Research Paper

Attenuation of lipopolysaccharide (LPS)-induced cytotoxicity by tocopherols and tocotrienols

Keiko Nishio*, Masanori Horie1, Yoko Akazawa, Mototada Shichiri, Hitoshi Iwahashi2, Yoshihisa Hagihara, Yasukazu Yoshida, Etsuo Niki*

Health Research Institute, National Institute of Advanced Industrial Science & Technology (AIST), Ikeda 563-8577, Japan

A R T I C L E  I N F O

Article history:
Received 25 October 2012
Received in revised form 26 October 2012
Accepted 26 October 2012

Keywords:
Lipid peroxidation
Lipopolysaccharide (LPS)
Oxidative stress
Tocopherol
Tocotrienol
Vitamin E

A B S T R A C T

Lipopolysaccharide (LPS) induces host inflammatory responses and tissue injury and has been implicated in the pathogenesis of various age-related diseases such as acute respiratory distress syndrome, vascular diseases, and periodontal disease. Antioxidants, particularly vitamin E, have been shown to suppress oxidative stress induced by LPS, but the previous studies with different vitamin E isoforms gave inconsistent results. In the present study, the protective effects of α- and γ-tocopherols and α- and γ-tocotrienols on the oxidative stress induced by LPS against human lung carcinoma A549 cells were studied. They suppressed intracellular reactive oxygen formation, lipid peroxidation, induction of inflammatory mediator cytokines, and cell death. Tocopherols were incorporated into cultured cells much slower than tocotrienols but could suppress LPS-induced oxidative stress at much lower intracellular concentration than tocotrienols. Considering the bioavailability, it was concluded that α-tocopherol may exhibit the highest protective capacity among the vitamin E isoforms against LPS-induced oxidative stress.

© 2013 The Authors. Published by Elsevier B.V. Open access under CC BY-NC-ND license.

Introduction

Lipopolysaccharide (LPS), a highly conserved cell wall component of gram-negative bacteria, is known to initiate signaling cascade for inflammatory mediator expression including cytokines such as tumor necrosis factor α (TNF-α) and interleukin (IL)-6, inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX2), and nuclear factor-kappaB (NF-kB) [1]. It is accepted that dysregulated inflammatory responses result in various age-related diseases including acute respiratory distress syndrome (ARDS), neurodegenerative and vascular diseases [2] and periodontal disease [3,4]. Oxidative stress mediated by reactive oxygen/nitrogen species (ROS/RNS) has been implicated in the pathogenesis induced by LPS which accelerates the formation of nitric oxide (NO) [5] and prostaglandin E2 (PGE2) [6].

Consequently, the effects of antioxidants against LPS-induced oxidative stress have been studied extensively. Notably, the role of vitamin E against deleterious effects of LPS has been the subject of many in vitro and in vivo studies. In general, it has been observed that vitamin E suppresses inflammatory responses and oxidative damage induced by LPS in both cell culture systems and animal experiments [7–11]. Vitamin E is composed of eight isoforms: α, β, γ, and δ-tocopherols and α, β, γ, and δ-tocotrienols. Tocopherols contain a saturated phytyl side chain, while tocotrienols contain three double bonds in the side chain, and α, β, γ, and δ-forms differ in the number and position of methyl groups on the chromanol ring [12]. It was reported that α-tocopherol (αToc) effectively prevented interferon-gamma/LPS-induced dopaminergic neuron degeneration [13] and that αToc decreased LPS-induced lipid peroxidation and IL-6 in murine microglia and brain [14]. In a rat model of acute lung inflammation caused by intratracheally administered LPS, aerosol-administered αToc attenuated lung inflammation [15]. Furthermore, αToc was found to promote recovery from LPS-induced infection in aged mice [16], and a low embryo implantation rate due to systemic administration of LPS was partially reversed or prevented by αToc [17].
The relative preventive capacity of different vitamin E isoforms against LPS-induced inflammation and oxidative stress has been the subjects of many studies. It was found that γToc inhibited proinflammatory PGE₂ and leukotriene B₄ (LTB₄) production, decreased TNF-α, and attenuated inflammation-mediated damage more effectively than αToc [18,19]. Further, a combination of aspirin and γToc, but not a combination of aspirin and αToc, exerted some advantage over aspirin alone in terms of anti-inflammatory effects and attenuation of aspirin-induced adverse effects [20,21]. In a study on the effects of αToc or γToc against LPS-triggered non-alcoholic steatohepatitis (NASH) in an obese (ob/ob) mouse model, the intraperitoneal injection of LPS increased serum alanine aminotransferase (ALT) and both αToc and γToc decreased this response and also hepatic malondialdehyde (MDA) and TNF-α production [22].

