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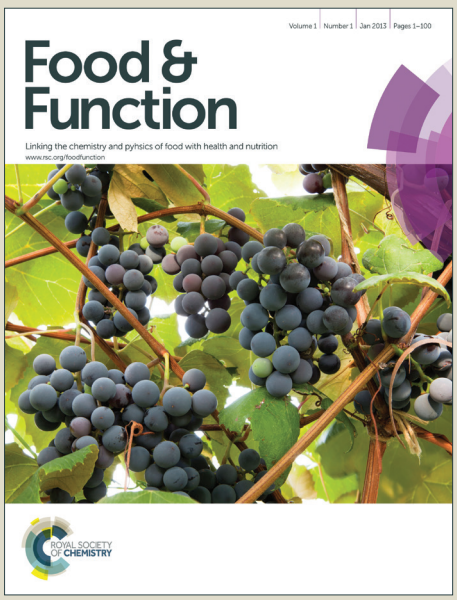

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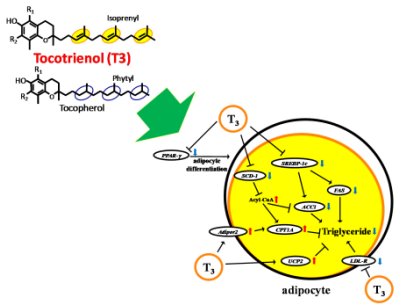

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Tocotrienol modulates crucial lipid metabolism-related genes in differentiated 3T3-L1 preadipocytes

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ABSTRACT

Obesity and other lipid metabolism-related diseases have become more prevalent in the recent years due to the drastic lifestyle change and dietary patterns. Unsaturated vitamin E, tocotrienol (T3), represents one of the most fascinating naturally-occurring compounds that have potential to influence a broad range of mechanism underlying abnormal lipid metabolism process. But however its efficacy and mechanism have been uncertain due to scarcity of data concerning the effect of T3 on lipid metabolism. In this study, we found out a series of entrancing experimental evidences on how T3 affects lipid metabolism in differentiated 3T3-L1 preadipocytes. Treatment of T3 (25 μM), especially δ and γ isomers, inhibited the accumulation of triglyceride and lipid droplets in differentiated 3T3-L1 cells. This manifestation was supported by mRNA and protein expressions of crucial lipid metabolism-related genes. The present study provided a novel set of data pertaining to the possibility of T3 as an anti-metabolic disorder agent.

Key Words: Tocotrienol; Tocopherol; Triglyceride; Lipid metabolism

1. Introduction

According to the world health organization (WHO), a change in lifestyle and habits which contributes to a change in dietary patterns have increased in the generation of lifestyle-related diseases especially those closely linked to heart, liver, obesity and other metabolic-related afflictions. Moreover, obesity in particular has become more prevalent in a certain social strata [1]. Though several approaches have been done regarding the amelioration of obesity, most of these undertakings largely unsuccessful. Several experimental trials concerning molecular regulation of some known lipid species such as triglyceride (TG) and cholesterol (Cho) via biologically active molecules are in route to reduce abnormal lipid accumulations.

The fat homeostasis is characterized between fat synthesis and fat breakdown in the biological system. Metabolic-related diseases such as obesity happen when the balance between energy intakes exceeds energy expenditure [2, 3]. Glucose generated from carbohydrates stimulates lipogenesis by making it a substrate for lipogenic process which is glycolytically converted to acetyl-CoA. This conversion then provides an ample substrate (acetyl-CoA) to be carboxylated by acetyl-CoA carboxylase (ACC) to malonyl-CoA of which it is elongated by fatty acid synthase (FAS) to create fatty acids of dependent types. Regulation of this complex interplay would consequently affect fat storage potential.

Since the discovery of adipose tissue as one of the sites that could synthesize fatty acids, this led to the initial conclusion that this tissue is the major site for lipogenesis [4]. Therefore, understanding the cellular mechanisms involved in the aberration of the homeostatic status of fat storage and usage in the adipose tissue could entail an array of

new insights regarding the possibility of future pharmacological target for the treatment of obesity and other metabolic-related diseases.

Recently, a handful of studies have been reported concerning the novel utilization of functional food components and nutrients as possible inhibitory agent against endogenous lipid accumulation generated by aberrant lipid metabolic process. For instance, catechin has been reported to inhibit adipocyte differentiation via down regulation of peroxisome proliferator-activated receptor (PPAR)-γ and CCAAT/enhancer binding protein (C/EBP)-α in 3T3-L1 cells [5]. Furthermore, administration of carotenoids and retinoids has been linked to have anti-adiposity through nuclear receptors regulation [6].

