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Original Article

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α-Tocopherol and γ-tocopherol decrease inflammatory response and insulin resistance during the interaction of adipocytes and macrophages

Sella Lee and Hye-Kyeong Kim §

Department of Food Science and Nutrition, The Catholic University of Korea, Bucheon 14662, Korea

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§Corresponding Author:
Hye-Kyeong Kim
Department of Food Science and Nutrition, The Catholic University of Korea, 43 Jibong-ro, Wonmi-gu, Bucheon 14662, Korea.
Tel. +82-2-2164-4314
Fax. +82-2-2164-4310
Email. hkyeong@catholic.ac.kr

ORCID iDs
Sella Lee
https://orcid.org/0009-0009-9555-7365
Hye-Kyeong Kim
https://orcid.org/0000-0003-1659-1709

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ABSTRACT

BACKGROUND/OBJECTIVES: The infiltration of macrophages into adipose tissue mediates chronic inflammation that is associated with insulin resistance in obesity. Although vitamin E is beneficial against insulin resistance, its impact on adipose tissue inflammation has not been elucidated. This study aims to investigate the effects of α-tocopherol and γ-tocopherol, major vitamin E isoforms, on the interaction between macrophages and adipocytes with regard to obesity-induced inflammation and insulin resistance.
MATERIALS/METHODS: Hypertrophied 3T3-L1 adipocytes were cocultured with RAW 264.7 macrophages and treated with α-tocopherol or γ-tocopherol at 12.5, 25, and 50 μM. The inflammatory cytokines (monocyte chemoattractant protein-1, tumor necrosis factor-α, and interleukin-6) and free fatty acid (FFA) release were measured by assay kits, and nuclear factor-kappaB (NF-κB) and c-Jun NH_2 terminal kinase (JNK) signals were evaluated by immunoblotting. Glucose uptake was measured with a fluorescent glucose derivative.
RESULTS: Treatment with α-tocopherol and γ-tocopherol restrained the coculture-induced increase in cytokines and FFA release. γ-Tocopherol exhibited greater suppression of inflammatory cytokines at 12.5 and 25 μM ($P < 0.001$). Both tocopherols inhibited NF-κB activation by limiting translocation of NF-κB (p65) to the nucleus, with γ-tocopherol showing a stronger effect compared to α-tocopherol. α-Tocopherol inhibited JNK phosphorylation at 50 μM, whereas γ-tocopherol did not. Furthermore, coculture with macrophages impaired glucose uptake in response to insulin, but both tocopherols restored insulin responsiveness ($P < 0.01$).
CONCLUSION: α-Tocopherol and γ-tocopherol effectively mitigate inflammation induced by adipocyte-macrophage interaction, thereby ameliorating coculture-induced insulin resistance. These findings suggest the therapeutic potential of tocopherols in managing obesity-related metabolic dysfunction.

Keywords: alpha-Tocopherol; gamma-tocopherol; inflammation mediators; insulin resistance; adipocyte

INTRODUCTION

Numerous studies have clarified that obesity leads to low-grade chronic inflammation, which is considered a potential factor linking obesity to insulin resistance [1,2]. Insulin resistance,

the defective response to insulin stimulation in the liver, muscle, and adipose tissue, results in hyperglycemia due to impaired insulin-mediated glucose uptake and leads to obesity-associated diseases, such as type 2 diabetes mellitus (T2DM), hypertension, and cardiovascular diseases [1,3]. In most cases, adipose tissue triggers obesity-related inflammation by releasing cytokines and free fatty acids (FFA) that affect the liver and muscle [4].

Adipose tissue comprises various cell types, with adipocytes and macrophages being key mediators of adipose tissue inflammation, insulin resistance, and metabolic dysfunction associated with obesity [5,6]. Hypertrophic adipocytes increase the release of non-esterified fatty acids (NEFA) through lipolysis and produce pro-inflammatory cytokines [7]. These obesity-induced alterations activate nuclear factor-kappaB (NF-κB) and c-Jun NH_2 terminal kinase (JNK) pathways, leading to heightened synthesis of inflammatory cytokines that promote the infiltration of pro-inflammatory macrophages and inhibit insulin signaling [2]. The infiltrated macrophages serve as the primary source of pro-inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6, and IL-1β, which can induce insulin resistance [8,9]. Consequently, inflammatory factors derived from macrophages, such as TNF-α, stimulate additional NEFA release from adipocytes [10]. This paracrine interaction between adipocytes and macrophages contributes to inflammatory changes and exacerbate insulin resistance in obese adipose tissue [11].

Epidemiological studies have shown a positive association between plasma vitamin E levels and insulin sensitivity, suggesting a preventive role in T2DM [12,13]. The major isoforms of vitamin E in the blood and tissues are α-tocopherol and γ-tocopherol. Supplementation with α-tocopherol has been found to increase insulin secretion in T2DM rats and improve insulin sensitivity in high-fat diet-fed obese mice, which is attributed to its antioxidative properties [14,15]. In contrast, γ-tocopherol has received less attention due to its lower antioxidant capacity despite its relatively higher dietary intake compared to α-tocopherol [16]. Nevertheless, the unique biological activities of γ-tocopherol support its potential efficacy against inflammation-associated diseases [17]. γ-Tocopherol was shown to decrease prostaglandin E_2 synthesis by inhibiting cyclooxygenase (COX) activity in macrophages and inhibit leukotriene synthesis by blocking 5-lipoxygenase translocation in neutrophils, whereas α-tocopherol had minimal or no effects [18,19]. Additionally, it has been effective in reducing inflammation-mediated damage, along with the pro-inflammatory eicosanoids, in a rat model of inflammation [20]. However, the specific impact of tocopherols on the interaction in adipose tissue to alleviate obesity-induced inflammation and insulin resistance remains unclear.

