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Elucidation of the novel biological function of tocotrienol on lipid metabolism and cancer regulations

著者	Burdeos Gregor Carpentero
学位授与機関	Tohoku University
学位授与番号	11301甲第15904号
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Chapter 1

Tocotrienol attenuates triglyceride accumulation in HepG2 cells and F344 rats

Introduction and objective

In 1922, tocopherol was discovered in green leafy vegetables as a micronutrient essential for reproduction [1]. More than 40 years later, tocotrienol was isolated from latex [2]. Structurally, these vitamin E 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. Vitamin E is synthesized in plastids of plants, and Toc is widely present in a variety of foods such as vegetable oils and nuts. But, T3-containing foods are limited. Rice bran, palm oil, and annatto seed are rich in T3 [3].

Toc and T3 are classified based on their ability (vitamin E activity) to prevent the resorption of rat fetuses. α-Toc displays the highest efficacy among the eight vitamin E, whereas α-T3 has about one third of the activity of α-Toc. Regardless, all forms of vitamin E are able to induce antioxidative effects and to act as protective agents against lipid peroxidation in biological membranes. In some model membrane studies, T3 has been reported to be a more potent antioxidant than Toc [4]. Moreover, 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 [5], and suppresses pathological angiogenesis [6,7] and cancer [8]. These unique effects of T3 could be partly explained by its abilities to induce cell cycle arrest [9], to activate p53 and caspase [10,11], to suppress adhesion molecules [12], to inhibit nuclear factor-κB [13], and to down-regulate c-Myc and telomerase [14].

Besides the above activities [5-14], T3 has also gained much attention for its lipid-lowering properties, especially reduction of cholesterol (Cho). The Cho-lowering effects have been observed in cell culture [15-17], animal [18-21], and human studies [22], and the mechanism may involve a repression of hepatic 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoAR) through a post-transcriptional process [15,16]. However, it should be noted that several research groups failed to observe significant changes in serum Cho profiles after supplementation of T3 to hypercholesterolemic patients [23-25] and hyperlipidemic patients with carotid atherosclerosis [26]. Therefore, the effect of T3 on Cho is still controversial and needs further studies.

On the other hand, although little attention has been paid to whether T3 affects other lipids except for Cho, some earlier studies have suggested a usefulness of T3 in improving triglyceride (TG) profiles. For instance, T3 supplementation has been reported to decrease serum TG levels in healthy human [27] and hypercholesterolemic

patients [28]. In support of these results, Zaiden et al. [29] recently reported an ability of T3 to reduce TG biosynthesis in human hepatoma cells (HepG2). Therefore, these studies [27-29] raise a novel possibility that reduction of TG may be a primary role for the lipid-lowering properties of T3. However, because there are few literatures that have evaluated the impact of T3 on TG at gene expression levels, further biological studies are needed to elucidate the mechanism(s) how T3 decreases TG.

In this study, we aimed to investigate how γ-T3 (most abundant isomer of rice bran T3, RBT3) suppresses TG levels by affecting TG-related gene expressions in normal and fat (oleic acid) loaded HepG2. In high fat diet fed rats, the TG-lowering properties of RBT3 was further evaluated, and compared with Cho-lowering activity. We confirmed that T3 attenuates TG accumulations by regulating fatty acid synthase (FASN), carnitine palmitoyltransferase 1 (CPT1A), and cytochrome P450 3A4

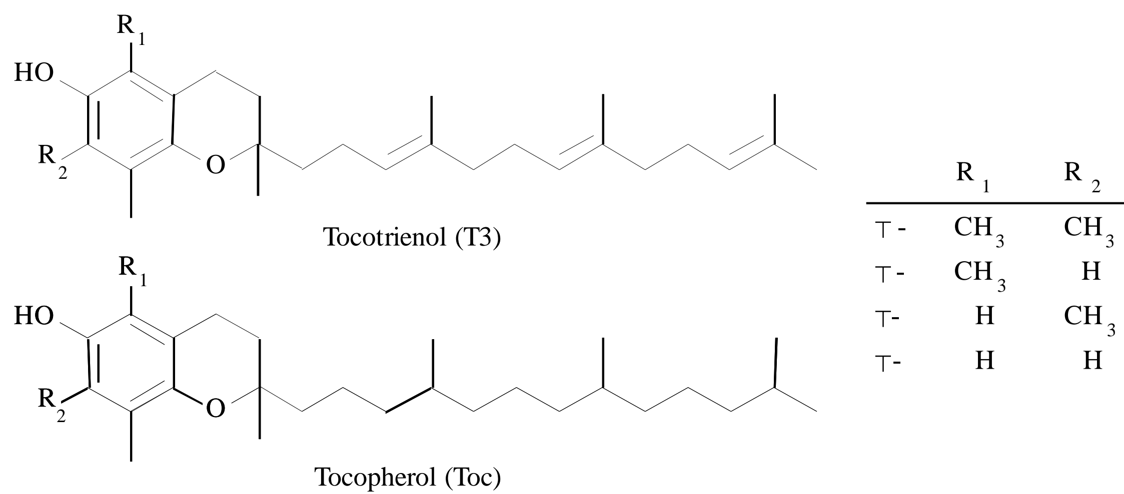


Fig. 1 Chemical structures of T3 and Toc.

Chapter 1: *In vitro* and *in vivo* attenuation of triglyceride accumulation by tocotrienol

Part 1. Attenuation of triglyceride accumulation by tocotrienol in HepG2 and F344 rats

1. Materials and Methods

1.1 Reagents and Cells

α-T3 and γ-T3 were purchased from Chromadex (Santa Ana, CA, USA). α-Toc was obtained from Sigma (St. Louis, MO, USA) and WST-1 reagent from Dojindo Laboratories (Kumamoto, Japan). All other reagents were of analytical grade. RBT3 was kindly provided by Sanwa Yushi Co. Ltd. (Tendo, Yamagata, Japan). RBT3 was composed of 82.3% T3 (31.4% α-T3, 50.5% γ-T3, and 0.4% δ-T3) and 6.0% Toc (1.9% α-Toc, 2.1% γ-Toc, and 2.0% δ-Toc) (w/w). Vitamin E-stripped corn oil was purchased from Acros Organics (Fairlawn, NJ, USA).

HepG2 were obtained from the RIKEN cell bank (Tsukuba, Japan). The cells were cultured in RPMI-1640 medium (containing 0.3 g/L L-glutamine and 2.0 g/L sodium bicarbonate, Sigma) supplemented with 10% fetal bovine serum (FBS; Biowest, Paris, France), 100 kU/L penicillin, and streptomycin (100 mg/L) (Gibco BRL

Rockville, MD, USA) at 37 °C in 5% CO_2/95% air atmosphere in a humidified incubator.

1.2 Preparation of Experimental Medium for Cell Culture Study

γ-T3 was dissolved in ethanol at a concentration of 50 μM. The stock solution was diluted with either 1% bovine serum albumin (BSA; Sigma)/RPMI-1640 (control test medium) or 1% BSA/RPMI-1640 containing 1 mM oleic acid as fat-loaded medium [30] 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 control medium.

1.3 Cell Viability Assay

For cell viability assays, HepG2 (1×10^5) were pre-incubated with 10% FBS/RPMI-1640 in 96 well culture plates. Twenty-four hours later, the cells were washed with PBS and medium was replaced with the experimental medium. As for

the control condition, HepG2 were incubated in the experimental medium without oleic acid together with 0-50 μM γ-T3. In fat-loaded condition, HepG2 were cultured with experimental medium containing 1 mM oleic acid (as fat-loaded medium) and γ-T3. After incubation for 24 h, the number of viable cells was determined using WST-1 reagent 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 the respiratory chain of active mitochondria of viable cells [31].

1.4 TG Analysis

Cellular TG concentrations were evaluated using Folch method [32]. Briefly, HepG2 (1×10^5) were pre-incubated with 10% FBS/RPMI-1640 in a 35 mm cell culture plate for 24 h, and were cultured under control and fat-loaded conditions as described above. After 24 h incubation, cells were washed with PBS and scrapped using rubber policeman, transferred into eppendorf tube (1.5 mL) and were centrifuged for 1,000*g* for

3 minutes. 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 (Bio-Rad Laboratories) [33]. Cellular TG was extracted using Folch method and was measured using commercial TG kit (Wako, Osaka, Japan).

1.5 Isolation of Total RNA and Analysis of mRNA Expression

After 24 h treatment of HepG2 (1×10^5) with the experimental medium, cells were lysed, scrapped with rubber police man, and transferred into a micro smashing tube. Total RNA was isolated with an RNeasy Plus Mini kit (Qiagen, Valencia, CA, USA) for real-time quantitative reverse transcription-PCR (RT-PCR). 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 *FASN, CPT1A, CYP3A4,* and *β-actin*. PCR conditions were 95 °C for 60 s, 95 °C for 5 s, and 65 °C for 30 s for 40 cycles.

1.6 Western Blotting Analysis

After HepG2 (1×10^5) both in control and fat-loaded conditions were incubated with test medium for 24 h, proteins were separated by SDS-PAGE (4-20% e-PAGEL; Atto, Tokyo, Japan). The protein bands were transferred to polyvinylidene fluoride membranes (Invitrogen, Carlsbad, CA, USA). After blocking for 1 h, membranes were incubated with primary antibodies for FASN, CPT1A, CYP3A4, and β-actin (Cell Signaling Technology, Beverly, MA, USA), 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 (BioRad, Hercules, CA, USA).

1.7 Animal Experiment

Male F344 rats (6 weeks of age) were obtained from CLEA (Tokyo, Japan), and housed in cages kept at 23 °C with a 12 h light:dark cycle. The rats were acclimated with MF rodent chow (5% of calories from fat; Oriental Yeast, Tokyo, Japan) and water for 1 week. After acclimatization, rats received *ad libitum* either MF or high fat diet

(35% of calories from fat (powdered beef tallow 15%/100 g and high oleic acid safflower oil 20%/100 g), High Fat Diet HFD32, CLEA). The high fat treated rats were fed with 5 mg of RBT3 (1.57 mg α-T3, 2.52 mg γ-T3, 0.02 mg δ-T3, 0.10 mg α-Toc, 0.11 mg γ-Toc, and 0.10 mg δ-Toc), 10 mg of RBT3 (3.14 mg α-T3, 5.04 mg γ-T3, 0.04 mg δ-T3, 0.19 mg α-Toc, 0.21 mg γ-Toc, and 0.20 mg δ-Toc), or 10 mg α-Toc once a day for 3 weeks by oral gavage using 200 μL vitamin E-stripped corn oil as a vehicle. Positive (high fat diet treated) and negative (MF rodent chow treated) control rats received only the vehicle (vitamin E-stripped corn oil). During the feeding period, weight of rats and food intake were recorded. At the end of the 3 week period, the rats were fasted for 12 h, weighed, and blood samples were collected by decapitation. Blood was treated with EDTA, and plasma was isolated by centrifugation at 1,000*g* for 15 min at 4 °C. Liver, heart, lung, spleen, kidney, and adipose tissues were removed and weighed. These protocols were reviewed by the Committee on the Ethics of Animal Experiments and carried out in accordance with the Animal Experiment Guidelines of Tohoku University.

1.8 Hepatic and Plasma Parameters

Livers were homogenized in aqueous solution containing 1 mM EDTA and 0.09% NaCl. The liver homogenates were then subjected to lipid extraction by Folch method [32]. Total lipid extracts were subjected to TG, total Cho (T-Cho), and phospholipid (PL) analyses using commercial kits (Wako).

For plasma samples, TG, T-Cho, and PL were measured by Mitsubishi Chemical Medicine Corporation (Tokyo, Japan).

1.9 Plasma and Liver Phospholipid Hydroperoxides

To examine oxidative stress, phospholipid hydroperoxides (PLOOH) in total lipid extracts from plasma and liver were measured by HPLC with chemiluminescence (CL) detection [34, 35]. PLOOH is a collective term for phospholipid hydroperoxides such as phosphatidylcholine hydroperoxide (PCOOH) and phosphatidylethanolamine hydroperoxide (PEOOH). The column was a Finepak SIL NH2-5 (4.6 × 250 mm; Japan Spectroscopic Co., Tokyo, Japan), the eluent was 2-propanol-methanol-water (135:45:20, v/v/v), and the flow rate was 1 mL/min. Post-column CL detection was

carried out using a CLD-100 detector (Tohoku Electronic Industries Co., Sendai, Japan). A mixture of luminol and cytochrome c in 50 mM borate buffer (pH 10.0) was used as a hydroperoxide-specific post-column CL reagent. Calibration was carried out using standard PLOOH.

1.10 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 $P < 0.05$.

1.11 Results and discussion

1.11.1 Effect of γ-T3 on HepG2 Proliferation

In order to investigate whether T3 affects TG concentrations, increasing amount of γ-T3 was treated to HepG2 for 24 h in both control as well as fat-loaded conditions. Before investigating the effect on TG, cytotoxic activity of γ-T3 was first evaluated.

As for the control condition, HepG2 proliferation was somewhat increased by γ-T3 treatment (1-5 μM), while 10-15 μM γ-T3 did not affect on the proliferation (**Fig. 2A**). As some papers have already reported [36], higher dose (over 15 μM) of γ-T3 showed cytotoxic effect (data not shown). Similar effect of γ-T3 was observed under fat-loaded condition, although the condition showed a more increased proliferation than control condition (**Fig. 2A**). Because 1-15 μM γ-T3 showed no cytotoxicity except for 15 μM γ-T3 in fat-loaded condition showing little decrease of proliferation, we utilized these γ-T3 concentrations in the next experiments for evaluating their effects on TG.