Vitamin E is the generic name for tocopherol (Toc) and tocotrienol (T3). Structurally, these vitamin E classes differ only in their side chains (Fig. 1). Toc has saturated phytyl side chain, while T3 contains unsaturated isoprenoid tail. To date, eight substances have been found in nature as vitamin E: α-, β-, γ-, and δ-Toc and α-, β-, γ-, and δ-T3. T3 has recently gained increasing interest due to its several health-promoting properties that differ somewhat from those of Toc. For example, T3 protects neuronal cells against oxidative damage, and have anti-angiogenesis, anti-tumor, and lipid-lowering activity [7-12]. However, it should be noted that only few paper have been published focusing on the biological effect of T3 on lipogenesis in the adipose tissue. T3 is reported to suppress adipocyte insulin-induced differentiation and Akt phosphorylation in 3T3-L1 preadipocytes [13]. It was also cited that γ-T3 isomer may improve obesity-related functional abnormalities in adipocytes by attenuating nuclear factor (NF)-κB activation and the expression of inflammatory adipokines [14]. Though these data emphasized the potency of T3 as anti-adipogenic and anti-inflammatory agents in adipocyte cells, it is

also worth to note that the lipid metabolic pathway is very complex as such regulation only of those genes (NF-κB and inflammatory adipokines) would not confirm the clinical usage of T3 as future anti-metabolic disease medicine. Because of the scarcity of data concerning the impact of T3 as potential lipid metabolism regulator in adipose tissues, further biological studies are needed to elucidate the mechanisms involve on how T3 affects the interaction of crucial genes in the lipid metabolism pathway in adipocyte cells. In this study, investigations concerning the modulation of T3 on lipid metabolism via multiple metabolic genes regulation in differentiated 3T3-L1 preadipocytes were clearly elucidated trough cellular, RT-PCR and western blotting analyses respectively.

2. Materials and methods

2.1 Reagents and cells

T3 isomers and α-Toc were kindly provided by Eisai Food & Chemical Co., Ltd (Tokyo, Japan) and were dissolved in ethanol at a concentration of 50 mM as stock solution. 3T3-L1 preadipocytes were obtained from the RIKEN cell bank (Tsukuba, Japan). The cells were cultured in DMEM medium (high glucose; Sigma, St. Louis, MO) containing 0.3 g/L L-glutamine and 2.0 g/L sodium bicarbonate supplemented with 10% fetal bovine serum (FBS; Biowest, Paris, France), 100 kU/L penicillin, and 100 mg/L streptomycin (Gibco BRL Rockville, MD) at 37 °C in 5% CO_2/95% air atmosphere in a humidified incubator. All reagents used in this study were of analytical grade.

2.2 Preparation of experimental medium for cell culture studies

T3 isomers and α-Toc stock solutions were diluted with 10% FBS/DMEM (high

glucose) medium to achieve the desired final concentration (0-50 μM). The final concentration of ethanol in the experimental medium was less than 0.1% (v/v), which did not affect cell viability. Medium with ethanol alone was similarly prepared and used as control medium.

2.3 Cell viability assay

For cell viability assays, 3T3-L1 preadipocytes (2×10^4 cells/well) were pre-incubated with 10% FBS/DMEM (high glucose) in 96 well culture plates. 24 h later, the cells were washed with phosphate buffered saline (PBS) and medium was replaced with the experimental medium. After incubation for 24 h, the number of viable cells was determined using WST-1 reagent according to the manufacturer's instructions (Dojindo Laboratories, Kumamoto, Japan). In brief, WST-1 reagent (10 μL) was added to the medium, and incubated at 37 °C for 3 h. Absorbance (450/655 nm) of the medium was measured with a microplate reader (Model 550, Bio-Rad Laboratories, Hercules, CA).

2.4 Adipocyte differentiation

3T3-L1 preadipocytes (2×10^4cells/plate) were seeded in a 35 mm cell culture plates supplemented with 10%FBS/DMEM (high glucose), and incubated until confluence. Differentiation was then initiated by 10%FBS/DMEM (high glucose) containing 0.25 μM dexamethasone, 0.5 mM 3-isobuthyl-1-methylxanthine and 10 μg/mL insulin in the presence or absence of T3 isomers and α-Toc. The cells were then further incubated for 6-8 din 10%FBS/DMEM (high glucose) containing10 μg/mL insulin in the presence or absence T3 and α-Toc. Medium change containing the latter was performed at every 2 d interval from the start of the differentiation. At day 8, cells were harvested for TG, T3 and Toc cellular uptake analyses.

2.5 TG analysis

Cellular TG concentrations were evaluated using Folch method [15]. Briefly, differentiated 3T3-L1 cells were washed with PBS and scrapped using rubber policeman, transferred into eppendorf tube (1.5 mL) and were centrifuged for 1000 *g* for 3 min. Cell pellets were transferred to micro smashing tube with the addition of 500 μL PBS buffer for homogenization. After homogenization, contents of the cellular protein were determined using Bradford protein assay [16]. Cellular TG was measured using commercial TG kit (Wako, Osaka, Japan).

2.6 Oil Red O staining

Differentiated 3T3-L1 cells were washed with PBS and were fixed using 4% formalin for 60 min. After fixation, the cells were washed with distilled water, stained with filtered oil red O working solution and washed further with distilled water. Visualization of the stained lipids was performed using photomicrograph system.

