Promoting Effect of α-Tocopherol on Beige Adipocyte Differentiation in 3T3-L1 Cells and Rat White Adipose Tissue

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Abstract: Thermogenic adipocytes that are distinct from classical brown adipocytes (beige adipocytes) were identified in 2012. Beige adipocytes are also called inducible brown adipocytes because their differentiation is induced by a number of physiological stimuli, including adrenaline or myokines. PPARγ is the master regulator of adipogenesis and promotes thermogenic adipocyte differentiation. A PPARγ agonist also promotes thermogenic adipocyte differentiation in mouse white adipose tissues. The vitamin E analog α-tocopherol promotes PPARγ expression and induces mRNA expression of target genes. This study investigated the effects of vitamin E analogs on thermogenic adipocyte differentiation in mouse preadipocytes and rat white adipose tissues. We determined the effects of vitamin E analogs (α-tocopherol and γ-tocopherol) on PPARγ, PGC-1α, and uncoupling protein 1 (UCP1) gene expression in 3T3-L1 cells. UCP1 expression and the mitochondrial contents were confirmed in the cells using immunofluorescence. In an in vivo study, male SD-IGS rats were fed a high-fat diet (HFD), α-tocopherol-enriched HFD, or γ-tocopherol-enriched HFD for 8 weeks before the analysis of PPARγ, PGC-1α, UCP1, and CD137 gene expression, and pathological examinations of white adipose tissues. The expression of PPARγ, PGC-1α, and UCP1 increased in 3T3-L1 cells following α-tocopherol treatment in a concentration-dependent manner. UCP1 expression and mitochondrial content also increased in α-tocopherol-treated cells. According to the histopathological examinations of rat white adipose tissues, multilocular cells were observed in the α-tocopherol intake group. Furthermore, the gene expression levels of PGC-1α, UCP1, and CD137 increased in the α-tocopherol intake group. Our results suggest that α-tocopherol promotes thermogenic adipocyte differentiation in mammalian white adipose tissues.

Key words: tocopherol, beige adipocyte, PGC-1α, UCP1

1 INTRODUCTION

The prevalence of obesity-related disease, including type 2 diabetes, hyperlipidemia, cardiovascular disease, and cancer, has increased in developed countries. To date, no country has successfully reduced its obese population; therefore the development of innovative treatments and prophylaxes is required. Adipose tissue is characterized as either white adipose tissue (WAT) or brown adipose tissue (BAT). WAT serves as the site for energy storage, whereas BAT functions as a radiator for thermogenesis. BAT is present in the supracleavicular and neck region of adult humans and can be detected using PET-CT. White adipocytes from WAT contain large lipid droplets and few mitochondria, whereas brown adipocytes from BAT possess multilocular lipid droplets and large numbers of mitochondria, which contain uncoupling protein 1 (UCP1). Activated UCP1 uncouples the respiratory chain, thereby facilitating rapid substrate oxidation.

In 2012, Wu et al. confirmed that thermogenic adipocytes found in mouse inguinal WAT are distinct from classical brown adipocytes and named them beige adipocytes. Beige adipocytes are defined by their multilocular lipid...
droplets, high mitochondrial content, and the expression of brown adipocyte-specific genes (Ucp1, Cidea, and Ppargc1a). Classical brown adipocytes are derived from a myf-5-positive, muscle-like cellular lineage, whereas beige adipocytes within white adipose depots are derived from a myf-5-negative, white adipocyte lineage. It was demonstrated that the vast majority of human BAT located in the suprACLavicular region is comprised of beige adipocytes.

Vitamin E has eight natural analogs, and each has a different number and position of methyl groups on the chroman ring, and the presence or absence of a double bond on the phytyl side chain. The tocopherol group members have a saturated phytyl side chain, whereas tocotrienols have an unsaturated phytyl side chain. Each group can occur in α-, β-, γ-, or δ- forms. Paolisso et al. reported that vitamin E supplementation influences insulin sensitivity in humans. In an in vitro study, Landrier et al. demonstrated that α-tocopherol and γ-tocopherol influence PPARγ expression as well as inducing the expression of target gene mRNAs. Vitamin E is thought to improve insulin sensitivity via its antioxidant capacity, but these findings suggest that other mechanisms may also be involved. PPARγ is the master regulator of adipogenesis and promotes thermogenic adipocyte differentiation by forming a transcriptional complex with transcription cofactors such as PGC-1α. In addition, a PPARγ agonist, rosiglitazone, was used as an internal control. Contents of the primer/probe mixtures are shown in Table 1.