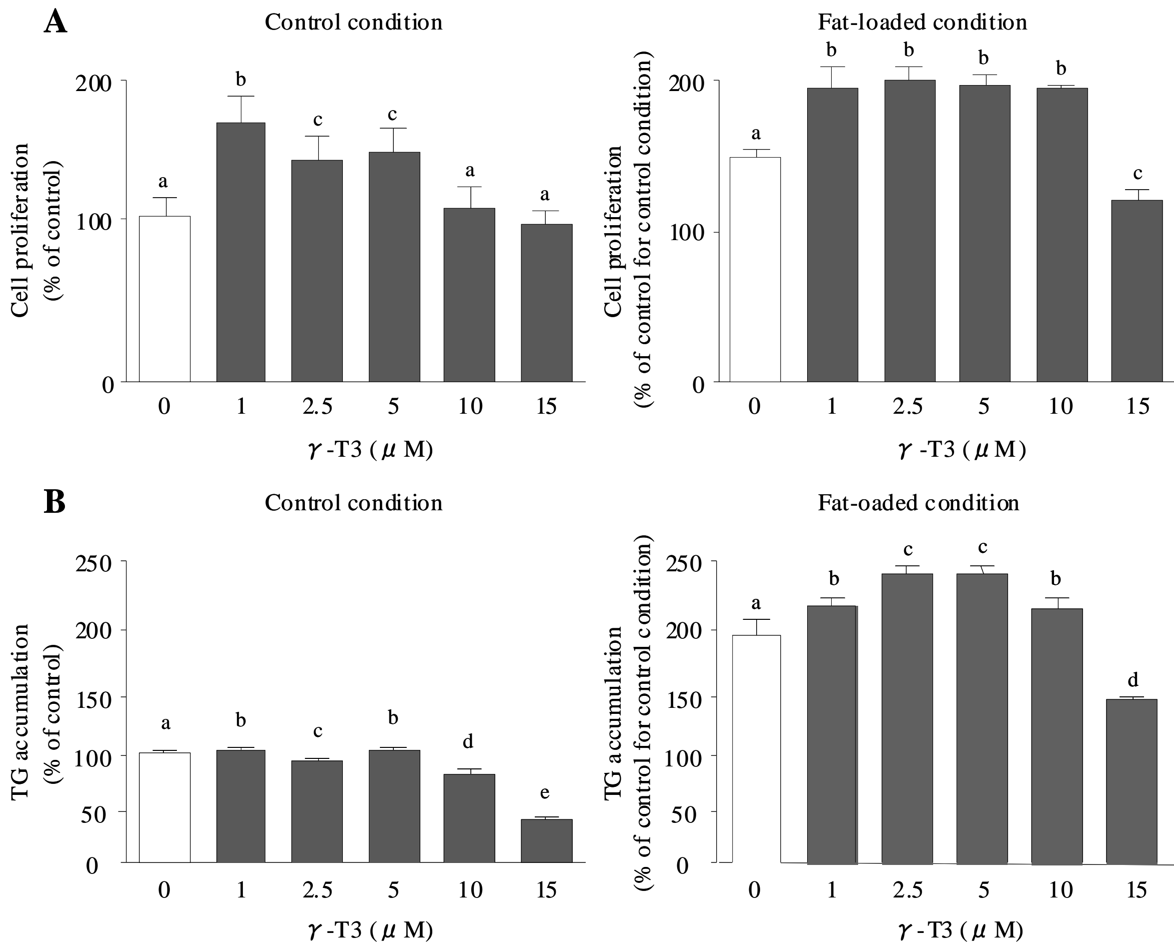


Fig. 2 Effect of increasing concentrations of γ-T3 on cell proliferation (A) and TG accumulation (B) of HepG2 in normal and fat overloaded conditions. Experimental procedures are shown in Materials and Methods. Data are expressed as mean ± SD (n = 6 for cell proliferation and n = 3 for TG accumulation). Means without a common letter differ, $P < 0.05$.

1.11.2 TG Accumulation is Attenuated with γ-T3 by Affecting Fatty Acid Synthase and β-Oxidation Genes and Proteins Expressions in HepG2

TG accumulation in HepG2 was evaluated by Folch method. For control condition, 1-5 μM γ-T3 showed a little effect on TG concentrations (**Fig. 2B**), probably due to somewhat increase of the proliferation. But, it was found that 10-15 μM γ-T3 revealed a marked decrease of TG concentration. For fat-loaded condition, 1-10 μM γ-T3 treated cells showed an increase in TG concentrations, whereas a significant decrease of TG was observed at 15 μM. The reason why 1-10 μM γ-T3 supplementation increases TG concentration may be due increased cell proliferation. These data clearly suggested the TG-lowering effect of T3, and we therefore investigated the effect of γ-T3 on TG-synthesis related gene (FASN) and β-oxidation genes (CPT1A and CYP3A4). Significant down-regulation of FASN as well as up-regulation of CPT1A and CYP3A4 genes were observed by γ-T3 treatment (over about 10 μM) in both control and fat-loaded conditions (**Fig. 3**). Similarly, γ-T3 attenuated the expression of FASN protein and at the same time induced the expression of CPT1A and CYP3A4 proteins (**Fig. 4**). Therefore, a possible mechanism of the TG-lowering effect of γ-T3 is mediated by down-regulation of FASN and up-regulation

of CPT1A and CYP3A4 genes.

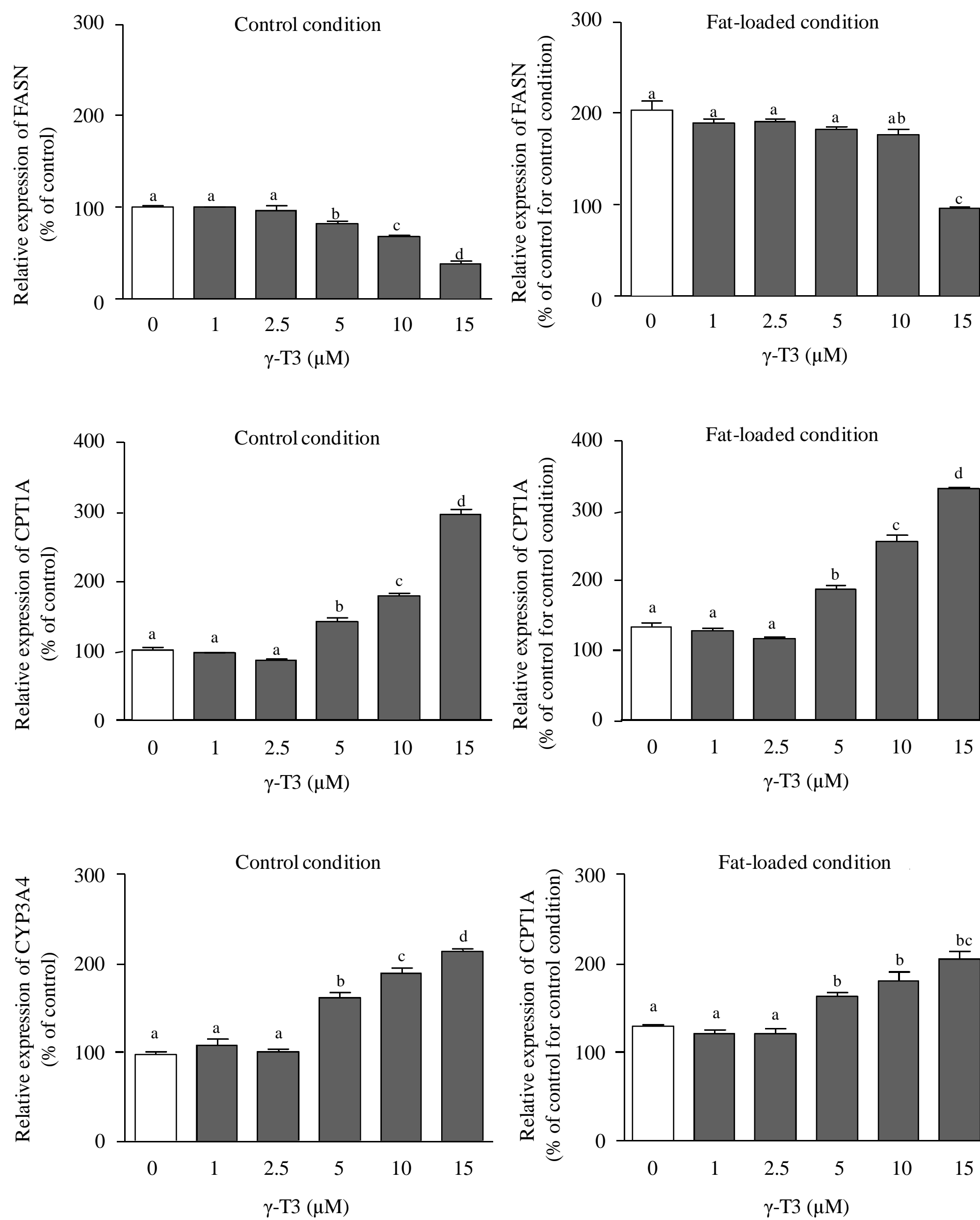


Fig. 3 Effect of increasing concentrations of γ-T3 on mRNA expression of FASN, CPT1A, and CYP3A4 genes in HepG2 in normal and fat overloaded conditions. Experimental procedures are shown in Materials and Methods. Data are expressed as mean ± SD (n = 6). Means without a common letter differ, $P < 0.05$.

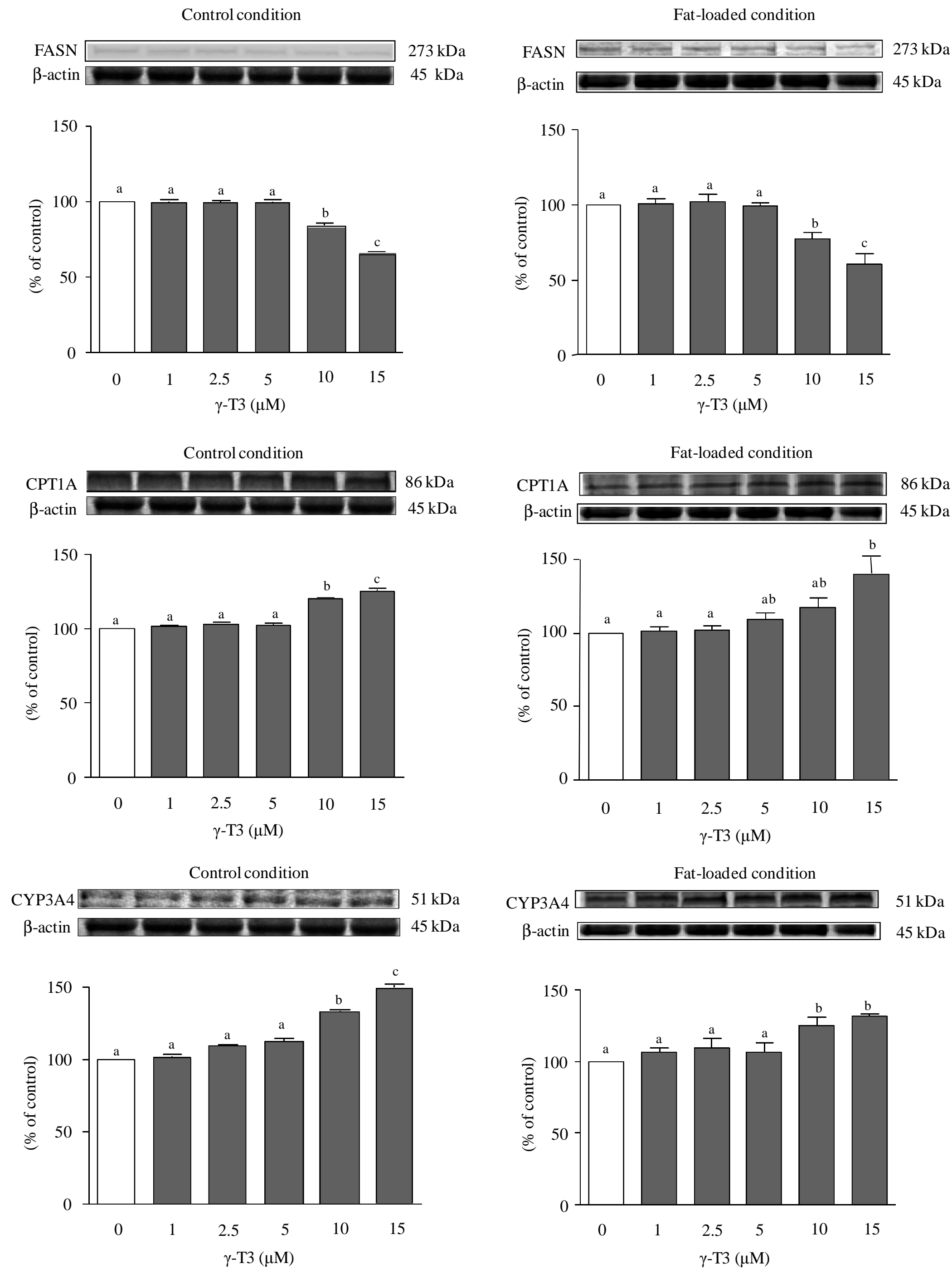


Fig. 4 Effect of increasing concentrations of γ-T3 on expression of FASN, CPT1A, and CYP3A4 proteins in HepG2 in normal and fat overloaded conditions. Experimental procedures are shown in Materials and Methods. Each Western blot is a representative example of data from three replicate experiments.

1.11.3 Dietary T3 Reduces TG and PLOOH accumulations in Liver and Plasma of Rats

To verify the TG-lowering effect of T3 in vivo, a rat study was conducted. Rats received either control MF or high fat diet with or without RBT3 for 3 weeks. High fat diet caused significant increase of the weight of rats, and the weight tended to decrease (P = 0.34) in 10 mg RBT3 group (**Table 2**). RBT3 supplementation did not affect the weight of organs, but adipose tissues, especially epididymal and mesenteric fats, showed reduction of the weight in 10 mg RBT3 group (**Table 1**). No differences were observed for food intake (data not shown). For liver parameters, high fat diet caused significant accumulation of TG and tended to induce PL (P = 0.11) accumulation, and also showed a significant accumulation of PLOOH (oxidative stress marker) (**Fig. 5**). It was found that these accumulations, especially TG and PLOOH, were significantly attenuated by 10 mg RBT3 supplemented group (**Fig. 5**). Similar results were observed for plasma (**Fig. 6**). In contrast, no significant differences were observed for liver and plasma T-Cho. For α-Toc supplemented group, no differences were found for lipids and PLOOH parameters in liver and plasma. These results indicated that the main lipid lowering effects of dietary T3 might be mediated by the

reduction of TG, thereby possibly attenuating the TG-induced PLOOH accumulation

[37]

Table 1. Body and tissue weights of rats (g).