2.7 T3 and Toc cellular uptake of 3T3-L1 differentiated cells

Cellular uptake of T3 and Toc in differentiated 3T3-L1 cells were measured using 4000 Qtrap LC-MS/MS (AB SCIEX, Tokyo, Japan). In brief, at day 8 after cellular differentiation, differentiated 3T3-L1 cells were washed with PBS, suspended in 2 mL of water in a micro smashing tube for homogenization. A 1 mL aliquot of 6% ethanolic pyrogallol and l mL of 1 μM ethanolic 2,2,5,7,8-pentamethyl-6-hydroxychroman (internal standard) were mixed with the cell suspension. The sample mixture was added with 0.2 mL of 60% aqueous KOH and incubated at 70 °C for 30 min. After the mixture was cooled down at room temperature, 1.5 mL of water and 5 mL of hexane were added for extraction. The samples were then centrifuged at 1000*g* for 5 min, and the upper hexane layer was collected and dried. The residue was reconstituted in 100 μL of hexane, and a portion of the aliquot (5 μL) was injected to LC-MS/MS for analysis.

153 Separation was performed at 40 °C using a silica column (ZORBAX Rx-SIL, 4.6 × 250
154 mm; Agilent, Palo Alto, CA). A mixture of hexane/1, 4-dioxane/2-propanol (100:4:0.5)
155 was used as the mobile phase at a flow rate of 1.0 mL/min. T3 and Toc were detected by
156 atmospheric pressure chemical ionization mode (APCI). MS/MS parameters were
157 optimized withT3 and Toc (Eisai Food & Chemical Co., Ltd) standards in APCI mode
158 (negative). T3 and Toc were determined using the multiple reaction monitor (MRM)
159 mode as follows: α-Toc, *m/z* 429.5>*m/z* 163.0; α-T3, *m*/z 423.4>*m/z* 163.1; β-T3, *m/z*
160 409.4>*m/z* 148.9; γ-T3, *m/z* 409.4>*m/z* 148.9; δ-T3, *m/z* 395.4>*m/z* 135.0. Cellular T3
161 and Toc contents were calculated in nmol/mg protein of differentiated 3T3-L1 cells.
162
163 **2.8Isolation of total RNA and analysis of mRNA expression**
164 Total cellular RNA was isolated with an RNeasyPlus Mini kit (Qiagen, Valencia, CA)
165 for real-time quantitative reverse transcription-PCR (RT-PCR). cDNA was synthesized
166 using a Ready-To-Go T-Primed First-Strand kit (GE Healthcare, Piscataway, NJ), and
167 PCR amplification was performed with a CFX96 Real-Time PCR Detection System
168 (Bio-Rad Laboratories, New South Wales, Australia) using SYBR Premix Ex Taq
169 (Takara Bio Inc., Shiga, Japan) and gene-specific primers for *FAS*, carnitine
170 palmitoyltransferase I (*CPT1*), stearoyl-CoA desaturase 1 (*SCD-1*), acetyl-CoA
171 carboxylase 1 (*ACC1*), sterol regulatory element-binding protein 1c (*SREBP 1c*),
172 adiponectin receptor 2 (*ADIPOR2*), uncoupling protein 2 (*UCP2*),
173 3-hydroxy-3-methyl-glutaryl-CoA reductase (*HMG-CoA-R*), low-density lipoprotein
174 receptor (*LDLR*), *PPAR-γ*, and beta actin (*β-actin*). PCR conditions were 95 °C for 60 s,
175 95 °C for 5 s, and 65 °C for 30 s for 40 cycles.
176 **2.9 Western blotting analysis**

Differentiated 3T3-L1 cells proteins were extracted and separated by SDS-PAGE (4-20% e-PAGEL; Atto, Tokyo, Japan). The protein bands were transferred to polyvinylidene fluoride membranes (Invitrogen, Carlsbad, CA). After blocking for 1 h, membranes were incubated with primary antibodies for FAS, CPT1, SCD-1, ACC1, SREBP 1c, UCP2, LDLR,PPAR-γ, and β-actin (Cell Signaling Technology, Beverly, MA), followed by horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology). ECL Plus (GE healthcare) was used for detection. Bands intensities were measured using Image Lab software version 3.0 (Bio-Rad Laboratories).

2.10 Statistical analysis

The data were expressed as the mean ± standard deviation (SD). One-Way ANOVA was performed, followed by the Bonferroni/Dunn test for multiple comparisons. Differences were considered significant at $P < 0.05$.

3. Results

3.1 Treatment of T3, especially δ and γ isomers, inhibits TG and lipid droplets accumulations in differentiated 3T3-L1 cells

Sample treated 3T3-L1 preadipocytes were subjected to WST-1 assay. Results showed that most of the T3 isomers exhibited cytotoxic effect to 3T3-L1 preadipocytes at higher doses (20-50 μM) except that of α-T3 which only showed significant cytotoxicity at 50 μM (Fig. 2). However, α-Toc did not show any cytotoxic effect to 3T3-L1 preadipocytes. Since most of the T3 isomers showed less cytotoxic effect at 25 μM, this concentration was used in the succeeding experiments.