### Table 1

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### EXPERIMENTAL PROCEDURES

#### 2.1 Materials

α-tocopherol and γ-tocopherol were purchased from Wako Pure Chemicals Industries Ltd. (Osaka, Japan). Rosiglitazone was purchased from Wako Pure Chemicals Industries Ltd. (Osaka, Japan). The rabbit anti-UCP1 antibody (ab10983) was purchased from Abcam, and MitoTracker Green FM was purchased from CST Japan K.K. (Tokyo, Japan).

#### 2.2 Cell culture and differentiation

3T3-L1 cells were cultured at 37°C with 5% CO₂ in high glucose Dulbecco’s modified Eagle’s medium (DMEM) containing 10% heat-inactivated fetal bovine serum, penicillin (100 U/mL), streptomycin (100 μg/mL), and L-glutamine (292 μg/mL). Cells were seeded in six-well plates at a density of 4×10⁵ cells per well and cultured until confluence and 3 days thereafter. Cells were maintained in high glucose DMEM supplemented with 10% fetal bovine serum, penicillin (100 U/mL), streptomycin (100 μg/mL), L-glutamine (292 μg/mL), insulin (10 μg/mL), dexamethasone (2.5 μM), 3-isobutyl-1-methylxanthine (0.5 mM) and T₃ (100 nM) for differentiation. Stock solutions of vitamin E analog were prepared at 10 mM-100 mM in DMSO. Each vitamin E analog (10 nM-100 μM final concentration) was added at the start of the induction of differentiation and the cells were cultured for 10 days. Rosiglitazone, a PPARγ agonist, as a positive control was added at 10 μM final concentration. Control cells were treated with equivalent DMSO (0.1% final concentration).

#### 2.3 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. A 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. Contents of the primer/probe mixtures are shown in Table 1.

#### 2.4 Immunofluorescent staining

Cells were briefly washed with phosphate-buffered saline (PBS) and fixed in 3.7% formaldehyde in PBS at room temperature for 15 min. Cells were then permeabilized for 5 min with 50 μg/mL digitonin and quenched with 50 mM NH₄Cl in PBS for 10 min, washed with PBS three times, and treated with blocking buffer (3% bovine serum albumin in PBS) for 45 min. Cells were incubated with primary antibodies for 2 h, then incubated with secondary antibodies and DAPI for 1 h, washed with PBS three times, and then mounted. To stain the mitochondria, cells were incubated with MitoTracker green for 30 min, washed three times.
with PBS, and then mounted.

2.5 Animals and diets
Male SD-IGS rats (4 weeks old) were purchased from Charles River Japan, Inc. They were housed individually in stainless steel wire netting cages and fed a commercial chow for 3 days during acclimation. The animals were kept in an environment controlled at 23±2°C and 55%±5% humidity, with a 12 h : 12 h light : dark cycle. Subsequently, rats were switched to the experimental diet for 8 weeks. The composition of the high-fat diet (HFD) is shown in Table 2. α-Corn starch, milk casein, sucrose, cellulose, mineral mix, and a vitamin mixture were obtained from Oriental Yeast Co. Ltd. (Tokyo, Japan). Soybean oil, l-cystine and t-butylhydroquinone were purchased from Wako Pure Chemicals Industries Ltd. (Osaka, Japan) and butter was purchased from Megmilk Snow Brand Co. Ltd. (Tokyo, Japan). The purity of α-tocopherol was above 96.1%. After a 12 h fast, all rats were killed under diethyl ether anesthesia, and the arterial blood and adipose tissues were removed for analysis. All animal experiments were conducted with the approval of the ethics committee, in accordance with the guidelines for the care and use of laboratory animals at Kanagawa Institute of Technology (Kanagawa, Japan).