Tocotrienols (Toc3) have received much attention recently because of a potential biological function distinct from tocopherols [23]. The tocotrienol-rich fraction of palm oil was found to inhibit induction of iNOS and COX2 expression and protect human monocyteic THP-1 cells from LPS-induced cell death [24]. α, γ, or δToc3 inhibited TNF-α secretion in LPS-stimulated RAW 264.7 cells and reduced serum levels of TNF-α after LPS treatment in BALB/c mice [11]. It was reported that low concentrations of δToc3 (<20 μM) blocked LPS-induced gene expression of TNF-α, IL-1β, IL-6, and iNOS, while higher concentrations (40 μM) increased gene expression of the latter in peritoneal macrophages as compared to control group [11]. Further, γToc3, γToc3 and δToc3 suppressed LPS-induced NO production in microglia, δToc3 being the most potent [25].

Thus, previous studies indicate contradictory outcomes for the anti-inflammatory effects of vitamin E isoforms [26]. One of the reasons may be ascribed to a marked difference in the rates of incorporation into cultured cells [27] and bioavailability of vitamin E isoforms [28]. Tocotrienols are incorporated into cultured cells much faster than the corresponding tocopherols [27,29,30], which makes the apparent efficacy of tocotrienols larger than tocopherols.

Materials and methods

Materials

Dulbecco's modified eagle medium (DMEM) was obtained from Gibco Invitrogen Corporation, (Grand Island, NY), fetal bovine serum from JRH Biosciences (Lenexa, KS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) from Nacalai Tesque (Kyoto, Japan), LPS (serotype Escherichia coli O55: B5; phenol extracted) and dichlorofluorescein diacetate (DCFH-DA) from Sigma-Aldrich, St. Louis, MO, and diphenyl-1-pyrenylphosphine (DPPP) from Dojindo (Kumamoto, Japan). The natural isoforms of vitamin E were kindly supplied by Eisai Co. Ltd. (Tokyo, Japan). Other chemicals were of the highest quality commercially available.

Determination of vitamin E uptake into cultured cells

Intracellular vitamin E was detected using HPLC systems with electrochemical detection as described previously [27]. Cell samples in PBS were mixed with chloroform/methanol (2:1, v/v), and then cellular vitamin E in the lower chloroform layer was analyzed with an HPLC using a post-column amperometric electrochemical detector (NANOSPACE SI-I, Shiseido, Japan) set at 800 mV combined with an octadecyl-bound silica column (LC18, 5 μm, 250 x 4.6 mm²; Sigma-Aldrich, Japan, Co.); methanol-tert-butyl alcohol (95:5, v/v) containing 50 mM sodium perchlorate was used as an eluent at a rate of 0.7 ml/min.

Measurement of intracellular oxidative stress

Intracellular ROS were detected using 2,7'-dichlorofluorescein diacetate (DCFH-DA; Sigma-Aldrich, St. Louis, MO). DCFH-DA was dissolved in dimethyl sulfoxide (DMSO) at 5 mM as a stock solution and stored at −20 °C. When used in an experiment, it was diluted 500 times with serum-free medium. After exposure of the cells to LPS for 6 h, the medium was changed to serum-free DMEM containing 10 μM DCFH-DA and incubated for 30 min at 37 °C. Cells were then washed once with PBS, collected by
0.25% trypsinization, washed once with PBS, and resuspended in 500 μl of PBS. Cell samples in PBS were excited with a 488-nm argon ion laser in a Cytomics FC500 flow cytometry system (Beckman Coulter, Inc., Brea, CA), and the DCF emission was measured at 525 nm. Data were collected from at least 5000 gated events.

