

Tiliroside, a glycosidic flavonoid, ameliorates obesity-induced metabolic disorders via activation of adiponectin signaling followed by enhancement of fatty acid oxidation in liver and skeletal muscle in obese–diabetic mice

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Abstract

Tiliroside contained in several dietary plants, such as rose hips, strawberry and raspberry, is a glycosidic flavonoid and possesses anti-inflammatory, antioxidant, anticarcinogenic and hepatoprotective activities. Recently, it has been reported that the administration of tiliroside significantly inhibited body weight gain and visceral fat accumulation in normal mice. In this study, we evaluated the effects of tiliroside on obesity-induced metabolic disorders in obese–diabetic KK-*A*^y mice. In KK-*A*^y mice, the administration of tiliroside (100 mg/kg body weight/day) for 21 days failed to suppress body weight gain and visceral fat accumulation. Although tiliroside did not affect oxygen consumption, respiratory exchange ratio was significantly decreased in mice treated with tiliroside. In the analysis of metabolic characteristics, it was shown that plasma insulin, free fatty acid and triglyceride levels were decreased, and plasma adiponectin levels were increased in mice administered tiliroside. The messenger RNA expression levels of hepatic adiponectin receptor (AdipoR)-1 and AdipoR2 and skeletal muscular AdipoR1 were up-regulated by tiliroside treatment. Furthermore, it was indicated that tiliroside treatment activated AMP-activated protein kinase in both the liver and skeletal muscle and peroxisome proliferator-activated receptor α in the liver. Finally, tiliroside inhibited obesity-induced hepatic and muscular triglyceride accumulation. These findings suggest that tiliroside enhances fatty acid oxidation via the enhancement adiponectin signaling associated with the activation of both AMP-activated protein kinase and peroxisome proliferator-activated receptor α and ameliorates obesity-induced metabolic disorders, such as hyperinsulinemia and hyperlipidemia, although it does not suppress body weight gain and visceral fat accumulation in obese–diabetic model mice.

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Keywords: Tiliroside; Insulin resistance; Fatty acid oxidation; Adiponectin; AMPK; PPAR α

1. Introduction

Obesity is characterized by an enhanced fat deposition in visceral and subcutaneous depots and is the underlying feature of several related metabolic defects including insulin resistance, type 2 diabetes, dyslipidemia, hypertension and cardiovascular diseases [1,2]. Obesity

causes excess fat accumulation in various tissues, most notoriously in adipose tissues, and also in other insulin-responsive organs such as the skeletal muscle and liver, predisposing one to the development of insulin resistance [3,4]. However, the molecular mechanisms underlying insulin resistance and obesity have not been fully clarified, and effective therapeutic approaches are currently of general interest.

Flavonoids belong to a polyphenol subclass, which are widely distributed in the plant kingdom, and there are more than 6400 known flavonoid compounds [5]. They are characterized by two or more aromatics rings, each bearing at least one aromatic hydroxyl and connected to a heterocyclic pyran [6]. The compounds are further divided into subclasses based on the link of the aromatic ring to the heterocyclic ring, their oxidation state and the functional groups attached to the heterocyclic ring [6]. Within each subclass, individual compounds are characterized by specific hydroxylation and conjugation patterns. Most flavonoids (with the exception of flavonols) occur naturally in the conjugated form mainly with sugar residues. Flavonoids are widespread in higher plants and widely distributed in dietary plants, such as fruit, vegetables, legumes, herbs, spices, stems,

Abbreviations: RER, respiratory exchange ratio; FFA, free fatty acid; HFD, high-fat diet; TG, triglyceride; HOMA-IR, homeostasis model assessment–insulin resistance; QUICKI, quantitative insulin-sensitivity check index; PAGE, polyacrylamide gel electrophoresis; AMPK, AMP-activated protein kinase; P-AMPK, phosphorylated AMPK; ACC, acetyl-CoA carboxylase; P-ACC, phosphorylated ACC; HMW, high molecular weight; MMW, middle molecular weight; LMW, low molecular weight; AdipoR, adiponectin receptor; WAT, white adipose tissue; BAT, brown adipose tissue; PPAR, peroxisome proliferator-activated receptor; ACO, acyl-CoA oxidase; UCP, uncoupling protein; CPT, carnitine palmitoyltransferase; MC, methylcellulose.

