

# Gamma-tocotrienol reduces the triacylglycerol level in rat primary hepatocytes through regulation of fatty acid metabolism

Chie Muto,<sup>1</sup> Rieko Yachi,<sup>2</sup> Yoshinori Aoki,<sup>3</sup> Taisuke Koike,<sup>3</sup> Osamu Igarashi<sup>1</sup> and Chikako Kiyose<sup>1,2,\*</sup>

<sup>1</sup>Department of Nutrition and Life science, Kanagawa Institute of Technology and <sup>2</sup>Department of Applied Chemistry, Graduate School of Kanagawa Institute of Technology, 1030 Shimo-ogino, Atsugi-shi, Kanagawa 243-0292, Japan

<sup>3</sup>Eisai Food and Chemical Co., Ltd., 2-13-10 Nihonbashi, Chuo-ku Tokyo 103-0027, Japan

(Received 6 September, 2012; Accepted 21 September, 2012; Published online 30 November, 2012)

The present study was carried out to investigate the effect of vitamin E analogs, especially gamma-tocotrienol ( $\gamma$ -T3), on hepatic TG accumulation and enzymes related to fatty acid metabolism in three types of rat primary hepatocytes: (1) normal hepatocytes, (2) hepatocytes incubated in the presence of palmitic acid (PA), and (3) hepatocytes with fat accumulation. Our results showed that  $\gamma$ -T3 significantly reduced the TG content of normal hepatocytes.  $\gamma$ -T3 also increased the expression of carnitine palmitoyltransferase 1 (CPT1A) mRNA, and tended to reduce that of sterol regulatory element binding protein 1c (SREBP-1c) mRNA. In addition,  $\gamma$ -T3 markedly suppressed the gene expression of both C/EBP homologous protein (CHOP) and SREBP-1c induced by PA. As these two genes are located downstream of endoplasmic reticulum (ER) stress, their suppression by  $\gamma$ -T3 might result from a decrease of ER stress. Moreover,  $\gamma$ -T3 suppressed the expression of interleukin 1 $\beta$  (IL-1 $\beta$ ), which lies downstream of CHOP signaling. Taken together, our data suggest that  $\gamma$ -T3 might prevent hepatic steatosis and ameliorate ER stress and subsequent inflammation in the liver.

**Key Words:** tocotrienol, fatty liver, SREBP-1c, CPT1A, CHOP

Non-alcoholic fatty liver disease (NAFLD) is a clinical entity that includes a spectrum of conditions ranging from simple steatosis to steatohepatitis (NASH), cirrhosis, and liver cancer. NAFLD has been described as the hepatic manifestation of metabolic syndrome, and affected patients show histopathological changes similar to those of alcoholic fatty liver disease despite the absence of a history of excessive alcohol intake. The incidence of NAFLD has been increasing dramatically in not only Western countries but also the Asia-Pacific region.<sup>(1)</sup> The so-called “two-hit theory” has been widely accepted as explaining the pathogenetic mechanism of NASH. The “first hit” is excessive fat accumulation in the liver induced by a high daily intake of energy and fat; thereafter, the “second hit” is characterized by induction of oxidative stress and inflammatory cytokines as a result of fat accumulation and deteriorating insulin resistance.

Natural vitamin E is divided into tocopherols (Toc) and tocotrienols (T3). T3 molecules differ from Toc molecules in that the former possess three double bonds in the phytyl tail. These molecules are further divided into four isomers —  $\alpha$ ,  $\beta$ ,  $\gamma$ , and  $\delta$  — according to the number and position of the methyl group on the chroman ring. Although Toc is present in various foodstuffs such as nuts and seeds, green and yellow vegetables, and fish eggs, foodstuffs contain T3 are not as numerous, rice oil, palm oil, corn and rice bran being the main sources. T3 has been reported to decrease the plasma cholesterol concentration,<sup>(2)</sup> to inhibit the

growth of breast cancer,<sup>(3)</sup> and to suppress angiogenesis.<sup>(4)</sup>  $\gamma$ -T3 in particular has been shown to be a potent inhibitor of nuclear factor- $\kappa$ B (NF- $\kappa$ B) activation, leading to anti-angiogenic, anti-proliferative, pro-apoptotic, anti-metastatic, anti-inflammatory, and immunomodulatory effects.<sup>(5)</sup>

Recently, it has been reported that daily supplementation of vitamin E (800 IU/day) for 96 weeks ameliorated certain parameters of NASH, including the serum levels of alanine and aspartate aminotransferases (ALT and AST), hepatic steatosis, and lobular inflammation,<sup>(6)</sup> although the mechanism responsible was unclear. Our previous study using rats showed that T3 was more effective than  $\alpha$ -Toc or  $\gamma$ -Toc for suppressing the increases in plasma ALT and the hepatic content of triacylglycerol (TG) elicited by administration of carbon tetrachloride.<sup>(7)</sup> Moreover, Burdeos *et al.*<sup>(8)</sup> have reported that  $\gamma$ -T3 attenuated the accumulation of TG by regulating the gene and protein expressions of fatty acid synthase (FAS), carnitine palmitoyltransferase 1 (CPT1), and cytochrome P450 3A4 (CYP3A4) in the HepG2 human liver cell line.

On the other hand, some studies have shown that an increase of saturated fatty acids (SFAs) induces inflammation. For example, it has been reported that, in monocyte/macrophage cells, SFAs including lauric acid (C12:0) and palmitic acid (PA;C16:0) induce inflammation through the toll-like receptor-4 (TLR-4) signaling pathway, which is known as a receptor of lipopolysaccharide (LPS).<sup>(9)</sup> In addition, PA has been reported to induce endoplasmic reticulum (ER) stress and subsequent apoptosis.<sup>(10)</sup> Therefore, suppression of not only fat accumulation but also hepatic inflammation and ER stress induced by SFAs such as PA appears to be important for prevention of NASH.