Next, differentiated 3T3-L1 cells were treated with T3 or Toc for 8 d, and cellular TG

was extracted. TG levels were measured using TG measuring Kit (Wako), and expressed as mg/mg protein. T3 showed abrogation of TG in differentiated 3T3-L1 cells at dose dependent manner (Fig. 3). T3 isomers (δ-, γ-, and β-T3) attenuated TG levels at 25-50 μM concentrations, but α-T3 showed attenuation only at 50 μM. However, unexpectedly, there were no significant differences observed in the cellular uptake concentration of T3 and Toc (Fig. 4). This suggested that the molecular structure of δ-, γ-, and β-T3, rather than their amounts in the 3T3-L1 is an important determinant for their bioactivity. This possibility needs further investigation, because it has been generally known that δ- and γ-T3 exert bioactivity (e.g., cytotoxicity) at lower concentrations than other T3 and Toc isomers because of faster rate of cellular uptake. Moreover, this is the first data reported regarding the measurement of T3 and Toc cellular uptake in differentiated 3T3-L1 cells which might give valuable information as to the differing T3 and Toc cellular incorporation in different cell lines.

In order to confirm whether T3 or Toc affects the lipid droplets accumulation in differentiated 3T3-L1 cells, oil red O staining assay was performed. Cellular lipid droplets aggregations were significantly observed from 6 to 8 d after differentiation (Fig. 5). Differentiated 3T3-L1 cells treated with T3 showed significant reduction of lipid droplets with higher effect exhibited by δ-, γ-, and β-T3 isomers respectively. Though α-T3 also showed a less reduction of lipid droplets, its effect was comparable with other T3 isomers (Fig. 5). Moreover, α-Toc did not showed any observable reduction of lipid droplets in the differentiated cells.

3.2 Regulation of T3 to crucial lipid metabolism-related genes and proteins in differentiated 3T3-L1 cells

225 Effects of T3 or Toc on mRNA expression levels of some known crucial lipogenic
226 genes were investigated. Differentiated 3T3-L1 cells treated with or without T3 or Toc
227 at 8 d after differentiation were harvested and subjected to mRNA extraction. Selected
228 genes mRNA expressions were evaluated using RT-PCR. Expression of crucial lipid
229 metabolism-related genes such as *FAS*, *SCD-1*, *ACC1*, *SREBP 1c*, *LDLR*, and *PPAR-γ*
230 were significantly down regulated by δ- and γ-T3 (Fig. 6). On the other hand, β-T3
231 showed down regulation to *SCD-1*, *ACC1* and *LDLR* genes respectively. There were no
232 significant attenuation observed for α-T3 and α-Toc. In contrast, only δ- and γ-T3
233 significantly up-regulated *CPT1*, *ADIPOR2* and *UCP2* genes expressions (Fig. 6). In
234 this study, interestingly, gene that codes for Cho synthesis (*HMG-CoA-R*) was not
235 regulated by all experimental groups. This observation was in coherence with our
236 previous animal and cell culture studies [12]. We therefore hypothesized that the
237 reported lipid lowering effect of T3 might be mediated by its regulation to other crucial
238 lipid metabolism-related genes rather than *HMG-CoA-R* gene. The present study caters
239 an immense possibility that T3 can regulate a wide array of essential genes necessary
240 for lipid metabolism.

241 Finally, we extracted the proteins from differentiated 3T3-L1 cells incubated with
242 T3 and Toc for 8 d, and analyzed the proteins using customary western blotting
243 procedure. Protein blots revealed that δ- and γ-T3 markedly repressed FAS, SCD-1,
244 ACC1, SREBP 1c, LDLR, and PPAR-γ protein expressions (Fig. 7). Up regulation of
245 CPT1 and UCP2 proteins were also observed in δ- and γ-T3 treated 3T3-L1
246 differentiated cells. However, β-T3 did not showed remarkable regulation to all selected
247 proteins though it displayed significant mRNA regulation to *SCD-1*, *ACC1*, and *LDLR*
248 genes (Fig. 6). In the present study, both α-T3 and α-Toc did not show any significant

regulation to all the proteins (Fig. 7).

4. Discussion

The modulation of precise gene expression especially those that activates lipid metabolism process in response to nutritional factors has become an immense significance as to the search of novel functional food constituents that could physiologically rectify the aberrant expressions of these genes. In our previous studies, T3 significantly attenuated TG accumulation via lipogenic and β-oxidation genes regulations [12]. In order to unravel the profound mechanism involve regarding the bioactivity of T3 against lipid metabolism, we investigated the effect of T3 to crucial lipid metabolism-related genes that significantly influences this metabolic machinery in differentiated 3T3-L1 preadipocytes.

