Improvement Effects of Trehangelin A on High-Fat Diet Causing Metabolic Clinical Conditions

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The incidence of obesity has been dramatically increasing over the past 30–40 years in not only developed countries but also developing ones, with over 600 million people now obese.1,2) The excessive consumption of a high-fat diet in the current western lifestyle results in overnutrition, leading to metabolic diseases. Obesity accompanied by metabolic disorders is a major risk factor for various diseases, including cardiovascular disease, type 2 diabetes mellitus, hypertension, hyperlipidemia, coronary artery disease and even certain types of cancers.3–5) Obesity accompanied by metabolic disorders thus increases the risk of death and now constitutes a global health problem. Therefore, controlling metabolic clinical conditions caused by a high-fat diet can help slow the metabolic disease progression and reduce the risk of death.

Trehangelin (THG)-A, THG-B and THG-C were originally purified by solvent extraction from cultured broth in Polyomorpha pheophorbide rubra K07-0510 which was isolated from the root of an orchid plant on Iriomote Island, Okinawa, Japan. The structures of these trehangelins comprise a trehalose moiety and two angelic acid moieties.6) In addition, the THG-A biosynthetic gene cluster of P. rubra K07-0510 was identified.7) Trehangelins have anti-oxidative effect that act to prevent hemolysis of red blood cells (RBCs) induced by light-activated pheophorbide α in vitro.8) Furthermore, trehangelins have shown no cytotoxic activities at concentrations below 100 μg/mL. Of note, P. rubra K07-0510 produces markedly more THG-A than THG-B and THG-C, and the anti-oxidative effect of THG-A was shown to be the strongest among the trehangelins, as well as stronger than that of the same dose of ascorbic acid.6) Therefore, we decided to use THG-A in the present experiments.

Mice fed a chronic high-fat diet become obese with metabolic clinical conditions, including high serum levels of cholesterol, triglyceride and glucose. In addition, mice fed a high-fat diet reportedly have oxidative stress and free radicals in the liver, resulting in metabolic disorder, liver inflammation and hepatocyte damage.8–10) Therefore, oxidative stress caused by a high-fat diet in the early phase plays important roles in inducing liver pathogenic development, including liver inflammation, steatosis, fibrotic liver and ultimately nonalcoholic steatohepatitis (NASH) and cirrhosis.

Based on these previous findings, in the present study, we explored whether or not THG-A treatment in the early phase affects obesity or the progression of metabolic clinical conditions caused by a high-fat diet using a mouse model of obesity.

MATERIALS AND METHODS

THG-A THG-A was purified from culture broth of P. rubra K07-0510 as previously described.6) THG-A freeze-dried powder was made into aliquots and stored at 4°C. The aliquots of THG-A freeze-dried powder were dissolved with sterile phosphate-buffered saline (PBS) before used.

Mice Five-week-old male C57BL/6 mice were purchased...
from Oriental Yeast Co., Ltd. (Tokyo, Japan). All mice were housed under specific pathogens free conditions according to the animal protocol guidelines of the Institutional Animal Care and Use Committee of Showa University (Protocol No. 09007).

High-Fat Diet and Control Diet High-fat diet (Cat# D12492) and a control diet of equivalent caloric content to the high-fat diet (Cat# D12450J) were purchased from Research Diet Inc. (NJ, U.S.A.). New diets were switched from the old diet twice a week. Mice were given ad libitum access to diet during experiment.

Mice Treatment and Sample Collection Experiments began with six-week-old mice given either high-fat diet or control diet for eight weeks. Week 0 was defined as the week at which the high-fat diet was started. In some groups, mice were intraperitoneally administered 0.4mg/0.2mL/mouse (roughly equivalent to 20mg/kg) of THG-A or PBS (as the control group) 3 times per week. The body weights of the mice were measured every week.

