Delta-Tocopherol Suppresses the Dysfunction of Thermogenesis due to Inflammatory Stimulation in Brown Adipocytes

Rieko Tanaka-Yachi¹,²*, Rena Otsu¹, Chie Takahashi-Muto³, and Chikako Kiyose⁴

¹ Department of Applied Biochemistry, Kanagawa Institute of Technology, Kanagawa 243-0292, JAPAN
² Department of Pharmacology, National Research Institute for Child Health and Development, Okura 2101, Setagaya-ku, Tokyo 157-8535, JAPAN
³ Department of Clinical Nutrition, Kitasato Junior College of Health and Hygienic Sciences, Niigata 949-724, JAPAN
⁴ Department of Nutrition and Life Science, Kanagawa Institute of Technology, Kanagawa 243-0292, JAPAN

Abstract: Brown adipose tissue (BAT) functions as a radiator for thermogenesis and helps maintain body temperature and regulate metabolism. Inflammatory signals have been reported to inhibit PGC-1α activation and UCP1-mediated thermogenesis in brown adipocytes. Inflammation is mainly caused by cell hypertrophy and macrophage invasion due to obesity, and invading macrophages secrete inflammatory cytokines, including TNF-α, IL1β, and IL6, which suppress the thermogenesis in BAT. Tocopherol is a lipidsoluble vitamin with anti-inflammatory effects and is expected to contribute to the suppression of inflammation in adipose tissue. In this study, we investigated the protective effect of tocopherols, α-tocopherol (α-toc) and δ-tocopherol (δ-toc), against brown adipocyte inflammation and thermogenesis dysfunction.

Inflammatory stimulation by TNF-α, a major inflammatory cytokine, significantly decreased the protein expression levels of UCP1 and PGC-1α in rat primary brown adipocytes. The pre-incubation of α-toc or δ-toc significantly suppressed the decrease in UCP1 and PGC-1α expression and lipid accumulation. Additionally, α-toc and δ-toc suppress the induction of ERK1/2 gene expression, implying that an anti-inflammatory effect is involved in this protective effect. We fed mice a high-fat diet for 16 weeks and investigated the effects of α-toc and δ-toc in the diet. Intake of α-toc and δ-toc significantly suppressed weight gain and hypertrophy of brown adipocytes. Our results suggest that α-toc and δ-toc suppress the dysfunction of thermogenesis in brown adipocytes due to inflammation and contribute to the treatment of obesity and obesity-related metabolic diseases.

Key words: tocopherol, brown adipocyte, inflammation, thermogenesis, UCP1

1 Introduction

Adipose tissue is characterized as either white adipose tissue (WAT) or brown adipose tissue (BAT). WAT serves as an energy storage site, while BAT acts as a radiator for thermogenesis. Classical brown adipocytes are derived from myf-5-positive, muscle-like cellular lineage. BAT has been recognized as an essential tissue for thermoregulation in infancy, but it has also been detected in the supraclavicular and neck region of adult humans using PET-CT. There is a negative correlation between BAT mass and body mass index, so it is gaining attention as a potential target for anti-obesity studies. Brown adipocytes possess large numbers of mitochondria, which contain the uncoupling protein 1 (UCP1), and activated UCP1 uncouples the respiratory chain, facilitating rapid substrate oxidation. Peroxisome proliferator-activated receptor γ co-activator-1 α (PGC1-α) plays a crucial role in the regulation of mitochondrial biogenesis and UCP1 gene expression. Activated PGC1-α regulates thermogenic gene expression through its interaction with Peroxisome Proliferator-Activated Receptor γ (PPARγ) and other transcriptional factors. Inflammatory signals have been reported to inhibit PGC-1α activation and UCP1-mediated thermogenesis in brown adipocytes. Inflammation of brown adipocytes is mainly caused by cell hypertrophy and macrophage invasion due to obesity. Invading macrophages secrete inflammatory cytokines, including TNF-α, IL1β, and IL6, which suppress the thermogenesis and inhibit BAT functions. The decrease in thermo-
genesis due to the dysfunction of brown adipocytes further reduces energy consumption, resulting in a vicious cycle. Maintaining the function of brown adipocytes is a critical issue in preventing obesity and metabolic diseases.