In the present study we investigated the effect of  $\gamma$ -T3 on hepatic fat accumulation, which is the etiological basis of NASH and a complicating factor related to the progression of NAFLD. Using rat primary hepatocytes, we focused on how  $\gamma$ -T3 affected the TG content and expression of two genes related to fatty acid metabolism: CPT1A and sterol regulatory element-binding protein-1c (SREBP-1c). These effects were compared among three types of hepatocytes: normal hepatocytes, hepatocytes that had been cultured in the presence of PA, and hepatocytes that had accumulated fat. In addition, in rat primary hepatocytes cultured with PA, we investigated the effects of  $\gamma$ -T3 on expression of the genes for TLR-4, interleukin-1 $\beta$  (IL-1 $\beta$ ), and C/EBP homologous protein (CHOP), which are related to inflammation and ER stress.

\*To whom correspondence should be addressed.  
E-mail: kiyose@bio.kanagawa-it.ac.jp

**Table 1.** Assay ID and reference sequence number of primer probe mixtures used in TaqMan Gene Expression Assays (Applied Biosystems) are shown

	Symbol	Assay ID	RefSeq
SREBP-1c	<i>Srebf1</i>	Rn01495769_m1	XM_213329.5
CPT1A	<i>Cpt1a</i>	Rn00580702_m1	NM_031559.2
TLR-4	<i>Tlr4</i>	Rn00569848_m1	NM_019178.1
IL1 $\beta$	<i>Il1b</i>	Rn99999009_m1	NM_031512.2
CHOP	<i>Ddit3</i>	Rn00492098_g1	NM_001109986.1/ NM_024134.2
$\beta$ -Actin	<i>Actb</i>	Rn00667869_m1	NM_031144.2

SREBP-1c; sterol regulatory element binding protein 1c, CPT1A; carnitine palmitoyltransferase 1, TLR-4; toll like receptor 4, IL1 $\beta$ ; interleukin 1 $\beta$ , CHOP; C/EBP homologous protein.

## Materials and Methods

**Materials.** Gamma-T3 (98.7%) was kindly donated by Eisai Food & Chemical Co. Ltd. (Tokyo, Japan). Dietary vitamin E-stripped corn oil was purchased from Funabasi Farm Co. (Chiba, Japan). Dietary *tert*-butylhydroquinone, L(-)-cysteine, cholesterol, and sodium cholate were purchased from Wako Pure Chem. Ind. Ltd. (Osaka, Japan). Other purified foodstuffs were purchased from Oriental Yeast Co. Ltd.

**Experimental animals.** Male Sprague-Dawley (SD)-IGS rats were purchased from Charles River Japan, Inc. (Kanagawa, Japan). They were housed individually in stainless steel wire mesh cages and fed a commercial chow for three days. The temperature and humidity were controlled at  $23 \pm 2^\circ\text{C}$  and  $55 \pm 5\%$ , respectively, with a 12 h/12 h light-dark cycle. A seven-week-old rat used for preparation of normal primary hepatocytes was soon laparotomized for perfusion. Separately, a five-week-old rat used for preparation of fat-accumulated primary hepatocytes was fed a high fat and cholesterol diet based on the AIN-93G diet, containing 20% (w/w) vitamin E-stripped corn oil, 1% (w/w) cholesterol, and 0.25% (w/w) sodium cholate, for two weeks.

All experiments were conducted in accordance with the guidelines for the care and use of laboratory animals at Kanagawa Institute of Technology.

**Hepatocyte isolation and culture.** After the rats had been laparotomized under diethyl ether anesthesia, hepatocytes were collected by *in situ* perfusion of the liver with collagenase according to the method of Tenoutasse.<sup>(11)</sup> Briefly, the livers were perfused with 200 ml of Hank's buffer (pH 7.5), followed by the same volume of collagenase buffer (pH 7.5). The livers were then isolated and placed in DMEM-F12 (-) FBS medium (Sigma-Aldrich Co. LLC., Tokyo, Japan) containing penicillin/streptomycin (100 U/ml and 100 mg/ml), and immediately transferred to a clean bench. The filtered hepatocytes were washed twice with the medium, and then seeded at a density of  $2 \times 10^6$  per 60-mm dish or  $4 \times 10^5$  per well of a 12-well collagen-coated plate in DMEM-F12 (+) FBS medium supplemented with 10% fetal bovine serum (FBS), triiodothyronine ( $1 \times 10^{-9}$  M), hydrocortisone ( $5 \times 10^{-8}$  M), ethanolamine ( $1 \times 10^{-6}$  M), ornithine ( $4 \times 10^{-4}$  M), lactic acid ( $1.8 \times 10^{-5}$  M), and penicillin/streptomycin (100 U/ml and 100 mg/ml). The cells were grown at  $37^\circ\text{C}$  in a humidified atmosphere containing 5% CO<sub>2</sub>. The medium was changed after the first 4 h to remove unattached cells, and culture was then continued for 20 h.

At 24 h after cell seeding, 50  $\mu\text{M}$   $\gamma$ -T3 (final concentration) was added to the hepatocytes in DMEM-F12 (+) BSA medium containing 0.5% bovine serum albumin (BSA), insulin ( $1.75 \times 10^{-7}$  M), triiodothyronine ( $1 \times 10^{-9}$  M), ornithine ( $4 \times 10^{-4}$  M), lactic acid ( $1.8 \times 10^{-5}$  M), and penicillin/streptomycin (100 U/ml and 100 mg/ml), followed by incubation for 6 h. The medium was then changed to DMEM-F12 (+) BSA or the same medium supplemented with 0.5 mM PA (final concentration). The hepatocytes were then further incubated for 8 h for RNA analysis and for 18 h for measurement of cellular lipid.