After being administered high-fat diet or control diet for 8 weeks, mice were sacrificed under CO2 gas inhalation after fasting for 12h. Their blood was collected from the heart, and their serum was frozen until measurement for alanine transaminase (ALT), total-cholesterol (T-CHO), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), glucose, insulin adiponec tin and interleukin (IL)-6 concentrations. At the same time, their livers were removed in order to measure the mRNA expression of pro-inflammatory cytokines and COL1A1 and COL1A2, which encode α1 and α2 chain in type I collagen, respectively, as fibrotic liver markers. The right hepatic lobe was also embedded in OCT compound for histology and quickly frozen.

Measurements of ALT, T-CHO, TG, LDL-C, HDL-C and Glucose Concentrations in Serum The serum levels of ALT, T-CHO, TG, LDL-C, HDL-C and glucose were measured by Oriental Yeast Co., Ltd. in brief, the ALT levels were measured by enzyme kinetics for photometric absorbance. The concentrations of T-CHO and TG were determined by an enzymatic assay. The levels of LDL-C and HDL-C were obtained from a direct assay. Glucose in the serum was measured by a HK-G6PDH assay.

Enzyme-Linked Immunosorbent Assay (ELISA) The serum levels of insulin or adiponecin were measured by a mouse insulin ELISA kit (Mercodia, Uppsala, Sweden) or by a mouse adiponecin simplestep ELISA kit (Abcam, Cambridge, U.K.), respectively, according to the manufacturer’s protocol. The concentrations of IL-6 in serum and liver were determined using mouse IL-6 ELISA development kit according to the manufacturer’s instruction (R&D systems, MN, U.S.A.).

Histological Analyses of Fatty Liver To determine the degree of hepatic steatosis, frozen embedded liver specimens were cut into sections with a microtome and then stained with oil red O at Soshiki Kagaku, Lab. Inc. (Kanagawa, Japan). The histology was evaluated by a pathologist.

RNA Extraction and Quantitative Real-Time PCR To isolate total RNA from the liver, liver tissues were homogenized in a bead-based tissue homogenizer (MagNA Lyser Green Beads and MagNA Lyser Instrument; Roche Diagnostics, Mannheim, Germany). The total RNA in the liver homogenates was purified using RNeasy Mini, and then cDNA was synthesized using QuantiTect Reverse Transcription according to the manufacturer’s protocol (QIAGEN, Hilden, Germany). The probes and primers for quantitative real-time PCR used in this study were TagMan gene expression assays for mouse IL-1β (Assay ID: Mm00434228_m1), mouse IL-6 (Assay ID: Mm00446190_m1), mouse tumor necrosis factor (TNF)-α (Assay ID: Mm00443258_m1), mouse COL1A1 (Assay ID: Mm00801666_g1), mouse COL1A2 (Assay ID: Mm00483888_m1) and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Assay ID: Mm99999915) (Thermo Fisher Scientific, MA, U.S.A.). Quantitative real-time PCR was performed using a LightCycler 480 probe Master and LightCycler 480 instrument with the included software program (Roche Diagnostics). Each sample was calibrated to the internal standard (GAPDH) level and normalized to the average value of control samples.

Statistical Analyses The statistical analyses were performed using the Microsoft Excel software program (Microsoft, WA, U.S.A.). The statistical significance of the findings was calculated using the unpaired t-test for all experimental analysis. p-Values of less than 0.05 were considered to be statistically significant. All values are presented as the mean ± standard deviation.

RESULTS

THG-A Administration Did Not Affect the Body Weight Gain or Adipose Weight in the Epididymis of Mice Fed a High-Fat Diet or Control Diet First, mice that had been intravenously administrated 400mg/kg of THG-A did not exhibit any pathological or toxic symptoms (data not shown), suggesting that the intraperitoneally administration of 20mg/kg of THG-A in this study was a reasonably safe dose. As shown in Fig. 1A, the body weight gains in mice fed a high-fat diet were significantly greater than in those fed a control diet during the experiment. However, the body weight gains in mice fed a high-fat diet or control diet treated with THG-A were similar to those in mice fed a high-fat diet or control diet treated with PBS, respectively (Figs. 1B, C). Correspondingly, although the adipose weights in the epididymis of mice fed a high-fat diet were significantly greater than those of mice fed a control diet, THG-A treatment did not have any marked effect on the adipose weights in the epididymis of mice fed either diet for eight weeks (Fig. 1D).