The objective of this study is to examine whether α-tocopherol and γ-tocopherol could exert an antidiabetic effect by interrupting the interaction between adipocytes and macrophages within obese adipose tissue. We investigated the direct effects of these tocopherols on inflammatory mediators, the release of FFA, and insulin-stimulated glucose uptake through a coculture of hypertrophied 3T3-L1 adipocytes and RAW 264.7 macrophages to model obese adipose tissue.

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MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), bovine calf serum (BCS), penicillin-streptomycin (PS), and 2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl) amino]-D-glucose (2-NBDG) were purchased from Invitrogen (Carlsbad, CA, USA). The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay kit was obtained from Promega (Madison, WI, USA). The enzyme-linked immunosorbent assay (ELISA) kits for TNF-α, monocyte chemoattractant protein-1 (MCP-1), and IL-6 were procured from BD Biosciences (San Diego, CA, USA). Nuclear and cytoplasmic extraction reagents and the assay kit for NEFA were provided by Thermo Fisher Scientific (Rockford, IL, USA) and FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan), respectively. Antibodies specific for phospho-JNK, total-JNK, and nuclear NF-κB p65 were purchased from Cell Signaling Technology (Beverly, MA, USA). The antibody for lamin B was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and antibodies for α-tubulin and glyceraldehyde 3-phosphate dehydrogenase were sourced from Ab Frontier (Seoul, Korea). α-Tocopherol and γ-tocopherol were purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO). DMSO, isobutylmethylxanthine, dexamethasone, insulin, and Bradford reagent for protein quantitation were also procured from Sigma-Aldrich.

Cell culture

The 3T3-L1 mouse embryo fibroblasts and RAW 264.7 macrophage cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and Korean Cell Line Bank (KCLB, Seoul, Korea), respectively. The cell culture followed a protocol previously described [21]. Briefly, preadipocytes were cultured in DMEM with 10% BCS until 2 days post-confluence and then differentiated with an induction medium containing 10% FBS, 0.5 μM isobutylmethylxanthine, 1 μM dexamethasone, and 167 nM insulin in DMEM (D0). After 2 days (D2), the medium was replaced with 10% FBS/DMEM containing 167 nM insulin. The cells were maintained in this medium for another 2 days and then cultured in 10% FBS/ DMEM thereafter, resulting in mature adipocytes with noticeable lipid droplets by day 6 (D6). For experiments, adipocytes with sustained lipid accumulation between day 14 (D14) and day 20 (D20) were used as hypertrophied 3T3-L1 adipocytes. Adipocytes and macrophages were cocultured in a contact system. RAW 264.7 macrophages were cultured with 10% FBS/ DMEM and plated onto serum-starved hypertrophied 3T3-L1 cells at a density of 1×10^6 cells/mL. The coculture was incubated in serum-free DMEM for 24 h. As a control culture, 3T3-L1 cells and RAW 264.7 macrophages were separately cultured under identical conditions and mixed 1 h before assays. The cocultured cells were treated with specified concentrations of tocopherol (either α-tocopherol or γ-tocopherol) or 0.1% DMSO as a control. All medium contained 1% v/v PS and cells were maintained at 37°C in humidified 5% CO_2 atmosphere.

Cell viability assay

The MTS assay was conducted to determine non-toxic concentrations of tocopherols. Tocopherol concentrations (12.5, 25, and 50 μM) were chosen based on plasma concentrations and previous research on cell lines [18,22]. The 3T3-L1 cells were seeded in a 96-well plate, cultured until they became hypertrophied mature adipocytes on D14, and subsequently exposed to media containing either α-tocopherol or γ-tocopherol. RAW 264.7 macrophages were introduced into a 96-well plate at a density of 2.5×10^5 cells per well, and each tocopherol was added after 24 h of culture. After incubating for 24 h, the viability of both

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cells was assessed by the MTS assay. The formazan concentration was measured at 490 nm with a microplate reader (ELx808; BioTek, Winooski, VT, USA), which is directly related to the number of viable cells.

Measurement of inflammatory cytokine production

Hypertrophied 3T3-L1 cells and RAW 264.7 macrophages were cocultured for 24 h and then treated with either α-tocopherol or γ-tocopherol (12.5, 25, and 50 μM). To capture sustained overall cytokine production, the cell culture supernatants were collected after 12 h of incubation, following previous work [23], and the concentrations of MCP-1, TNF-α, and IL-6 were measured using ELISA kits according to the manufacturer's instructions.