PA-supplemented medium was prepared by adding PA (sodium salt) solution to DMEM-F12 (+) BSA medium at 0.5 mM (palmitic acid : BSA = 1 : 6.6 by molar ratio), equilibrated by shaking for 1 h at  $37^\circ\text{C}$ , and sterilized using a syringe filter before use.<sup>(12)</sup>

**Cell toxicity assay.** The influence of  $\gamma$ -T3 and PA on cytotoxicity was examined by MTT assay. This assay is based on the ability of viable cells to metabolize water-soluble 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into an insoluble formazan salt.<sup>(13)</sup> After the seeded hepatocytes had been cultured in 12-well plates for 24 h, 10–50  $\mu\text{M}$   $\gamma$ -T3 and 0.025–0.5 mM PA were added to them separately, and incubation was continued for a further 24 h. The medium was then changed by adding 120  $\mu\text{l}$  of MTT (5 mg/ml in PBS) to 1.2 ml of medium, and incubation was continued at  $37^\circ\text{C}$  for 4 h. After the MTT reaction, the medium was removed and the insoluble formazan crystals were dissolved in DMSO. Finally, the absorbance was measured at 570 nm.

**RNA isolation and analysis.** The hepatocytes in 12-well plates were incubated with  $\gamma$ -T3 for 6 h and then cultured under normal conditions or in the presence of PA for an additional 8 h as described above. Total RNA was extracted with RNA iso Plus reagent (TAKARA BIO INC., Shiga, Japan). RNA purity was determined from the A258/A280 ratio. Total RNA was reverse-transcribed to complementary DNA (cDNA) using a high-capacity RNA-to-cDNA™ kit (Life Technologies Corporation, Tokyo, Japan). Next, the cDNA was quantified using a 7500 Fast Real-Time PCR system and a real-time PCR kit (TaqMan® Gene Expression Assays, Life Technologies Corporation) based on the manufacturer's instructions, using  $\beta$ -actin as an endogenous control. Details of the primer/probe mixture of SREBP-1c, CPT1A, TLR-4, CHOP, IL-1 $\beta$ , and  $\beta$ -actin are shown in Table 1. The PCR run consisted of an initial denaturation at  $95^\circ\text{C}$  for 20 s, 40 cycles of denaturation at  $95^\circ\text{C}$  for 3 s, and annealing/elongation at  $60^\circ\text{C}$  for 30 s.

**Measurement of hepatocyte lipid content.** The hepatocytes in 60-mm dishes were incubated with  $\gamma$ -T3 for 6 h and then cultured under normal conditions or in the presence of PA for an additional 18 h. After the cells had been washed with PBS twice, 1.5 mL of hexane/2-propanol (3:2 by vol.) solvent was added to the dishes, and the incubation was continued at  $4^\circ\text{C}$  for 15 min. The solvent was then collected in a test tube and the same extraction was repeated one more time. After centrifugation of the test tube and its content at 3000 rpm and  $4^\circ\text{C}$  for 5 min, 2 ml of the upper layer was collected, transferred to a centrifuge tube, and the solvent was distilled using a rotary evaporator. The extract was re-dissolved in 150  $\mu\text{l}$  of 2-propanol and 1 ml of 2% Triton/2-propanol, then centrifuged at 3000 rpm and  $4^\circ\text{C}$  for 5 min. The final sample was obtained by emulsifying the upper layer at  $70^\circ\text{C}$  for 20 min. The concentration of TG was measured using TG E-test Wako (Wako Pure Chem. Ind. Ltd.). The amounts of hepatic lipids were expressed as mg lipid per mg protein. The protein fraction was collected with 0.5 M sodium hydrate, and quantified by the Lowry method.<sup>(14)</sup>

**Oil red-O staining.** Observations of lipid droplets in normal and fat-accumulated hepatocytes were made using oil red-O staining. After hepatocytes seeded on 60-mm dishes had been cultured for 48 h, they were washed with PBS, and then fixed with 10% formalin solution. The hepatocytes were then washed with distilled water, and incubated with 0.3% oil red-O in 60% 2-propanol for 30 min. The stained hepatocytes were washed again with distilled water several times, and then observed using a light microscope.

**Statistical analysis.** All data are presented as mean  $\pm$  SD. Significance of differences in assay values was evaluated using one-way ANOVA, followed by the Bonferroni multiple comparison post-hoc test or independent *t* test, using SPSS ver.11.0 for Windows. Differences at  $p < 0.05$  were considered to be statistically significant.

## Results

**Cytotoxicity of  $\gamma$ -T3 and PA.** First, the cytotoxicity of  $\gamma$ -T3 or PA on rat primary hepatocytes was assessed by incubation of the cells with various doses for 24 h, and then measuring their viability using the MTT assay. Hepatocytes incubated with  $\gamma$ -T3 (50  $\mu$ M) or PA (0.5 mM) showed no reduction of viability