In this study, T3 isomers but not α-Toc showed a significant cytotoxic effect to 3T3-L1 preadipocytes at 30-50 μM (Fig. 2). Moreover, as to the reason why α-Toc did not show any cytotoxic effect to the cells is still unknown. But such manifestation has been observed in different cell lines such as human hepatocellular carcinoma (HepG2) [12]. Moreover, T3-induced TG attenuation was significantly observed in differentiated 3T3-L1 cells with higher efficacy to that of δ- and γ-T3 isomers (25μM) (Fig. 3). Conversely, although T3 significantly attenuated TG in 3T3-L1 cells, the concentrations of T3 and Toc cellular uptake were not significantly different (Fig.4). Furthermore, since 3T3-L1 cells were incubated with T3 and Toc for such a long time (8 days), this may raise the possibilities of the role of T3 metabolites in the present experimental condition as such further studies are needed to elucidate the mechanism behind this observed experimental phenomenon.

To date, very few data have shown the ability of some T3 isomers such as α- and γ-T3 in the reduction of cellular TG in adipocyte cell lines [13], but however it is also important to note that α-Toc failed to show significant TG attenuation (Fig. 3). Interestingly, lipid droplets accumulation in differentiated 3T3-L1 cells were also markedly reduced by T3 (Fig. 5). In its simplest form, lipid droplets controls the storage and hydrolysis of neutral lipids including TG or Cho esters and its regulation is of vital importance in the development of lipid-related diseases [17]. Therefore, T3, but not Toc, may attenuate TG accumulation by reducing lipid droplets in the differentiated preadipocytes. Our findings provided a new information regarding the effect of T3 (all isomers) as well as Toc on the TG levels in differentiated 3T3-L1 cells.

T3 has been increasingly known as potential compound that can reduce TG accumulation. For instance, it was reported that T3 suppresses TG accumulations and cellular differentiations in preadipocytes cell lines via Akt phosphorylation and transcription factor C/EBPα down regulation [13]. But however it is very vital to note that lipid metabolic process involved a complex system of genetic and enzymatic links that are directly or subsidiarily interplaying to sustain lipid homeostasis. In the present study, T3 significantly modulated an array of genes that are crucial for lipid metabolism. Among these genes, T3 observably showed regulation to those genes that code for lipid biosynthesis (*FAS*, *SCD-1*, *ACC1*, *ADIPOR2* and *LDLR*) and β-oxidation (*CPT1* and *UCP2*). Additionally, transcription factors such as *SREBP 1c* and *PPAR-γ* were also markedly regulated by T3 (Fig. 6). Moreover, down regulation of cleaved SREBP1c was also observed in T3 treated differentiated 3T3-L1 cells (date not shown) which further justifies that the suppression of T3 to SREBP 1c was firstly initiated by its non-phosphorylated membrane-bound precursor thereby deactivating its transcription

process. *FAS* and *SREBPs* are both noted to be the culprit of lipid synthesis, and regulations of these genes are known to induce dramatic reductions of fat accumulations [18, 19]. Specifically, this physiological role of SREBP-1c in lipogenesis was initially proposed for its direct control of lipogenic genes such as those that codes for FAS and acetyl coenzyme-A carboxylase (ACC) in the liver and adipose tissue [20]. Thus, the dramatic reduction of TG observed in differentiated 3T3-L1 cells maybe associated with the multiple modulation of *SREBP 1c*, *FAS* and *ACC* genes transcription by T3 (Fig. 6). Moreover, reports showed that *SCD-1* inhibition results in the buildup of acyl-CoAs which diminishes the inhibition of CPT shuttle and will allow fat transportation to the mitochondria for breakdown via β-oxidation [21, 22]. Therefore, T3-induced down regulation of *SCD-1* gene expression may partly explain the up regulation of *CPT1* gene expression thereby enhancing β-oxidation process in 3T3-L1 differentiated cells. Interestingly, uncoupling protein 2 (*UCP2*), a mitochondrial gene that functions as uncouplers of oxidative phosphorylation thus dissipating energy as heat, was also up regulated by T3 [23]. The mechanism of this new discovery is still unknown. However, although the exact molecular mechanism of action is still contentious, it is reported that *UCP2* expression is activated by the peroxisome proliferator-activated receptors (PPARs) [24]. Contrary to our findings, T3 administration to 3T3-L1 differentiated cells significantly down regulated *PPAR-γ* and up regulated *UCP2* genes expressions (Fig. 6). Whether the up regulation *UCP2* gene expression is directly associated with PPAR transcription factor activation or by other unknown mechanism, this issue is still open for further research.

Besides this regulation of T3 to lipid metabolism-related genes transcription, protein expressions of these genes were also significantly regulated by T3 (Fig. 7). Importantly,

β-T3 was not able to show significant regulation to selected proteins even though it exhibited significant mRNA regulation to *SCD-1*, *ACC1*, and *LDLR* genes (Fig. 6). This might employ that the effect of β-T3 to these genes is confined only at mRNA level. This observation may relate to the processes between transcription and translation regulation. The correlation between transcription and translation level can vary sometimes as there are many steps and factors involved in the two processes. An mRNA may have a low expression profile, but may be stable and efficiently translated. To date, to the best of our knowledge, this is the first data reported concerning the wide regulation of T3 to crucial lipid metabolism-related protein expressions in differentiated preadipocytes.

