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## Research Paper

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

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## ABSTRACT

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  $\alpha$ - and  $\gamma$ -tocopherols and  $\alpha$ - and  $\gamma$ -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  $\alpha$ -tocopherol may exhibit the highest protective capacity among the vitamin E isoforms against LPS-induced oxidative stress.

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## 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  $\alpha$  (TNF- $\alpha$ ) and interleukin (IL)-6, inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX2), and nuclear factor-kappaB (NF- $\kappa$ B) [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 also

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 (PGE<sub>2</sub>) [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:  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ -tocopherols and  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ -tocotrienols. Tocopherols contain a saturated phytyl side chain, while tocotrienols contain three double bonds in the side chain, and  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$ -forms differ in the number and position of methyl groups on the chromanol ring [12]. It was reported that  $\alpha$ -tocopherol ( $\alpha$ Toc) effectively prevented interferon-gamma/LPS-induced dopaminergic neuron degeneration [13] and that  $\alpha$ Toc 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  $\alpha$ Toc attenuated lung inflammation [15]. Furthermore,  $\alpha$ Toc 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  $\alpha$ Toc [17].

**Abbreviations:** DCFH, Dichlorofluorescein; DPPPP, Diphenyl-1-pyrenylphosphine; LPS, Lipopolysaccharide; MTT, 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide; NF- $\kappa$ B, Nuclear factor-kappaB; ROS, Reactive oxygen species; SP-D, Pulmonary surfactant protein D; Toc, Tocopherol; Toc3, Tocotrienol; TNF- $\alpha$ , Tumor necrosis factor  $\alpha$ .

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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  $\gamma$ Toc inhibited proinflammatory PGE<sub>2</sub> and leukotriene B<sub>4</sub> (LTB<sub>4</sub>) production, decreased TNF- $\alpha$ , and attenuated inflammation-mediated damage more effectively than  $\alpha$ Toc [18,19]. Further, a combination of aspirin and  $\gamma$ Toc, but not a combination of aspirin and  $\alpha$ Toc, 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  $\alpha$ Toc or  $\gamma$ Toc 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  $\alpha$ Toc and  $\gamma$ Toc decreased this response and also hepatic malondialdehyde (MDA) and TNF- $\alpha$  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 monocytic THP-1 cells from LPS-induced cell death [24].  $\alpha$ ,  $\gamma$ , or  $\delta$ Toc3 inhibited TNF- $\alpha$  secretion in LPS-stimulated RAW 264.7 cells and reduced serum levels of TNF- $\alpha$  after LPS treatment in BALB/c mice [11]. It was reported that low concentrations of  $\delta$ Toc3 (< 20  $\mu$ M) blocked LPS-induced gene expression of TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and iNOS, while higher concentrations (40  $\mu$ M) increased gene expression of the latter in peritoneal macrophages as compared to control group [11]. Further,  $\alpha$ Toc3,  $\gamma$ Toc3 and  $\delta$ Toc3 suppressed LPS-induced NO production in microglia,  $\delta$ Toc3 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. This also suggests that the capacity of antioxidants should be assessed based upon the actual intracellular concentrations rather than those added into the cell culture medium. In most previous studies, intracellular concentrations of antioxidants added into the culture medium were not measured. The present study was performed aiming at elucidating the preventive effects of four kinds of vitamin E isoforms,  $\alpha$ Toc,  $\gamma$ Toc,  $\alpha$ Toc3, and  $\gamma$ Toc3, against the oxidative stress and cytotoxicity induced by LPS using human lung carcinoma A549 cultured cells. The intracellular concentrations of four vitamin E isoforms were measured and considered in assessment of their preventive effects.

## 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.

### Cell culture and determination of cell viability

Human lung carcinoma A549 cells, used as a model of lung tissue cells considering the effect of LPS in ARDS, were purchased from the Riken BioResource Center (Tsukuba, Japan). Cells were routinely cultured in DMEM containing 10% heat-inactivated fetal bovine serum (FBS; CELlect GOLD; MP Biomedicals Incorporated, Solon, OH), 100 units/mL of penicillin, 100  $\mu$ g/mL of streptomycin, and 250 ng/mL of amphotericin B (Nacalai Tesque Inc., Kyoto, Japan).