Intracellular lipid hydroperoxides were measured using DPPP [32]. DPPP was dissolved in DMSO at 5 mM as a stock solution and stored at −20 °C. When used in an experiment, it was diluted 100-times with serum-free medium. After exposure of the cells to LPS for 6 h, the medium was changed to serum-free DMEM containing 50 μM DPPP and incubated for 30 min at 37 °C. Cells were then washed once with PBS, harvested by trypsinization, washed once with PBS, and re-suspended in 3 ml of PBS. Cell samples in PBS were excited with a 351-nm argon ion laser and the fluorescence emission of DPPP oxide was measured at 380 nm using an FR-5300PC spectrophotometer (Shimadzu Corporation, Kyoto, Japan). After measurement, the cells were collected and measured for protein concentration. DPPP oxide level was adjusted by cellular protein concentration.

Protein extraction and Western blot analysis

To obtain total cell extracts, treated cells were collected, washed with ice-cold PBS, and resuspended in lysis buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 50 mM NaF, 5 mM ethylenediamine tetraacetic acid (EDTA), 0.5% Triton X-100, and 1 mM Na3VO4 with a protease inhibitor cocktail tablet (Roche Diagnostics, Penzberg, Germany) at 4 °C for 30 min. Nuclei and unlysed cellular debris were removed by centrifugation at 15,000g for 5 min. Equal amounts of protein (from 10 to 50 μg) were loaded and separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). In all cases, the detection of specific proteins in the total cell extracts was carried out by Western blot analysis, following the previously described procedure [27].

Real time polymerase chain reaction

The expression of target genes was determined using Real Time polymerase chain reaction (PCR) as described previously [33]. Total RNA was isolated from cells using the RNeasy mini kit (Qiagen GmbH, Hilden, Germany). cDNA synthesis was carried out with a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA). Real Time-PCR system was conducted using a Step One Real Time-PCR system (Applied Biosystems), and PCR amplification was detected with power SYBR Green PCR master mix (Applied Biosystems). Human ribosomal protein L32 (Ribol32) was used as an endogenous control. The primers for amplification were: Ribol32, 5′-GTA ACT GGC GGA AAC CCA-3′.

**Fig. 1.** Production of reactive oxygen species (ROS) as measured by DCFH induced by LPS added into the A549 cell culture medium. (A) Fluorescence (FL) intensity from DCFH as described in Materials and methods. Closed square: 0.6 mg/ml LPS; open circle: 0.75 mg/ml LPS. (B) Effects of vitamin E isoforms preincubated for 24 h on the intracellular ROS levels.

### A

<table>
<thead>
<tr>
<th>α-Toc (μM)</th>
<th>α-Toc3 (μM)</th>
<th>γ-Toc (μM)</th>
<th>γ-Toc3 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>1</td>
<td>5</td>
<td>0.25</td>
</tr>
<tr>
<td>1</td>
<td>0.75</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

**Relative FL intensity**

- **p < 0.001, **p < 0.005, *p < 0.01, **p < 0.025, *p < 0.05 compared with controls without LPS.**

### B

**Relative ROS level**

- 0.6 mg/ml LPS × 6h

- **p < 0.001, **p < 0.005, *p < 0.01, **p < 0.025, *p < 0.05 compared with controls without LPS.**

**Fig. 1.** Production of reactive oxygen species (ROS) as measured by DCFH induced by LPS added into the A549 cell culture medium. (A) Fluorescence (FL) intensity from DCFH as described in Materials and methods. Closed square: 0.6 mg/ml LPS; open circle: 0.75 mg/ml LPS. (B) Effects of vitamin E isoforms preincubated for 24 h on the intracellular ROS levels. **p < 0.001, **p < 0.005, *p < 0.01, **p < 0.025, *p < 0.05 compared with controls without LPS.
(forward), 5'-AGA TCT GGC CCT TGA ATC TTC-3' (reverse); TNF-α, 5'-TGC TCC TCA CCC ACA CCA T-3' (forward), 5'-GGT GGT TGA CCT TGG TCT GGT A-3' (reverse); IL-8, 5'-AAA CCA CCG GAA GGA ACC A-3' (forward), 5'-CAC GGC CAG CTT GGA AGT-3' (reverse); HO-1, 5'-AGC TCC TGC AAC TCC TCA AAG A -3' (reverse). The results shown in Fig. 2 indicate that lipid hydroperoxides increased at the initial stage and then decreased, probably because of the reduction by peroxidases. αToc, γToc3, γToc, and γToc3 all suppressed the formation of lipid hydroperoxides significantly.