2.6 Quantitative analysis of vitamin E analogs using HPLC
The quantity of vitamin E in adipose tissue was measured using Ueda’s method⁷, as described previously⁸. The extracted vitamin E was dissolved in 0.2 mL of n-hexane and analyzed by HPLC. The HPLC system comprised a pump, degasser, column oven, and detector (LC-20AD, DGU-20A3, CTO-20A, and RF-10AXL; Shimadzu Co., Kyoto, Japan). The analytical conditions were as follows: column = Capcell pak NH₂ column (4.6 mm I.D. × 250 mm; Shiseido, Tokyo, Japan); mobile phase = n-hexane: 2-propanol (98:2); flow rate = 1.0 mL/min; detection wavelength = 325 nm.

2.7 Pathological examination of the adipose tissue by hematoxylin-eosin (HE) staining
Adipose tissue specimens were paraffin embedded by Sapporo General Pathology Laboratory Co. Ltd. (Hokkaido, Japan). Sections were stained with HE and observed using an optical microscope at ×100 or ×400 (Olympus Co., Tokyo, Japan).

2.8 Immunohistochemistry
Sections of adipose tissue were treated with blocking buffer (3% bovine serum albumin in PBS) for 45 min. Cells were incubated with the primary antibodies for 2 h, incubated with the secondary antibodies for 1 h, washed three times with PBS, and then mounted.

2.9 Confocal microscopy
Images were obtained using a laser scanning microscope with a 63 × 1.4 Plan-Apochromat oil immersion lens. Alexa Fluor 488 was excited with a 30-mW argon laser emitting at 488 nm and the emissions were detected using a 505–530 nm band-pass filter.

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

<table>
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<th>Table 2</th>
<th>The composition of the experimental diets [g].</th>
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<td>Soybean oil</td>
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<td>Butter</td>
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<td>Cellulose</td>
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<td>t-Buthylhydroquinone</td>
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<td>α-Tocopherol</td>
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<tr>
<td>γ-Tocopherol</td>
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3 RESULTS
3.1 α-tocopherol increases PPARγ, PGC-1α, and UCP1 mRNA expression in a concentration-dependent manner

The expression levels of PPARγ, PGC-1α, and UCP1 genes were measured in 3T3-L1 cells using real-time PCR (Fig. 1A). These genes expression were significantly increased by rosiglitazone treatment. As reported previously, we confirmed that α-tocopherol induced the expression of PPARγ mRNA. On the other hand, γ-tocopherol did not affect the expression of PPARγ mRNA in this study. Expression of PGC-1α mRNA was significantly increased in α-tocopherol-treated cells. Next, we examined the effect of α-tocopherol on mitochondrial thermogenic genes. UCP1 mRNA expression was increased 1.7-fold in α-tocopherol-treated cells. On the other hand, γ-tocopherol addition did not affect the expression of UCP1 mRNA.

Fig. 1 Effects of vitamin E analogs on the expression of transcriptional factor and UCP1 genes in 3T3-L1 cells. (A) Expression levels of the PPARγ (Pparg), PGC-1α (Ppargc1α) and UCP1 (Ucp1) genes in 3T3-L1 cells. Cells were cultured with 10 μM tocopherols or 10 μM rosiglitazone. Control cells were treated with DMSO. (B) Dose-dependent effects of α-tocopherol on PPARγ, PGC-1α and UCP1 gene expression. Gene expression levels were measured by real-time PCR and normalized against that of β-actin. The values represent the mean ± SD based on two samples. Statistical analysis was performed using one-way ANOVA followed by Bonferroni’s post hoc test (*p<0.05, **p<0.01, ***p<0.001 vs control).
not affect the expression of these genes. Next, we examined changes in the expression of PPARγ, PGC-1α, and UCP1 genes after the addition of different concentrations of α-tocopherol. We found that the expression of PGC-1α, and UCP1 mRNA increased in a concentration-dependent manner when the concentration of α-tocopherol was more than 1 μM (Fig. 1B). Thus, it is clear that α-tocopherol induces the expression of PGC-1α and UCP1 mRNA.

### 3.2 α-tocopherol promotes UCP1 expression and increases mitochondrial content

We determined the intracellular expression of UCP1 protein by immunofluorescent staining. As shown in Fig. 2A, staining of UCP1 was not observed in control cells, whereas staining was observed in cells treated with rosiglitazone or α-tocopherol. Thermogenic adipocytes are characterized by their high mitochondrial content. Therefore, we determined the mitochondrial content of cells by staining the mitochondria. We observed extensive mitochondria staining in cells treated with rosiglitazone or α-tocopherol (Fig. 2B).