Cells were incubated in humidified atmosphere of 5% carbon dioxide and 95% air at 37 °C. In order to assess the effect of vitamin E isoforms, cells were treated with Toc and Toc3 at different concentrations for 24 h, and then LPS was added to the medium.

The cell viability was measured by three assays, lactate dehydrogenase (LDH) release assay, MTT assay, and Annexin V/PI double staining assay. LDH release assay was conducted along the protocol outlined by the manufacture for the Cytotoxic Detection Kit (Roche Diagnostics, Penzberg, Germany). Maximum LDH release was determined by the incubation of cells with 1% Triton X-100. Data are expressed as a percentage of maximum LDH release, after subtraction of background determined from serum medium alone [27].

To determine mitochondrial function, which is another indicator of cell viability, the MTT assay was conducted as described previously [27]. Briefly, cells were incubated with 0.5 mg/ml MTT solution in fresh medium at 37 °C for 2 h. Isopropyl alcohol containing 0.04 N HCl was added to the culture medium (3:2, v/v), and they were mixed by pipette until the formazan was completely dissolved. The optical density of formazan was measured at 570 nm using a Multiskan Ascent plate reader (Thermo Lab-systems, Helsinki, Finland).

The apoptotic cell death was measured from the extent of phosphatidylserine (PS) exposure [31], which was measured by the binding of annexin V-FITC according to the protocol outlined by the manufacture in the Apoptosis Detection-kit (Medical & Biological Laboratories). The treated cells were stained with annexin V-FITC and propidium iodide (PI), followed by analysis with a Cytomics FC500 Flow Cytometry System (Beckman Coulter, Inc., Miami, FL) with a 488 nm argon laser. Data were collected from 10,000 events.

### 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-1, Shiseido, Japan) set at 800 mV combined with an octadecyl-bounded silica column (LC18, 5  $\mu$ m, 250  $\times$  4.6 mm<sup>2</sup>, 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  $\mu$ M 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  $\mu$ l 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 DPPH [32]. DPPH was dissolved in DMSO at 5 mM as a stock solution and stored at  $-20^{\circ}\text{C}$ . 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  $\mu\text{M}$  DPPH and incubated for 30 min at  $37^{\circ}\text{C}$ . 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 DPPH 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. DPPH oxide level was adjusted by cellular protein concentration.

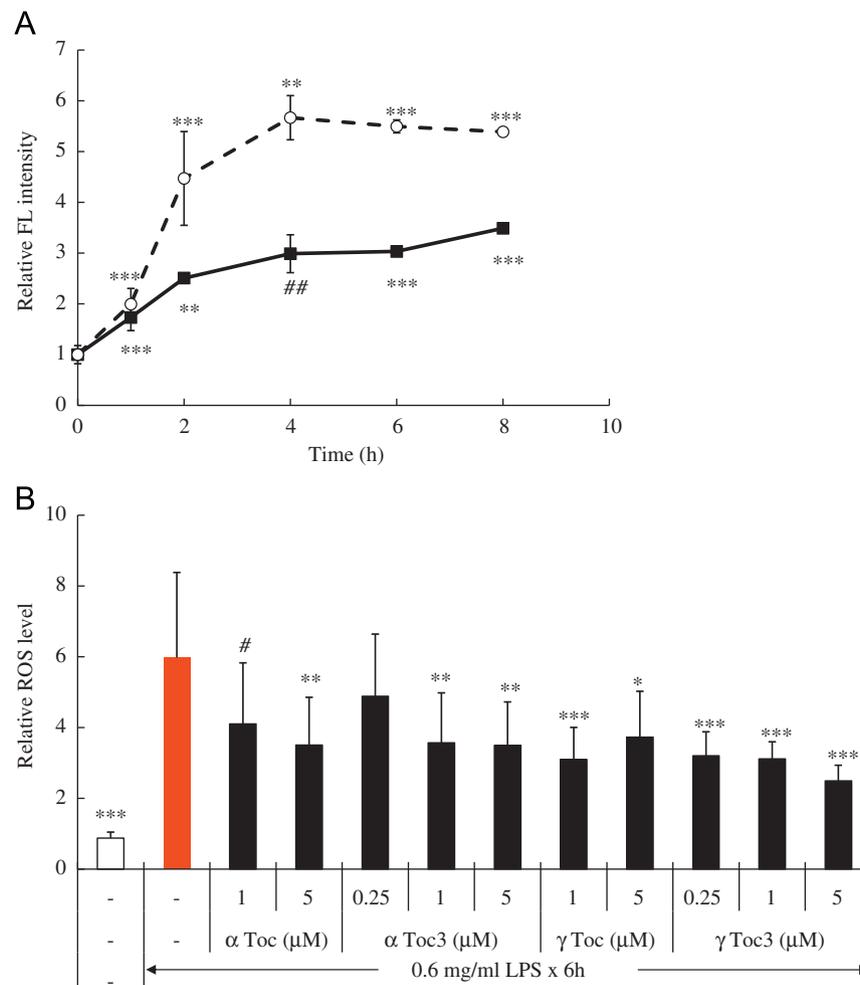
#### Protein extraction and Western blot analysis