The modulation of T3 to these crucial lipid metabolism-related proteins accentuates the possible reason as to the reported lipid-lowering effect of T3 [12, 25]. Furthermore, the underlying mechanism on how T3 (especially δ and γ isomers) significantly regulated both mRNA and protein expressions of crucial lipid metabolism-related genes can somehow be explained on its individual isomer molecular structure differences. This assumption has been currently in experimental development in our laboratory. Generally, adhering to these valuable findings, regulation of T3 on both mRNA and protein expressions of lipid metabolism related-genes would create a possible down and upstream modulation of interacting genes necessary for lipid metabolism which eventually could lead to the amelioration of abnormal lipid accumulations caused by aberrant lipid metabolic process. A thorough study on how these regulated genes by T3 affects the other interplaying genes necessary for the total cellular process of lipid metabolism are of great importance future studies to provide a probable lipid metabolism regulation genetic profile for T3.

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Conflict of Interest

The authors declare that they have no conflict of interest

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Figure legends

Fig. 1 Chemical structures of T3 and Toc.

Fig. 2 Effect of increasing concentrations of T3 or α-Toc (0-50 μM) on cell proliferation of 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were incubated with T3 or α-Toc for 24 h, and then cell proliferation was evaluated with WST-1 assay. Experimental procedures are shown in Materials and methods. Data are expressed as mean ± SD (n = 6). Means without a common letter differ, $P< 0.05$.

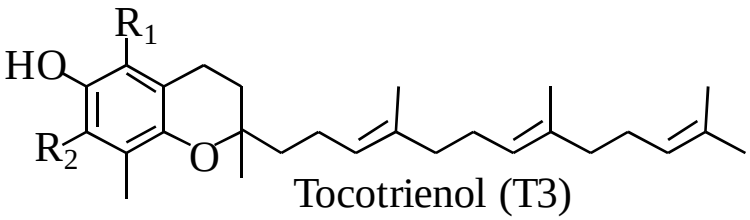
Fig. 3 Effect of increasing concentrations of T3 or α-Toc (0-50 μM) on TG levels of 3T3-L1 cells at 8 d after differentiation. Experimental procedures are shown in Materials and methods. Data are expressed as mean ± SD (n = 3). Means without a common letter differ, $P< 0.05$.

Fig. 4 Cellular uptake of T3 or Toc (25 μM) at 8 d after differentiation. Below detection limit of T3 and Toc cellular uptake were observed for control cells. Experimental procedures are shown in Materials and methods. Data are expressed as mean ± SD (n = 3).

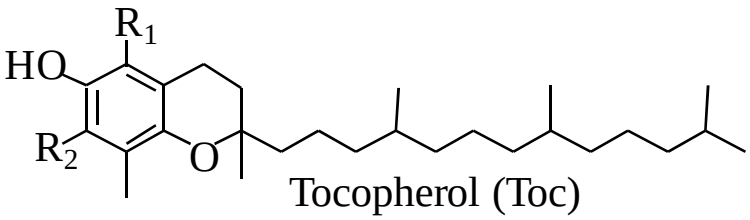
Fig. 5 Oil red O stained lipid droplets of 3T3-L1 cells treated with T3 or α-Toc (25 μM) from 0, 6, and 8 d after differentiation. Scale bar= 30 μm. Experimental procedures are shown in Materials and methods.

448 **Fig. 6** Effect of T3 or α-Toc (25 μM) on mRNA expression of *FAS*, *CPT1*, *SCD-1*,
449 *ACC1*, *SREBP 1c*, *ADIPOR2*, *UCP2*, *HMG-CoA-R*, *LDLR*, and *PPAR-γ* genes in
450 3T3-L1 cells at 8 d after differentiation. Experimental procedures are shown in
451 Materials and methods. Data are expressed as mean ± SD (n = 3). Means without a
452 common letter differ, $P < 0.05$.
453
454 **Fig. 7** Effect of T3 or α-Toc (25 μM) on FAS, CPT1, SCD-1, ACC1, SREBP 1c, UCP2,
455 LDLR, PPAR-γ, and β-actin protein expressions in 3T3-L1 cells at 8 d after
456 differentiation. Experimental procedures are shown in Materials and methods. Each
457 Western blot is a representative example of data from three replicate experiments.
458