#### 3.3 Body weight, fat weight, and vitamin E content of rat plasma and adipose tissues

There were no significant differences in final body weight and fat weight among the groups (Table 3). α-tocopherol content increased in the WAT and plasma of the α-tocopherol treatment group, and the γ-tocopherol

![Figure 2](image-url) **Fig. 2** Effects of α-tocopherol on the expression of UCP1 and the mitochondrial contents. (A) 3T3-L1 cells were cultured with 10 μM tocopherols or 10 μM rosiglitazone. Control cells were treated with DMSO. Cells were fixed, permeabilized, and stained for UCP1 (in green) and nuclei with DAPI (in blue), before examination with confocal microscopy. Scale bar, 20 μm. (B) 3T3-L1 cells were cultured with 10 μM tocopherols. Control cells were treated with DMSO. Cells were fixed, permeabilized, and stained for mitochondria with Mito Tracker (in green) and nuclei with DAPI (in blue), before examination with confocal microscopy. Scale bar, 10 μm or 20 μm.
content increased in the rat tissues of the α-tocopherol treatment group (Tables 4, 5).

### 3.4 α-tocopherol intake promotes expression of PGC-1α, UCP1, and CD137 mRNA

We measured the expression of PPARγ, PGC-1α, UCP1, and CD137 mRNA in rat subcutaneous fat (Fig. 3). The mRNA expression cannot be accurately quantified in perirenal fat because adipose browning occurs locally (data not shown). For this reason, we measured gene expression in subcutaneous fat. Contrary to our expectations, PPARγ expression did not increase after α-tocopherol intake according to our in vivo study. PGC-1α expression increased significantly after α-tocopherol intake. Moreover, UCP1 and CD137 (a beige adipocyte marker) expression increased significantly in the HFD + α-tocopherol group.

### 3.5 α-tocopherol intake promotes the browning of rat adipose tissue

Beige adipocytes in WAT are defined by their multilocular lipid droplet morphology. We performed pathological examinations of rat perirenal fat by HE staining. We found that multilocular cells were observed in the HFD + α-tocopherol group (Fig. 4A), but the histology of the HFD + γ-tocopherol group did not change compared with the HFD group. We identified UCP1-positive cells by UCP1 staining and found increased numbers of UCP1-positive cells in the perirenal fat of α-tocopherol intake rats (Fig. 4B). These findings suggested that the browning of WAT was induced by α-tocopherol intake.

### 4 DISCUSSION

In this study, we examined the effects of vitamin E analogs on thermogenic adipocyte differentiation in mouse adipocytes and rat adipose tissue. In a recent study, it was reported that treatment with T3 and rosiglitazone induced UCP1 expression in 3T3-L1 cells (9). In this study, UCP1 expression was examined in 3T3-L1 cells. However, it was not positively correlated with the results in our study. We assumed that differences in cell culture conditions could be a reason for this discrepancy. Further studies are needed to elucidate the mechanism behind the difference in the results.

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**Table 3** Body weight and fat weight in rats after experimental diet ingestion.

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<th>Perirenal fat [g]</th>
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<td>HFD + α-toc</td>
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<td>HFD + γ-toc</td>
<td>498.55 ± 21.31</td>
<td>18.05 ± 2.93</td>
<td>13.39 ± 2.40</td>
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**Table 4** Contents of α-tocopherols in subcutaneous fat and plasma.

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<th>Subcutaneous fat (μg / g)</th>
<th>Plasma (μg / mL)</th>
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<td>HFD</td>
<td>8.22 ± 0.79</td>
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<td>HFD + α-toc</td>
<td>18.31 ± 6.75</td>
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<td>HFD + γ-toc</td>
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**Table 5** Contents of γ-tocopherols in subcutaneous fat and plasma.