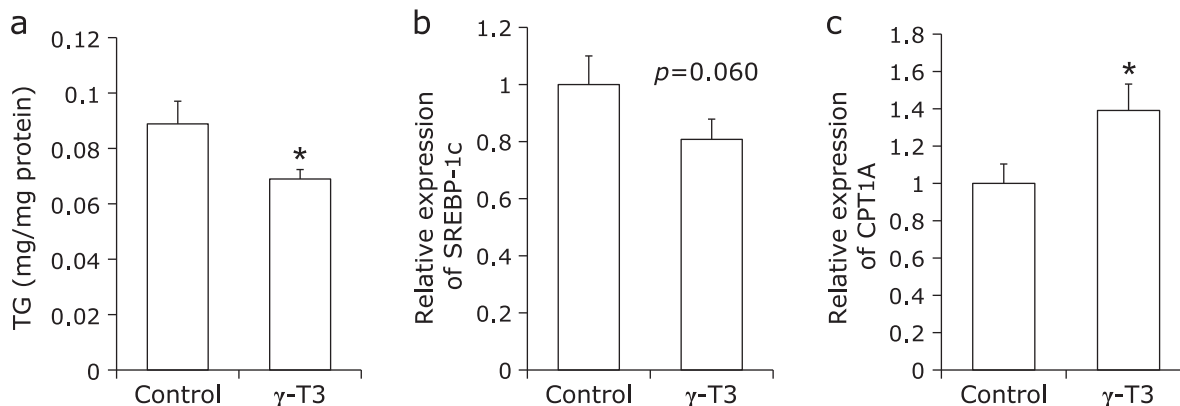
(105  $\pm$  1.26% and 103  $\pm$  1.81% vs control, respectively). Therefore, these concentrations of  $\gamma$ -T3 and PA were used in the subsequent studies.

### Effects of $\gamma$ -T3 on TG content and expression of mRNAs related to hepatic TG metabolism in normal hepatocytes.

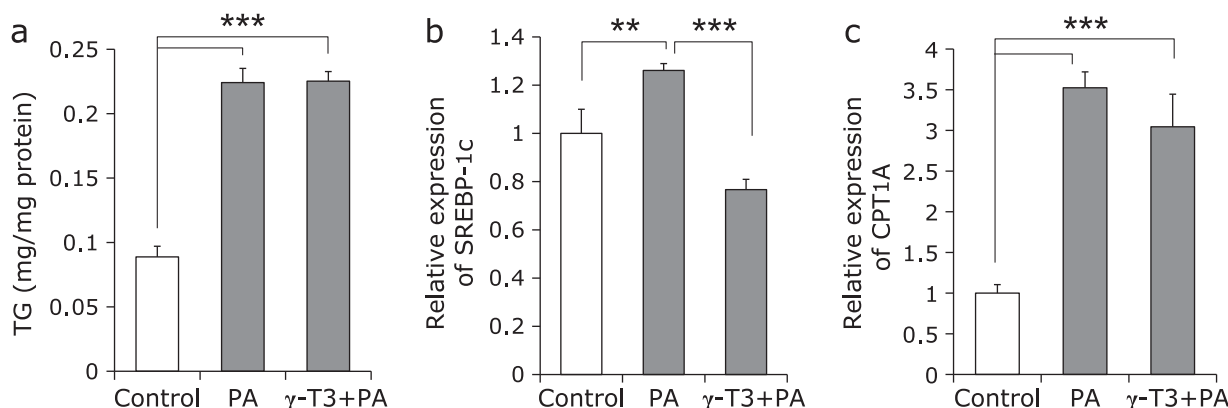
Hepatic TG content was significantly reduced by  $\gamma$ -T3 (50  $\mu$ M) in normal primary hepatocytes (Fig. 1a). To elucidate the effect of  $\gamma$ -T3 on enzymes related to fatty acid metabolism, especially SREBP-1c and CPT1A, expression of their mRNAs in the hepatocytes was measured. It was found that expression of SREBP-1c mRNA tended to be reduced by  $\gamma$ -T3 (Fig. 1b) whereas that of CPT1A mRNA was significantly increased (Fig. 1c). This suggested that  $\gamma$ -T3 might accelerate TG catabolism and suppress TG synthesis in the liver.

### Effects of $\gamma$ -T3 on TG content and expression of mRNAs related to hepatic TG metabolism in hepatocytes incubated with PA.

Next, we examined the effect of  $\gamma$ -T3 on PA-induced TG accumulation in normal hepatocytes.  $\gamma$ -T3 did not suppress the TG accumulation elevated by PA addition (Fig. 2a). However,  $\gamma$ -T3 significantly suppressed the increase of SREBP-1c mRNA expression induced by PA (Fig. 2b). Expression of CPT mRNA was also increased by PA, and no differences were observed after addition of  $\gamma$ -T3 (Fig. 2c).



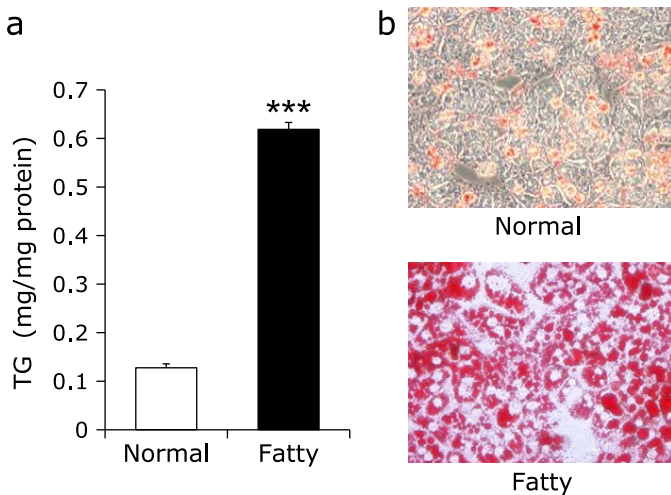
**Fig. 1.** Effects of  $\gamma$ -T3 on normal hepatocytes obtained by perfusion from a 7-week-old rat and then cultured under normal conditions. The effects on TG content (a) and expression of mRNAs for SREBP-1c (b) and CPT1A (c) are shown. The hepatocytes were incubated with 50  $\mu$ M  $\gamma$ -T3 for 6 h and medium was changed. The hepatocytes were collected after 8 h and 18 h for RNA analysis and TG measurement, respectively. Data are expressed as mean  $\pm$  SD ( $n = 3$ ), \* $p < 0.05$  (independent *t* test).