	Body weight	Liver	Heart	Lung	Spleen	Kidney	Perinephiric fat	Mesenteric fat	Epididymal fat
Control MF	192±9.3 [a]	6.54±0.62 [a]	0.70±0.07 [a]	0.83±0.08 [a]	0.47±0.03 [a]	1.26±0.10 [a]	2.50±0.65 [a]	2.24±0.49 [a]	2.81±0.82 [a]
High fat diet	213±10.5 [b]	6.40±0.60 [a]	0.71±0.04 [a]	1.01±0.14 [a]	0.46±0.05 [a]	1.34±0.08 [a]	4.93±0.95 [b]	3.77±0.43 [b]	4.46±0.92 [b]
5 mg RBT3	219±9.7 [b]	6.78±0.71 [a]	0.71±0.05 [a]	1.18±0.22 [a]	0.50±0.03 [a]	1.39±0.09 [a]	4.98±0.31 [b]	3.79±0.27 [b]	4.73±0.68 [b]
10 mg RBT3	206±15.3 [ab]	6.19±0.54 [a]	0.67±0.05 [a]	0.88±0.19 [a]	0.46±0.02 [a]	1.30±0.11 [a]	3.73±0.65 [c]	2.97±0.30 [a]	3.93±0.60 [a]
10 mg α-Toc	202±11.8 [ab]	6.35±0.51 [a]	0.66±0.06 [a]	1.08±0.16 [a]	0.47±0.04 [a]	1.31±0.07 [a]	3.76±0.59 [c]	3.76±0.81 [b]	3.99±0.61 [a]

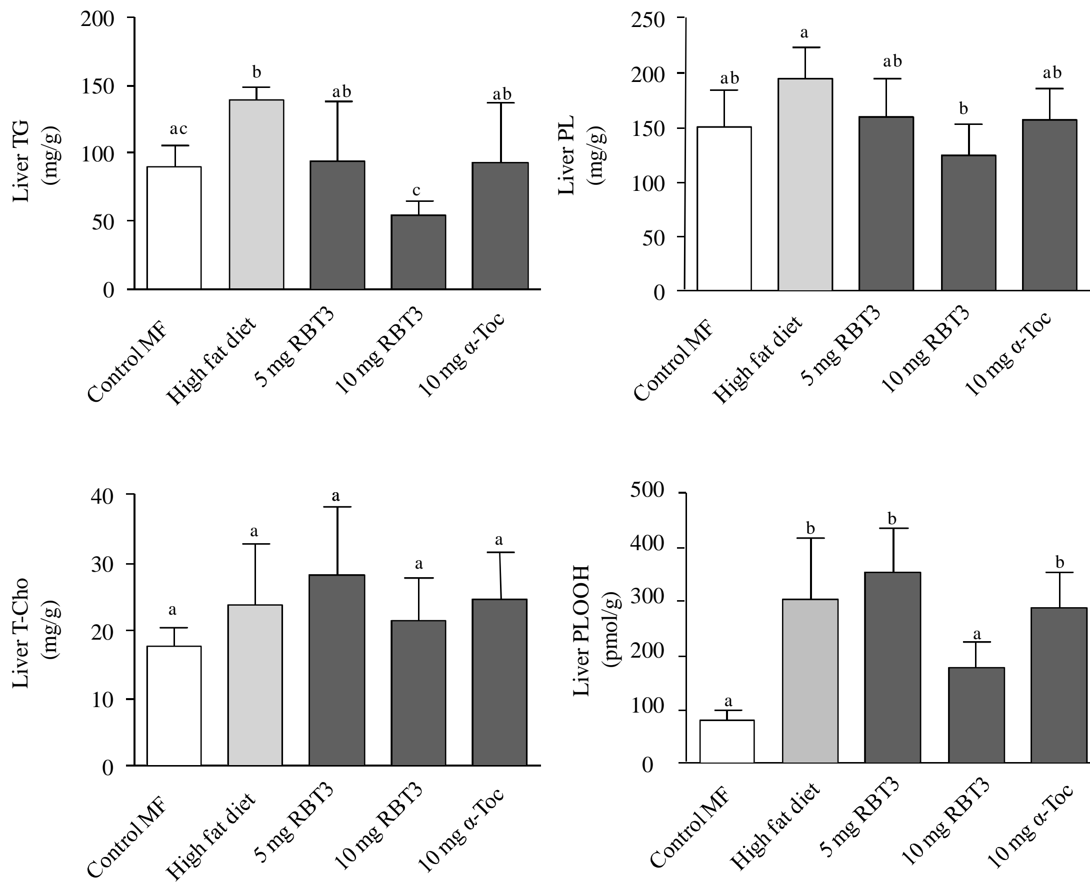


Fig. 5 Effect of RBT3 supplementation on liver lipids (TG, PL, and T-Cho) and oxidative parameter (PLOOH) of rats receiving control MF or high fat diet for 3 weeks. Experimental procedures are shown in Materials and Methods. Because phosphatidylcholine hydroperoxide (PCOOH) and phosphatidylethanolamin hydroperoxide (PEOOH) are major forms of PLOOH in liver [32, 33], PLOOH is calculated as sum of PCOOH and PEOOH. Data are expressed as mean ± SD (n = 7). Means without a common letter differ, $P < 0.05$.

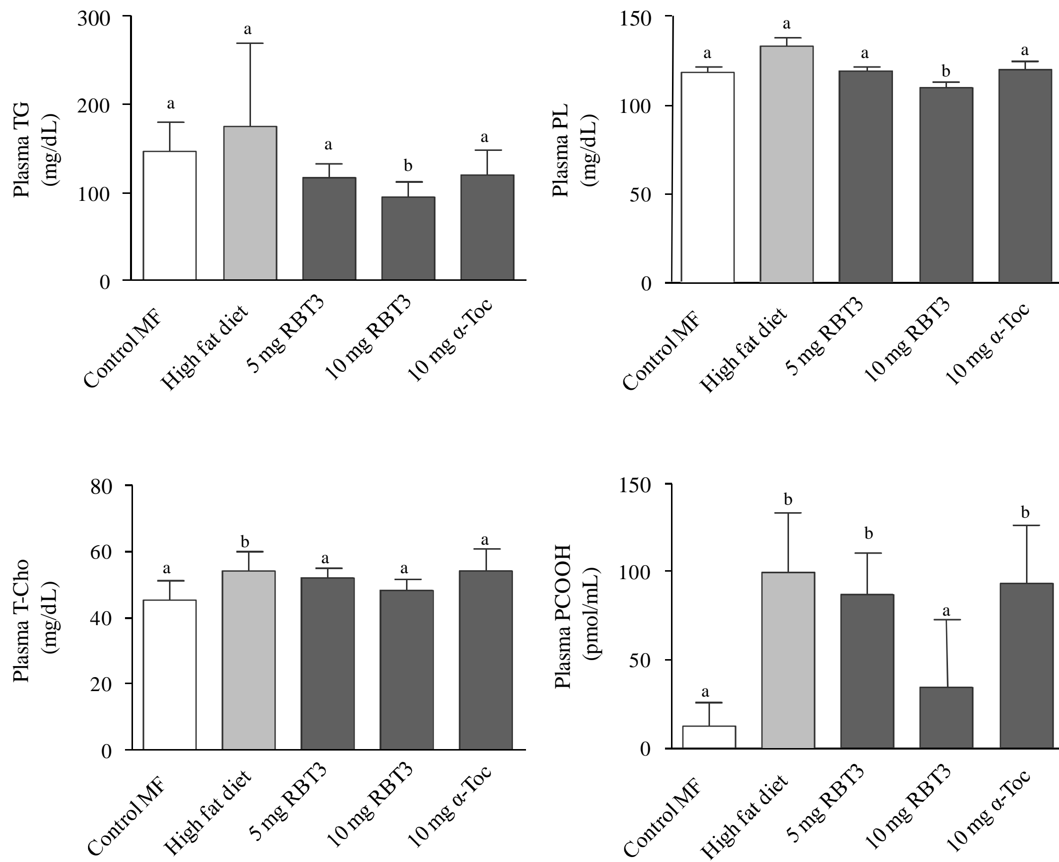


Fig. 6 Effect of RBT3 supplementation on plasma lipids (TG and PL) and oxidative parameter (PLOOH) of rats receiving control MF or high fat diet for 3 weeks. Experimental procedures are shown in Materials and Methods. Because PCOOH is a major form of PLOOH in plasma [32, 33], PCOOH content is presented as PLOOH. Data are expressed as mean ± SD (n = 7). Means without a common letter differ, $P < 0.05$.

1.11.4 Discussion

TG accumulation has been linked to cascade different afflictions such as coronary artery diseases. In addition, the involvement of oxidative stress (i.e., PCOOH formation) probably due to high TG concentration in vivo has been reported [37]. On the other hand, a novel possibility pertaining to the attenuating effect of T3 on TG accumulation has been published in some (but not many) studies [27-29]. Since the specific target of T3 in the lipogenic pathway is still vague, further biological studies are needed to elucidate the mechanism(s) on how T3 decreases TG. In the present study, we confirmed TG-lowering effect of T3 in both *in vivo* and *in vitro* studies, and the effect of T3 could be partly explained by its ability to down-regulate hepatic fatty acid synthase gene and up-regulate β-oxidation genes. These results support a hypothesis that reduction of TG may be the primary benefit caused by dietary T3 [29].

In this study, similar to previous papers [36], higher dosage of γ-T3 (over 15 μM) caused cytotoxicity to HepG2 possibly due to apoptosis induction (**Fig. 2A**). However, low dosage of γ-T3 (1-5 μM) somewhat increased HepG2 proliferation (**Fig. 2A**). The reasons of the induced cellular proliferation still remain unknown, but such increased proliferation was found when cells were incubated with certain antioxidants such as

catechin [38]. Consequently, because 1-15 μM γ-T3 showed no obvious cytotoxicity, we utilized these γ-T3 concentrations in the next experiments for evaluating their effects on TG.

For TG concentration, addition of oleic acid as fat inducer showed around 1.5 fold increase of TG in HepG2 as compared to control condition (**Fig. 2B**). Such increase was markedly attenuated by 15 μM γ-T3. Similar effect was observed to its control condition counterpart. To date, to the best of our knowledge, there has been one report focusing on the effect of T3 on TG in cell culture study. Zaiden et al. [29] reported that treatment of γ-T3 and δ-T3 (each 20 μM) reduces TG synthesis and VLDL secretion in HepG2, however this study did not measure the actual TG content of the cells. The present study provides quantitative TG results in the cells after administration of γ-T3, and significant attenuation of TG was observed at lesser concentrations of γ-T3 (15 μM).

In the present study, we provided new information regarding the possible mechanism of action of γ-T3 for its TG lowering property by down regulation of FASN and up regulation of CPT1A and CYP3A4 in both mRNA and protein levels (**Figs. 3 and 4**). Way et al. [39] showed that down regulation of FASN, key enzymes for lipogenesis by Puerh tea supplementation can attenuate lipid accumulation such as TG

in HepG2 cells. On the other hand, β-oxidation genes such as CPT1A and CYP3A4 have different interplaying roles in lipid metabolism. For instance, CPT1A, rate-limiting enzyme for fatty acid oxidation in all tissues was up-regulated in HepG2 upon consumption of polyunsaturated fatty acids, polyphenol and L-carnitine which led to reduced plasma lipid concentrations in human study [40]. Moreover, CYP3A4, one of the most important enzymes involved in the metabolism of xenobiotics in the body, has been reported to participate in lipid metabolism by regulation of 25-hydroxycholesterol in mouse liver cells derived cell line [41]. In support to these reports [39-41], administration of γ-T3 in HepG2 significantly down regulated FASN and up-regulated CPT1A and CYP3A4 genes thereby attenuating TG accumulations (**Figs. 2B and 3**). In addition, similar effects were observed in the protein expression of these genes (**Fig. 4**).

In support to cell culture study, 10 mg RBT3 diet significantly attenuated the accumulation of TG in both liver and plasma of rats (**Figs. 5 and 6**). T3 has been reported to have a potent antioxidative effect [4]. High fat diet has been reported to increase oxidative stress and resulted in increase of plasma and liver PLOOH [37]. It is therefore conceivable that T3 can markedly reduce high fat diet-induced PLOOH accumulation in vivo (**Figs. 5 and 6**). In contrast, dietary RBT3 failed to affect T-Cho

concentrations (**Figs. 5 and 6**). Concerning the reported Cho lowering effect of T3, Querishi et al. [42] actually demonstrated that the effect of T3 was attenuated by α-Toc. Therefore, the unchanged Cho levels may be due to the presence of α-Toc in the RBT3 (around 0.6 mg for 10 mg RBT3) used in this study. On the other hand, α-Toc did not show any attenuation of TG (**Figs. 5 and 6**). In support to this peculiarity, a study published by Punithavathi et al. [43] showed no effect of α-Toc supplementation alone on TG in an isoproterenol-induced myocardial infarcted Wistar rats. Moreover, our study [43] reported that α-Toc attenuated the cytotoxic effect of δ-T3 in human colorectal adenocarcinoma cells. Present study may implicate that among all vitamin E family isomers, a more detailed research between the interactions of these isomers is of great interest. In addition, since the amount of α-T3 present in RBT3 is considerably high, α-T3 was then subjected to proliferation and TG assays to remove the doubt that this T3 isomer might contributed in the TG attenuating effect of T3 (γ-T3). It was found out that α-T3 did not show any cytotoxicity nor attenuated the TG accumulations in HepG 2 cells (**Supplementary figure 1**). Interestingly, supplementation of RBT3 (10 mg/day) as well as α-Toc showed significant reduction of the weight of both perinephric and epididymal fats of rats (**Table 1**), and therefore we now investigating the possible mechanism behind this interesting attenuating effect of

α-Toc on adipose tissue deposition.

The lipid biosynthesis has a broad array of interplaying genes in its pathway thereby a thorough testing of any possible genes that regulates lipid synthesis and nailing down these genes would provide a more adequate knowledge about the right target for the discovery of lipid-related diseases medicine. The TG attenuating effect of T3 in both *in vivo* and *in vitro* in this study provides a novel data about the new TG lowering function of T3.

Chapter 1: *In vitro* and *in vivo* attenuation of triglyceride accumulation by tocotrienol

Part 2. γ-Tocotrienol Attenuates Triglyceride through Effect on Lipogenic Gene Expressions in Mouse Hepatocellular Carcinoma Hepa1-6

1.2. Materials and methods

1.2.1 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), 100 kU/L penicillin, and 100 mg/L streptomycin (Gibco) at 37 °C in 5% CO_2/95% air atmosphere in a humidified incubator.

1.2.2 Preparation of experimental medium

γ-T3 was purchased from Chromadex (Santa Ana, CA, USA), and dissolved in ethanol at a concentration of 50 μM. The stock solution was diluted with10% 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 control medium.

1.2.3 Cell viability assay

For cell viability assay, Hepa 1-6cells (1×10^5) were pre-incubated with 10% FBS/DMEM in 96 well culture plates. Twenty-four hours later, the cells were washed with PBS and 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) 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.). WST-1 is a tetrazolium salt that is converted into the soluble formazan salt by succinate-tetrazoliumreductase of the respiratory chain of active mitochondria of viable cells.

1.2.4 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. After24 h incubation, cells were washed with PBS, scrapped using rubber policeman, transferred onto eppendorf tube, and centrifuged for 1,000 *g* for 3 min. Cell pellets were transferred to micro smashing tube with the addition of 500µLPBS. After homogenization, contents of the cellular protein were determined using Bradford protein assay (Bio-Rad Laboratories). Cellular TG and Cho were extracted using Folch method [32] and were measured using commercial kit (Wako).

1.2.5 mRNA analysis

For real-time quantitative reverse transcription-PCR (RT-PCR) analysis, after 24 h treatment of Hepa 1-6 (1×10^5) with the experimental medium, cells were lysed, scrapped with rubber police man, and homogenized. Total RNA was isolated from cell homogenate with an RNeasy Plus Mini kit (Qiagen). cDNA was synthesized using a Ready-To-Go T-Primed First-Strand kit (GE Healthcare), and PCR amplification was

performed with a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories) 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*), carnitinepalmitoyltransferase 1A (*Cpt1a*) and β-actin. PCR conditions were 95 °C for 60 s, 95 °C for 5 s, and 65 °C for 30 s for 40 cycles.

1.2.6 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 $P < 0.05$.

1.2.7 Results and discussion

1.2.7.1 Effect of γ-T3 on Hepa 1-6 cells Proliferation

Hepa 1-6 cells are adherent and epithelial cells growing as 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, alpha1-antitrypsin, alpha-fetoprotein, and amylase), and are used by researchers as a good model of lipid metabolism related experiments [44].

