**Note**

**γ-Tocotrienol Attenuates Triglyceride through Effect on Lipogenic Gene Expressions in Mouse Hepatocellular Carcinoma Hepa 1-6**

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**Summary** Vitamin E is the generic name for tocopherol (Toc) and tocotrienol (T3), which have saturated and unsaturated side chains, respectively. Such differences allow T3 to be different from Toc in terms of their functions. T3 has been known to attenuate cholesterol (Cho) level by inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoAR). Recent reports also showed the efficacy of T3 in improving triglyceride (TG) profiles in both in vivo and in vitro studies. However, the mechanism involved in this biological activity is still unclear and needs to be further investigated. In the present study, we elucidated the effect of γ-T3 on lipid levels and lipogenic gene expressions in mouse hepatocellular carcinoma Hepa 1-6. γ-T3 showed attenuation of TG through effect on fatty acid synthase, sterol regulatory element-binding transcription factor 1, stearoyl CoA desaturase 1, and carnitine palmitoyl transferase 1A gene expression in Hepa 1-6. In contrast, the Cho level remained unchanged. These results expanded our previous finding of lipid-lowering effects of T3, especially for TG. Therefore, T3 is a potential lipid-lowering compound candidate with realistic prospects for its use as a therapy for lipid-related diseases in humans.

**Key Words** tocotrienol, triglyceride, lipogenic genes

Vitamin E is the generic name for tocopherol (Toc) and tocotrienol (T3), which have saturated and unsaturated side chains, respectively (Fig. 1A). Toc is widely present in a variety of foods, whereas T3-containing foods are limited. Rice bran, palm oil, and annatto seed are rich in T3 (1). T3 has recently gained increasing interest due to its several health-promoting properties such as antioxidative, neuroprotective and anti-cancer effects (2–4). Besides these biological activities, T3 has also achieved much attention for its lipid-lowering properties, especially the reduction of cholesterol (Cho) in both cell culture and animal studies, and the mechanisms may involve a repression of hepatic 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoAR) (5).

On the other hand, while little attention has been given to whether T3 affects other lipids besides Cho, some earlier studies suggested the efficacy of T3 in improving triglyceride (TG) profiles. For instance, T3 supplementation has been reported to decrease serum TG levels in healthy humans and hypercholesterolemic patients (6, 7). In support of these results, we and other researchers recently reported the ability of T3 to reduce TG biosynthesis in human hepatoma HepG2 cells (8, 9). Thus, these studies would entail the novel possibility that reduction of TG may be one of the primary roles for the lipid-lowering properties of T3. However, to the best of our knowledge, there is no study on the effect of T3 using liver cell lines other than HepG2. Moreover, there are few publications that evaluate the lipid-lowering effect of T3 at gene expression levels. Therefore, in this present study, we aimed to confirm the TG-lowering properties of γ-T3 and investigate how γ-T3 regulates lipogenic gene expression in mouse hepatocellular carcinoma Hepa 1-6 mainly by using PCR analysis.

**Materials and Methods**

**Cell culture.** Hepa 1-6 cells were obtained from the RIKEN cell bank (Tsukuba, Japan). The cells were cultured in DMEM medium containing 0.3 g/L L-glutamine and 2.0 g/L sodium bicarbonate (St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Biowest, Paris, France), 100 kU/L penicillin, and 100 mg/L streptomycin (Gibco BRL Rockville, MD, USA) at 37°C in 5% CO2 /95% air atmosphere in a humidified incubator.

**Preparation of experimental medium.** γ-T3 was purchased from Chromadex (Santa Ana, CA, USA), and dissolved in ethanol at a concentration of 50 mM. The stock solution was diluted with 10% FBS/DMEM medium to achieve the desired final concentration of γ-T3 (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 a control medium.

**Cell viability assay.** For cell viability assay, Hepa 1-6 cells (1×10^4) were pre-incubated with 10% FBS/DMEM

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in 96-well culture plates. Twenty-four hours later, the cells were washed with PBS and the medium was replaced with the experimental medium supplemented with or without γ-T3. After incubation for 24 h, the number of viable cells was determined using WST-1 reagent (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer’s instructions. 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, Inc., Hercules, CA, USA). WST-1 is a tetrazolium salt that is converted into the soluble formazan salt by succinate-tetrazolium reductase of active mitochondria of viable cells (10).