**Fig. 2.** Effects of  $\gamma$ -T3 on normal hepatocytes obtained by perfusion from a 7-week-old rat and then cultured in the presence of PA. The effects on TG content (a) and expression of mRNAs for SREBP-1c (b) and CPT1A (c) are shown. The hepatocytes were incubated with 50  $\mu$ M  $\gamma$ -T3 for 6 h, and then incubated with 0.5 mM PA. The hepatocytes were collected after 8 h and 18 h for RNA analysis and TG measurement, respectively. Data are expressed as mean  $\pm$  SD ( $n = 3$ ), \*\*\* $p < 0.001$ . \*\*\* $p < 0.001$  (one-way ANOVA, followed by the Bonferroni multiple comparison post-hoc test).

These results suggested that  $\gamma$ -T3 did not suppress the accumulation of TG caused by rapid inflow of PA into hepatocytes. However,  $\gamma$ -T3 suppressed the expression of SREBP-1c mRNA induced by addition of PA.

**Development of a model of fat accumulation in hepatocytes.** In order to compare the effects of  $\gamma$ -T3 between normal liver and fatty liver, we attempted to develop a model of fat accumulation in primary hepatocytes. The hepatocytes were obtained by perfusing the liver of a fatty rat that had been fed a high fat and cholesterol diet for two weeks. The TG content of fatty hepatocytes was found to be dramatically increased in comparison with normal hepatocytes (Fig. 3a). In addition, light microscopy after oil red-O staining demonstrated a number of red-stained fat droplets in fatty hepatocytes (Fig. 3b). Therefore, we decided to assess the effects of  $\gamma$ -T3 using these fatty hepatocytes.



**Fig. 3.** Comparison of fat accumulation between normal hepatocytes obtained by perfusion from a 7-week-old rat, and fatty hepatocytes obtained by perfusion from a 7-week-old rat that had been fed a high fat and cholesterol diet for 2 weeks beforehand. The effects on TG content (a) and observation of lipid droplets made using oil red-O staining (b) at 48 h after perfusion are shown. Data are expressed as mean  $\pm$  SD ( $n = 3$ ), \*\*\* $p < 0.001$  (independent  $t$  test).

**Effects of  $\gamma$ -T3 on TG content and expression of mRNAs related to hepatic TG metabolism in fatty hepatocytes.**

When fatty hepatocytes were cultured with  $\gamma$ -T3 at 50  $\mu$ M (final concentration), their TG content was not reduced, unlike the outcome in normal hepatocytes (Fig. 4a). In addition,  $\gamma$ -T3 did not affect the expression of mRNA for either SREBP-1c or CPT1A (Fig. 4 b and c). This suggested that  $\gamma$ -T3 was unable to attenuate the excessive accumulation of lipids in hepatocytes.

**Effects of  $\gamma$ -T3 on expression of mRNAs related to inflammation and ER stress in hepatocytes incubated with PA.**

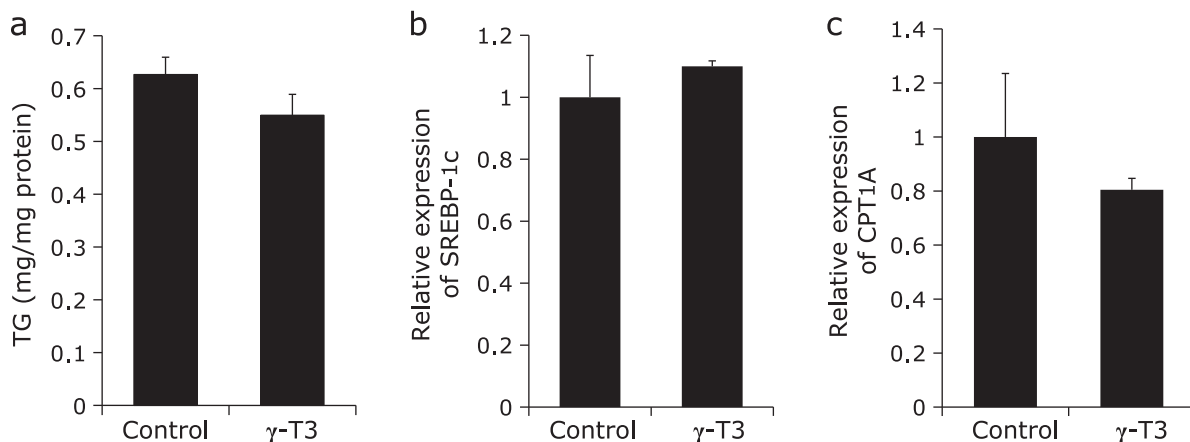
We also investigated the anti-inflammatory effect of  $\gamma$ -T3 on primary hepatocytes that had been incubated with PA. Whereas  $\gamma$ -T3 elicited no significant change in the expression of TLR-4 mRNA (Fig. 5a), it significantly suppressed the expression of CHOP mRNA, a marker of ER stress, that had been stimulated by addition of PA (Fig. 5b). Interestingly,  $\gamma$ -T3 reduced the expression of IL-1 $\beta$  mRNA irrespective of whether or not hepatocytes had been cultured in the presence of PA (Fig. 5c).

These results suggested that PA induced the expression of CHOP mRNA through an increment of ER stress, and that ER stress was significantly suppressed by  $\gamma$ -T3.