We first evaluated the cytotoxicity of γ-T3 to Hepa 1-6 by using WST-1 reagent. Hepa 1-6 showed a somewhat increase in cell proliferation by γ-T3 treatment (1-15 μM) (**Fig. 7**). The reasons of the induced cellular proliferation still remain unknown, but such increased proliferation was found when cells were incubated with certain antioxidants such as catechin [38].Higher dose of γ-T3 (30-50 μM) caused cytotoxic effect possibly due to apoptosis induction, as some papers have already reported [36].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).

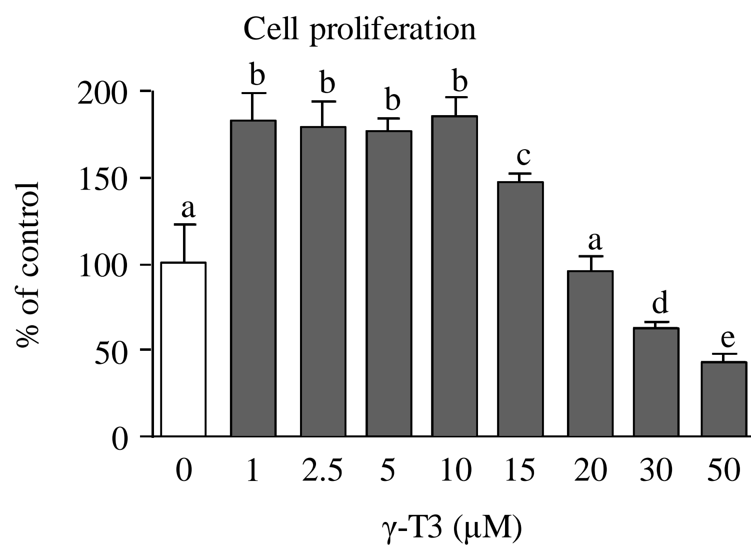


Fig. 7 Effect of increasing concentrations of γ-T3 on cell proliferation. Cell proliferation data are expressed as mean ± SD (n = 6). Means without a common letter differ, $P < 0.05$.

1.2.7.2 Effect of T3 on TG and Cho accumulations on Hepa 1-6 cells

Cellular TG and Cho were extracted by Folch procedure [32], and measured by enzymatic methods. 1-5 μM γ-T3 showed no significant differences for TG concentrations; however, it was found that 10-15μM γ-T3 revealed a significant decrease in TG levels (**Fig. 8A**). The results were in concordance with our previous findings of the TG-lowering effect of T3 in HepG2 cells [45].As for the Cho, no significant differences were observed among all the concentrations of γ-T3 **(Figs. 8B**). As mentioned in the introductory paragraphs, current reports have elucidated the inhibitory effect of T3 on lipid accumulation with common emphasis to Cho [17]. However, it should be noted that some reports showed no attenuation of Cho by T3 [25].Thus, the present findings imply the possibility that the lipid-lowering effect of T3 might be primary mediated by the reduction of TG rather than Cho. The possibility requires further investigation, since TG and Cho are one of the two most abundant lipids in the biological system and attenuation of these two lipid classes would be a great target for clinical application.

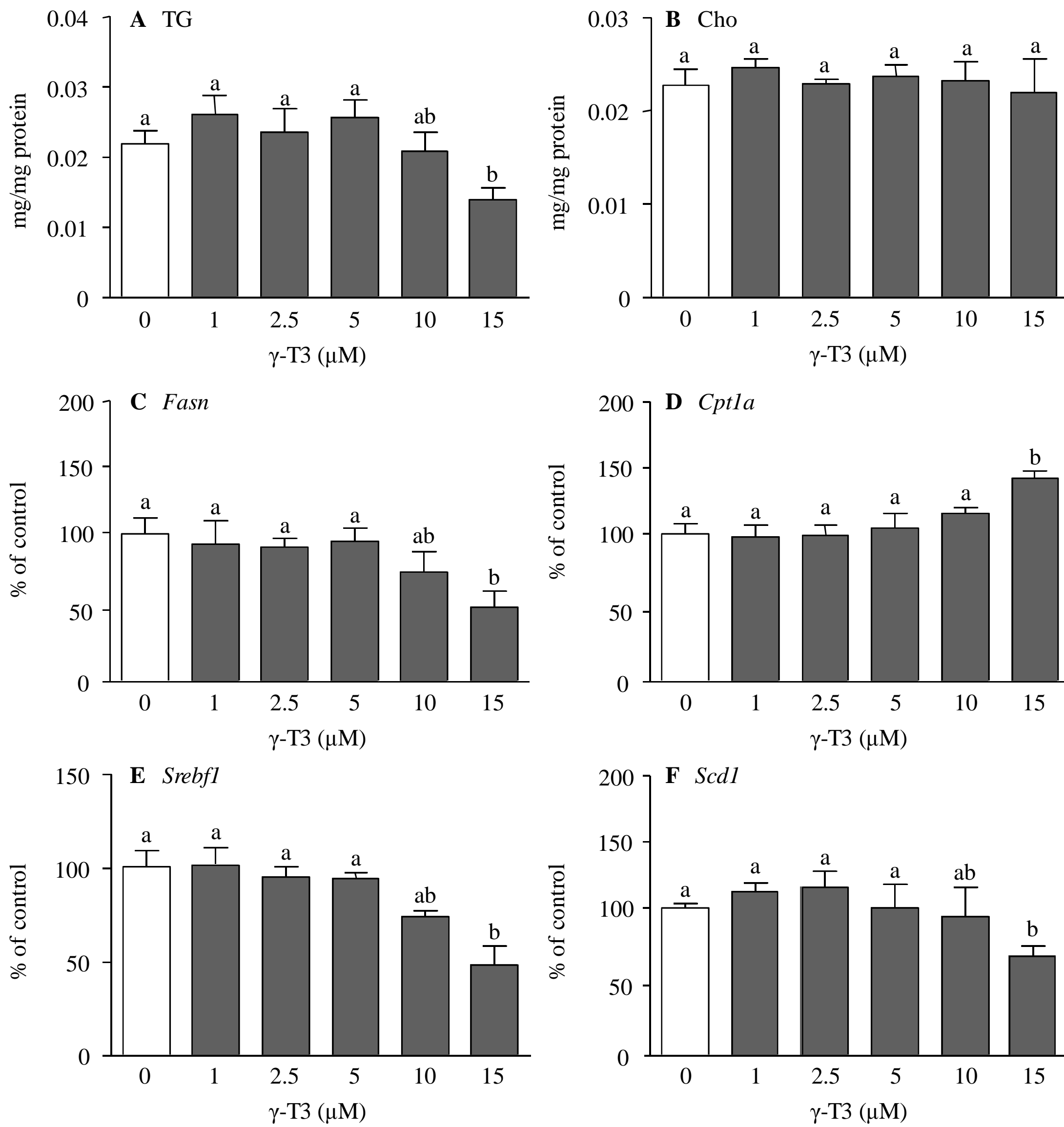


Fig. 8 Effect of increasing concentrations of γ-T3 on lipids levels and mRNA expressions. A. TG. B. Cho. C. *Fasn* gene. D. *Cpt1a* gene. E. *Srebf1* gene. F. *Scd1* gene. Data are expressed as mean ± SD (n = 3). Means without a common letter differ, $P < 0.05$.

1.2.7.3 Effect of T3 on Hepa 1-6 cells lipogenic genes

To evaluate the mechanisms underlying the TG-lowering effect of γ-T3, we next investigated mRNA expression levels of known lipogenic and β-oxidation genes namely *Fasn*, *Srebf1*, *Scd1*, and *Cpt1a*. Like our previous study using HepG2 cells [45], significant down regulation of *Fasn* and up regulation of *Cpt1a* genes were observed in 10-15 μM γ-T3-treated Hepa 1-6 (**Figs. 8 C-D**). In addition, we found down regulation of *Srebf1*and *Scd1* (**Figs. 8 E-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 possible mechanisms of the TG-lowering effect of γ-T3in Hepa 1-6 cells.

1.2.7.4 Discussion

In this study, among T3 isomers, effect of γ-T3on TG levels and lipogenic gene expressions in Hepa1-6 was evaluated. This is because the isomer is suggested to be potent in the regulation of lipid metabolism [29]. Another reason is that the isomer is abundant in natural sources such as rice bran [3]. Now, we are comparing the effect of T3 isomers using several cells (e.g., Hepa1-6, 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 study.

In conclusion, γ-T3 showed attenuation of TG through effect on *Fasn*, *Srebf1*, *Scd1*, and *Cpt1a* genes expression in Hepa 1-6. The result expanded our previous finding of lipids-lowering effect of T3, especially 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 of the expression of these genes which will led to lipid related afflictions. Therefore, understanding the complexity of lipid biosynthesis at gene expression level is of vital importance for the development of natural product medicine (e.g., T3) against lipid related diseases. Consequently, further studies pertaining to the lipid-lowering effect

and mechanism of T3 is recommended to provide ample evidences for a realistic prospect of its use as a human therapy.

CHAPTER 2

Modulation of tocotrienol to crucial lipid metabolism-related genes in 3T3-L1 differentiated preadipocytes

Introduction and objective

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 [46]. 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 [47, 48]. 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 [49], this led to the initial conclusion that this tissue is the major site for lipogenesis. 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 [50].Furthermore, administration of carotenoids and retinoids has been linked to have anti-adiposity through nuclear receptors regulation [51].

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 [22, 5, 6, 7, 8, and 45]. 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 [52]. 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 [53].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 3T3-L1 differentiated preadipocytes were clearly elucidated trough cellular, RT-PCR and western blotting analyses respectively.

Chapter 2: Modulation of tocotrienol to crucial lipid metabolism-related genes in 3T3-L1 differentiated cell lines.

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), 100 kU/L penicillin, and 100 mg/L streptomycin (Gibco BRL) 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 study

T3 isomers and α-Toc stock solutions were diluted with10% 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-L1preadipocytes (2×10^4) 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).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).

2.4. Adipocyte differentiation

3T3-L1 preadipocytes (2×10^4) were seeded in a 35 mm cell culture plates supplemented with 10%FBS/DMEM (high glucose), and incubated for 3 d. 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) containing only 10 μg/mL insulin at 2 d interval from the start of the differentiation.

2.5. TG analysis

Cellular TG concentrations were evaluated using Folch method [32]. Briefly, 3T3-L1 (2×10^4) preadipocytes 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 [33]. Cellular TG was measured using commercial TG kit (Wako).

2.6. Oil red O staining

Differentiated 3T3-L1 preadipocytes 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. Isolation of total RNA and analysis of mRNA expression

Total cellular RNA was isolated with an RNeasyPlus Mini kit (Qiagen) for real-time quantitative reverse transcription-PCR (RT-PCR). cDNA was synthesized using a Ready-To-Go T-Primed First-Strand kit (GE Healthcare), and PCR amplification was performed with a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories) using SYBR Premix Ex Taq (Takara Bio Inc.) and gene-specific primers for *FAS*, carnitine palmitoyltransferase I(*CPT1*), stearoyl-CoA desaturase 1(*SCD-1*),acetyl-CoA

carboxylase 1 (*ACC1*), sterol regulatory element-binding protein 1c (*SREBP 1c*),adiponectin receptor 2 (*ADIPOR2*), uncoupling protein 2 (*UCP2*), 3-hydroxy-3-methyl-glutaryl-CoA reductase (*HMG-CoA-R*), low-density lipoprotein receptor (*LDLR*),*PPAR-γ*, and beta actin (*β-actin*). PCR conditions were 95 °C for 60 s, 95 °C for 5 s, and 65 °C for 30 s for 40 cycles.

2.8. Western blotting analysis

Differentiated 3T3-L1 preadipocytes proteins were extracted and separated by SDS-PAGE (4-20% e-PAGEL; Atto). The protein bands were transferred to polyvinylidene fluoride membranes (Invitrogen).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, 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).

2.9 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 $P < 0.05$.

2.10 Results and discussion

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

First of all, 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. 9**). However, α-Toc did not show any cytotoxic effect to 3T3-L1 preadipocytes. 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) [45]Since most of the T3 isomers showed less cytotoxic effect at 25 μM, this concentration was used in the succeeding experiments.

Next, 3T3-L1 differentiated preadipocytes 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 preadipocytes at dose dependent manner (**Fig. 10**). T3 isomers (δ-, γ-, and β-T3)

attenuated TG levels at 25-50 μM concentrations, but α-T3 showed attenuation only at50 μM. Structurally, α-T3has 5- and 7-methyl groups attached to its chroman ring, so it is possible that α-T3 could hardly pass through cell membranes. The poor incorporation of α-T3 into 3T3-L1 differentiated preadipocytes may explain its lower inhibitory effect on cellular TG levels. To date, few data have shown the ability of some T3 isomers such as γ- and α-T3 in the reduction of cellular TG in adipocyte cell lines [52], but however it is also important to note that α-Toc failed to show significant TG attenuation (**Fig. 10).** Our findings provided a new information regarding the effect of T3 (all isomers) as well as Toc on the TG levels in 3T3-L1 differentiated preadipocytes.

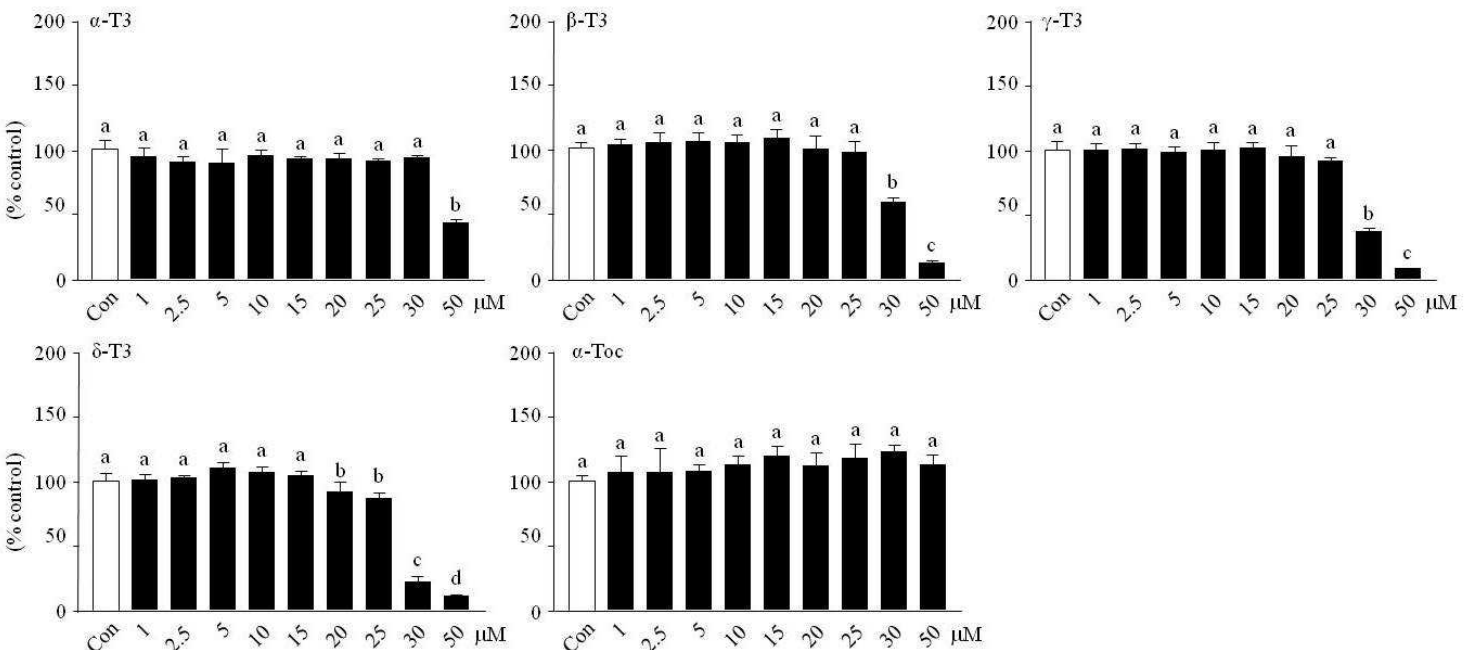


Fig. 9 Effect of increasing concentrations of T3 or α-Toc (0-50 μM) on cell proliferation of 3T3-L1 preadipocytes.3T3-L1 preadipocytes were incubated withT3 or α-Toc for 24 h, and then cell proliferation was evaluated with WST-1 assay. Experimental procedures are shown in Materials and methods. Data are expressed as mean ± SD (n = 6). Means without a common letter differ, $P < 0.05$.

