAAT/enhancer-binding protein (C/EBP) β (sc-7962), C/EBPδ (sc-636) and secondary antibodies as well as C/EBPβ small interfering RNA (siRNA) (sc-29862) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Inhibitors of mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK) (U0126), p38 mitogen-activated protein kinase (MAPK) (SB202190) and NF-κB (parthenolide) were from Calbiochem (La Jolla, CA, USA). c-Jun NH(2)-terminal protein kinase (JNK) inhibitor (SP600125), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and all other chemicals were from Sigma. Cell culture media were from American Type Culture Collection (ATCC) (Manassas, VA, USA).

2.2. Cell culture

Murine RAW264.7 macrophages from ATCC were routinely maintained in Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum (FBS). Confluent cells were seeded and allowed to attach overnight at 7×10^5 or 5×10^6 per well in a 24-well or a 6-well plate, respectively. Vitamin E stock solutions were initially made in dimethyl sulfoxide (DMSO) and then diluted in 10 mg/ml of fatty-acid-free bovine serum albumin. Cells were incubated in DMEM with 1% FBS containing 0.05% DMSO (control) or vitamin E forms for 14–16 h and stimulated with LPS (0.1 μg/ml) or other stimuli. Cell viability was determined using cellular dehydrogenase/reductase activity by MTT assays [18,19].

2.3. Preparation of BMDMs

BMDMs from mice were prepared according to a published protocol [20]. The protocol on animal use was approved by the Animal Care and Use Committee at Purdue University and was strictly followed. Briefly, bone marrow was obtained by flushing femur from 7-week-old to 8-week-old c57BL/6 black mice from Harlan (Indianapolis, IN, USA). Suspension cells were cultured in DMEM containing 10% FBS with penicillin (100 U/ml), streptomycin (100 μg/ml) and M-CSF (100 U/ml). Cells were cultured in 10-cm dish for 5 days, followed by replacing fresh medium for additional 2 days. Attached cells were harvested by non-enzymatic dissociation solution (Sigma) and characterized using flow cytometry or re-seeded into 24- or 6-well plate for subsequent studies.

2.4. Characterization of BMDMs with flow cytometry

BMDMs were characterized by flow cytometry. Briefly, cells were blocked with mouse immunoglobulin G (Sigma) before stained with phycoerythrin (PE)-conjugated anti-mouse F4/80/EMR1 (#FAB5580P) or PE-conjugated isotype control (#IC006P) (R&D Systems, Minneapolis, MN, USA) for 30 min at room temperature. Cells were then washed and analyzed by Cell Lab Quanta SC-MPL flow cytometer (Beckman Coulter, Brea, CA, USA) with excitation at 488 nm.

2.5. Analyses of cytokines

Cytokine accumulation in the media was quantitatively measured using immunoassay kits from R&D Systems or eBioscience (San Diego, CA, USA) according to the manufacturers' instructions. In some experiments, relative levels of selected cytokines and chemokines were determined by proteome profiler mouse cytokine array kit (Catalog #ARY006; R&D Systems).

2.6. Western blot

Cells were lysed in a lysis buffer containing Tris-EDTA, 1% sodium dodecyl sulfate (SDS), 1 mM dithiothreitol, 2 mM sodium vanadate and protease inhibitor cocktails (Sigma). Cytosolic and nuclear proteins were extracted using a Pierce kit (Pierce, Rockford, IL, USA). The resulting solution was heated at 95°C for 10–15 min. Proteins (25–50 μg) were loaded on 10% precast SDS-polyacrylamide gel electrophoresis gels. Resolved proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA) and probed by antibodies. Membranes were exposed to chemiluminescent reagent (Perkin Elmer, Waltham, WA, USA) and visualized on a Kodak film. In all the experiments, immunoblotting was first performed with antibodies for proteins of interest. PVDF membranes were then stripped with antibody-stripping solution from EMD Millipore Corporation and re-immunoblotted with antibodies for internal controls such as β-actin.

2.7. Knockdown of C/EBPβ by siRNA transfection

Mouse C/EBPβ siRNA and control siRNA were purchased from Santa Cruz Biotechnology. Transfections were performed according to the manufacturer's instructions with lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Briefly, 75% confluent RAW cells were transfected with 10 μl of lipofectamine and 10 μl of 10 μM siRNA in 1 ml Opti-MEM Reduced Serum Medium (Catalog #51985-091; Invitrogen) for 6 h. The transfection mixture was replaced with fresh DMEM containing 10% FBS for 16 h. Cells were then stimulated with LPS for 8 h. Media and cells were used for cytokine analysis and Western blot, respectively.

2.8. Statistical analyses

One-way analysis of variance and Student's *t* tests were used in statistical analyses.

3. Results

3.1. γ-TE inhibited LPS-stimulated IL-6 but had no effect on up-regulation of TNF-α, IL-10 or COX-2 in RAW264.7 macrophages

In confluent RAW264.7 macrophages, LPS treatment led to marked increase of proinflammatory cytokines including IL-6 (4–16 ng/ml) and TNF-α (24–33 ng/ml) as well as an antiinflammatory cytokine IL-10 (1–12 ng/ml). Vitamin E forms differentially inhibited LPS-induced IL-6, and γ-TE was much stronger than tocopherols in this activity (Fig. 1A and B). These observations are consistent with a previous study [17]. On the other hand, γ-TE or tocopherols had no significant effects on LPS-stimulated increase of TNF-α or IL-10 (Fig. 1C and D). Consistent with our previous work [10,11], tocopherols or γ-TE did not significantly affect LPS-induced up-regulation of cyclooxygenase-2 (COX-2) (Fig. 1E), a key enzyme catalyzing proinflammatory eicosanoids [21]. In all these studies, γ-TE treatment had no obvious impact on cell viability based on cell morphological examination and MTT assays (data not shown).

3.2. NF-κB is necessary for IL-6 production and γ-TE inhibited NF-κB activation in RAW276.4 macrophages

To understand the mechanism underlying the observed effects by γ-TE, we examined potential involvement of several signaling pathways using specific inhibitors. Chemical inhibition of p38 MAPK, but not NF-κB, extracellular signal-regulated kinase, PKC or JNK, led to significant suppression of LPS-induced COX-2 up-regulation (Fig. 2A). This suggests that p38 activation plays a key role in COX-2 induction in RAW cells. Interestingly, several previous studies reported that p38 MAPK is important to COX-2 induction in various types of cells [22,23]. Here we found that γ-TE showed no significant effects on LPS-stimulated p38 activation (Fig. 2B), which is consistent with its lack of impact on COX-2 induction in RAW264.7 cells.

Using the similar approaches, we found that blocking LPS-induced NF-κB activation by parthenolide almost completely abolished IL-6 production, whereas inhibitors of JNK, MEK and p38 MAPK had moderate or no influence on IL-6 (Fig. 3A). These results strongly suggest that NF-κB is important for LPS-stimulated IL-6. Interestingly, while LPS caused immediate increase of IκBα phosphorylation, a critical step for NF-κB activation [24], even stronger IκBα phosphorylation was seen after 60-min LPS stimulation (Fig. 3B). We reason that the second activation may be caused by cytokine-induced re-stimulation subsequent to the initial Toll-like receptor-mediated activation [25]. The importance of the delayed NF-κB activation in IL-6 induction is evident by the observation that addition of parthenolide after LPS treatment resulted in similar suppression of IL-6 to that caused by cells pretreated with parthenolide prior to LPS stimulation (Fig. 3C). We found that γ-TE inhibited LPS-stimulated IκBα phosphorylation at the 60-min activation rather than at initial activation (Fig. 3D). In addition to