Lipolysis assay

Lipolysis was measured by the NEFA released into the culture medium from adipocytes [10]. Hypertrophied 3T3-L1 cells were cocultured with RAW 264.7 macrophages for 24 h. After washing with phosphate-buffered saline (PBS), cells were incubated in Krebs–Ringer bicarbonate buffer (119 mM NaCl, 4.8 mM KCl, 1.28 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $7H_2O{\cdot}MgSO_4$, 0.25 mM $NaHCO_3$, 5 mM glucose, and 4% bovine serum albumin; pH 7.4) containing either α-tocopherol or γ-tocopherol (12.5, 25, and 50 μM) for 12 h. The concentration of FFA in the culture medium was measured by an acyl-coenzyme A oxidase-based colorimetric assay kit, and the protein concentration of the pellets was measured for calibration.

Western blot analysis

After coculture of hypertrophied 3T3-L1 cells and RAW 264.7 macrophages for 24 h, the cells were treated with the indicated concentrations of either α-tocopherol or γ-tocopherol for 12 h. For the assessment of JNK and NF-κB activation, lipopolysaccharide (LPS, 0.1 μg/mL) was added 30 min before the end of treatment, based on a previous study of time-course inflammatory response following LPS exposure [24]. To determine NF-κB translocation, cytoplasmic and nuclear fractions were separated using extraction reagents according to the manufacturer's instructions. The whole cells or cellular fractions were lysed in a cold RIPA buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, and 1 μg/mL leupeptin; pH 7.4) and kept on ice for 15 min. After centrifugation at 10,000 ×*g* at 4°C for 10 min, lysates with an equal amount of protein (20–30 μg) were separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). These membranes were blocked with 5% skim milk in PBS/0.1% Tween 20 for 1 h and then incubated overnight with the primary antibodies at 4°C. After PBS rinsing, the membrane was incubated with a secondary horseradish peroxidase-conjugated antibody for 1 h at room temperature. Immunoblots were visualized using an enhanced chemiluminescence system (Ab Frontier) and quantified with a FluorChem densitometer along with the ImageJ program (National Institutes of Health, Bethesda, MD, USA).

Glucose uptake assay

3T3-L1 cells were plated in 96-well fluorescence plates (Corning Inc., Corning, NY, USA) and differentiated into mature adipocytes. RAW 264.7 cells were then added to the hypertrophied adipocytes. After 24 h of coculture in serum-free DMEM, the cells were treated with either α-tocopherol or γ-tocopherol (12.5, 25, and 50 μM) for 6 h. The time point was chosen from a preliminary test. Following treatment, the cells were starved in glucose-free PBS for 1 h, and then 100 nM insulin was introduced along with 20 μM of the fluorescent glucose derivative,

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2-NBDG. After incubation for 2 h, the cells were washed with cold PBS to remove residual free 2-NBDG. The fluorescence retained within the cell monolayers was measured using a fluorescence microplate reader (Synergy Mx; BioTek) at an excitation wavelength of 465 nm and an emission wavelength of 540 nm.

Statistical analysis

Data were analyzed by 1-way analysis of variance, followed by Duncan's multiple range test using SAS software (version 9.4; SAS Institute, Inc., Cary, NC, USA). The results were presented as means ± SD, and all experiments were carried out at least 3 times. *P*-values of less than 0.05 were considered to be significant.

RESULTS

Effect of α-tocopherol and γ-tocopherol on cell viability

As shown in **Fig. 1**, both α-tocopherol and γ-tocopherol did not suppress the viability of hypertrophied mature adipocytes and RAW 264.7 macrophages at concentrations up to 50 μM. Therefore, we used the non-cytotoxic range of tocopherols in all subsequent experiments.

α-Tocopherol and γ-tocopherol reduce inflammatory cytokines production in the coculture of adipocytes and macrophages

Fig. 2 shows the effect of α-tocopherol and γ-tocopherol on inflammatory responses in the coculture of adipocytes and macrophages. The separately cultured adipocytes and macrophages exhibited very low secretion of pro-inflammatory cytokines, including TNF-α, MCP-1, and IL-6. Coculture of hypertrophied 3T3-L1 adipocytes and RAW 264.7 macrophages resulted in a remarkable surge in these levels. However, the coculture-induced increase was inhibited by both α-tocopherol and γ-tocopherol, even at the lowest concentration of 12.5 μM ($P < 0.001$). When comparing the effects of α-tocopherol and γ-tocopherol, the inhibitory effects of γ-tocopherol on TNF-α and MCP-1 were greater than those of α-tocopherol at 12.5 and 25 μM. The production of TNF-α was suppressed by 50 μM of both tocopherols compared to that of separated cultured cells, and γ-tocopherol had the same effect even at 25 μM. In