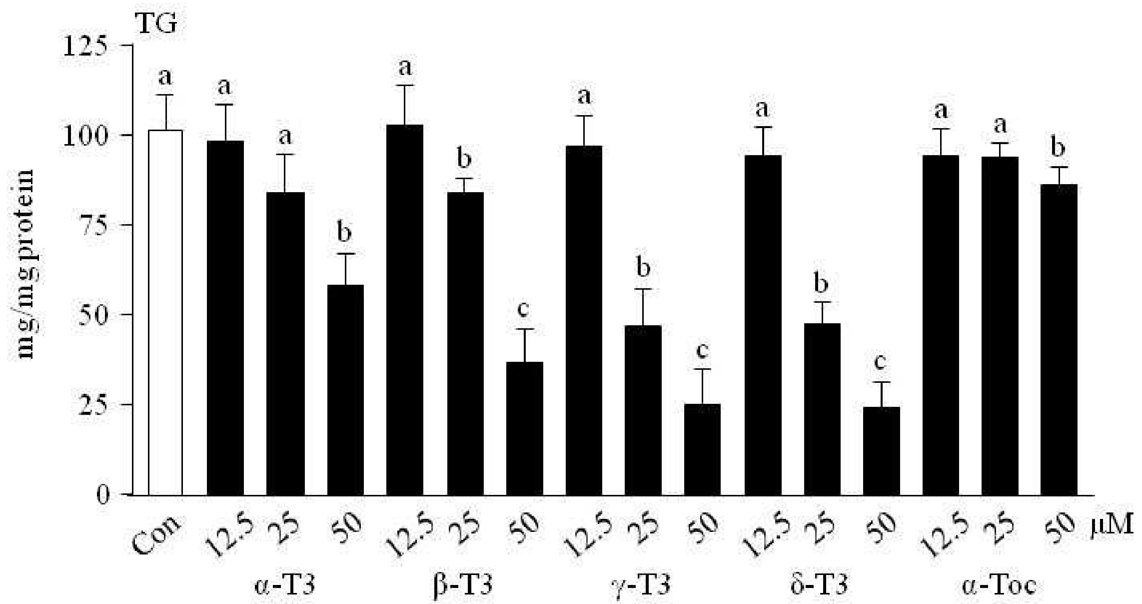


Fig. 10 Effect of increasing concentrations of T3 or α-Toc (0-50μM) on TG levels of differentiated 3T3-L1 preadipocytes at 8 d after differentiation. Experimental procedures are shown in Materials and methods. Data are expressed as mean ± SD (n = 3). Means without a common letter differ, $P < 0.05$.

In order to confirm whether T3 or Toc affects the lipid droplets accumulation in 3T3-L1 differentiated preadipocytes, oil red O staining technique was performed. Cellular lipid droplets aggregations were significantly observed from 6 to 8 d after differentiation (**Fig. 11**). Differentiated preadipocytes 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. Moreover, α-Toc did not showed any observable reduction of lipid droplets in the differentiated preadipocytes. 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 [54]. Therefore, T3, but not Toc, may attenuate TG accumulation by reducing lipid droplets in the differentiated preadipocytes.

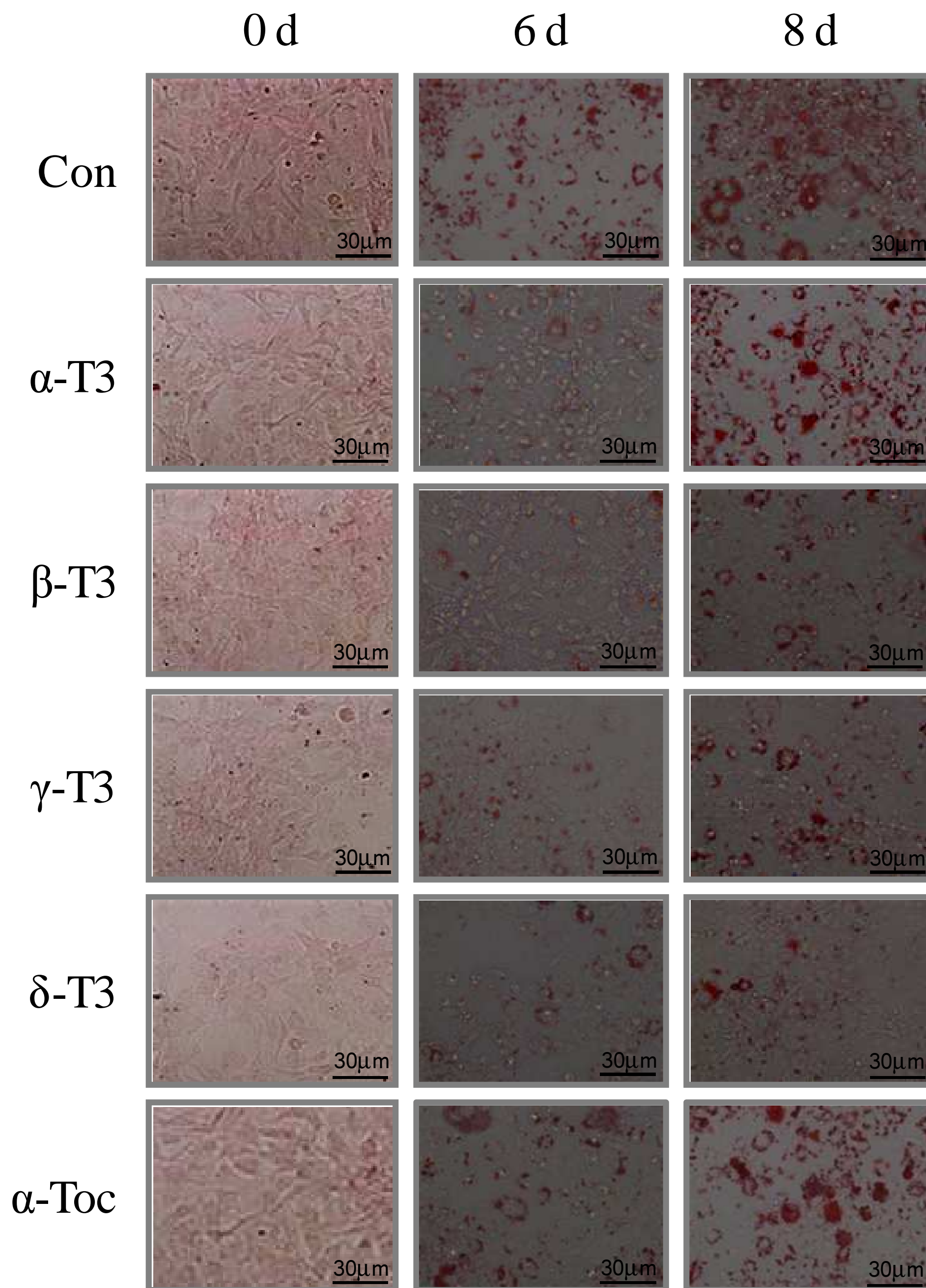


Fig. 11 Oil red O stained lipid droplets of differentiated 3T3-L1 preadipocytes treated with T3 or α-Toc (25 μM) from 0, 6, and 8 d after differentiation. Scale bar= 30 μm. Experimental procedures are shown in Materials and methods.

2.10.2. Regulation of T3 to crucial lipid metabolism-related genes and proteins in 3T3-L1 differentiated preadipocytes

Effects of T3 or Toc on mRNA expression levels of some known crucial lipogenic genes were investigated. Differentiated 3T3-L1 preadipocytes treated with or without T3 or Toc at 8 d after differentiation were harvested and subjected to mRNA extraction. Selected genes mRNA expressions were evaluated using RT-PCR. Expression of crucial lipid metabolism-related genes such as *FAS*, *SCD-1*, *ACC1*, *SREBP 1c*, *LDLR*, and *PPAR-γ* were significantly down regulated by δ- and γ-T3 (**Fig. 12**). On the other hand, β-T3 showed down regulation to *SCD-1*, *ACC1* and *LDLR* genes respectively. There were no significant attenuation observed for α-T3 and α-Toc. In contrast, only δ- and γ-T3 significantly up-regulated *CPT1*, *ADIPOR2* and *UCP2* genes expressions (**Fig.12**). *FAS* and *SREBPs* are both noted to be the culprit of lipid synthesis and regulation of these genes are known to induce dramatic reductions of fat accumulations [55, 56]. Moreover, reports showed that *SCD-1* inhibition results in the buildup of acyl-CoAs which in turn cause a feedback suppression to ACC. This cascading reaction then diminishes the inhibition of CPT shuttle and will allow fat transportation to the mitochondria for breakdown via β-oxidation [57, 58]. In this study, interestingly, gene

that codes for Cho synthesis (*HMG-CoA-R*) was not regulated by all experimental groups. This observation was in coherence with our previous animal and cell culture studies [45]. We therefore then hypothesize that the reported lipid lowering effect of T3 might be mediated by its regulation to other crucial lipid metabolism-related genes rather than *HMG-CoA-R* gene. The present study caters an immense possibility that T3 can regulate a wide array of essential genes necessary for lipid metabolism.

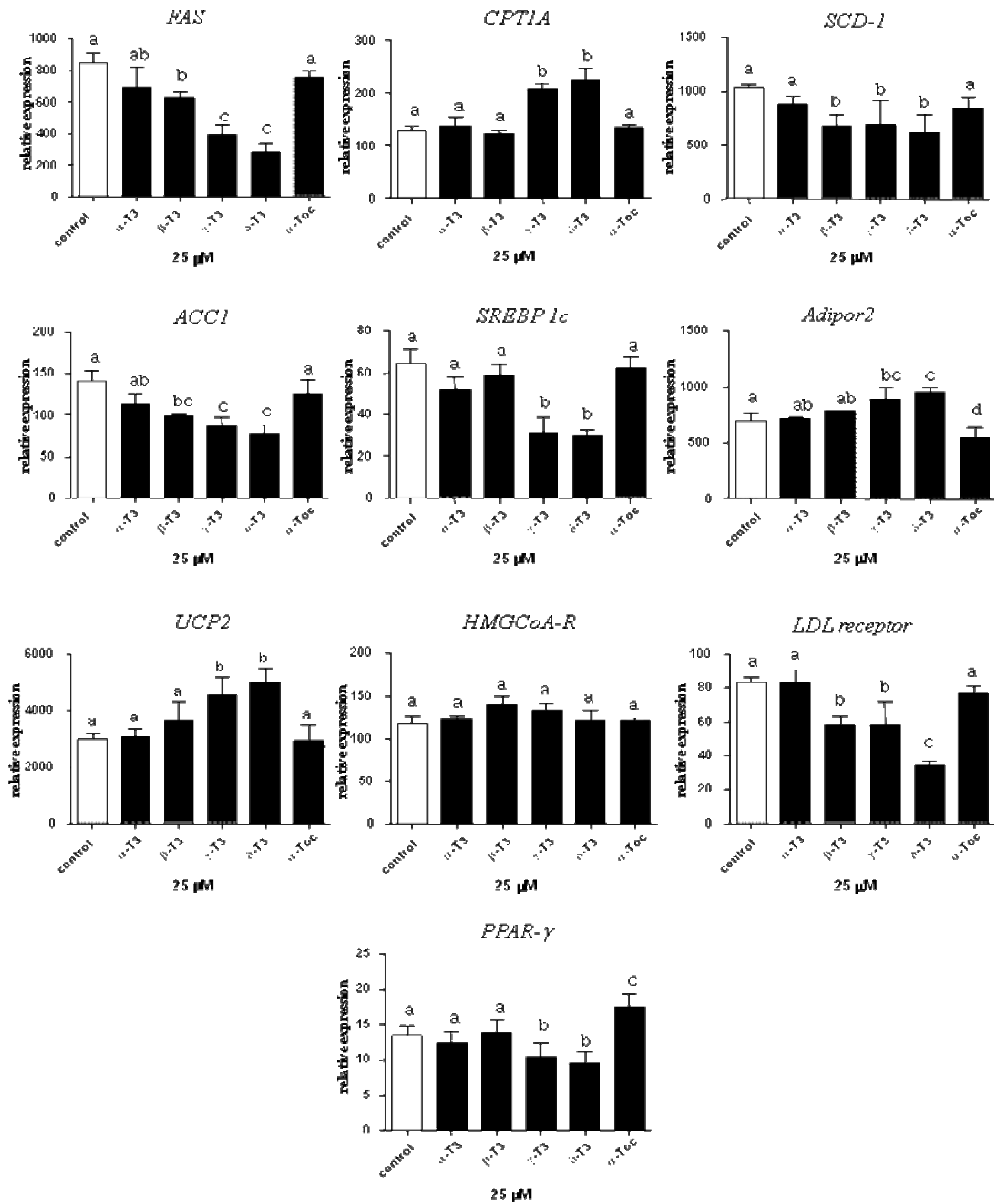


Fig. 12 Effect of T3 or α-Toc (25 μM) on mRNA expression of *FAS*, *CPT1*, *SCD-1*, *ACC1*, *SREBP 1c*, *ADIPOR2*, *UCP2*, *HMG-CoA-R*, *LDLR*, and *PPAR-γ* genes in differentiated 3T3-L1 preadipocytes at 8 d after differentiation. Data are expressed as mean ± SD (n = 3). Means without a common letter differ, $P < 0.05$.

Finally, we extracted the proteins from differentiated 3T3-L1 preadipocytes incubated with T3 and Toc for 8 d, and analyzed the proteins using customary western blotting procedure. Protein blots revealed that δ- and γ-T3 markedly repressed FAS, SCD-1, ACC1, SREBP 1c, LDLR, and PPAR-γ protein expressions (**Fig. 13**). Up regulation of CPT1 and UCP2 proteins were also observed in δ- and γ-T3 treated 3T3-L1 differentiated preadipocytes. However, β-T3 did not showed remarkable regulation to all selected proteins though it displayed significant mRNA regulation to *SCD-1*, *ACC1*, and *LDLR* genes (**Fig. 12**). 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. In the present study, both α-T3 and α-Toc did not show any significant regulation to all the proteins **(Fig. 13). T**his 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 [29, 45].