459 Fig. 1



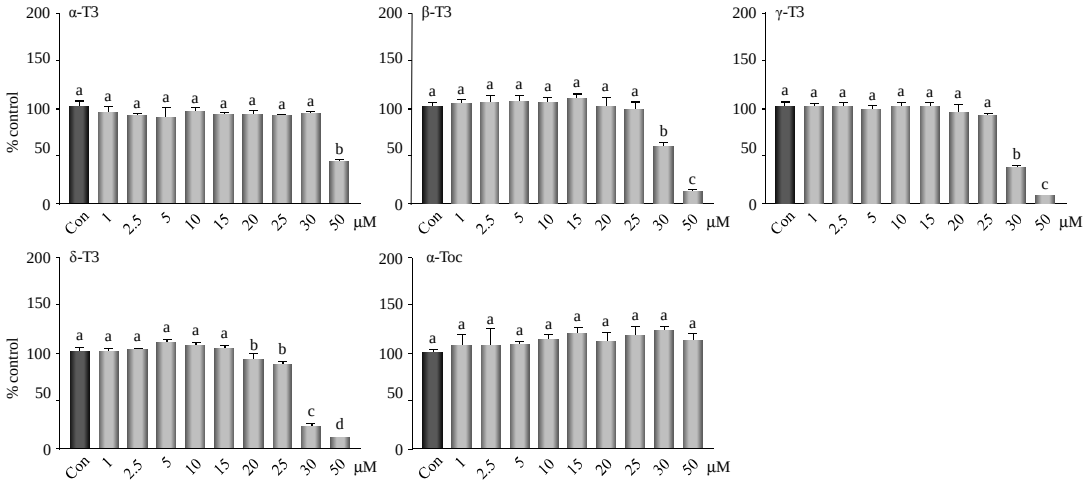
Tocotrienol (T3)



Tocopherol (Toc)

	R_1	R_2
α-	CH_3	CH_3
β-	CH_3	H
γ-	H	CH_3
δ-	H	H

460 Fig. 2



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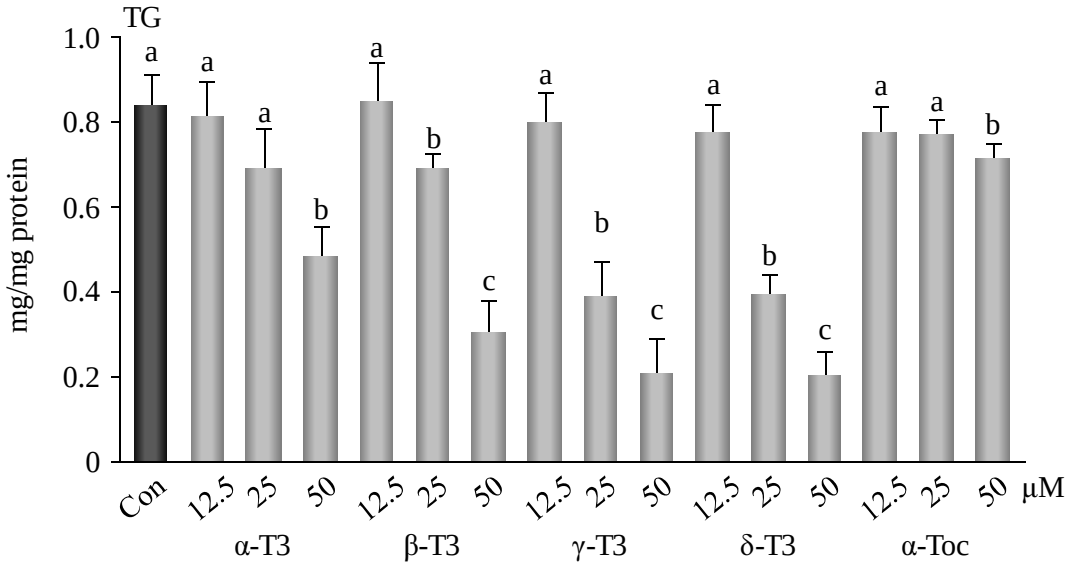
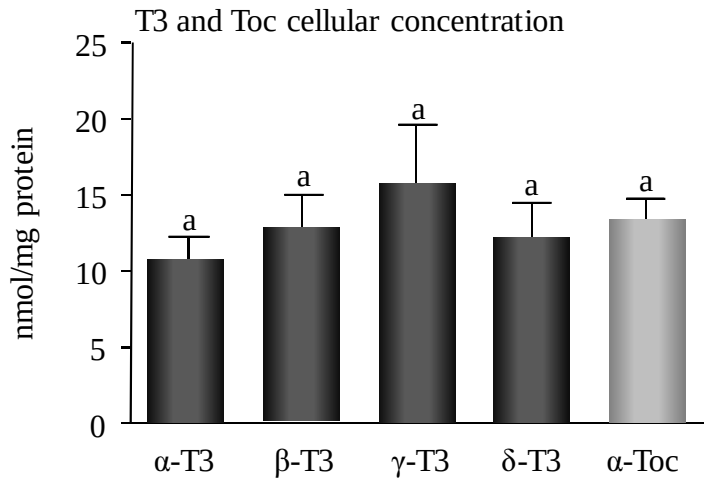
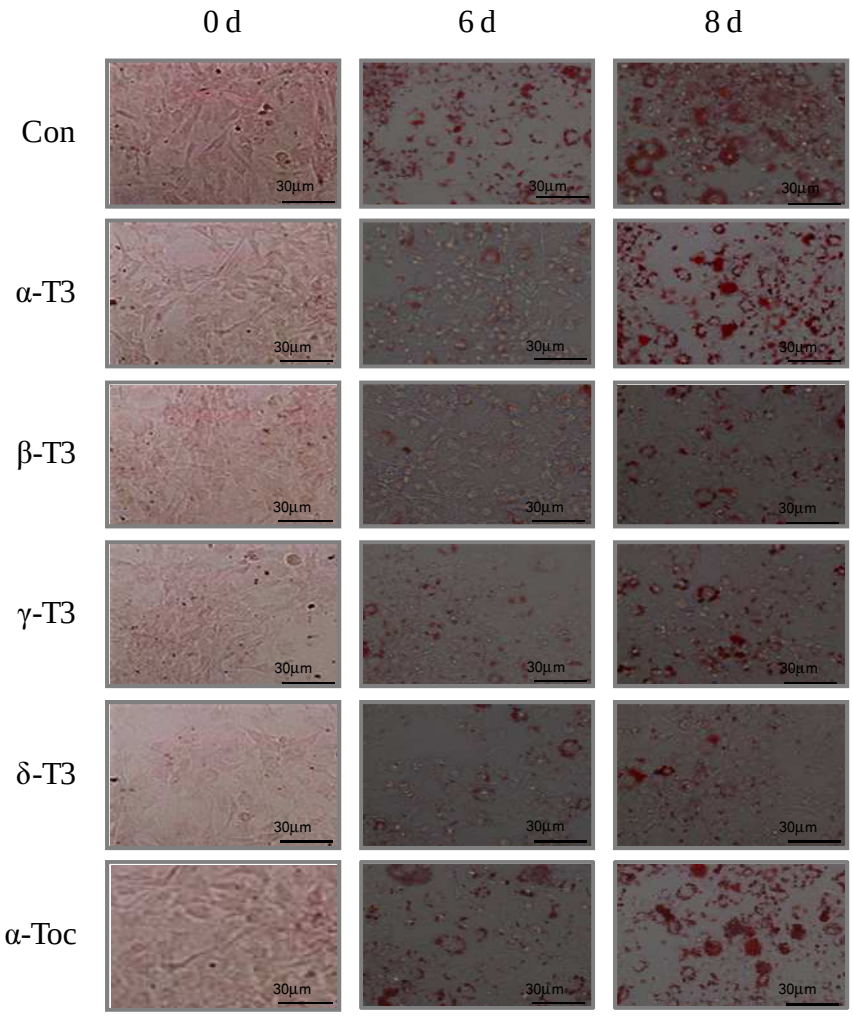