Tocopherols (toc) occur naturally in the form of four analogs (α-, β-, γ-, and δ-), each differing in the number and position of the methyl groups on the chroman ring. We previously reported that α-tocopherol (α-toc) and δ-tocopherol (δ-toc) enhance thermogenesis in 3T3-L1 cells. Treatment of α-toc or δ-toc significantly increases the expression level of UCP1 and the number of mitochondria in 3T3-L1 cells. It has been proposed that PGC-1α activation is involved in this mechanism, but it is unknown whether the anti-inflammatory effects of tocopherols are involved. There are several reports on the anti-inflammatory effect of tocopherol via the suppression of inflammatory signaling in adipocyte, liver, heart, etc. In this study, we examined whether α-toc and δ-toc protect the function of brown adipocytes through anti-inflammatory effects.

2 Experimental procedures

2.1 Materials

We purchased α-tocopherol, γ-tocopherol, dibutylhydroxytoluene (BHT), and TNF-α recombinant protein from FUJIFILM Wako Pure Chemical Corporation. The rabbit anti-UCP1 antibody (ab10983) was purchased from Abcam PLC, while the rabbit anti-PGC-1α antibody (AB3242) was purchased from Merck & Co., Inc. Anti-pSer (1CS) was purchased from Enzo Life Sciences, Inc.

2.2 Cell culture and differentiation

The rat primary brown adipocyte culture kit (BAT10) was purchased from Cosmo Bio Co., Ltd. Cells were maintained at 37°C with 5% CO₂. Cells were seeded and cultured according to the manufacturer’s instruction. In brief, cells were seeded in 12-well plates at a density of 8 × 10⁴ cells per well and cultured in a growth medium (BATGM, Cosmo Bio Co., Ltd.) for four days postconfluence. Then, the cells were cultured in a differentiation medium (BATDM, Cosmo Bio Co., Ltd.) in order to induce differentiation into mature brown adipocytes. Vitamin E analogs or BHT (10 μM final concentration) were added at the start of the differentiation induction, and the cells were cultured for two days. Mock cells were treated with equivalent DMSO (0.1% final concentration). Then, 10 ng/mL of TNF-α was treated to induce inflammation, and cells were collected 24 h later.

2.3 Immunoprecipitation and immunoblotting

Total protein was extracted from cells using RIPA buffer (CST Japan K.K.). Proteins were separated in a 4%–20% Mini-PROTEAN TGX Gel (Bio-Rad Laboratories, Inc.) before being transferred to polyvinylidene difluoride membranes. The membranes were blocked for 5 min using EveryBlot blocking buffer (Bio-Rad Laboratories, Inc.) and then incubated overnight with primary antibodies. The concentrations used in immunofluorescence staining and Western blotting were in accordance with the recommended protocol for each antibody. After washing the membranes three times with 1× TTBS, they were incubated for 1 h at room temperature with the secondary antibody-conjugated HRP. The membranes were then washed three times with 1× TTBS, treated with reagents in Clarity Western ECL substrate (Bio-Rad Laboratories, Inc.), and detected with iBright FL1000 imaging system (Thermo Fisher Scientific K.K.). PGC-1α protein was isolated from total protein using an immunoprecipitation (IP) kit (BioVision Inc.) according to the manufacturer’s protocol. Total PGC-1α protein and phosphorylated PGC-1α were detected by Western blotting. The intensity of western blot bands was quantified using Image J quantification tool. The quantified data were normalized with α-tubulin.

2.4 Measurement of mRNA expression using real-time PCR

Total RNA was extracted using Sepasol RNA II (Nacalai Tesque Inc.). The RNA quantity and purity were determined based on the absorbance at 258/280 nm. Total RNA was reverse-transcribed into cDNA using the ABI high-capacity RNA-to-cDNA kit according to the manufacturer’s protocol. The 7500 Fast Real-Time PCR system and real-time PCR kit (ABI TaqMan® Gene Expression Assays) were used according to the manufacturer’s instructions, and β-actin was used as an internal control. The assay IDs of the primer/probe mixtures in the TaqMan gene expression assays were as follows: b-actin (Actb); Mm00465997_m1, ERK1 (Mapk3); Mm01973540_m1, ERK2 (Mapk1); Mm00424797_m1, IL6 (Il6); Mm00461900_m1.

2.5 Animals and diets

The breeding conditions for mice are the same as previously reported. Male C57BL/6Jcl strain mice (three weeks old, n = 25) were purchased from CLEA Japan, Inc. and housed individually in plastic cages in an environment controlled at 23°C ± 2°C and 55% ± 5% humidity, with a 12 h light/dark cycle. Then, the mice were divided into four groups based on their average weight: the control group (Cont, n = 7), the high-fat and high-sucrose group (HFD, n = 6), the high-fat and high-sucrose diet + α-tocopherol group (HFD + α-toc, n = 6), and the high-fat and high-sucrose diet + δ-tocopherol group (HFD + δ-toc, n = 6). Table S1 shows the composition of the experimental diet. The feed and water were supplied ad libitum for 16 weeks. After a 16-h fast, all mice were euthanized under isoflurane anesthesia, and BAT were collected for analysis. All animal experiments were conducted with the approval of the ethics committee and in accordance with the guidelines for
the care and use of laboratory animals at Kanagawa Institute of Technology (Kanagawa, Japan. Approval number; 0147, December 26, 2018.).

