Effects of Various 5,7-Dihydroxyflavone Analogs on Adipogenesis in 3T3-L1 Cells

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We studied the effects of twelve 5,7-dihydroxyflavone analogs on adipogenesis in 3T3-L1 cells. Among the compounds, luteolin, diosmetin, and chrysoeriol partly inhibited adipogenesis by blocking the accumulation of triacylglycerol in the cells. Conversely, tricetin facilitated triacylglycerol accumulation in the cells. The induction of lipogenesis or lipolysis may depend on the number and bonding position of hydroxyl or methoxy groups on the B ring of 5,7-dihydroxyflavone. The mRNA expression levels of adipogenic and lipogenic genes were suppressed by luteolin treatment in the cells, while the mRNA levels of lipolytic genes were not affected. However, the expression levels of the adipogenic, lipogenic, and lipolytic genes, except for adipocyte protein 2 (aP2), were not affected by the addition of tricetin. Moreover, luteolin suppressed glucose transporter type 4 (GLUT4) gene and protein levels. These results indicate that luteolin decreased triacylglycerol levels in 3T3-L1 cells during adipogenesis through the suppression of adipogenic/lipogenic and GLUT4 genes and GLUT4 protein.

Key words flavonoid; adipogenesis; lipolysis; lipogenesis; glucose transporter type 4 (GLUT4)

Adipocytes are associated with energy homeostasis in mammals.1 Energy is stored as lipids in the cells, and released in response to nutritional signals or energy shortages.2 Furthermore, adipocytes in adipose tissue play an important role in the progress of obesity. Adipose tissues in obesity are characterized by increases in the number and size of adipocytes. Thus, excessive accumulation of lipids in the adipose tissue may lead to obesity.3 Moreover, obesity is associated with type 2 diabetes mellitus, hypertension, cardiovascular diseases, and cancer.4 Recent studies suggest that adipocytes are also endocrine cells that secrete some hormones, namely adipocytokines, and these may regulate the metabolism of lipids in adipocytes in many organs.5

Adipogenesis is regulated by complex processes via changes in gene expression, hormonal sensitivity, and so forth. Three transcriptional factor families, peroxisome proliferator-activated receptors (PPAR), CCAAT/enhancer binding proteins (C/EBPs), and sterol regulatory-element binding proteins (SREBPs), are considered important factors for the regulation of adipogenesis.6 These factors may regulate the expression of various genes leading to the differentiation of adipocytes.7 On the other hand, some types of proteins may be responsible for the accumulation and release of lipid droplets in differentiated adipocytes.8 For example, acetyl-CoA carboxylase-1 (ACC1), fatty acid synthase (FAS), stearoyl-CoA desaturase-1 (SCD1), and sterol regulatory-element binding proteins-1c (SREBP-1c) may induce lipogenesis,9 while adipocyte triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), monoacyl glyceride lipase (MGL), and perilipin may induce lipolysis.10 For instance, it is known that lipid droplets generated by lipogenesis are decreased by lipolysis. Therefore, the regulation of genes and proteins related to lipogenesis is important for combating obesity research into the elucidation of antilipogenic components continues.

Flavonoids are a typical family of natural phenol compounds. They are found in fruits, vegetables, seeds, bark, roots, plant stems, flowers, tea, and wine.11 The existence of flavonoids was well known before they were isolated due to their positive influences on health, and more than 4000 flavonoids have been isolated to date.12 Many flavonoids, comprising aglycons, glycosides, and methylated derivatives, are major components of the colors of flowers, fruits, and leaves.13 Flavonoid aglycons consist of a benzene ring (A) fused with a six-membered heterocyclic ring (C) bearing a phenyl group (B) at the 2-position. Flavonoids are classified as flavonols, flavones, catechins, flavanones, anthocyanidins, and isoflavones, but the mechanisms of adipogenesis by flavonoids have not yet been clarified.14 Conversely, the biological activities of the constituents of plants, including antimicrobial, antiviral, antiatherosclerotic, antitumor, anti diabetic, anti-inflammatory, antioxidant, antiaging, antihypertensive, antiobesity, and antiplatelet activities, have been recognized for centuries.15

There are many reports on the promotion or suppression of adipogenesis by flavonoid analogs. For example, Nomura et al. evaluated the effects of 24 compounds including flavonols, flavones, catechins, flavanones, and isoflavones on adipogenesis, and discovered the inhibitory effects on adipogenesis of apigenin, luteolin, kaempferol, quercetin, fisetin, genistein, flavanone, slybin, and flavonol.16 In addition, Fujimori and Shibano reported that avicularin, a flavonoid glycoside, sup-

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presses the uptake of glucose by adipocytes via inhibition of C/EBP-activated GLUT4. Other studies by Yamaguchi et al. and Zhang et al. investigated the antiadipogenic effect of p-hydroxycinnamic acid, and the promotion of adipogenesis by isoprenylated flavonoids, respectively. In the present study, we evaluated the effects of twelve 5,7-dihydroxyflavone analogs on adipogenesis in 3T3-L1 cells to confirm their structure–activity relationships.