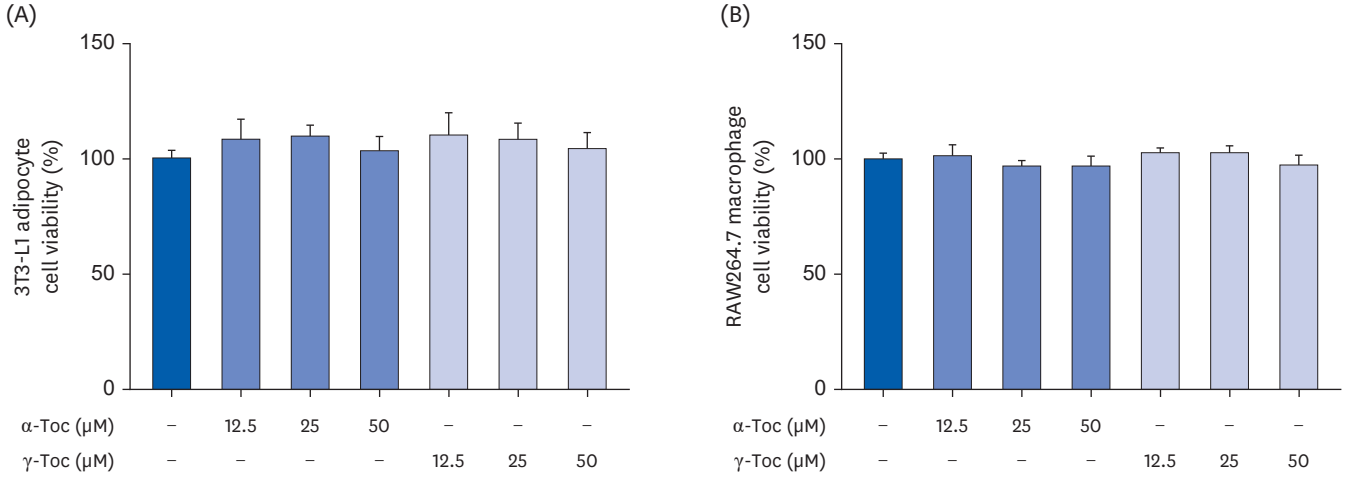

Fig. 1. Effect of α-Toc and γ-Toc on the viability of 3T3-L1 cells and RAW 264.7 macrophages. Cell viability of (A) 3T3-L1 adipocytes and (B) RAW 264.7 macrophages was measured using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay after 24 h exposure to either α-Toc or γ-Toc. The values are presented as the means ± SD (n = 6).
α-Toc, α-tocopherol; γ-Toc, γ-tocopherol.

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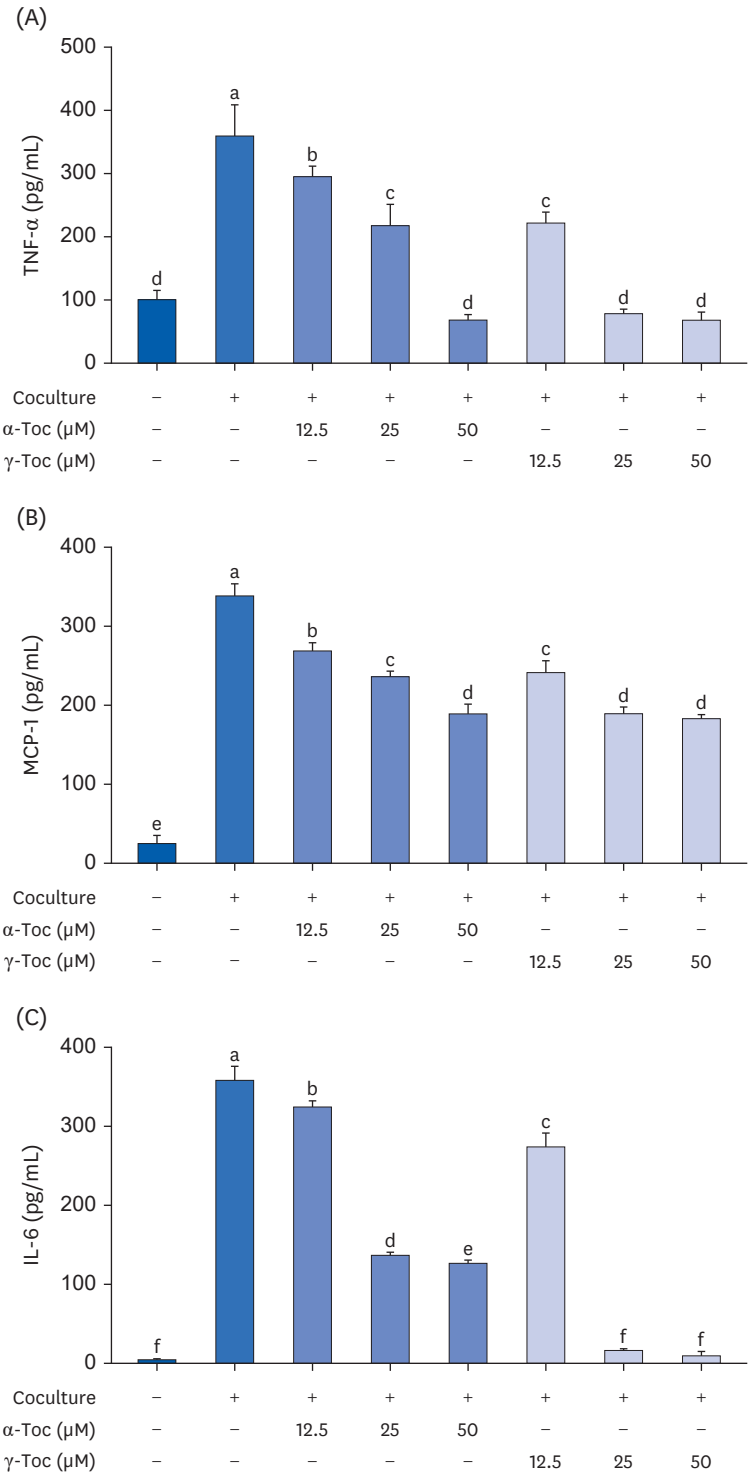