Lipid analysis. Cellular lipid and protein concentrations were measured as follows: Hepa 1-6 (1 × 10^5) were incubated with experimental medium in a 35 mm cell culture plate. After 24 h incubation, the cells were washed with PBS, scraped with rubber policeman, and homogenized. Total RNA was isolated from cell homogenate with an RNeasyPlus Mini kit (Qiagen, Valencia, CA, USA). cDNA was synthesized using a Ready-To-Go T-Primed First-Strand kit (GE Healthcare, Piscataway, NJ, USA), and PCR amplification was performed with a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, New South Wales, Australia) using SYBR Premix Ex Taq (Takara Bio Inc., Shiga, Japan) and gene-specific primers for fatty acid synthase (Fasn), sterol regulatory element-binding transcription factor 1 (Srebf1), stearoyl CoA desaturase 1 (Scd1), carnitine palmitoyl transferase 1A (Cpt1a) and β-actin (ACTB) (Table 1). PCR conditions were 95˚C for 60 s, 95˚C for 5 s, and 65˚C for 30 s for 40 cycles.

Results and Discussion
Hepa 1-6 cells are adherent and epithelial cells growing as a monolayer. This mouse hepatoma cell line was derived from the BW7756 tumor that arose in a C57L mouse. The cells secrete several liver-specific products (e.g. albumin, α1-antitrypsin, α-fetoprotein, and amylase), and are used by researchers as a good model of lipid metabolism (12).
We first evaluated the cytotoxicity of γ-T3 to Hepa 1-6 by using WST-1 reagent. Hepa 1-6 showed a slight increase in cell proliferation with γ-T3 treatment (1–15 μM) (Fig. 1B). The reasons for the induced cellular proliferation still remain unknown, but such increased proliferation was found when cells were incubated with certain antioxidants such as catechin (13). A higher dose of γ-T3 (30–50 μM) caused a cytotoxic effect, possibly due to apoptosis induction, as some papers have already reported (14). Since 1–15 μM γ-T3 showed no cytotoxicity, experiments for TG and Cho levels were conducted utilizing these concentrations (1–15 μM γ-T3).

Cellular TG and Cho were extracted by the Folch procedure (11), and measured by enzymatic methods. One to 5 μM γ-T3 showed no significant differences for TG concentrations; however, it was found that 10–15 μM γ-T3 revealed a significant decrease in TG levels (Fig. 2A). The results were in concordance with our previous findings of the TG-lowering effect of T3 in HepG2 cells (9). As for the Cho, no significant differences were observed among any concentrations of γ-T3 (Fig. 2B). As mentioned in the introductory paragraphs, current reports have elucidated the inhibitory effect of T3 on lipid accumulation with common emphasis on Cho (5). However, it should be noted that some reports showed no attenuation of Cho by T3 (15). Thus, the present findings imply the possibility that the lipid-lowering effect of T3 is primary mediated by the reduction of TG rather than Cho. The possibility requires further investigation, since TG and Cho are two most abundant lipids in the biological system and attenuation of these two lipid classes would be a great target for clinical application.

To evaluate the mechanisms underlying the TG-lowering effect of γ-T3, we next investigated the mRNA expressions of known lipogenic and β-oxidation genes namely Fasn, Srebf1, Scd1, and Cpt1a. Like our previous study using HepG2 cells (9), significant down regulation of Fasn and up regulation of Cpt1a genes were observed in 10–15 μM γ-T3-treated Hepa 1-6 (Fig. 2C and D). In addition, we found down regulation of Srebf1 and Scd1 (Fig. 2E and F), which are some of the key genes for lipid metabolism. Interestingly, Scd1, a gene that catalyzes a rate-limiting step in the synthesis of unsaturated fatty acids, was first suggested in our present study to be down regulated by γ-T3. Therefore, the regulation of these lipogenic genes (Fasn, Srebf1, Scd1, Cpt1a) might be the mechanisms of the TG-lowering effect of γ-T3 in Hepa 1-6 cells.

In this study, among T3 isomers, the effect of γ-T3 on TG levels and lipogenic gene expressions in Hepa 1-6 was evaluated. This is because the isomer is suggested to be potent in the regulation of lipid metabolism (8). Another reason is that the isomer is abundant in natu-
eral sources such as rice bran (1). Now, we are comparing the effect of T3 isomers using several cells (e.g., Hepa 1-6, HepG2, and 3T3-L1 cells), and initially found that γ-T3 and δ-T3 showed potent effects on TG levels. These results will be presented in the near future as a different story.

In conclusion, γ-T3 showed attenuation of TG through effect on Fasn, Srebf1, Scd1, and Cpt1a gene expression in Hepa 1-6. The result expanded our previous finding of the lipid-lowering effect of T3, especially for TG. The lipid biosynthesis has a multiple interplaying genes that work together to maintain the lipid homeostasis in the biological system and aberration of its cellular environment would cause dramatic change in the expression of these genes which would lead to lipid-related afflictions. Therefore, understanding the complexity of lipid biosynthesis at the gene expression level is of vital importance for the development of natural medicine products (e.g., T3) against lipid-related diseases. Consequently, further studies pertaining to the lipid-lowering effect and mechanism of T3 are recommended to provide ample evidence for a realistic prospect of its use as a human therapy.

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REFERENCES