MATERIALS AND METHODS

Chemicals and Reagents Rosiglitazone [ROS; a type of thiazolidinedione (TZD)] and Dulbecco’s modified Eagle’s medium (DMEM), chrysin, and luteolin were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Fetal bovine serum (FBS), calf serum, and BODIPY 493/503 were purchased from Life Technologies (Carlsbad, CA, U.S.A.). Insulin, dexamethasone (DEX), 3-isobutyl-1-methylxanthine (IBMX), and berberine were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.).

Preparation of 5,7-Dihydroxyflavone Analogs A series of 5,7-dihydroxyflavonones (2, 3, 5–12) was prepared by a convenient synthetic method as previously described. To gain insight into the influence of hydroxylation and methylation in the B ring, we synthesized a series of 5,7-dihydroxyflavones through the condensation reactions of tert-butylidimethylsilyl (TBS)-protected acetophenone and methyl benzoates, followed by acid cyclodehydration. The condensation reaction of TBS-protected acetophenone and methyl benzoates performed using 8 equiv. of lithium bis(trimethylsilyl)amide (LiHMDS) in tetrahydrofuran (THF) at −78°C and with a temperature increase to room temperature for 1 h produced intermediates comprising mixtures of tautomers. These intermediates were subjected to acid cyclodehydration and deprotection with 0.5% H2SO4 in acetic acid at 100°C for 4.5 h. These reaction conditions resulted in the formation of 5,7-dihydroxyflavones, with the chemical structures depicted in Fig. 1.

Cell Culture Murine 3T3-L1 preadipocytes were propagated in DMEM supplemented with 10% calf serum until 80% confluence (day 0) and the medium was replaced with DMEM containing a 10% FBS, MDI mixture (a mixture of 0.5 mM 3-isobutyl-1-methylxanthine (M), 0.1 μM dexamethasone (D), and, 2 μM insulin (I)), with or without one of 5,7-dihydroxyflavone analogs or reference compound. After 48 h (day 2), the medium was replaced with DMEM containing 10% FBS and 5 μg/mL insulin. After 48 h (day 4), the medium was replaced with DMEM containing 10% FBS and 10% TR. Thereafter, the medium was exchanged every other day. 0.1 μM or 2.7 nM of ROS or berberine (BER) was used as positive or negative reference compound, respectively. Cells were maintained in a humidified atmosphere of 5% CO2 at 37°C.

Cell Toxicity Assay 3T3-L1 cells were seeded in 96-well plate with DMEM supplemented with 10% calf serum until 80% confluence. Various concentration of dimethyl sulfoxide (DMSO) solution of 5,7-dihydroxyflavone analogs were added followed by incubation for 48 or 192 h. Cytotoxicity was measured by the use of Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the instruction by the manufacturer. Absorbance was measured at 450 nm by using Sunrise Absorbance Reader (Tecan, Männendorf, Switzerland).

Measurement of Intracellular Triacylglycerol Level Intracellular triacylglycerol levels in 3T3-L1 cells at day 8 were measured by using of E-test WAKO Triglyceride Kit (Wako Pure Chemical Industries, Ltd.) according to the instruction by the manufacturer. A hundred nanograms per milliliter of BODIPY 493/503 were added to culture medium followed by incubation of 10 min. Images were taken by a fluorescent cell imager (ZO; Bio-Rad, Hercules, CA, U.S.A.).