2.6 Pathological examination of the adipose tissue by hematoxylin-eosin (HE) staining

BAT specimens were paraffin-embedded by Sapporo General Pathology Laboratory Co. Ltd. (Hokkaido, Japan). Sections were stained with HE and observed using an optical microscope (Olympus Co., Tokyo, Japan).

2.7 Statistical analysis

All data were expressed as mean ± SD. Statistical analyses were performed using one-way ANOVA, followed by Bonferroni’s post hoc test using Kaleida Graph v.4.5 (Hulinks Inc., Tokyo, Japan). Differences were considered significant at p < 0.05.

3 Results

3.1 α- and δ-tocopherol suppresses the reduction of thermogenic protein expression and inhibit lipid accumulation

Figure 1A depicts the time course of cell culture. Primary brown adipocytes cultured under each condition were treated with TNF-α for 24 h and then analyzed. The morphology of differentiated rat primary brown adipocytes was shown in Fig. 1B.

The stimulation of TNF-α resulted in the enlargement of the lipid droplets, but the size of the lipid droplets did not change in cells supplemented with α-toc or δ-toc, compared with mock cells. This result indicates that α-toc and δ-toc may suppress the decrease in energy expenditure and inhibit lipid accumulation by TNF-α. TNF-α-induced inflammatory stimulation significantly reduced the protein expression levels of UCP1 and PGC-1α in brown adipocytes. The pre-incubation of α-toc or δ-toc significantly suppressed the decrease in UCP1 due to inflammation (Fig. 2A). Also, δ-toc was effective in maintaining PGC-1α expression (Fig. 2B). PGC-1 is a transcriptional cofactor activated by phosphorylation. As a result, proteins were extracted from cells stimulated with TNF-1α, and PGC-1α protein was isolated by immunoprecipitation to quantify the phosphorylation state. The addition of α-toc or δ-toc improved the phosphorylation of PGC-1α more than twice (Fig. 2C).

3.2 α- and δ-tocopherol suppress the inflammatory signals in brown adipocytes

ERK1/2 is a typical inflammatory signaling factor, which is activated downstream of TNF-α. ERK1/2 attenuates thermogenic function in brown adipocytes by suppressing the activation of the promoter region of UCP1. Here, the addition of TNF-α markedly induced the expression of ERK1/2 genes, Mapk3 and Mapk1, in primary cultured brown adipocytes. The addition of α-toc or δ-toc significantly suppressed the increased expression of these inflammatory signaling factors (Figs. 3A, 3B). These results indicate that α-toc and δ-toc improve the UCP1 expression by suppressing inflammatory signaling. Interleukin-6 (IL6) is an adipokine involved in inflammation and insulin resistance. Transcription of IL6 is induced by activation of NFκB downstream of TNF-α signaling. The expression of

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**Fig. 1** Cell culture condition and cell morphology. (A) The time course of cell culture. (B) Primary brown adipocyte morphology. Arrows indicate the enlarged lipid droplets by TNF-α stimulation. Scale bar, 20 μm.
IL6 tended to increase by TNF-α stimulation, but the gene expression was low level in primary brown adipocytes and there was no significant difference (Fig. 3C).

3.3 Ingestion of tocopherols suppresses cell hypertrophy in mouse BAT

High-fat diet-induced obesity causes inflammation in brown adipocytes, resulting in cell hypertrophy. We fed mice a high-fat diet for 16 weeks and investigated the effects of α-toc and δ-toc in the diet. The weight of BAT did not differ between the groups (Fig. 4A). The HDF group exhibited large lipid droplet accumulation and balloon-like hypertrophy, but normal morphology was observed in groups ingesting α-toc or δ-toc (Fig. 4B). This result indicates that α-toc and δ-toc also protect BAT functions in vivo.