Fig. 2. Effect of α-Toc and γ-Toc on inflammatory responses in the coculture of adipocytes and macrophages. Hypertrophied 3T3-L1 adipocytes were cocultured with RAW 264.7 macrophages for 24 h in the contact system and treated with either α-Toc or γ-Toc for 12 h. The production of (A) TNF-α, (B) MCP-1, and (C) IL-6 was measured in the coculture medium using enzyme-linked immunosorbent assay. The data are presented as the means ± SD (n = 3) and are representative of results obtained from 3 independent experiments. The different superscripts indicate a significant difference by analysis of variance, followed by Duncan's test ($P < 0.001$).
TNF-α, tumor necrosis factor-alpha; Coculture +, adipocytes cocultured with macrophages; Coculture –, adipocytes and macrophages were separately cultured and mixed before the assay; α-Toc, α-tocopherol; γ-Toc, γ-tocopherol; MCP-1, monocyte chemoattractant protein-1; IL-6, interleukin-6.

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addition, γ-tocopherol was more effective than α-tocopherol in inhibiting IL-6 production at all concentrations, reducing coculture-induced IL-6 production by 95.6% and 97.4% at 25 and 50 μM, respectively. These results demonstrate the anti-inflammatory effects of tocopherols in the coculture-induced inflammatory response, with a greater effect of γ-tocopherol than α-tocopherol.

α-Tocopherol and γ-tocopherol suppress lipolysis in adipocytes cocultured with macrophages

To investigate the effect of tocopherols on FFA release, which is closely associated with insulin resistance, the levels of NEFA secreted into the culture medium were measured. As depicted in **Fig. 3**, NEFA release was enhanced by 50% in a coculture of hypertrophied 3T3-L1 adipocytes with RAW 264.7 macrophages compared to the control culture. However, this increase was significantly suppressed by tocopherol treatment ($P < 0.001$). The suppressive impact was comparable for both α-tocopherol and γ-tocopherol, with NEFA levels recovering to those of the control culture upon treatment with 25 and 50 μM of tocopherols.

Effect of α-tocopherol and γ-tocopherol on JNK activation and NF-κB signaling

To elucidate the mechanisms that modulate the inflammatory response induced by coculture with tocopherols, the effects of tocopherols on JNK phosphorylation and NF-κB signaling pathway were examined. As shown in **Fig. 4A**, the coculture of hypertrophied adipocytes with macrophages led to a modest increase in JNK phosphorylation. This phosphorylation was further stimulated approximately 2.4-fold with LPS treatment. However, treatment with 50 μM of α-tocopherol resulted in the suppression of the increase in JNK phosphorylation, whereas γ-tocopherol did not exhibit any effect across all concentrations ($P < 0.001$). The effect of tocopherols on NF-κB signaling is shown in **Fig. 4B**. NF-κB activation occurs through the degradation of the cytoplasmic inhibitor κB proteins (IκB)–NF-κB complex, releasing NF-κB subunits. This leads to the nuclear translocation of NF-κB and its subsequent binding to the promoter regions of target genes encoding pro-inflammatory mediators [25]. To assess NF-κB translocation from the cytosol to the nucleus, we measured the NF-κB p65 subunit

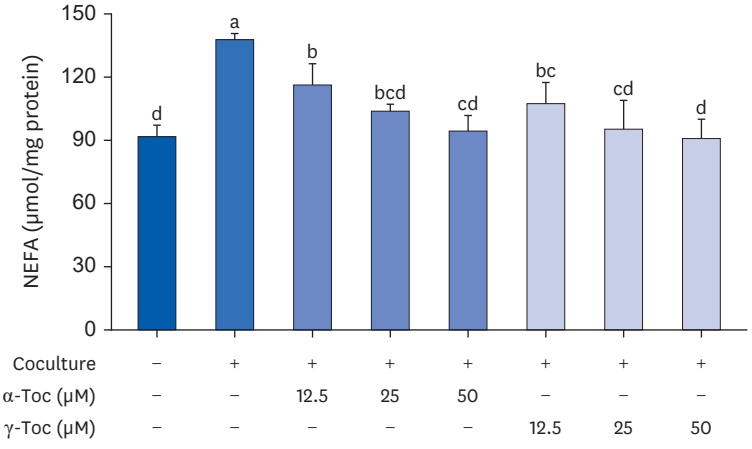

Fig. 3. Effect of α-Toc and γ-Toc on free fatty acid release in coculture of adipocytes and macrophages.
Hypertrophied 3T3-L1 adipocytes were cocultured with RAW 264.7 macrophages for 24 h in the contact system and treated with either α-Toc or γ-Toc for 12 h. The concentration of NEFA in the coculture medium was measured by a NEFA kit. The data are presented as means ± SD of 3 independent experiments. The different superscripts indicate a significant difference by analysis of variance, followed by Duncan's test ($P < 0.001$).
NEFA, non-esterified fatty acid; Coculture +, adipocytes cocultured with macrophages; Coculture –, adipocytes and macrophages were separately cultured and mixed before the assay; α-Toc, α-tocopherol; γ-Toc, γ-tocopherol.