mRNA Expressions of Adipogenesis-Related Genes in 3T3-L1 Cells We quantitated mRNA of adipogenesis-related genes in the various flavonoids-treated 3T3-L1 cells using semi-quantitative real time reverse transcription (RT)-PCR (qPCR) as previously reported. We quantitated mRNA of the genes as follows: peroxisome proliferator-activated receptor γ (PPARγ), CCAAT/enhancer binding protein α (C/EBPα), adipocyte fatty acid-binding protein 2 (aP2), FAS, ACC1, SCD1, SREBP-1c, ATGL, HSL, MGL, perilipin, and glucose transporter type 4 (GLUT4). Total RNA was isolated from the differentiated 3T3-L1 cells using an available kit (ISOGEN reagent, Wako Pure Chemical Industries, Ltd., Tokyo, Japan). cDNAs were synthesized from 1 μg total RNA to final reaction volume of 20 μL using an available kit (Transcriptor First Strand cDNA Synthesis Kit; Roche Diagnostics, Mannheim, Germany). Reverse transcription was performed at 55°C for 10 min after annealing at 29°C for 10 min, followed by heat denaturation at 85°C for 5 min. The mRNA was quantitated using a RT-PCR system (Light Cycler 1.5, Roche Diagnostics) with THUNDERBIRD SYBR qPCR Mix (TOYOBO, Osaka, Japan) and the primer sequences and PCR conditions were followed as previously described. The expression level of the target genes was normalized to that level of β-actin.

Detection of Proteins Differentiated (day 8) 3T3-L1 cells onto 6-well plates were placed on ice and each well was washed with phosphate buffered saline (PBS), and subsequently lysed with 150 μL of 20 mM Tris–HCl buffer (pH 8.0) containing 150 mM NaCl, 2 mM ethylenediaminetetraacetic acid (EDTA), 1% Nonidet P-40 (w/v), 1% sodium deoxycholate (w/v), 0.1% sodium dodecyl sulfate (w/v), 50 mM NaF, 0.1% aprotinin (w/v), 0.1% leupeptin (w/v), 1 mM Na3VO4, and 1 mM phenylmethylsul-
Phosphoryl fluoride (PMSF). Cell lysates were collected by using a cell scraper and centrifuged at 15,000 × g for 30 min at 4°C. The supernatant was collected and the overall protein concentration was determined by a Protein Assay Reagent Kit (Cytoskeleton, Denver, CO, U.S.A.) with BSA as the standard.

Supernatant fluids containing proteins (20 µg) were mixed with lithium dodecyl sulfate (LDS) sample buffer (Invitrogen Corp., Carlsbad, CA, U.S.A.) and incubated for 5 min at 80°C. Proteins in the samples were separated on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and the proteins in gels were electroblotted onto polyvinylidene fluoride (PVDF) filters (Fluorotrans membrane W, 0.2 µm; Nihon Genetics, Tokyo, Japan). Immunoblotting analysis was performed by using monoclonal antibodies against GLUT4 (Cell Signaling Technology, Lake Placid, NY, U.S.A.) as the primary antibodies, followed by reaction with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) antibodies from Sigma-Aldrich. (St. Louis, MO, U.S.A.) Primary and secondary antibodies were diluted 1000 or 3000 times for use, respectively. The blots were developed by the enhanced chemiluminescence method (Western Lightning ECL Pro; PerkinElmer, Inc., Waltham, MA, U.S.A.).

**Statistical Analysis** The results were expressed as mean ± standard deviation (S.D.). The significant difference between the groups compared were determined using ANOVA followed by Tukey test.

### RESULTS

**Effects of Various 5,7-Dihydroxyflavone Analogs during Adipogenesis in 3T3-L1 Cells** Using these compounds (Fig. 1), we examined cell survival among 3T3-L1 cells co-cultured with the analogs for 6 d (Fig. 2). Although some compounds induced cytotoxicity at 30 µM, none of them induced cytotoxicity at 10 µM. Therefore, the adipogenesis effects on 3T3-L1 cells of each compound were evaluated at 5 and 10 µM under the conditions mentioned in Materials and Methods. Differentiation of 3T3-L1 cells to adipocytes was achieved within 8 d in the presence of each 5,7-dihydroxyflavone analog at a concentration of 5 and 10 µM, and the accumulation of intracellular lipid levels were measured by E-Test WAKO Triglyceride Kit (Fig. 3). When ROS or berberine (BER) with MDI mixture (a mixture of 0.5 mM 3-isobutyl-1-methyl xanthine (M), 0.1 µM dexamethasone (D), and, 2 µM insulin (I)) was added to the medium, lipid accumulation levels increased or decreased, respectively. Lipid accumulation levels decreased significantly when luteolin, diosmetin, or chrysoeriol with MDI mixture was added to the medium compared with addition of MDI mixture only, but the addition of tricetin or 4'-methoxytricetin up-regulated the levels of intracellular lipids. From the images shown in Fig. 4, it was considered that the size of 3T3-L1 cells was diminished following the addition of ROS with MDI mixture. On the other hand, the addition of tricetin with MDI mixture led to an increase in the level of intracellular lipids, but unlike ROS, miniaturization of the adipocytes did not
occur. The results suggested that the effects of 5,7-dihydroxyflavone analogs on adipogenesis in 3T3-L1 cells differ greatly according to the number and types of functional groups binding to the B ring.