4 Discussion

In this study, we examined the effects of vitamin E analogs, α-toc and δ-toc, on inflammation and dysfunction in brown adipocytes. Macrophages invade and release inflammatory cytokines into BAT that has become hypertrophied due to obesity. In particular, TNF-α or IL-1β-mediated inflammatory signals inhibit heat produc-
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The pre-incubation of α-toc or δ-toc significantly suppressed the decrease in UCP1 and PGC-1α expression and inhibit lipid accumulation in rat primary brown adipocytes. As fat-soluble antioxidants, vitamin E analogs are well known to be involved in the protection of various cells. Interestingly, adding BHT, a lipid-soluble antioxidant similar to vitamin E analogs, had no protective effect on thermogenesis in brown adipocytes. These results indicate that tocopherols protect brown adipocytes via a pathway independent of antioxidative effects.

TNF-α binds to receptors and transmits inflammatory signals via ERK, JNK, NFκB and p38 MAPK. Inhibition of

Fig. 3 Effects of α-tocopherol and δ-tocopherol on gene expression of inflammation signaling factors. (A, B) Gene expression of ERK1/2 and IL6. The cells were stimulated with TNF-α under each condition, and total RNA was extracted. Real-time PCR was used to measure the expression of the ERK (Mapk3), ERK2 (Mapk1), and IL6 (Il6) genes, which were normalized against that of β-actin. The values represent the mean ± SD (n = 3). Statistical analysis was performed using a one-way ANOVA, followed by Bonferroni’s post hoc test (*p < 0.05).

Fig. 4 Effects of α-tocopherol and δ-tocopherol on histology of mice brown adipose tissue. (A) Weight of mice brown adipose tissue. (B) Hematoxylin-eosin staining of mice brown adipose tissue. Original magnification ×400, arrows indicate enlarged lipid droplets.

Addition by suppressing UCP1 transcription⁵. In this study, TNF-α-induced inflammatory stimulation significantly reduced the protein expression levels of UCP1 and PGC-1α in rat primary brown adipocytes. This result indicates that the decrease in thermogenic ability due to inflammation can be reproduced in an in vitro culture system. Brown adipocytes have a large number of mitochondria and rapidly convert energy into heat. Lipid droplets of brown adipocytes do not grow larger than those of white adipocytes. The enlargement of lipid droplets due to TNF-α indicates a decrease in energy consumption due to a decrease in thermogenic ability.
CREB by the MEK/ERK pathway suppresses cAMP-dependent transcription of UCP1 in adipocytes. We showed that at the ERK pathway is suppressed by α-toc and δ-toc in this study. However, this result may only be part of the mechanism. JNK and NFkB suppresses the differentiation and function of adipocytes by suppressing the expression of PPARγ. Reports that α-tocopherol and δ-tocopherol upregulate PPARγ expression suggest that maintenance of PPARγ expression is also involved in the mechanism. On the other hands, the role of p38 MAPK in adipocytes is complex. While p38 MAPK suppresses insulin signals, it activates UCP1 transcription by promoting phosphorylation of PGC-1α. Multiple inflammatory signals may indirectly be involved in UCP1 expression, further detailed studies of various signaling pathways are needed to elucidate the mechanism on the effect of tocopherol.

Ingested tocopherols accumulate in the liver and adipose tissue. Based on these findings, the role of vitamin E in suppressing inflammation in the liver and adipose tissue has been investigated for several years. However, there are no reports on the function of vitamin E in BAT. In this study, the ingestion of α-toc and δ-toc suppressed fat accumulation and hypertrophy of brown adipocytes in mice. We previously reported that α-toc and δ-toc intake significantly suppressed weight gain in these mice (Table S2). The results of this study suggest that the maintenance of thermogenesis and energy consumption in BAT is involved in the anti-obesity effect of α-toc and δ-toc ingestion. The effects of vitamin E analogs on macrophage invasion and inflammatory signals in vivo in BAT warrant further investigation. Recent studies have made it possible to induce differentiation from ES cells and iPS cells to brown adipocytes. The effects of food ingredients on the function of human brown adipocytes will be further investigated in the future.

5 Conclusion

Conclusively, we suggest that α-tocopherol and δ-tocopherol suppress the decrease of thermogenic ability in brown adipocytes due to inflammation. Also, ingestion of α-tocopherol and δ-tocopherol suppresses brown adipocyte hypertrophy and weight gain in mice, so these vitamin E analogs may contribute to the treatment of obesity and obesity-related metabolic diseases.

Conflict of Interest Statement

The authors declare no competing interests.

Author Contributions

R. T.-Y. and C. K.; Accomplished conceptualization, a draft of experiment, resources, writing, and editing. R. O.; Investigation and data curation. C. T.-M.; Writing (reviewing and editing). All authors approved the manuscript.

Data Availability Statement

The datasets analyzed during the current study available from the corresponding author on reasonable request.

Supporting Information

This material is available free of charge via the Internet at doi: 10.5650/jos.ess22184

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