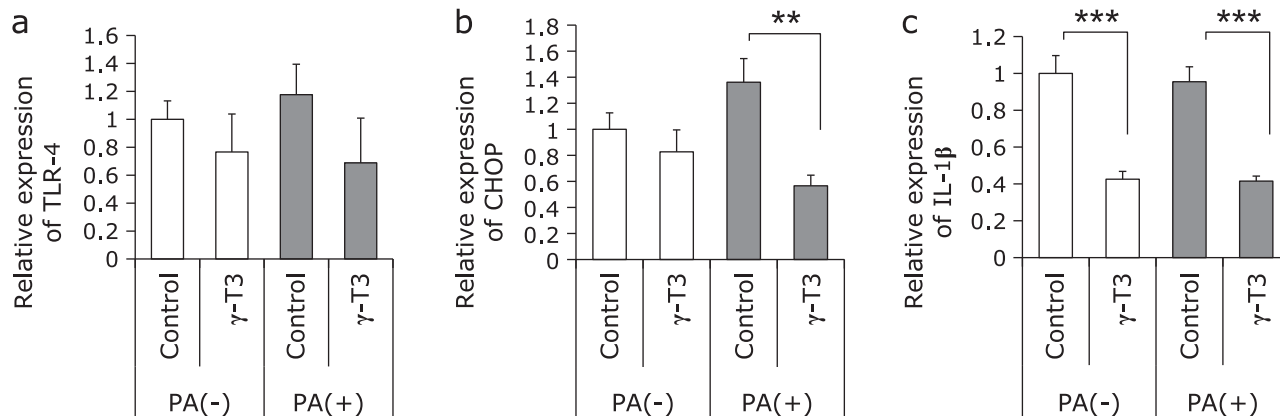
**Discussion**

In the present study using rat primary hepatocytes, we examined the effects of  $\gamma$ -T3 on TG content and the expression of genes for enzymes involved in fatty acid metabolism (SREBP-1c and CPT1A) and factors related to ER stress and inflammation (TLR-4, CHOP and IL-1 $\beta$ ). These effects were compared among 1) normal hepatocytes, 2) hepatocytes cultured with PA, and 3) hepatocytes with fat accumulation in order to investigate any effects of  $\gamma$ -T3 relevant to the prevention or amelioration of fatty liver disease.

Our result showed that  $\gamma$ -T3 reduced the TG content of normal hepatocytes (Fig. 1a). Using SD rats, we had previously demonstrated the suppressive effect of a T3 mixture on hepatic TG accumulation resulting from administration of carbon tetrachloride.<sup>(7)</sup> Burdeos *et al.*<sup>(8)</sup> have also reported that a T3 mixture from rice bran reduced hepatic TG in F344 rats, and also that  $\gamma$ -T3 at 10–15  $\mu$ M decreased the TG content of HepG2 cells. They suggested that the TG-lowering effect of  $\gamma$ -T3 in HepG2 cells could be partly explained by its ability to down-regulate the hepatic fatty acid synthase gene (FAS) and up-regulate  $\beta$ -oxidation genes (CPT1A and CYP3A4). In the present study we



**Fig. 4.** Effects of  $\gamma$ -T3 on fatty hepatocytes obtained by perfusion from a 7-week-old rat that had been fed a high fat and cholesterol diet for 2 weeks beforehand. The effects on TG content (a) and expression of mRNAs for SREBP-1c (b) and CPT1A (c) are shown. The hepatocytes were incubated with 50  $\mu$ M  $\gamma$ -T3 for 6 h and medium was changed. The hepatocytes were collected after 8 h and 18 h for RNA analysis and TG measurement, respectively. Data are expressed as mean  $\pm$  SD ( $n = 3$ ), (independent  $t$  test).



**Fig. 5.** Effects of  $\gamma$ -T3 on normal hepatocytes obtained by perfusion from a 7-week-old rat and then cultured in the presence of PA. The effects on expression of mRNAs for TLR-4 (a), CHOP (b), and IL-1 $\beta$  (c) are shown. The hepatocytes were incubated with 50  $\mu$ M  $\gamma$ -T3 for 6 h, and then incubated with 0.5 mM PA. The hepatocytes were collected after 8 h and 18 h for RNA analysis and TG measurement, respectively. Data are expressed as mean  $\pm$  SD ( $n = 3$ ), \*\* $p < 0.01$ . \*\*\* $p < 0.001$  (one-way ANOVA, followed by the Bonferroni multiple comparison post-hoc test).

confirmed the TG-lowering effects of  $\gamma$ -T3 in rat primary hepatocytes. However, we also found another mechanism by which  $\gamma$ -T3 reduced the hepatic TG content. In normal rat primary hepatocytes, up-regulation of CPT1A and down-regulation of SREBP-1c were considered to be the main mechanisms responsible for the TG-lowering effect (Fig. 1 b and c). CPT1, located in mitochondria, is the rate-limiting enzyme for fatty acid oxidation. It has three isoforms, CPT1A, CPT1B, and CPT1C, and CPT1A is the isoform known to exist in the liver. On the other hand, SREBPs are located on the ER membrane, and operate as nuclear transcription factors. There are three isoforms, SREBP-1a, -1c, and -2. SREBP-1c in particular has a greater role in regulating fatty acid synthesis than it does in cholesterol synthesis. In addition, Yahagi *et al.*<sup>(15)</sup> have shown that absence of SREBP-1 markedly reduced the hepatic TG content of leptin-deficient (*Lep<sup>ob/ob</sup> × Srebp-1<sup>-/-</sup>*) mice relative to *Lep<sup>ob/ob</sup> × Srebp-1<sup>+/+</sup>* mice. Furthermore, using wild type and SREBP-1c-null mice, Ji *et al.*<sup>(16)</sup> demonstrated that the predominant mechanism of hepatic TG accumulation in mice fed alcohol intragastrically required the presence of SREBP-1c. Therefore, it appears that SREBP-1c plays important roles in the synthesis of not only fatty acid but also TG. Moreover, in a human clinical study, Kohjima *et al.*<sup>(17)</sup> have evaluated the expression of fatty acid metabolism-related genes in patients with NAFLD ( $n = 26$ ) and normal liver ( $n = 10$ ), and found that in NAFLD SREBP-1c gene expression was increased and CPT1A gene expression was decreased. In other words, in order to ameliorate NAFLD, down-regulation of the SREBP-1c gene and up-regulation of the CPT1A gene appear to be very important. Therefore, our present data support the notion that  $\gamma$ -T3 has a capacity to ameliorate NAFLD through regulation of these genes.