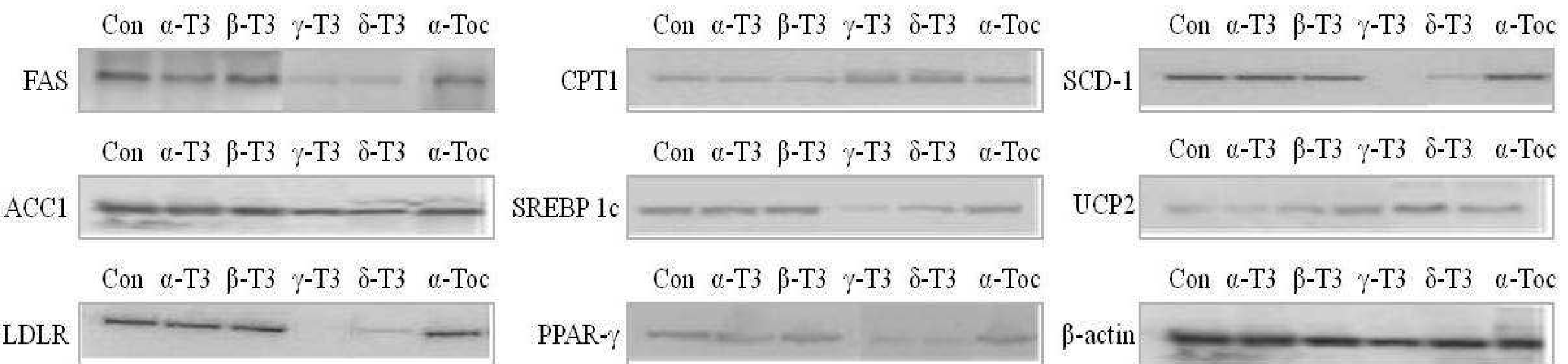


Fig. 13 Effect of T3 or α-Toc (25 μM) on FAS, CPT1, SCD-1, ACC1, SREBP 1c, UCP2, LDLR, PPAR-γ, and β-actin protein expressions in differentiated 3T3-L1 preadipocytes at 8 d after differentiation. Experimental procedures are shown in Materials and methods. Each Western blot is a representative example of data from three replicate experiments.

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 lipid metabolism-related diseases. 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 to provide a probable lipid metabolism regulation genetic profile of T3.

2.10.3 Discussion

The present study elucidated information as to the effect of T3 on lipid metabolism-related genes and proteins in differentiated 3T3-L1 preadipocytes. Among all the T3 isomers, δ-, γ- and β-T3 showed a marked significance in attenuating cellular TG levels in differentiated 3T3-L1 preadipocytes by modulating the gene and protein expressions concerning lipid metabolism. β-T3 on the other hand showed only significant modulation to selected lipid metabolism related genes mRNA expression, but failed to show modulation in its protein expressions. This peculiarity is hypothesized that β-T3 effect on these genes was confined only at mRNA level. In the current experimental condition, gene concerning Cho synthesis (*HMG-CoA-R*) was not regulated by T3 or Toc. Both α-T3 and α-Toc did not demonstrate any significant regulation to TG level, mRNA and protein expressions of lipid metabolism-related genes. Up to present, there were only very few data reporting the potency of T3 to regulate lipid metabolism related genes in 3T3-L1 preadipocytes[52, 53] as such the present study provided a new set of evidences concerning the possibility of T3 as an anti-metabolic disorder candidate.

CHAPTER 3.

γ and δ Tocotrienol suppresses hepatocellular liver carcinoma cell line proliferation via proto-oncogene *Ras*- associated upstream signaling regulation

Introduction and objective

T3 has recently gained increasing interest due to its several health-promoting properties that differ somewhat from those of Toc. Reports stated that T3 protects suppresses pathological angiogenesis, and cancer [7, 8]. These unique effects of T3 could be partly explained by its abilities to induce cell cycle arrest [9], to activate p53 and caspase [10,11]. Most of these mechanisms were considerably associated to the regulation of *Ras-Raf-MEK-ERK* oncogenic pathway.

Within the context of these effects regarding mechanism of T3 against cancer, it should be noted that there were a handful of information regarding the effect of T3 in the upstream signaling of this oncogenic pathway. Since the immense effect of T3 on *Ras-Raf-MEK-ERK* oncogenic pathway has been the crucial regulatory factor for this oncogenic pathway cascades, it is worth speculating that these manifestations might be first at hand being ubiquitously regulated by its upstream associated signaling process. In this study, we investigated whether T3 suppresses Human hepatocellular carcinoma cell line proliferation via regulation of *Ras*-associated upstream signaling gene and protein expressions. These data will be of vital importance in the elucidation of the regulatory potential of T3 on *Ras-Raf-MEK-ERK* oncogenic pathway which may in turn gives tangible information as to the application of T3 for clinical benefits.

Chapter 3: γ and δ Tocotrienol suppresses hepatocellular liver carcinoma cell line proliferation via proto-oncogene *Ras*- associated upstream signaling regulation

3. Materials and methods

3.1 Reagents and cells

γ and δ T3 isomers were kindly provided by Eisai Food & Chemical Co., Ltd and were dissolved in ethanol at a concentration of 50 mM as stock solution. HepG2 cells were obtained from the RIKEN cell bank (Tsukuba, Japan). The cells were cultured in RPMI/1640 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), 100 kU/L penicillin, and 100 mg/L streptomycin (Gibco BRL) at 37 °C in 5% CO_2/95% air atmosphere in a humidified incubator. All reagents used in this study were of analytical grade.

3.2 Preparation of experimental medium

γ and δ T3 isomers stock solutions were diluted with10% FBS/RPMI-1640 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.

3.3 Cell proliferation assay

For cell viability assays, HegG2 cells (1×10^5) were pre-incubated with 10% FBS/RPMI-1640 in 96 well culture plates. Twenty-four hours later, the cells were washed with 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. 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.).

3.4 Isolation of total RNA and analysis of mRNA expression

Total cellular RNA was isolated with an RNeasyPlus Mini kit (Qiagen) for real-time quantitative reverse transcription-PCR (RT-PCR). cDNA was synthesized using a Ready-To-Go T-Primed First-Strand kit (GE Healthcare), and PCR amplification was performed with a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories,) using SYBR Premix Ex Taq (Takara Bio Inc.) and gene-specific primers for GTPase Hras (*Hras*), growth factor receptor-bound protein 2 (*Grb2*), son of sevenless homolog, 1 (*Sos-1*), phospho proto-oncogene c-Src family (*Phospho-Src Tyr416*), phospho-Shc (*Phospho-Shc Tyr 239/240*) and beta actin (*β-actin*). PCR conditions were 95 °C for 60 s, 95 °C for 5 s, and 65 °C for 30 s for 40 cycles.

3.5 Western Blotting Analysis

After HepG2 (1×10^5) cells were incubated with test medium for 24 h, proteins were separated by SDS-PAGE (4-20% e-PAGEL; Atto). The protein bands were transferred to polyvinylidene fluoride membranes (Invitrogen). After blocking for 1 h, membranes were incubated with primary antibodies for Hras, Grb2, Sos-1, Phospho-Src.

Phospho-Shc and β-actin (Cell Signaling Technology, Beverly, MA, USA), 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 (BioRad).

3.6 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 $P < 0.05$.

3.7 Results and discussion

3.7.1 Effect of γ and δT3 on HepG2 Proliferation

Cytotoxic activity of both γ and δ T3 including α-Toc were first evaluated. HepG2 proliferation was significantly inhibited at higher dose of T3 (15-50 μM) at dose dependent manner. Among all the T3 isomers (γ and δ) concentrations, significant inhibition of HepG2 cells proliferation were already observed at 15 μM. However, α-Toc did not show any significant inhibitory effect to HepG2 cells at all concentrations **(Fig.14).** Since both T3 isomers showed significant inhibitory effect to HepG2 at 15 μM, T3 concentrations ranging from 5-20 μM were utilized in the succeeding experiments.

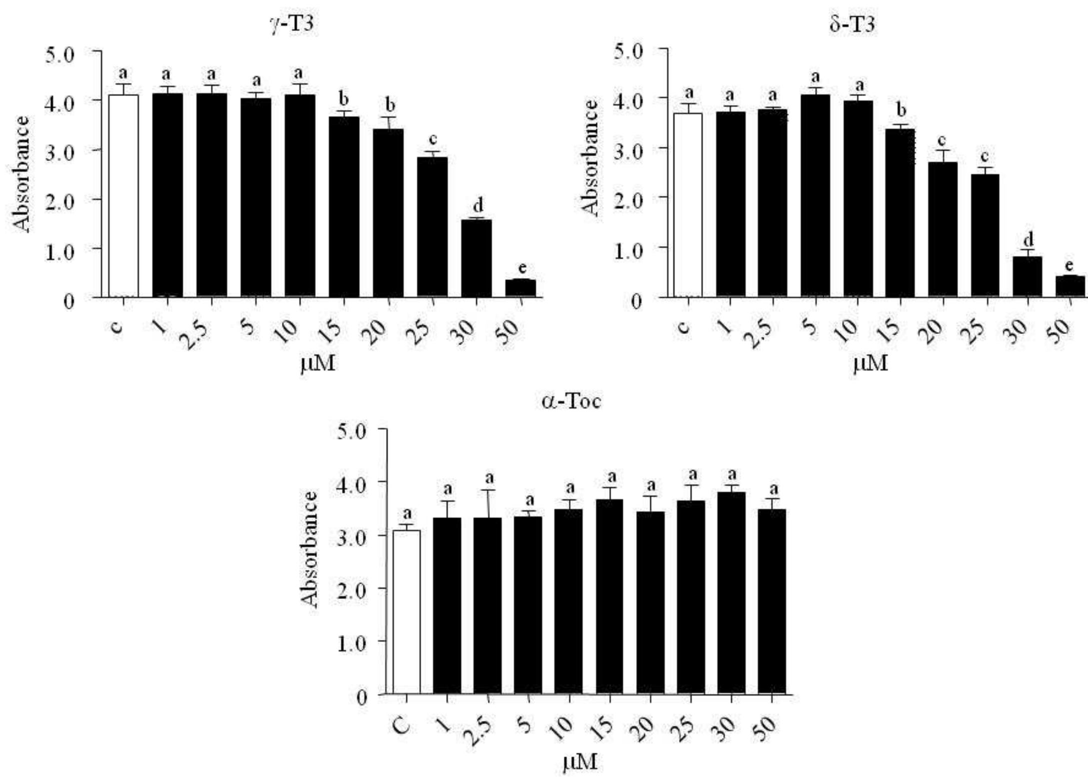


Fig. 14 Effect of increasing concentrations of γ and δ T3 including α- Toc on HepG2 cell proliferation. Cell proliferation data are expressed as mean ± SD (n = 6). Means without a common letter differ, $P < 0.05$.

3.7.2 γ and δ T3 regulated proto-oncogene Hras and Ras- associated upstream signaling genes expressions

γ and δ T3 treatment to hepatocellular carcinoma cells showed significant cytotoxic effect at higher dose (15-50 μM δ and γ T3 concentrations). Down regulation of proto-oncogene *Hras* gene expressions was significantly observed in HepG2 cells for both T3 isomers. Additionally, *Ras* upstream genes signaling cohorts (*Grb2*, *Sos-1, Phospho-Src Tyr41* and *Phospho-Shc Tyr 239/240*) were also significantly down regulated upon administration of both γ and δT3 isomers at dose dependent manner **(Figs. 15&16)**.

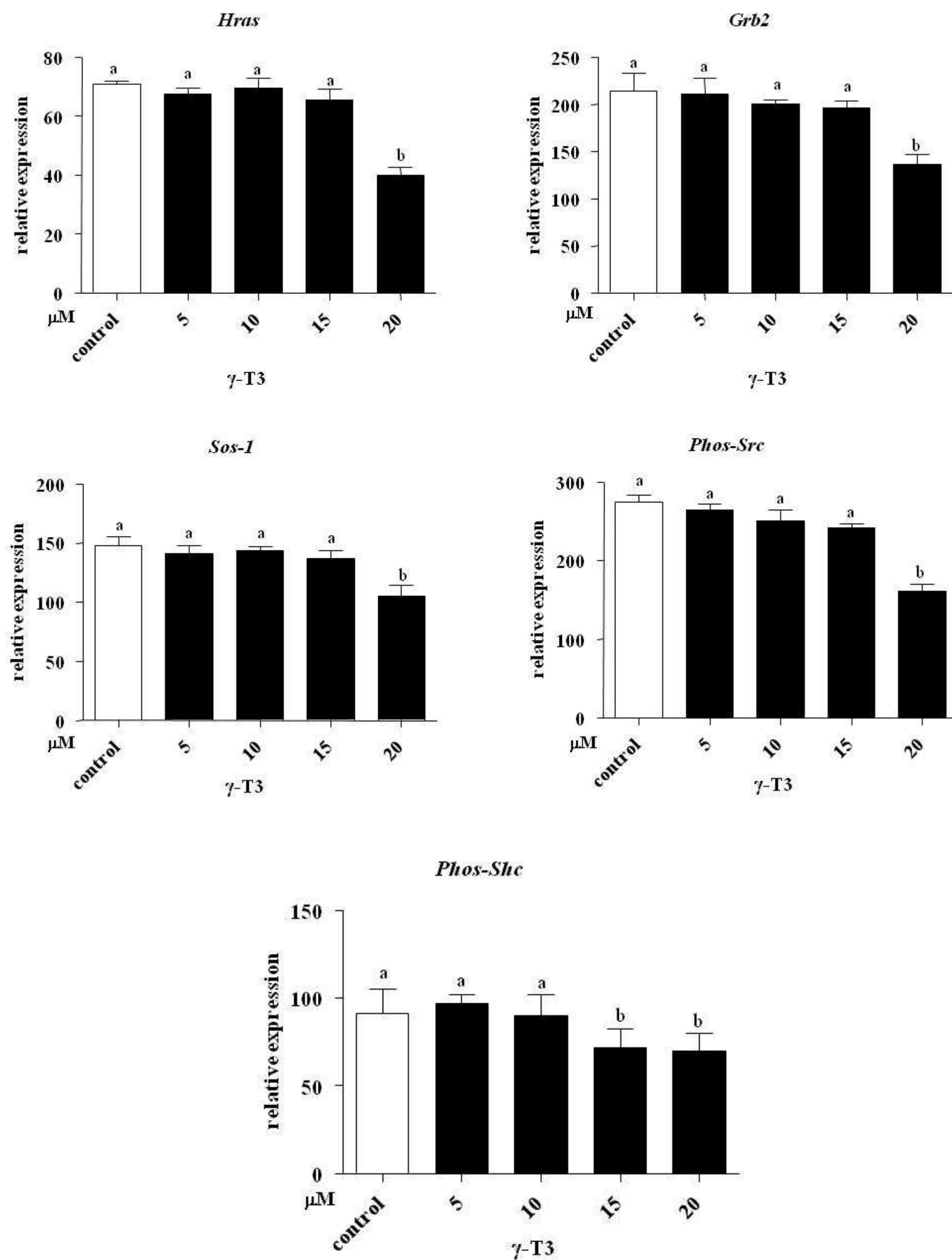


Fig. 15 Effects of γ-T3 (0-20 μM) on mRNA expressions of *Hras*, *Grb2*, *Sos-1*, *Phos-Src* and *Phos-Shc* genes in HepG2. Data are expressed as mean ± SD (n = 3). Means without a common letter differ, $P < 0.05$.