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  $\text{Na}_2\text{VO}_4$  with a protease inhibitor cocktail tablet (Roche Diagnostics, Penzberg, Germany) at  $4^{\circ}\text{C}$  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  $\mu\text{g}$ ) 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. \*\*\* $p < 0.001$ , \*\* $p < 0.005$ , \* $p < 0.01$ , ## $p < 0.025$ , # $p < 0.05$  compared with controls without LPS.

(forward), 5'-AGA TCT GGC CCT TGA ATC TTC-3' (reverse); TNF- $\alpha$ , 5'-TGC TCC TCA CCC ACA CCA T-3' (forward), 5'-GGA 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'-GGG TGA TAG AAG AGG CCA AGA CT-3' (forward), 5'-AGC TCC TGC AAC TCC TCA AAG A -3' (reverse).

### Statistical analysis

All results were expressed as mean  $\pm$  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

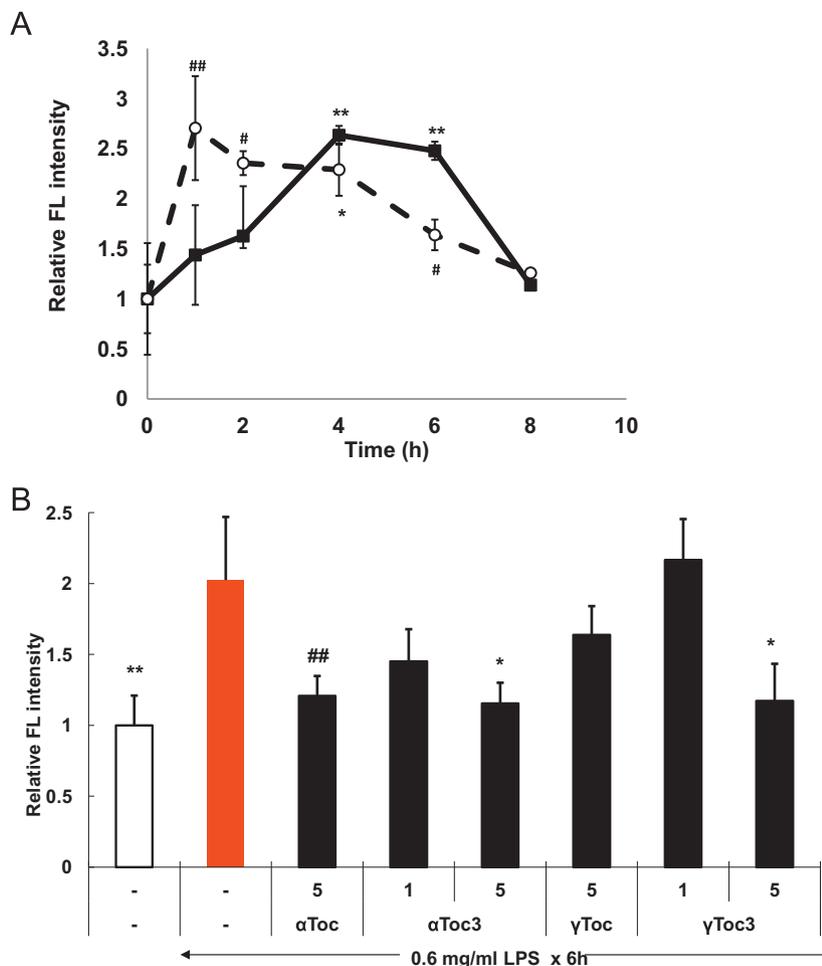
Firstly, the LPS-induced oxidative stress and cytotoxicity against A549 cells and the preventive effects of vitamin E were studied. The addition of LPS into the cell culture medium increased intracellular ROS and lipid hydroperoxides six times and twofold as measured by DCFH and DPPP assay respectively (Figs. 1 and 2). The ROS level increased and plateaued at 4 h after addition of LPS.  $\alpha$ Toc,  $\alpha$ Toc3,  $\gamma$ Toc, and  $\gamma$ Toc3 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