To examine this possibility in greater depth, we compared the effects of  $\gamma$ -T3 among three types of hepatocytes: 1) normal hepatocytes; 2) hepatocytes cultured with PA, and 3) hepatocytes that had accumulated fat. In normal hepatocytes  $\gamma$ -T3 might be expected to suppress TG synthesis through down-regulation of SREBP-1c, and then activate beta-oxidation of remaining fatty acid by up-regulation of CPT1A. However, in a situation where large amounts of free fatty acids are flowing into hepatocytes, TG synthesis would advance at a pace faster than suppression of SREBP-1c by  $\gamma$ -T3. Furthermore, in a situation where TG accumulation in hepatocytes had already advanced, any improvement of steatosis by  $\gamma$ -T3 might be difficult because of the inability of SREBP-1c and CPT1A to control the form of TG.

On the other hand, acceleration of very low-density lipoprotein (VLDL) secretion is also important for reduction of the hepatic TG

content. In this study, we examined the expression of apolipoprotein B (ApoB) mRNA, but found it was unaffected by  $\gamma$ -T3 (data not shown). By contrast, Zaidan *et al.* have shown that  $\gamma$ -T3 and  $\delta$ -T3 down-regulated the protein expression of both ApoB100 and diacylglycerol acyltransferase 2 (DGAT2) in HepG2 cells. As DGAT2 plays a key role in TG biosynthesis, and ApoB plays a role in VLDL synthesis in the liver, down-regulation of these proteins would be considered to ameliorate arteriosclerosis. However, in the context of fatty liver disease, excessive suppression of ApoB expression would not be desirable. At the present time, it remains to be clarified how  $\gamma$ -T3 is involved in VLDL synthesis and secretion. Therefore, further studies will be needed to examine the effect of  $\gamma$ -T3 on secretion of TG from the liver to plasma.

In this study, we also found that  $\gamma$ -T3 attenuated the increased expression of CHOP elicited by culture of hepatocytes in the presence of PA (Fig. 5b). CHOP signaling is induced by ER stress, which in turn leads to apoptosis. Endo *et al.*<sup>(18)</sup> have reported that CHOP plays an essential role in TLR-4 pathway signaling during inflammation, and suggested that treatment with LPS would stimulate TLR-4, thus inducing ER stress. Meanwhile, some previous studies have demonstrated that SFAs such as PA induce inflammation through the TLR-4 signaling pathway, as in the case for LPS.<sup>(9,19)</sup> Furthermore, Wei *et al.*<sup>(10)</sup> have shown that PA-induced ER stress and subsequent apoptosis in liver cells are mediated by CHOP signaling. Our present result suggested that PA increased CHOP signaling by inducing ER stress, and we found that  $\gamma$ -T3 markedly suppressed the increase of ER stress that had been induced by PA. However, TLR-4 was not significantly changed (Fig. 5a), perhaps because of a signaling time lag. With regard to ER stress and inflammation, Endo *et al.*<sup>(18)</sup> have reported that expression of CHOP induced by ER stress activated caspase-11 and -1 signaling, and then increased the activation of pro-IL-1 $\beta$  to mature IL-1 $\beta$  in lung cells. Therefore, ER stress would induce further production of inflammatory cytokines, including IL-1 $\beta$ , and in fact we clarified that  $\gamma$ -T3 reduced the gene expression of IL-1 $\beta$  irrespective of whether PA was present or absent (Fig. 5c). These results suggested that  $\gamma$ -T3 suppressed the inflammatory cytokines induced by TLR-4 and CHOP signaling. At the present time, however, it is unclear why  $\gamma$ -T3 reduced the expression of the IL-1 $\beta$  gene under normal conditions. It will therefore be necessary to confirm how  $\gamma$ -T3 directly affects IL-1 $\beta$  gene expression.

In attempting to address the relationship between ER stress and TG accumulation, Wand *et al.*<sup>(20)</sup> have demonstrated that ER stress

activates SREBP-1c, and that this is implicated in  $\beta$ -cell glucolipototoxicity. That is, ER stress induces TG accumulation through an increase of SREBP-1c. Taken together, the data suggest that suppression of ER stress is very important for breaking the vicious cycle between fat accumulation and inflammation in the liver, and that  $\gamma$ -T3 controls fatty acid metabolism and inflammation through amelioration of ER stress.

In summary, we have clarified that  $\gamma$ -T3 significantly reduces the TG content of normal rat primary hepatocytes through up-regulation of CPT1A and down-regulation of SREBP-1c. We have also shown that  $\gamma$ -T3 suppresses the gene expression of both SREBP-1c and CHOP induced by PA. As these two genes are located downstream of ER stress, the suppressive effects of  $\gamma$ -T3 on SREBP-1c and CHOP might result from a decrease of ER stress. Our present findings suggest that  $\gamma$ -T3 might prevent steatosis and ameliorate hepatic ER stress and subsequent inflammation.