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<td>HFD + γ-toc</td>
<td>11.53 ± 6.33</td>
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Fig. 3 Effects of vitamin E analogs on the expression of transcription factor genes and beige adipocyte genes in rat white adipose tissues. Total RNA was extracted from rat subcutaneous adipose tissues. Expression of the PPARγ (Pparg), PGC-1α (Ppargc1a), UCP1 (Ucp1) and CD137 (Tnfrsf9) genes was measured using real-time PCR and normalized against that of β-actin. The values represent the mean ± SD based on five-six rats. Statistical analysis was performed using a one-way ANOVA followed by Bonferroni’s post hoc test (*p < 0.05, **p < 0.01 vs control).
α-Tocopherol promotes beige adipocyte differentiation

expression was not increased by rosiglitazone or vitamin E analogs in the absence of T3 as reported previously (data not shown). Thus, it is considered that PPARγ and TR activation is necessary for thermogenic adipocyte differentiation. As reported previously, we confirmed that α-tocopherol induced the expression of PPARγ mRNA. However, unlike previous reports, γ-tocopherol did not affect the expression of PPARγ mRNA in this study. We do not know the detailed reasons, so it should be investigated different culture conditions and the presence or absence of T3.

The expression of UCP1 and PGC-1α was increased in a concentration-dependent manner by α-tocopherol. In agreement with the results of our in vitro study, the expression of UCP1 and PGC-1α mRNA increased in the WAT of α-tocopherol intake rats. Multilocular cells, such as brown adipocytes, were observed in the WAT of α-tocopherol intake rats, which suggests that the browning of WAT was induced. However there were no significant differences in final body weight and fat weight among the groups. Beige adipocytes are activated by exposure to cold temperatures and exercise, so weight loss of α-tocopherol-fed rats might be expected in response to repetitive cold exposure or chronic exercise. All rats were fed a HFD in this study. We believe that it is necessary to repeat these investigations with a normal diet.

The antioxidant ability of γ-tocopherol is thought to be stronger than that of α-tocopherol\(^{[10]}\). However, it is possible that the antioxidant activity of α-tocopherol affected beige adipocyte differentiation, while that of γ-tocopherol is not effective at differentiating beige adipocytes in vivo and in vitro. Therefore, antioxidant activity is not necessarily proportional to these effects.

PGC-1α gene expression increased significantly in vitro and in vivo, thus the influence of PGC-1α expression and activation should be considered rather than PPARγ-dependent regulation. Many factors control the functions of PGC-1α, including protein kinases such as S6K, AMPK, and MAPK, which can phosphorylate and activate PGC-1α\(^{[11-13]}\). It has been reported that α-tocopherol...
promotes p38 MAPK phosphorylation in vivo, suggesting that MAPK activation contributes to PGC-1α upregulation in adipocytes. Recently, Liu et al. demonstrated that tocotrienol enhances AMPK activation in human prostate cancer cells. Moreover, Wu SJ et al. reported that tocotrienol induced cell cycle arrest and apoptosis via activation of AMPK signaling pathways in 3T3-L1 cells. Thus, we suggest that it is necessary to reexamine whether the activation of AMPK is induced by other homologs. Furthermore, PGC-1α combines with other transcriptional factors when it is deacetylated by SIRT1. Therefore, it is necessary to investigate the deacetylation state of PGC-1α.

Many proteins are regulated by PGC-1α signaling. In particular, irisin, a novel hormone identified in 2012, induces the differentiation and activation of beige adipocytes. Irisin is secreted from muscles and adipocytes in a PGC-1α-dependent manner. Irisin secretion may be promoted if PGC-1α expression is increased by α-tocopherol in skeletal muscle cells. FGF21 is a hormone-like protein, classified as a member of the GGF family, and is expressed mainly in the liver and pancreas. FGF21 promotes the expression of thermogenic genes and lipolysis in BAT and beige adipose tissue. The administration of FGF21 lowers the plasma lipid level and improves obesity, insulin resistance, and fatty liver. Therefore, it is necessary to examine the involvement of secretory proteins that depend on PGC1-α.

5 CONCLUSION

In conclusion, we suggest that α-tocopherol promotes thermogenetic adipocyte differentiation in mammalian white adipocytes. We also suggest that PGC-1α upregulation promotes adipocyte differentiation. However, the detailed mechanism that underlies these effects remains unclear. It is necessary to examine the involvement of secretory proteins that depend on PGC1-α.

ACKNOWLEDGEMENT

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References