THG-A Administration Improved the Metabolic Markers and the Decreased Adiponecin Concentration in Serum Induced by a High-Fat Diet To determine whether or not THG-A treatment improves metabolic markers in serum, mice were fed a high-fat diet for eight weeks with or without THG-A administration. In mice fed a high-fat diet for eight weeks without THG-A, the T-CHO, TG, LDL-C and glucose concentrations in serum after fasting were significantly higher than those of mice fed a control diet without THG-A (Fig. 2). In addition, the ALT concentration tended to be higher in the serum of mice fed a high-fat diet than in mice fed a control diet. However, the increased serum levels of TG and glucose caused by a high-fat diet were significantly improved by THG-A administration. Interestingly, THG-A administration increased the HDL-C level in serum compared to PBS administration in mice fed both diets. Moreover, as
shown in Fig. 3, the decreased serum levels of adiponectin caused by a high-fat diet were also significantly recovered by THG-A treatment. The levels of serum IL-6 and hepatic IL-6 were under limit of detection by ELISA (data not shown). No groups of mice showed liver steatosis on histological evaluation with oil red staining (Fig. 4). These results suggest that THG-A administration can improve metabolic markers and the decreased levels of adiponectin in serum caused by a high-fat diet.

**THG-A Administration Suppressed Inflammation in the Liver Caused by a High-Fat Diet** We next investigated whether or not a high-fat diet caused liver inflammation...
and the effects of THG-A treatment on this inflammation. As shown in Fig. 5, mice fed a high-fat diet for eight weeks showed a significantly higher mRNA expression of pro-inflammatory cytokines of IL-6 and TNF-α in the liver than mice fed a control diet. In addition, the expression of IL-1β mRNA also tended to be high in the liver of mice fed a high-fat diet. However, this high expression of IL-6 mRNA in the liver was significantly suppressed by THG-A administration and the high levels of TNF-α mRNA and IL-1β mRNA were attenuated as well. These results indicated that liver inflammation caused by a high-fat diet could be suppressed by THG-A treatment.

**THG-A Administration Ameliorated Fibrotic Liver Progression Caused by a High-Fat Diet**

As chronic liver
inflammation is known to induce liver fibrosis, the mRNA expression of COL1A1 and COL1A2, which are encode the α1 and α2 chains in type I collagen was measured by quantitative real-time PCR. As shown in Fig. 6, the expression of COL1A1 mRNA was not markedly different in mice fed a high-fat diet compared to mice fed a control diet. However, the expression of COL1A2 mRNA in mice fed a high-fat diet was significantly higher than in those fed a control diet. Furthermore, the increased expression of COL1A2 mRNA in the liver caused by a high-fat diet tended to be suppressed by THG-A administration. These results suggested that fibrotic liver progression caused by a high-fat diet might be suppressed by THG-A treatment.

DISCUSSION

In the present study, we explored whether or not THG-A treatment affects obesity with metabolic disorders caused by a chronic high-fat diet. We found that although THG-A administration did not affect the body weight gain or epididymal adipose volume, THG-A treatment did improve the slightly deteriorated metabolic markers in serum caused by a high-fat diet, including the increased levels of TG and glucose. Interestingly, the serum levels of HDL-C, which is recognized as beneficial cholesterol protecting against cardiovascular disease by transporting T-CHO, TG and LDL-C from arteries, were increased by THG-A administration, regardless of the diet. The decreased serum level of adiponectin, which is beneficial adipokine against obesity, caused by a high-fat diet was also recovered by THG-A treatment. Furthermore, the increased mRNA expression of pro-inflammatory cytokines, such as IL-6 and TNF-α, caused by a high-fat diet were suppressed by THG-A treatment. Our finding also suggested that THG-A treatment might alleviate fibrotic liver progression caused by a high-fat diet.

Excess and for long-term consumption of a high-fat diet causes obesity, inflammation, oxidative stress, increased plasma TG and hyperglycemia and other metabolic disorders, ultimately leading to NASH and cirrhosis.