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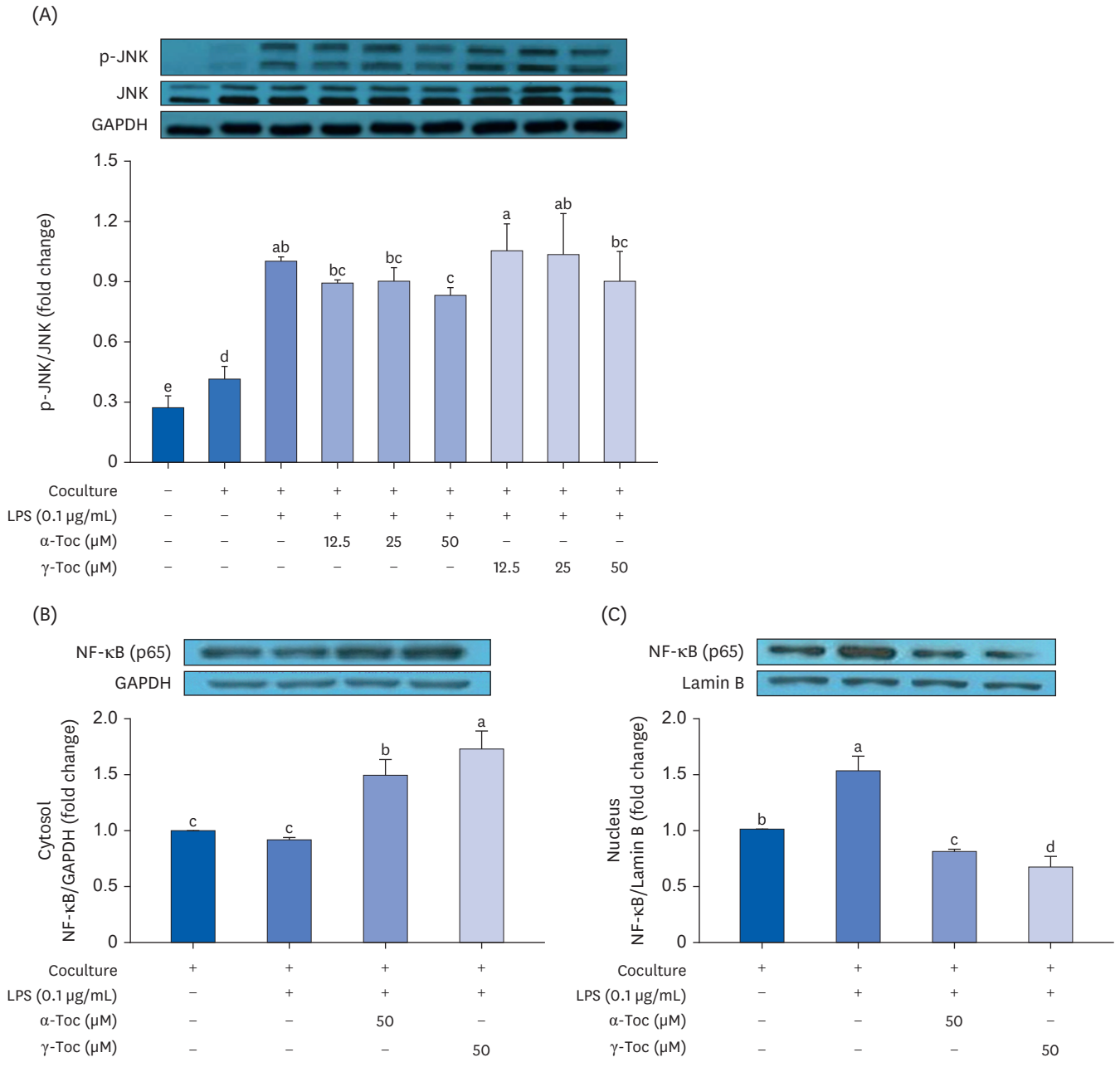

Fig. 4. Effect of α-Toc and γ-Toc on (A) JNK activation and (B) NF-κB signaling in the coculture of adipocytes and macrophages. Hypertrophied 3T3-L1 adipocytes were cocultured with RAW 264.7 macrophages for 24 h and treated with either α-Toc or γ-Toc for 12 h. After stimulating with LPS (0.1 μg/mL) for 30 min, cytosolic and nuclear levels of NF-κB p65 subunit were measured to assess the translocation of NF-κB from the cytoplasm to the nucleus for activation. The protein levels of JNK and phosphorylated JNK were measured by western blot analysis. The data are represented as means ± SD from 3 independent experiments. Means without the same letters are significantly different by analysis of variance, followed by Duncan's test ($P < 0.01$).
p-JNK, phospho-c-Jun NH_2 terminal kinase; JNK, c-Jun NH_2 terminal kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Coculture +, adipocytes cocultured with macrophages; Coculture –, adipocytes and macrophages were separately cultured and mixed before the assay; LPS, lipopolysaccharide; LPS (0.1 μg /mL) +, treated with LPS; LPS (0.1 μg/mL) –, not treated with LPS; α-Toc, α-tocopherol; γ-Toc, γ-tocopherol; NF-κB, nuclear factor-kappaB.