results shown in Fig. 2 indicate that lipid hydroperoxides increased at the initial stage and then decreased, probably because of the reduction by peroxidases.  $\alpha$ Toc,  $\alpha$ Toc3,  $\gamma$ Toc, and  $\gamma$ Toc3 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).  $\alpha$ Toc,  $\alpha$ Toc3,  $\gamma$ Toc, and  $\gamma$ Toc3 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  $\alpha$ Toc,  $\gamma$ Toc,  $\alpha$ Toc3, and  $\gamma$ Toc3 into A549 cells were measured. As observed previously for Jurkat cells [27,29] and primary cortical neuron cells [30],  $\alpha$ Toc3 and  $\gamma$ Toc3 were incorporated into A549 cells at a much faster rate than  $\alpha$ Toc and  $\gamma$ Toc (Fig. 4).

LPS treatment increased TNF- $\alpha$  about 10-fold, which was reduced to about 50% by  $\alpha$ Toc,  $\gamma$ Toc,  $\alpha$ Toc3, and  $\gamma$ Toc3, but the difference in the effect between the four isoforms was small (Fig. 5A). IL-8 was increased about eight times and  $\alpha$ Toc,  $\gamma$ Toc,  $\alpha$ Toc3, and  $\gamma$ Toc3 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  $\alpha$ Toc,  $\gamma$ Toc,  $\alpha$ Toc3, and  $\gamma$ Toc3 was small (Fig. 5D).