LPS-induced cell death was assessed by the three methods described in the Methods section. The cellular viability decreased with increasing LPS concentrations (Fig. 3). αToc, γToc3, γToc, and γToc3 suppressed LPS cytotoxicity, but the effects were not significant except for the apoptotic cell death. The difference between the effects of vitamin E isoforms was not significant. It has been known that tocotrienols are incorporated into cultured cells more rapidly than tocopherols [27,29,30], which affects their apparent capacity to prevent oxidative stress. The rates of uptake of αToc, γToc, αToc3, and γToc3 into A549 cells were measured. As observed previously for Jurkat cells [27,29] and primary cortical neuron cells [30], αToc3 and γToc3 were incorporated into A549 cells at a much faster rate than αToc and γToc (Fig. 4).

LPS treatment increased TNF-α about 10-fold, which was reduced to about 50% by αToc, γToc, αToc3, and γToc3, but the difference in the effect between the four isoforms was small (Fig. 5A). IL-8 was increased about eight times and αToc, γToc, αToc3, and γToc3 reduced the level similarly (Fig. 5B). Heme oxygenase-1 (HO-1) also was increased about 1.5 times, but the effect of vitamin E was small (Fig. 5C). Pulmonary surfactant protein D (SP-D), a member of the collectin protein family which

Statistical analysis

All results were expressed as mean ± SD of at least three independent experiments. The statistical significance of difference between determinations was calculated by Student’s t-test and an analysis of variance (ANOVA) using Dunnett test for multiple comparisons.

Results

Firstly, the LPS-induced oxidative stress and cytotoxicity against A549 cells and the preventive effects of vitamin E were studied. The addition of LPS into the cell culture medium increased intracellular ROS and lipid hydroperoxides six times and twofold as measured by DCFH and DPPP assay respectively (Figs. 1 and 2). The ROS level increased and plateaued at 4 h after addition of LPS. αToc, αToc3, γToc, and γToc3 all reduced the level of ROS significantly. DPPP is a useful probe for detection of lipid hydroperoxides, but not for hydrogen peroxide [32,34]. The rates of uptake of αToc, γToc, αToc3, and γToc3 into A549 cells were measured. As observed previously for Jurkat cells [27,29] and primary cortical neuron cells [30], αToc3 and γToc3 were incorporated into A549 cells at a much faster rate than αToc and γToc (Fig. 4).

LPS treatment increased TNF-α about 10-fold, which was reduced to about 50% by αToc, γToc, αToc3, and γToc3, but the difference in the effect between the four isoforms was small (Fig. 5A). IL-8 was increased about eight times and αToc, γToc, αToc3, and γToc3 reduced the level similarly (Fig. 5B). Heme oxygenase-1 (HO-1) also was increased about 1.5 times, but the effect of vitamin E was small (Fig. 5C). Pulmonary surfactant protein D (SP-D), a member of the collectin protein family which

Fig. 2. (A) Production of lipid hydroperoxides measured with DPPP induced by LPS in A549 cells as described in Materials and methods. Closed square: 0.6 mg/ml LPS; open circle: 0.75 mg/ml LPS. (B) Effects of vitamin E isoforms preincubated for 24 h. ***p < 0.001, **p < 0.005, *p < 0.01, #p < 0.025, *p < 0.05 compared with controls without LPS.
modulates LPS-induced inflammatory cell responses [35], was increased also, but the effect of αToc, γToc, αToc3, and γToc3 was small (Fig. 5D).

Collectively, the above results show that αToc, γToc, αToc3, and γToc3 suppressed the oxidative stress induced by LPS, but the difference in the capacity between the four vitamin E isoforms was not significant. Importantly, αToc and γToc were incorporated into cells much slower than αToc3 and γToc3 yet exerted similar preventive effects at much lower concentrations.