Effects of Luteolin or Tricetin on Expression of Adipogenesis-Related Genes

The expression levels of adipogenic genes, PPARγ, C/EBPα, and aP2, in 3T3-L1 cells differentiated by MDI mixture with or without ROS, BER, luteolin, or tricetin are shown in Fig. 5A. ROS, a positive reference compound, up-regulated the expression levels of aP2, PPARγ, and C/EBPα genes. BER, the negative reference compound, and luteolin at 10 μM down-regulated the gene expression of C/EBPα, aP2 and PPARγ. On the other hand, addition of tricetin was irrelevant to the expression levels of PPARγ and C/EBPα genes but the expression of aP2 gene was regulated.

Effects of Luteolin or Tricetin on Expression of Lipogenic Genes

The effects of each compound on the levels of lipogenic related genes are shown in Fig. 5B. Although a significant difference was observed only for SREBP-1c, ROS showed a tendency to induce the expression of four lipogenic-related genes. Stimulation by tricetin with MDI mixture showed up-regulated the expression levels of ACC1, FAS, and SREBP-1c genes but statistically not significant. BER and luteolin down-regulated the levels of the four lipogenic genes.

Effects of Luteolin or Tricetin on Expression of Lipolytic Genes

ROS significantly induced the expression of four lipolytic genes. BER significantly suppressed the levels of lipolytic genes except for ATGL. On the other hand, luteolin and tricetin were irrelevant to the expression levels of lipolytic genes.

Effects of Luteolin and Tricetin on the Expression of Protein and Gene of GLUT4

GLUT4 is accumulated in intracellular organelles (endosomes) in an insulin-unstimulated state. Once insulin binds with the receptor in the cell membrane, insulin receptor substrate, phosphoinositide 3-kinase (PI3K), Akt, and so on are activated in turn, followed by translocation of GLUT4 to the cell membrane. GLUT4 in the cell membrane uptakes glucose from the blood. Moreover, the translocation of GLUT4 to the cell membrane is closely related to adipogenesis. The expression of the GLUT4 protein and gene in 3T3-L1 cells differentiated under the same conditions as those in Fig. 5 are shown in Fig. 6. ROS and BER up-regulated or down-regulated the GLUT4 gene and
protein levels, respectively (up-regulation and down-regulation of GLUT4 protein were not statistically significant). Luteolin showed significant regulatory effects on GLUT4 protein and gene levels. Tricetin, on the other hand, did not affect the expression of the GLUT4 gene and the protein levels were slightly up-regulated but not significantly.

DISCUSSION

We examined the positive and negative effects on adipogenesis, lipogenesis, or lipolysis of twelve 5,7-dihydroxyflavone analogs in 3T3-L1 cells. We found that luteolin, diosmetin, and chrysoeriol partially inhibited adipogenesis by blocking triacylglycerol accumulation in cells. In contrast, tricetin facilitated triacylglycerol accumulation in cells. The induction of lipogenesis or lipolysis depends on the number and bonding positions of hydroxyl or methoxy groups on the B ring of 5,7-dihydroxyflavone. The expression of mRNA levels suggested that luteolin suppressed adipogenic and lipogenic genes in the cells, while lipolytic genes were not affected. However, the expression levels of adipogenic genes except aP2 were not affected by the addition of tricetin. On the other hand, the tricetin stimulation showed that the tendency of the up-regulation of the expression levels of ACC1, FAS, and SREBP-1c genes was seen, although it was not significant. In addition, luteolin suppressed the levels of GLUT4 gene and protein. Thus, luteolin decreased triacylglycerol levels in 3T3-L1 during adipogenesis through the suppression of adipogenic/lipogenic and GLUT4 genes and GLUT4 protein.