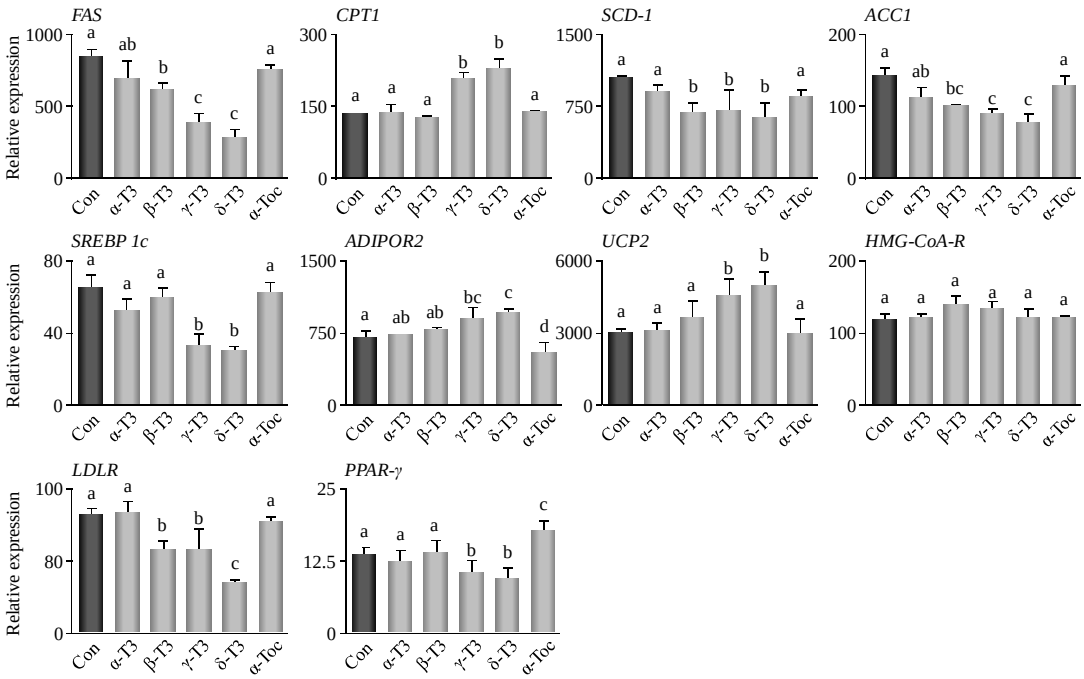
462 Fig. 4
463



464 Fig. 5



465 Fig. 6



466 Fig. 7