Discussion

LPS plays a pivotal role in the pathogenesis of various age-associated diseases by accelerating the production of ROS/RNS and inflammation-related mediators [1,5,6,36]. In the present study, the accelerated production of ROS/RNS was confirmed from an increase in the fluorescence from DCFH in the LPS-treated A549 cells. DCFH has been used frequently for detection of ROS/RNS. As discussed in recent articles [37,38], care should be taken in the detection, characterization and quantification of ROS/RNS with DCFH, but the results shown in Figs. 1 and 2 suggest that LPS induced the production of active species that could be suppressed by vitamin E and initiate lipid peroxidation. Further, an increase in lipid hydroperoxides was observed as assessed with DPPP, which is a specific probe for lipid hydroperoxides [32,34].

As described above, the protective effects of vitamin E against LPS-induced oxidative stress have been observed in many previous studies, although the relative efficacy of different vitamin E isoforms varied. It was reported that peripherally administered αToc and γToc were equally effective at suppressing the production of F(2)-isoprostanes and F(4)-neuroprostanes, oxidation products from arachidonic and docosahexaenoic acid.

**Fig. 3.** Cytotoxicity of LPS on A549 cells and preventive effects of vitamin E isoforms as measured by (A), (D) MTT, (B), (E) LDH, and (C), (F) Annexin V-FITC and propidium iodide assay as described in Materials and methods. **p < 0.001, ***p < 0.005, **p < 0.01, ***p < 0.025, *p < 0.05 compared with controls without LPS.
respectively, caused by kainite, while αToc, but not γToc, was effective at suppressing these lipid peroxidation products induced by the LPS-induced innate immune response [39]. In other studies, γToc was found to be more potent than αToc [18–20]. Further, it was also reported that tocotrienols were superior to tocopherols [11,40].

In the present study, αToc, αToc3, γToc, and γToc3 all suppressed the cell death, production of ROS, lipid peroxidation, and expression of TNF-α and IL-8 induced by treatment of LPS to A549 cells, although the difference in relative potency between the four isoforms expressed solely as the added concentration to the media was not significantly different used. As shown above, one of the important and yet often overlooked issue is the marked difference in the rate of incorporation of vitamin E isoforms into cultured cells. Tocotrienols are incorporated into cultured cells much faster than tocopherols, probably due to the shorter side chain length. It may be noted that the rate of incorporation of chromanol into erythrocytes decreases significantly with increasing side chain length [41] and that αToc and αToc3-fortified cells showed similar cellular distribution [29]. The results described above show that αToc and γToc exerted similar preventive effects as αToc3 and γToc3 at much lower intracellular concentrations than those of αToc3 and γToc3.

It should be noted that vitamin E may function as signaling mediator as well as radical-scavenging antioxidant [42,43]. It has been proposed that vitamin E is capable of modulating signal transduction, gene expression, and activity of several enzymes by non-antioxidant function [43,44]. The present study shows that vitamin E is capable of suppressing LPS-induced oxidative stress but does not show explicitly whether non-antioxidant functions may also play a role.

In humans, αToc exhibits the highest affinity toward α-tocopherol transfer protein [45], and, furthermore, tocotrienols are metabolized faster than tocopherols, which make αToc the most abundant vitamin E isoform in humans [28]. The chemical reactivity toward peroxyl radicals of tocopherols and the corresponding tocotrienols are similar and α-forms exhibit higher reactivity than other forms [46].

Taken together we conclude that αToc exerts the most potent protective effect against LPS toxicity despite its slower rate of uptake by cells.

Fig. 4. Uptake of vitamin E into A549 cultured cells. Different concentrations of tocopherols and tocotrienols were treated with 2 × 10^5 cells/ml A549 cells for 24 h and their intracellular concentrations were measured as described in the Methods section. N.D.: not detected.

Fig. 5. Effects of pre-treatment of vitamin E homologs on cytokine and antioxidant enzyme expression induced by 0.6 mg/ml LPS. Cells pre-treated with vitamin E homologs were harvested for total RNA isolation and real-time PCR analysis. The expression levels of TNF-α, IL-8, HO-1 and SP-D were normalized to that of Ribo-L32 mRNA and are shown relative to those of the control under static conditions. ***p < 0.001, **p < 0.005, *p < 0.01, #p < 0.025, *p < 0.05 in comparison with the LPS-treated cells (Turkey, ANOVA).
Conflict of interest

The authors report no conflicts of interest.