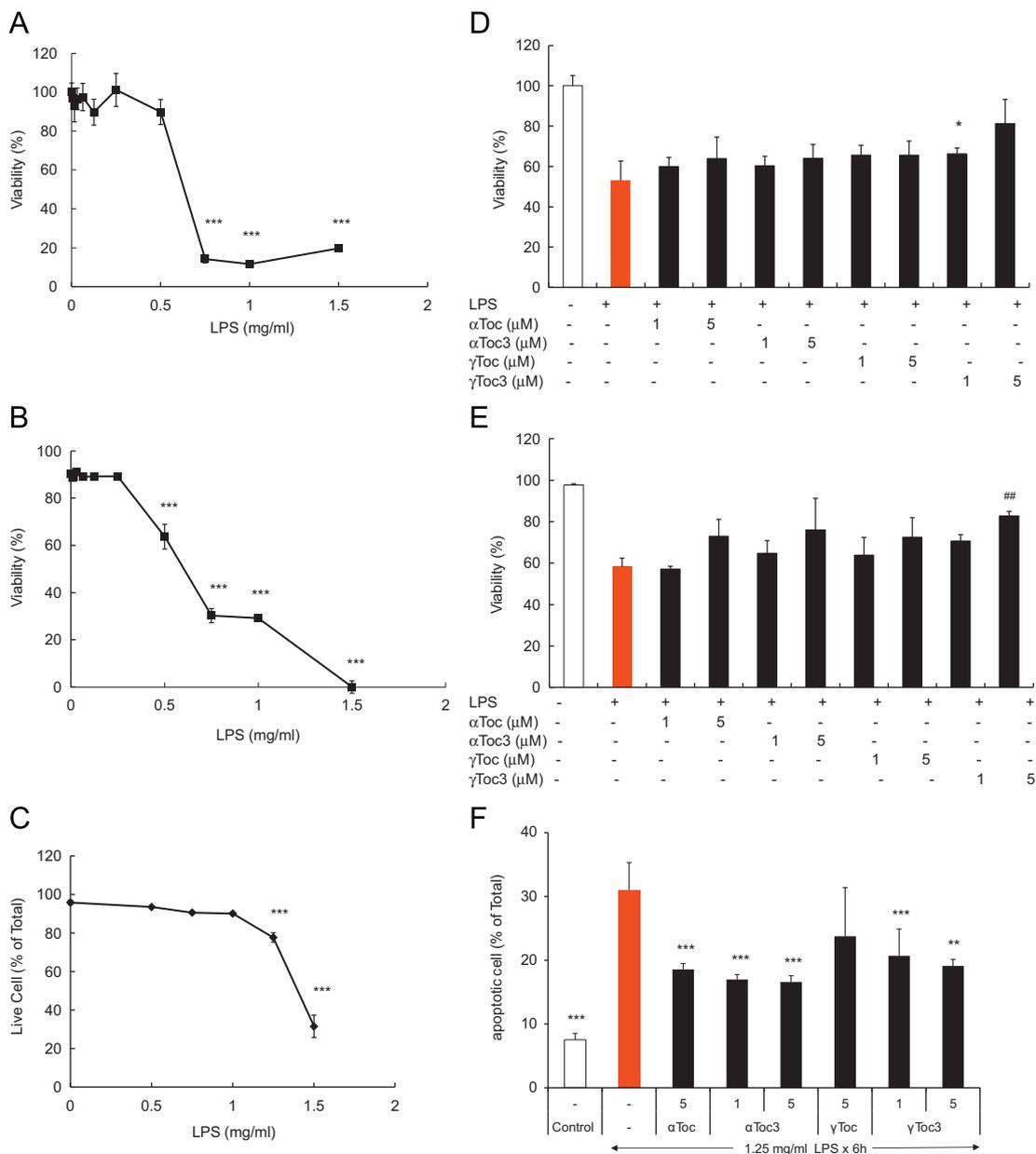
Collectively, the above results show that  $\alpha$ Toc,  $\gamma$ Toc,  $\alpha$ Toc3, and  $\gamma$ Toc3 suppressed the oxidative stress induced by LPS, but the difference in the capacity between the four vitamin E isoforms was not significant. Importantly,  $\alpha$ Toc and  $\gamma$ Toc were incorporated into cells much slower than  $\alpha$ Toc3 and  $\gamma$ Toc3 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].

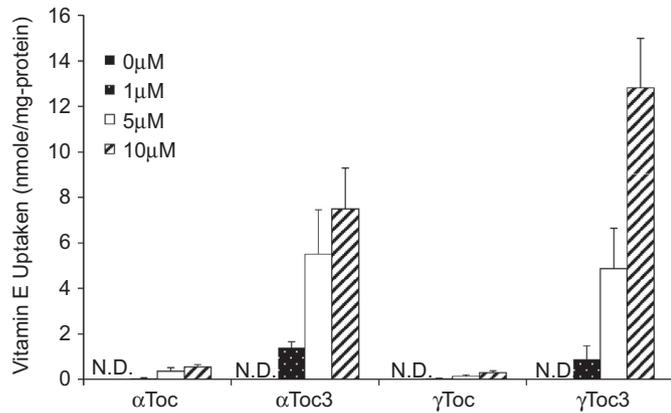
As described above, the protective effects of vitamin E against LPS-induced oxidative stress have been observed in many previous studies, although the relative efficacy of different vitamin E isoforms varied. It was reported that peripherally administered  $\alpha$ Toc and  $\gamma$ Toc were equally 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  $\alpha$ Toc, but not  $\gamma$ Toc, was effective at suppressing these lipid peroxidation products induced by the LPS-induced innate immune response [39]. In other studies,  $\gamma$ Toc was found to be more potent than  $\alpha$ Toc [18–20]. Further, it was also reported that tocotrienols were superior to tocopherols [11,40].