in cytosolic and nuclear fractions. LPS treatment to induce IκBα degradation increased the nuclear content of p65 by 1.5-fold. However, when treated with 50 μM of α-tocopherol or γ-tocopherol, both exhibiting apparent anti-inflammatory effects in **Fig. 2**, the nuclear translocation of the NF-κB p65 protein decreased by 47% and 57%, respectively, compared to the LPS-treated coculture control ($P < 0.001$).

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α-Tocopherol and γ-tocopherol stimulate glucose uptake in insulin-resistant adipocytes

The effect of tocopherols on glucose uptake in the coculture of adipocytes and macrophages is depicted in **Fig. 5**. In hypertrophied 3T3-L1 adipocytes without coculture, insulin increased the incorporation of 2-NBDG, a fluorescent glucose derivative. However, in cocultured cells, there was no significant change in glucose uptake in response to insulin, indicating the development of insulin resistance. Treatment with tocopherols effectively reversed the coculture-induced insulin resistance ($P < 0.01$). α-Tocopherol demonstrated a dose-dependent increase in 2-NBDG uptake, which increased by 37%, 54%, and 80% at 12.5, 25, and 50 μM of α-tocopherol, respectively, compared to the insulin-treated coculture control. Comparing the effects of α-tocopherol and γ-tocopherol, the stimulatory effect of γ-tocopherol on glucose uptake in cocultured cells was more pronounced than that of α-tocopherol at 12.5 μM but similar for both tocopherols at higher concentrations.

DISCUSSION

Interactions between adipocytes and adipose-infiltrating macrophages contribute to chronic inflammation in obese adipose tissue, which can induce systemic insulin resistance. In the present study, we investigated the effects of α-tocopherol and γ-tocopherol on inflammatory responses and adipocyte function during the coculture of 3T3-L1 adipocytes and RAW264.7 macrophages. The results showed that both tocopherols not only suppress inflammatory changes induced by coculture but also improve coculture-induced insulin resistance by increasing insulin-stimulated glucose uptake into adipocytes.

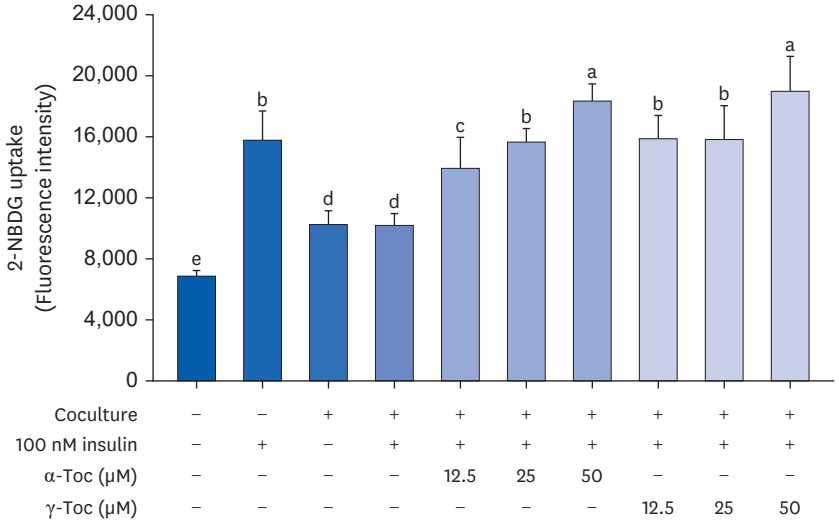

Fig. 5. Effect of α-Toc and γ-Toc on insulin-induced glucose uptake in the coculture of adipocytes and macrophages. Hypertrophied 3T3-L1 adipocytes were cocultured with RAW 264.7 macrophages for 24 h and treated with either α-Toc or γ-Toc for 6 h. Cells were incubated with insulin (100 nM) and a glucose derivative, 2-NBDG (20 μM), for 2 h before the end of incubation, and then the fluorescence was measured for glucose uptake. The data are represented as means ± SD (n = 6). The different superscripts indicate a significant difference by analysis of variance, followed by Duncan's test ($P < 0.01$).
2-NBDG, 2-deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl) amino]-D-glucose; Coculture +, adipocytes cocultured with macrophages; Coculture −, adipocytes not cocultured; 100 nM insulin +, treated with 100 nM insulin; 100 nM insulin −, not treated with insulin; α-Toc, α-tocopherol; γ-Toc, γ-tocopherol.