THG-A was originally isolated from the cultured broth of P. rubra K07-0510, and the structure was determined. One of the biological activities of THG-A is its anti-oxidative activity against hemolysis of RBCs induced by light-activated pheophorbide α activation. Notably, the anti-oxidative activity of THG-A is stronger than that of the same dose of ascorbic acid. Anti-oxidative molecules, such as vitamin C and vitamin E, can reportedly protect against liver pathogenicity caused by an excessive high-fat diet, as the oxidative stress and free radicals induced by a high-fat diet cause inflammation in the liver and hepatocyte damage. In addition, mice fed a high-fat diet have lower levels of superoxide dismutase activity and catalase activity in the liver than those fed a control diet which also leads to oxidative stress. Furthermore, the production of pro-inflammatory cytokines, including IL-6 and TNF-α in the liver and adipose tissue can activate Kupffer cells, which are the main source of transforming growth factor (TGF)-β in the liver, followed by COL1A1 and COL1A2 gene activation to cause liver fibrosis. The suppression of liver inflammation by THG-A treatment can thus inhibit fibrotic liver progression and hepatic functional deterioration. THG-A associated with obesity also decreases insulin sensitivity and has been implicated in the development of type 2 diabetes. The reduction of oxidative stress in liver may therefore be responsible for inhibiting pathogenic development, which is first step in progression. Indeed, we showed that THG-A treatment in mice fed a high-fat diet suppressed pro-inflammatory cytokine production in the liver and may have mildly reduced the expression of COL1A2 mRNA.

The serum levels of metabolic markers, especially TG and glucose, were increased by a high-fat diet and subsequently decreased by THG-A treatment. High levels of metabolic markers in serum are risk factors for a number of diseases, including cardiovascular disease, type 2 diabetes mellitus, hypertension, hyperlipidemia, coronary artery disease and certain cancers. Although the mechanisms by which THG-A treatment reduces the increased metabolic marker levels caused by a high-fat diet remain unclear, these increased levels may attribute to the imbalance between lipid and/or glucose production and expenditure in the liver caused by inflammation and oxidative stress. Moreover, recently, the signal pathways of energy and nutrition control in liver, involving
nuclear receptors, have been reported. Among their nuclear receptors, peroxisome proliferator-activated receptor (PPAR) α, PPARβ/δ or PPARγ are responsible for lipid and glucose metabolism and they may be targets of therapeutic drugs development for fatty liver diseases.2,6) Long-chain fatty acids and eicosanoids can activate PPARα and PPARβ/δ,2,28 and arachidonic acid metabolites are ligand for PPARγ.29) PPARα activation induces fatty acid catabolism and clearance in liver and prevents inflammation through expression of inhibitor of nuclear factor kappa B (NF-κB).30) PPARα activation also increases serum and hepatic fibroblast growth factor (FGF) 21 which can enhance an expression of glucose transporter 1 leading to maintaining of normal blood glucose level.31) PPARβ/δ activation by the ligands increases carbohydrate catabolism with enhanced glucose-6-phosphate dehydrogenase activity in liver and muscle resulting in reduced blood glucose level.32) Furthermore, PPARβ/δ activation also protect from liver fibrosis.33) PPARγ is highly expressed in an adipose tissue and macrophage. The beneficial effects of PPARγ activation against a high-fat diet are to store fatty acids in adipose tissue as triacylglycerol from a circulation. PPARα activation is also to attenuate an inflammation by inhibition of NF-κB activity and to increase an adiponectin secretion which can enhance glucose up take in peripheral tissues.34,35) Based on these previous reports, one possible mechanism of THG-A may target PPARs for improvement of metabolic disorder, although whether THG-A exerts its effect directly or indirectly remains unclear.

In conclusion, to our knowledge, this is the first report of THG-A treatment improving metabolic clinical conditions caused by a high-fat diet, including ameliorating increased TG and glucose concentrations in serum, inflammation in the liver and fibrotic liver progression. Future studies should determine the detailed mechanisms underlying the effects of THG-A against metabolic clinical conditions. We are also curious about whether or not the oral administration of THG-A affects metabolic clinical conditions caused by a high-fat diet.

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Conflict of Interest The authors declare no conflict of interest.

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