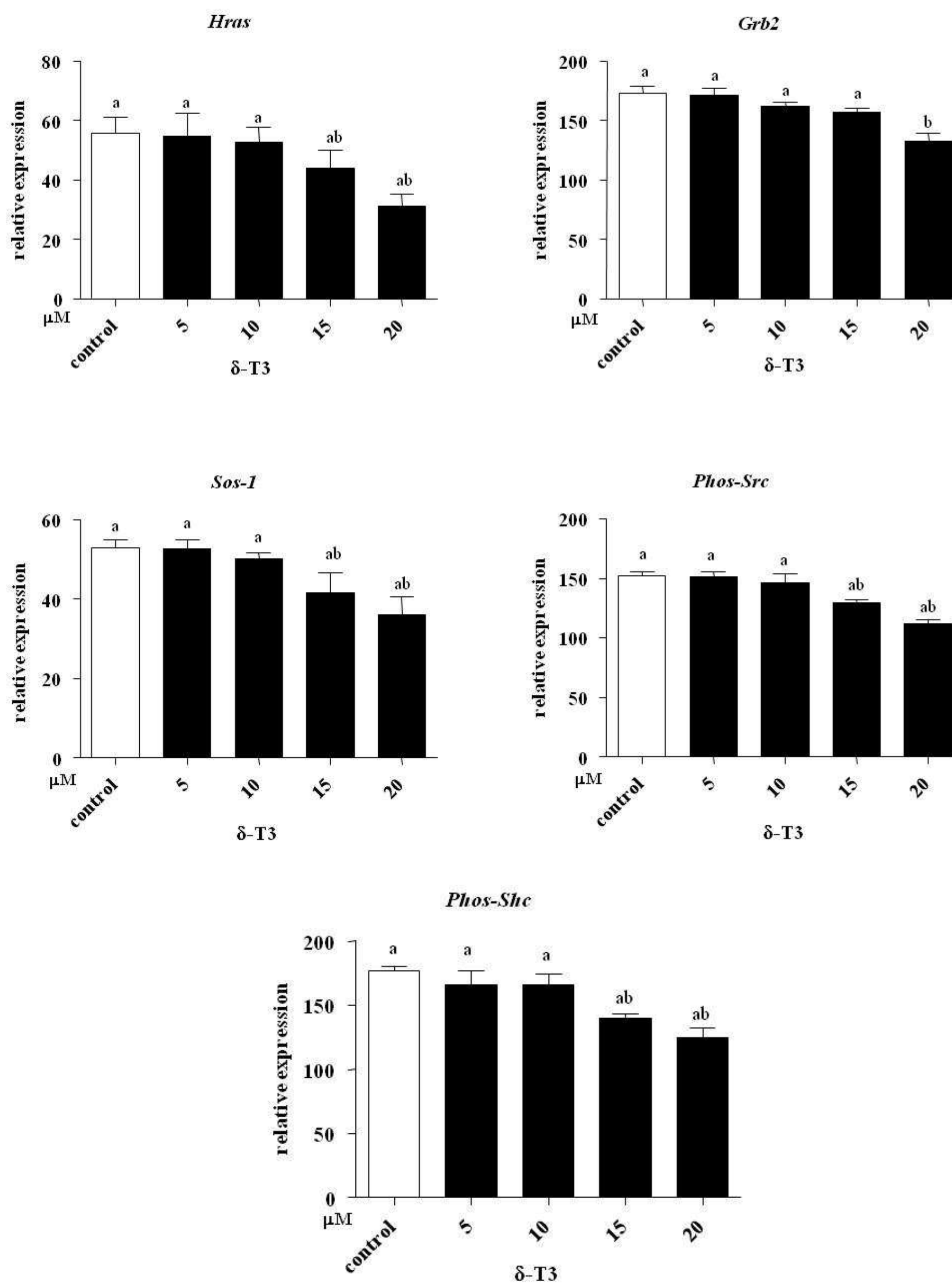


Fig. 16 Effects of δ-T3 (0-20 μM) on mRNA expressions of *Hras*, *Grb2*, *Sos-1*, *Phos-Src* and *Phos-Shc* genes in HepG2. Data are expressed as mean ± SD (n = 3). Means without a common letter differ, $P < 0.05$.

3.7.2 γ and δ T3 regulated proto-oncogene Hras and Ras- associated upstream protein expressions

Proto-oncogene Hras and its *Ras*-associated upstream signaling genes protein expressions were measured via western blotting analysis. γ and δ T3 showed marked down regulation of Hras, Grb2, Sos-1, Phos-SRC and Phos Shc protein expressions at dose dependent manner **(Figs. 17&18).**

Therefore, the current findings demonstrated a novel mechanism of the anti-cancer effect of T3 specifically γ and δ T3 isomers via proto-oncogene *Ras* upstream signaling regulation. Furthermore, since *Ras* activation directly influences the activity of Ras-Raf-MEK-ERK oncogenic pathway, it is then hypothesized that the reported anti-cancer mechanisms of T3 via Ras-Raf-MEK-ERK pathway oncogenic cascades to various cancer cell lines might be mediated by the upstream regulation of this pathway.

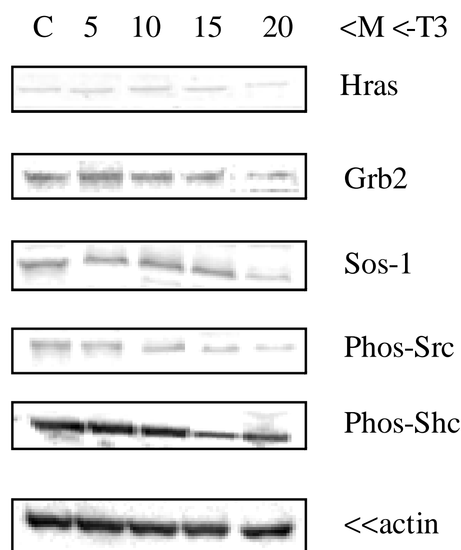


Fig. 17 Effects of γ-T3 (0-20 μM) on protein expressions of Hras, Grb2, Sos-1, Phos-Src and Phos-Shc genes in HepG2. Each western blot is a representative example of data from three replicates experiments

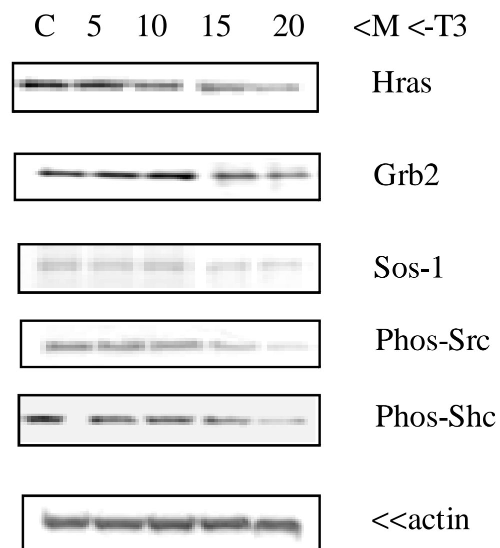


Fig. 18 Effects of δ-T3 (0-20 μM) on protein expressions of Hras, Grb2, Sos-1, Phos-Src and Phos-Shc genes in HepG2. Each western blot is a representative example of data from three replicates experiments

3.7.3 Discussion

The cytotoxic effect of T3 to various cancer cell lines is very valuable information. The major scientific claims regarding its mechanisms on cancer regulation were mainly focused on its ability to affect some major molecular oncogenic pathways (AKT signaling, EGF pathways, p53 pathways, etc) [7]. Because of the complexity of the cancer signaling process, it is of vital importance to perform an expansive research pertaining to the effect of T3 on different genes that are interacting with these pathways. For instance, *Ras* genes are one of the key switch genes for cancer progression in *Ras-Raf-MEK-ERK* oncogenic pathway that is crucial in the regulation of cellular proliferation process in response to growth factor stimulation [59]. Interestingly, our present *in vitro* data showed that T3 specifically δ and γ T3 significantly regulated the mRNA and protein expressions of *Hras* gene; one of the isoforms of *Ras* gene family in human hepatocellular carcinoma cell line. Moreover, γ and δ T3 also markedly regulated the *Hras*-associated upstream signaling genes. Since *Ras* activation directly influences the activity of *Ras-Raf-MEK-ERK* oncogenic pathway, it is then hypothesized that the reported anti-cancer mechanisms of T3 via *Ras-Raf-MEK-ERK* pathway oncogenic cascades to various cancer cell lines might be mediated by the

upstream regulation of this pathway.

General conclusions

T3 as one of the groups of a naturally-occurring vitamin E family has been considerably attracted the attention of some research scientists due its promising biological activity that may even surpass its potency to Toc, the most commonly known and studied vitamin E group. One of the recently dynamic researches on T3 was focused on its lipid metabolism and cancer regulations. In our studies, we found out a series of novel functions and mechanisms of T3 on lipid metabolism regulation [45]. Both *in vitro* and *in vivo* experimental data suggested that T3 affects lipid accumulation by multiple regulations of lipid metabolism-related genes. Moreover, the ability of T3 to reduce oxidative stress due to fat-induced oxidative damage has been a crucial factor to its total novel biological function against lipid accumulation. Aberrant lipid metabolic process could generate multiple diseases that are linked to obesity and other metabolic-related diseases. Therefore, understanding the cellular mechanisms involve in the aberration of the homeostatic status of fat storage and usage in the biological systems and nailing down this pathological signaling abnormalities could entail an array of new insights regarding the possibility of future pharmacological target for the treatment of obesity and other metabolic-related diseases.

Besides this novel bioactivity rendered by T3, it is also very important to note that T3 has been reported to display a remarkable cytotoxic effect to various cancer cell lines. The major scientific claims regarding its mechanisms on cancer regulation were mainly focused on its ability to affect some major molecular oncogenic pathways (AKT signaling, EGF pathways, p53 pathways, etc) [7]. Because of the complexity of the cancer signaling process, it is of vital importance to perform an expansive research pertaining to the effect of T3 on different genes that are interacting with these pathways. For instance, *Ras* genes are one of the key switch genes for cancer progression in *Ras*-*Raf*-*MEK*-*ERK* oncogenic pathway that is crucial in the regulation of cellular proliferation process in response to growth factor stimulation [59]. Interestingly, our present *in vitro* data showed that T3 specifically γ and δ T3 significantly regulated the mRNA and protein expressions of *Hras* gene; one of the isoforms of *Ras* gene family in human hepatocellular carcinoma cell line. Moreover, γ and δ T3 also markedly regulated the *Hras*-associated upstream signaling genes. Since *Ras* activation directly influences the activity of *Ras*-*Raf*-*MEK*-*ERK* oncogenic pathway, it is then hypothesized that the reported anti-cancer mechanisms of T3 via *Ras*-*Raf*-*MEK*-*ERK* pathway oncogenic cascades to various cancer cell lines might be mediated by the upstream regulation of this pathway.

References

1. Evans HM, Bishop KS (1922) On the existence of a hitherto unrecognized dietary factor essential for reproduction. Science 56:650-651

2. Dunphy PJ, Whittle KJ, Pennock JF, Morton RA (1965) Identification and estimation of tocotrienols in Hevea latex. Nature 207:521-522

3. Sookwong P, Nakagawa K, Murata K, Kojima Y, Miyazawa T (2007) Quantitation of tocotrienol and tocopherol in various rice brans. J Agric Food Chem 55:461-466

4. Serbinova E, Kagan V, Han D, Packer L (1991) Free radical recycling and intramembrane mobility in the antioxidant properties of α-tocopherol and α-tocotrienol. Free Radic Biol Med 10:263-275

5. Khanna S, Roy S, Slivka A, Craft TKS, Chaki S, Rink C, Notestine MA, DeVries AC, Parinandi NL, Sen CK (2005) Neuroprotective properties of the natural vitamin E α-tocotrienol. Stroke 36:2258-2264

6. Nakagawa K, Shibata A, Yamashita S, Tsuzuki T, Kariya J, Oikawa S, Miyazawa T (2007) In vivo angiogenesis is suppressed by unsaturated vitamin E, tocotrienol. J Nutr 137:1938-1943

7. Shibata A, Nakagawa K, Sookwong P, Tsuzuki T, Oikawa S, Miyazawa T (2008) Tumor anti-angiogenic effect and mechanism of action of δ-tocotrienol. Biochem Pharmacol 76:330-339

8. Hiura Y, Tachibana H, Arakawa R, Aoyama N, Okabe M, Sakai M, Yamada K (2009) Specific accumulation of γ- and δ-tocotrienols in tumor and their antitumor effect in vivo. J Nutr Biochem 20:607-613

9. Srivastava JK, Gupta S (2006) Tocotrienol-rich fraction of palm oil induces cell cycle arrest and apoptosis selectively in human prostate cancer cells. Biochem Biophys Res Commun 346:447-453

10. Agarwal MK, Agarwal ML, Athar M, Gupta S (2004) Tocotrienol-rich fraction of palm oil activates p53, modulates Bax/Bcl2 ratio and induces apoptosis independent of cell cycle association. Cell Cycle 3:205-211

11. Shah S, Sylvester PW (2004) Tocotrienol-induced caspase-8 activation is unrelated to death receptor apoptotic signaling in neoplastic mammary epithelial cells. Exp Biol Med 229:745-755

12. Theriault A, Chao JT, Gapor A (2002) Tocotrienol is the most effective vitamin E for reducing endothelial expression of adhesion molecules and adhesion to monocytes. Atherosclerosis 160:21-30

13. Ahn KS, Sethi G, Krishnan K, Aggarwal BB (2007) γ-Tocotrienol inhibits nuclear factor-κB signaling pathway through inhibition of receptor-interacting protein and

TAK1 leading to suppression of antiapoptotic gene products and potentiation of apoptosis. J Biol Chem 282:809-820

14. Eitsuka T, Nakagawa K, Miyazawa T (2006) Down-regulation of telomerase activity in DLD-1 human colorectal adenocarcinoma cells by tocotrienol. Biochem Biophys Res Commun 348:170-175

15. Pearce BC, Parker RA, Deason ME, Qureshi AA, Wright JJ (1922) Hypocholesterolemic activity of synthetic and natural tocotrienols. J Med Chem 35:3595-3606

16. Parker RA, Pearce BC, Clark RW, Gordon DA, Wright JJ (1993) Tocotrienols regulate cholesterol production in mammalian cells by post-transcriptional suppression of 3-hydroxy-3-methylglutaryl-coenzyme A reductase. J Biol Chem 268:11230-11238