In the present study,  $\alpha$ Toc,  $\alpha$ Toc3,  $\gamma$ Toc, and  $\gamma$ Toc3 all suppressed the cell death, production of ROS, lipid peroxidation, and expression of TNF- $\alpha$  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



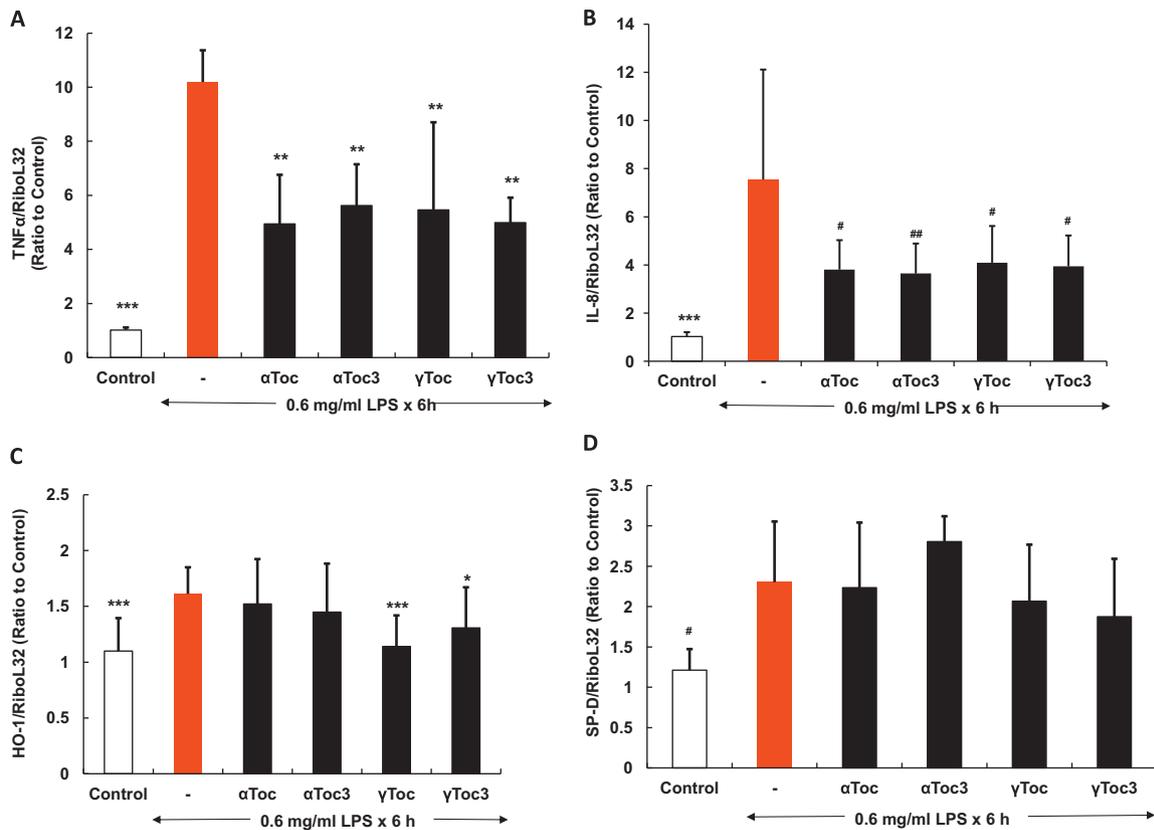
**Fig. 4.** Uptake of vitamin E into A549 cultured cells. Different concentrations of tocopherols and tocotrienols were treated with  $2 \times 10^5$  cells/ml A549 cells for 24 h and their intracellular concentrations were measured as described in the Methods section. N.D.: not detected.

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 chromanols into erythrocytes decreases significantly with increasing side chain length [41] and that  $\alpha$ Toc and  $\alpha$ Toc3-fortified cells showed similar cellular distribution [29]. The results described above show that  $\alpha$ Toc and  $\gamma$ Toc exerted similar preventive effects as  $\alpha$ Toc3 and  $\gamma$ Toc3 at much lower intracellular concentrations than those of  $\alpha$ Toc3 and  $\gamma$ Toc3.

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

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



**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- $\alpha$ , IL-8, HO-1 and SP-D were normalized to that of Ribol.32 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).

## Contribution of authors

KN has carried out most of the experiments. All the authors were involved in the design of the study and interpretation of data. KN and EN edited the manuscript. All the authors have read and approved the final version.

## Conflict of interest

The authors report no conflicts of interest.

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