Adipocytes can be both large and small. Small adipocytes secrete beneficial adipocytokines such as adiponectin, improve insulin resistance, regulate glucose secretion from the liver, and improve diabetes mellitus. ROS, a type of thiazolidinedione used as the positive reference in the present study, induces the differentiation of adipocytes from large to small via activation of PPARγ.26) Conversely, it has been confirmed that BER, used as a negative control in the present study, suppresses the synthesis and accumulation of lipids through the activation of AMPK.27) Among the twelve 5,7-dihydroxyflavone analogs, luteolin, diosmetin, and chrysoeriol down-regulated, while tricetin up-regulated intracellular lipid accumulation. ROS induced both an accumulation of intracellular lipids and the miniaturization of adipocytes, but tricetin only induced the accumulation of intracellular lipids (Fig. 4). Therefore, tricetin may induce obesity.

We showed the effects of each compound on the expression levels of adipogenic genes. Luteolin and BER inhibited the accumulation of intracellular lipids via the inhibition of adipogenic and lipogenic gene expression levels (Fig. 5). Luteolin significantly inhibited the expression of C/EBP, aP2, and PPARγ genes but ROS induced up-regulation. Because the stimulation by tricetin with MDI mixture showed that the up-regulation tendency of the expression levels of ACC1, FAS, and SREBP-1c genes was found. However, it could not rule out the possibility that the accumulation of intracellular lipids by tricetin may be induced by the expression of such genes. Further studies are needed to clarify the reason for the up-regulation of intracellular lipids by tricetin, and the inhibition of aP2 adipogenic gene expression, because insulin resistance and the promotion of obesity may be induced by aP2.28)

Luteolin and BER significantly inhibited the expression of lipogenic genes, namely, those that induce lipid production. However, unlike BER, luteolin did not suppress lipolytic genes. Moreover, luteolin regulated the expression levels of GLUT4 genes and proteins. Therefore, it was deduced that luteolin controlled lipid accumulation in 3T3-L1 cells via inhibition of the expression of both adipogenic and lipogenic genes. On the other hand, the possibility that BER showed a certain kind of toxicity because it regulated the levels of all adipogenic, lipogenic, and lipolytic genes, has not fully been
Since it is recognized that the cytotoxicity (IC50) of BER against 3T3-L1 cells is less than 10 µM,29) the effects on adipogenesis-related genes in 3T3-L1 cells measured at 2.7 nM in the present study are considered reliable.

In the present study, the effects of the numbers and types of functional groups binding to the B ring of 5,7-dihydroxyflavone during adipogenesis of 3T3-L1 cells were assessed. Tricetin with three hydroxy groups showed lipogenic effects. On the other hand, luteolin with two hydroxy groups, diosmetin, and chrysoeriol with hydroxy and methoxy groups showed lipolytic effects.

To date, it has been reported that flavonoids control insulin-stimulated glucose uptake via modulation of insulin receptor tyrosine kinases and/or the PI3K pathway.30) Some reports have clarified that flavonoids such as myricetin, quercetin, catechins, gallates, genistein, and naringenin regulate insulin-stimulated glucose uptake in adipocytes.31) Bazuine et al. found that genistein, an isoflavone, directly inhibits insulin-stimulated glucose uptake in differentiated 3T3-L1 cells.32) On the other hand, naringenin (flavanone) regulates insulin-stimulated glucose uptake through activation of the PI3K pathway.33) Nomura et al. investigated the effects of 24 flavonoids, including flavones, flavonols, isoflavones, flavanones, flavonans, and flavanonols, on insulin-stimulated glucose uptake in MC3T3-G2/PA6 adipocytes.16) We could not find any other reports with results that overlap those of this study.

Obesity and obesity-induced diabetes mellitus, cancer, and hypertension through excessive energy intake are major issues around the world. The methods of obesity-regulation proposed so far can be classified into two kinds, namely, regulation of appetite by controlling the central nervous system, and regulation of lipid-uptake from the intestinal tract. The ultimate antiobesity medicine has not yet been developed, despite the substantial efforts of pharmaceutical companies around the world for more than a decade. The major reason for the difficulties in developing antiobesity treatments is the physiological desire of humans to eat.

Previous reports suggested that vegetable diets that include flavonoids show antiobesity and/or antidiabetic effects as mentioned in the Introduction.34,35) Conclusively, the effects of the compounds with these effects differ greatly according to the bonded functional groups. The effects of vegetable diets on obesity and diabetes mellitus will be examined in the near future, following investigation of the effects of each flavonoid and the dietary flavonoid constituents.

**Conflict of Interest** The authors declare no conflict of interest.
REFERENCES