17. Song BL, DeBose-Boyd RA (2006) Insig-dependent ubiquitination and degradation of 3-hydroxy-3-methylglutaryl coenzyme a reductase stimulated by δ- and γ-tocotrienols. J Biol Chem 281:25054-25061

18. Watkins T, Lenz P, Gapor A, Struck M, Tomeo A, Bierenbaum M (1993) γ-Tocotrienol as a hypocholesterolemic and antioxidant agent in rats fed atherogenic diets. Lipids 28:1113-1118

19. Khor H, Chieng D (1996) Effect of dietary supplementation of tocotrienols and tocopherols on serum lipids in the hamster. Nutr Res 16:1393-1401

20. Black TM, Wang P, Maeda N, Coleman RA (2000) Palm tocotrienols protect ApoE +/- mice from diet-induced atheroma formation. J Nutr 130:2420-2426

21. Qureshi AA, Salser WA, Parmar R, Emeson EE (2001) Novel tocotrienols of rice bran inhibits atherosclerotic lesions in C57BL/6 ApoE-deficient mice. J Nutr. 131:2606-2618

22. Qureshi AA, Qureshi N, Wright JJ, Shen Z, Kramer G, Gapor A, Chong YH, DeWitt G, Ong A, Peterson DM (1991) Lowering of serum cholesterol in hypercholesterolemic humans by tocotrienols (palmvitee). Am J Clin Nutr 53:1021S-1026S

23. Wahlqvist ML, Krivokuca-Bogetic Z, Lo CS, Hage B, Smith R, Lukito W (1992) Differential serum responses of tocopherols and tocotrienols during vitamin supplementation in hypercholesterolaemic individuals without change in coronary risk factors. Nutr Res 12:S181-S201

24. Mensink RP, van Houwelingen AC, Kromhout D, Hornstra G (1999) A vitamin E concentrate rich in tocotrienols had no effect on serum lipids, lipoproteins, or platelet function in men with mildly elevated serum lipid concentrations. Am J Clin Nutr 69:213-219

25. O'Byrne D, Grundy S, Packer L, Devaraj S, Baldenius K, Hoppe PP, Kraemer K, Jialal I, Traber MG (2000) Studies of LDL oxidation following alpha-, gamma-, or

delta-tocotrienyl acetate supplementation of hypercholesterolemic humans. Free Radic Biol Med 29:834-845

26. Tomeo AC, Geller M, Watkins TR, Gapor A, Bierenbaum ML (1995) Antioxidant effects of tocotrienols in patients with hyperlipidemia and carotid stenosis. Lipids 30:1179-1183.

27. Tan DT, Khor HT, Low WH, Ali A, Gapor A (1991) Effect of a palm-oil-vitamin E concentrate on the serum and lipoprotein lipids in humans. Am J Clin Nutr 53:1027S-1030S

28. Qureshi AA, Bradlow BA, Brace L, Manganello J, Peterson DM, Pearce BC, Wright JJ, Gapor A, Elson CE (1995) Response of hypercholesterolemic subjects to administration of tocotrienols. Lipids 30:1171-1177

29. Zaiden N, Yap WN, Ong S, Xu CH, Teo VH, Chang CP, Zhang XW, Nesaretnam K, Shiba S, Yap YL (2010) Gamma delta tocotrienols reduce hepatic triglyceride synthesis and VLDL secretion. J Atheroscler Thromb 17:1019-1032

30. Ishiyama M, Tominaga H, Shiga M, Sasamoto K, Ohkura Y, Ueno K (1996) A combined assay of cell viability and in vitro cytotoxicity with a highly water-soluble tetrazolium salt, neutral red and crystal violet. Biol Pharm Bull 19:1518-1520

31. Folch J, Lees M, Stanley GHS (1957) A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 226:497-509

32. Bradford, MM (1976) Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding Anal. Biochem. 72: 248–254

33. Miyazawa T, Suzuki T, Fujimoto K, Yasuda K (1992) Chemiluminescent simultaneous determination of phosphatidylcholine hydroperoxide and phosphatidylethanolamine hydroperoxide in the liver and brain of the rat. J Lipid Res 33:1051-1059

34. Kinoshita M, Oikawa S, Hayasaka K, Sekikawa A, Nagashima T, Toyota T, Miyazawa T (2000) Age-related increase in plasma phosphatidylcholine

hydroperoxide concentrations in normal subjects and patients with hyperlipidemia Clin Chem 46:822-828

35. Wada S, Satomi Y, Murakoshi M, Noguchi N, Yoshikawa T, Nishino H (2005) Tumor suppressive effects of tocotrienol in vivo and in vitro. Cancer Lett 2:181-191

36. Asai A, Nakagawa K, Miyazawa T (1999) Antioxidative effects of turmeric, rosemary and capsicum extracts on membrane phospholipid peroxidation and liver metabolism in mice. Biosci Biotecnol Biochem 12:2118-122

37. Zhang D, Al-Hendy M, Richard-Davis D, Montgometry-Rice V, Rajaratnam V, Al-Hendy A (2010) Antiproliferative and proapoptotic effects of epigallocatechin gallate on human leiomyoma cells. Fertil Steril 5:1887-1893

38. Way TD, Lin HY, Kuo DH, Tsai SJ, Shieh JC, Wu JC, Lee MR, Lin JK (2009) Pu-erh tea attenuates hyperlipogenesis and induces hepatoma cells growth arrest

through activating AMP-activated protein kinase (AMPK) in human HepG2 cells. J Agric Food Chem 12: 5257-5264

39. Radler U, Stangl H, Lechner S, Lienbacher G, Krepp R, Zeller E, Brachinger M, Eller-Berndl D, Fischer A, Anzur C, Schoerg G, Mascher D, Laschan C, Anderwald C, Lohninger A (2011) A combination of (ω-3) polyunsaturated fatty acids, Polyphenols and L- carnitine reduces the plasma lipid levels and increases the expression of genes involved in fatty acid oxidation in human peripheral blood monoclear cells and hepG2. Ann Nutr Metab 58:133-140

40. Honda A, Miyazaki T, Ikegami T, Iwamoto J, Maeda T, Hirayama, Saito Y, Teramoto T, Matsuzaki Y (2011) Cholesterol 25-hydroxylation activity of CYP3A4. J Lipid Res 52:1509-1516

41. Quereshi AA, Pearce BC, Nor R.M, Gapor A, Peterson DM, Elson C.E (1996) Dietary α-tocopherol attenuates the impact of γ-tocotrienol on hepatic 3-hydroxy-3-methylglutaryl A reductase activity in chickens. J Nutr 126:292-301

42. Punithavathiand VR, Prince PS (2009) Combined effects of quercetin and α-tocopherol on lipids and glycoprotein components in isoproterenol induced myocardial infarcted wistar rats. Chem Biol Interact 181:322-327

43. Shibata A, Nakagawa K, Sookwong P, Tsuduki T, Asai A, Miyazawa T (2010) α-Tocopherol attenuates the cytotoxic effect of δ-tocotrienol in human colorectal adenocarcinoma cells. Biochem Biophys Res Commun 397:214-219

44. Kobayashi M, Kawashima H, Takemori K, Ito H, Murai A, Masuda S, Yamada K, Uemura D, Horio F (2012) Ternatin, a cyclic peptide isolated from mushroom, and its derivative suppress hyperglycemia and hepatic fatty acid synthesis in spontaneously diabetic KK-A(y) mice. BiochemBiophys Res Commun 427:299-304

45. Burdeos GC, Nakagawa K, Kimura F, Miyazawa T (2012) Tocotrienol attenuates triglyceride accumulation in HepG2 cells and F344 rats. Lipids 47: 471-481.

46. Kopelman PG. (2000) Obesity as a medical problem. Nature, 404, 635-643.

47. Spiegelman BM & Flier JS (2001) Obesity and the regulation of energy balance. Cell, 104, 531-543

48. Flier JS. (2004) Obesity wars: molecular progress confronts an expanding epidemic.Cell, 116, 337-3503

49. Furuyashiki T, Nagayasu H, Aoki Y, Bessho H, Hashimoto T, Kanazawa K et al. (2004) Tea catechin suppresses adipocyte differentiation accompanied by down-regulation of PPARgamma2 and C/EBPalpha in 3T3-L1 cells. Bioscience, Biotechnology, and Biochemistry, 68, 2353-2359

50. Shapiro B& Wertheimer E (1948) The synthesis of fatty acids in adipose tissue *in vitro*. Journal of Biological Chemistry, 173, 725-728

51. Kawada T, Kamei Y, Fujita A, Hida Y, Takahashi N, Sugimoto E et al. (2000) Carotenoids and retinoids as suppressors on adipocyte differentiation via nuclear receptors. Biofactors, 13, 103-109

52. Uto-Kondo H, Ohmori R, Kiyose C, Kishimoto Y, Saito H, Igarashi O et al. (2009) Tocotrienol suppresses adipocyte differentiation and Akt phosphorylation in 3T3-L1 preadipocytes. The Journal of Nutrition, 139, 51-57

53. Matsunaga T, Shoji A, Gu N, Joo E, Li S, Adachi T et al. (2012) γ-Tocotrienol attenuates TNF-α-induced changes in secretion and gene expression of MCP-1, IL-6 and adiponectin in 3T3-L1 adipocytes. Molecular Medicine Reports, 5, 905-909

54. Greenberg AS, Coleman RA, Kraemer FB, McManaman JL, Obin MS, Puri V et al. (2011) The role of lipid droplets in metabolic disease in rodents and humans. The Journal of Clinical Investigation, 121, 2102-2110

55. Alberts AW, Strauss AW, Hennessy S &Vagelos PR (1975) Regulation of synthesis of hepatic fatty acid synthetase: binding of fatty acid synthetase antibodies to polysomes. Proceedings of the National Academy of Sciences of the United States of America, 7, 3956-3960

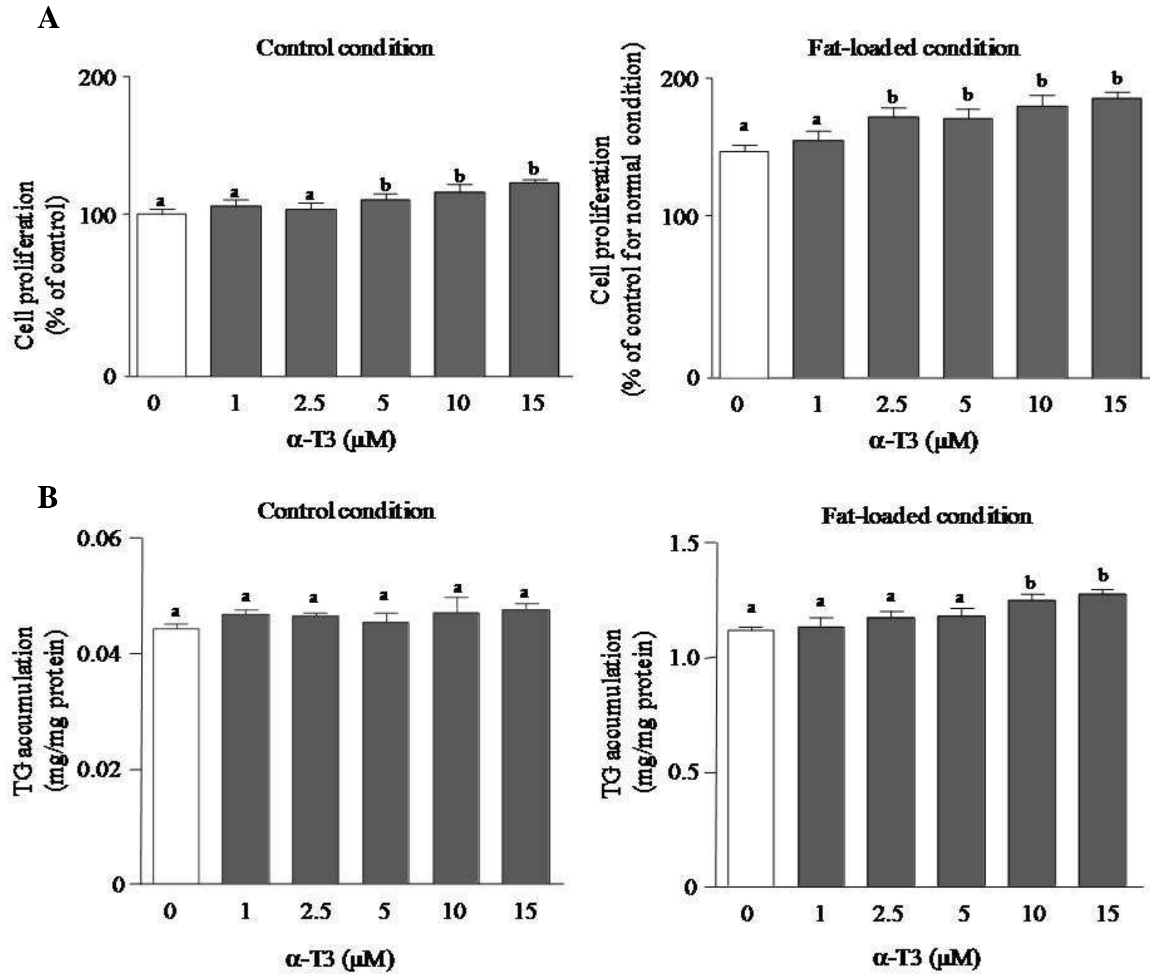
56. Yang F, Vought BW, Satterlee JS, Walker AK, Jim Sun ZY, Watts JL et al. (2006) An ARC/Mediator subunit required for SREBP control of cholesterol and lipid homeostasis. Nature, 442, 700-704

57. Cohen P, Ntambi JM, & Friedman JM (2003) Stearoyl-CoA desaturase-1 and the metabolic syndrome. Current Drug Targets-Immune, Endocrine &Metabolic Disorder, 3, 271-280

58. Cohen P, & Friedman JM (2004) Leptin and the control of metabolism: role for stearoyl-CoA desaturase-1 (SCD-1). Journal of Nutrition, 134, 2455S-2463S

59. Robinson MJ & Cobb MH (1997) Mitogen-activated protein kinase pathways. Current Opinion on Cell Biology, 9, 180-186

Supplementary figure 1



Supplementary figure 1. Effect of increasing concentrations of α-Toc on cell proliferation (A) and TG accumulation (B) of HepG2 in normal and fat overloaded conditions. Experimental procedures are shown in Materials and Methods. Data are expressed as mean ± SD (n = 6 for cell proliferation and n = 3 for TG accumulation). Means without a common letter differ, $P < 0.05$.

List of original publications described in this dissertation

1. Burdeos GC, Nakagawa K, Kimura F, Miyazawa T (2012) Tocotrienol attenuates triglyceride accumulation in HepG2 cells and F344 rats. Lipids 47: 471-481.
2. Gregor, C.B., Nakagawa, K., Watanabe, A., Kimura, F., Miyazawa, T, (2013) γ-Tocotrienol attenuates triglyceride through effect on lipogenic gene expressions in mouse hepatocellular carcinoma Hepa 1-6. J. Nutr. Sci. Vitaminol.59, *148-151.*

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