## Acknowledgments

This work was supported by The Nippon Foundation, Japan.

## References

- 1 Amarapurkar DN, Hashimoto E, Lesmana LA, *et al.* How common is non-alcoholic fatty liver disease in the Asia-Pacific region and are there local differences? *J Gastroenterol Hepatol* 2007; **22**: 788–793.
- 2 Qureshi AA, Bradlow BA, Brace L, *et al.* Response of hypercholesterolemic subjects to administration of tocotrienols. *Lipids* 1995; **30**: 1171–1177.
- 3 Nesaretnam K, Stephen R, Dils R, Darbre P. Tocotrienols inhibit the growth of human breast cancer cells irrespective of estrogen receptor status. *Lipids* 1998; **33**: 461–469.
- 4 Nakagawa K, Shibata A, Yamashita S, *et al.* *In vivo* angiogenesis is suppressed by unsaturated vitamin E, tocotrienol. *J Nutr* 2007; **137**: 1938–1943.
- 5 Ahn KS, Sethi G, Krishnan K, Aggarwal BB. Gamma-tocotrienol inhibits nuclear factor-kappaB signaling pathway through inhibition of receptor-interacting protein and TAK1 leading to suppression of antiapoptotic gene products and potentiation of apoptosis. *J Biol Chem* 2007; **282**: 809–820.
- 6 Sanyal AJ, Chalasani N, Kowdley KV, *et al.* Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. *N Engl J Med* 2010; **362**: 1675–1685.
- 7 Yachi R, Igarashi O, Kiyose C. Protective effects of vitamin E analogs against carbon tetrachloride-induced fatty liver in rats. *J Clin Biochem Nutr* 2010; **47**: 148–154.
- 8 Burdeos GC, Nakagawa K, Kimura F, Miyazawa T. Tocotrienol attenuates triglyceride accumulation in HepG2 Cells and F344 rats. *Lipids* 2012; **47**: 471–481.
- 9 Lee JY, Sohn KH, Rhee SH, Hwang D. Saturated fatty acids, but not unsaturated fatty acids, induce the expression of cyclooxygenase-2 mediated through Toll-like receptor 4. *J Biol Chem* 2001; **276**: 16683–16689.
- 10 Wei Y, Wang D, Topczewski F, Pagliassotti MJ. Saturated fatty acids induce endoplasmic reticulum stress and apoptosis independently of ceramide in liver cells. *Am J Physiol Endocrinol Metab* 2006; **291**: E275–E281.
- 11 Tenoutasse S, Van Vliet G, Ledru E, Deal C. IGF-I transcript levels in

## Abbreviations

ApoB	apolipoprotein B
BSA	bovine serum albumin
CHOP	C/EBP homologous protein
CPT1A	carnitine palmitoyltransferase 1
ER	endoplasmic reticulum
FBS	fetal bovine serum
IL-1 $\beta$	interleukin 1 $\beta$
NAFLD	nonalcoholic fatty liver disease
NASH	nonalcoholic steatohepatitis
NF- $\kappa$ B	nuclear factor $\kappa$ B
PA	palmitic acid
SFA	saturated fatty acid
SREBP-1c	sterol regulatory element binding protein 1c
TG	triacylglycerol
TLR-4	Toll-like receptor 4
Toc	tocopherol
T3	tocotrienol

## Conflict of Interest

No potential conflicts of interest were disclosed.

- whole-liver tissue, in freshly isolated hepatocytes, and in cultured hepatocytes from lean and obese Zucker rats. *Horm Res* 2003; **59**: 135–141.
- 12 Listenberger LL, Ory DS, Schaffer JE. Palmitate-induced apoptosis can occur through a ceramide-independent pathway. *J Biol Chem* 2001; **276**: 14890–14895.
- 13 Twentyman PR, Luscombe M. A study of some variables in a tetrazolium dye (MTT) based assay for cell growth and chemosensitivity. *Br J Cancer* 1987; **56**: 279–285.
- 14 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; **193**: 265–275.
- 15 Yahagi N, Shimano H, Hasty AH, *et al.* Absence of sterol regulatory element-binding protein-1 (SREBP-1) ameliorates fatty livers but not obesity or insulin resistance in Lep(ob)/Lep(ob) mice. *J Biol Chem* 2002; **277**: 19353–19357.
- 16 Ji C, Chan C, Kaplowitz N. Predominant role of sterol response element binding proteins (SREBP) lipogenic pathways in hepatic steatosis in the murine intragastric ethanol feeding model. *J Hepatol* 2006; **45**: 717–724.
- 17 Kohjima M, Enjoji M, Higuchi N, *et al.* Re-evaluation of fatty acid metabolism-related gene expression in nonalcoholic fatty liver disease. *Int J Mol Med* 2007; **20**: 351–358.
- 18 Endo M, Mori M, Akira S, Gotoh T. C/EBP homologous protein (CHOP) is crucial for the induction of caspase-11 and the pathogenesis of lipopolysaccharide-induced inflammation. *J Immunol* 2006; **176**: 6245–6253.
- 19 Shi H, Kokoeva MV, Inouye K, Tzameli I, Yin H, Flier JS. TLR4 links innate immunity and fatty acid-induced insulin resistance. *J Clin Invest* 2006; **116**: 3015–3025.
- 20 Wang H, Kouri G, Wollheim CB. ER stress and SREBP-1 activation are implicated in beta-cell glucolipototoxicity. *J Cell Sci* 2005; **118**: 3905–3915.