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In this study, α-tocopherol and γ-tocopherol suppressed the coculture-induced increase in NEFA release and inflammatory cytokines, such as TNF-α, IL-6, and MCP-1. Coculture of hypertrophied 3T3-L1 adipocytes and RAW 264.7 macrophages results in marked upregulation of pro-inflammatory cytokines, indicating its usefulness as a model of obese adipose tissue [10,23,26]. TNF-α has been shown to act as an early inflammatory mediator to induce the expression of inducible nitric oxide synthase, COX-2, and IL-6 through NF-κB activation in 3T3-L1 adipocytes [26]. IL-6 is an inflammatory cytokine linked to the development of insulin resistance by impairing phosphorylation of insulin receptor and insulin receptor substrate-1 (IRS-1) through the induction of suppressor of cytokine signaling-3, a potent inhibitor of insulin signaling [27]. MCP-1 stimulates the infiltration of circulating macrophages, which are then activated by FFA to secrete inflammatory cytokines [28]. Among the macrophage-derived cytokines, TNF-α induces MCP-1 secretion and enhances FFA release in adipocytes [10,29]. Thus, the reduction of NEFA release and inflammatory cytokines by α-tocopherol and γ-tocopherol indicates that these tocopherols can directly suppress the inflammatory changes in obese adipose tissue by blocking the interaction between adipocytes and macrophages.

Unlike the similar suppression in NEFA release, the inhibitory effects of γ-tocopherol on inflammatory cytokine production were stronger than those of α-tocopherol at 12.5 and 25 μM. Particularly, the production of TNF-α and IL-6 was reduced to the level of the separately cultured control by 25 μM of γ-tocopherol. Although α-tocopherol is the most abundant form of vitamin E in the body, ranging from 18 to 25 μM in the plasma, supplementation with pharmacological doses of γ-tocopherol increased its plasma levels up to 30–40 μM [22,30,31]. Moreover, γ-tocopherol exhibits greater bioavailability in tissues compared to plasma, as evidenced by the lower concentration ratio of α-tocopherol to γ-tocopherol in tissues compared to plasma [32]. Previous studies have demonstrated that γ-tocopherol possesses anti-inflammatory effects on LPS-activated macrophages and alleviates inflammatory damage in *in vivo* models at physiological concentrations, whereas α-tocopherol exhibits a little to moderate effect at higher concentrations [18,20]. However, there has been no comparative study on the direct effect of these tocopherols on obesity-induced inflammatory changes. This study indicates that γ-tocopherol has a stronger anti-inflammatory effect than α-tocopherol in obese adipose tissue within a physiologically normal range.

A failure in glucose uptake into adipocytes and muscle in response to insulin is a major feature of insulin resistance. In this study, adipocytes cocultured with macrophages could not increase glucose uptake in response to insulin, but both α-tocopherol and γ-tocopherol restored the coculture-induced insulin resistance. Biological alterations in obese adipose tissue, such as excess FFA release, increased reactive oxygen species, and elevated pro-inflammatory cytokines, play a key role in obesity-induced insulin resistance, which is mediated by the JNK and NF-κB signaling pathways [5,6,33]. Activation of these pathways results in the enhanced production of inflammatory cytokines that contribute to pro-inflammatory macrophage infiltration and insulin resistance [34]. Additionally, JNK signaling can directly inhibit insulin signaling through inhibitory serine phosphorylation of IRS-1 [35]. This study found that LPS-induced NF-κB activation was inhibited by both tocopherols, with a relatively stronger effect seen with γ-tocopherol. Meanwhile, JNK phosphorylation in the coculture was suppressed by only α-tocopherol, not by γ-tocopherol. These results suggest that α-tocopherol and γ-tocopherol might exert their effects in different ways. Increased oxidative stress in adiposity has been identified as a mediator that impairs insulin signaling through the activation of the JNK pathway, and antioxidant

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supplementation has been effective in inhibiting JNK activation and improving insulin sensitivity [36,37]. Based on previous studies, it is conceivable that the anti-inflammatory effect of γ-tocopherol through the suppression of NF-κB activation and the potent antioxidative capacity of α-tocopherol to reduce oxidative stress are major mechanisms by which these tocopherols improve insulin sensitivity, respectively.

This study has a limitation in that we did not examine the efficacy of tocopherols in an *in vivo* system. However, this is the first study to report the direct effects of tocopherols on inflammatory responses and insulin resistance during the interaction of adipocytes and macrophages in obesity. Previous studies have shown that supplementation with α-tocopherol decreased oxidative stress and circulating cytokines and improved insulin sensitivity in high-fat diet-fed obese mice [15,38]. However, high doses of α-tocopherol deplete plasma and tissue γ-tocopherol [39], whereas γ-tocopherol supplementation leads to a marked increase in both tocopherols [40]. These findings suggest that γ-tocopherol supplementation may provide beneficial effects in combination, given the distinct activities of α-tocopherol and γ-tocopherol on insulin sensitivity. Considering the stronger anti-inflammatory and insulin-sensitizing effects of γ-tocopherol than α-tocopherol at physiological ranges in this study, it will be necessary to investigate the comparative effects of supplemented tocopherols on inflammatory responses and insulin sensitivity using an obese animal model in the future.

In conclusion, both α-tocopherol and γ-tocopherol directly suppressed pro-inflammatory cytokine production and FFA release but increased insulin-stimulated glucose uptake in hypertrophied adipocytes cocultured with macrophages. This implies that these tocopherols have therapeutic potential to alleviate obesity-induced insulin resistance by interfering with the interaction between adipocytes and macrophages that promote inflammation and adipocyte dysfunction.

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