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flowers, tea and red wine [6]. They are prominent components of citrus fruits and other food sources and are in many countries regularly consumed in a healthy diet [6,7]. They contribute to the flavor and pigmentation of fruit and vegetables in human diet [8], whereas they have important roles in plant growth, reproduction and pathogen and predator resistance [5]. Epidemiological studies suggest the health beneficial effects of dietary flavonoids through the reduction of the risk of carcinogenesis, hypertension, inflammation and cardiovascular diseases [9–11]. Flavonoids not only have antioxidant activity but also exert more specific effects on various cellular and molecular processes.

Recently, much attention has been focused on plant flavonoids that might be beneficial in reducing the risk of obesity and obesity-associated metabolic disorders. It has been revealed that several flavonoids could induce neutral lipid hydrolysis from lipid stores in adipose tissues and the liver through a mechanism by which the elevation of cyclic AMP levels is induced by the direct inhibition of phosphodiesterase [11]. Furthermore, several flavonoids inhibit intestinal glucose and fructose transport by glucose transporter 2 [12]. Indeed, dietary catechins and anthocyanins significantly decrease the weight of intraperitoneal adipose tissues [13,14]. Investigation of the metabolic effects of flavonoids might lead to more effective strategies for the treatment of obesity and obesity-associated metabolic disorders.

Tiliroside (kaempferol 3-O- β -D-glucopyranoside-6-*p*-coumaril ester) is a glycosidic flavonoid, and it is contained in several medicinal and dietary plants, such as linden, rose hip and strawberry [15–17]. Tiliroside possesses anti-inflammatory, antioxidant, anticarcinogenic, cytochrome P450 inhibitory and hepatoprotective activities [15–20]. Recently, it has been reported that the administration of tiliroside significantly inhibits body weight gain and visceral fat accumulation after fasting in nonobese mice [16]. However, it has not been clarified whether tiliroside ameliorates obesity-induced metabolic disorders, such as insulin resistance, hyperlipidemia and diabetes.

In the present study, we investigated the effects of tiliroside on obesity and obesity-induced metabolic disorders in an obese–diabetic mouse model, KK-*A*^y mice. Although the administration of tiliroside failed to suppress body weight gain and visceral fat accumulation, respiratory exchange ratio (RER) was significantly decreased in mice treated with tiliroside. Tiliroside administration improved dyslipidemia, insulin resistance and hypoadiponectinemia. It was revealed that tiliroside activated adiponectin signaling in the liver and skeletal muscle. These findings suggest that tiliroside stimulates fatty acid (FA) oxidation via the enhancement of adiponectin signaling and ameliorates obesity-induced metabolic disorders, such as insulin resistance and hyperlipidemia in spite of no effects on body weight gain and visceral fat accumulation in obese–diabetic model mice.

2. Materials and methods

2.1. Materials

Tiliroside was extracted and purified from the seeds of *Rosa canina* L. (Morishita Jintan Co., Osaka, Japan) known as “Dog Rose” as described previously [16]. Unless otherwise indicated, all chemicals were purchased from Sigma (St. Louis, MO, USA) or Nacalai Tesque (Kyoto, Japan) of guaranteed reagent grade or tissue culture grade.

2.2. Animals

All the mice were maintained in a temperature-controlled (23°C) facility with a constant 12-h light/dark cycle and were given free access to water. Seven-week-old female obese–diabetic KK-*A*^y mice (CLEA Japan, Tokyo, Japan) [21] were maintained for 21 days on a 60% high-fat diet (HFD) (D12492 diet; Research Diets Inc., St. Louis, MO USA). Once a day, tiliroside suspended in 0.5% methylcellulose (Wako Pure Chemicals, Osaka, Japan) was administered to KK-*A*^y mice orally at a concentration of 100 mg/kg body weight/day. Body weight and food intake were measured every day before administration. Ten or 11 days after the initial administration, oxygen consumption and RER were measured. At days 12 and 19, after fasting for 5 h, fresh urine was collected directly, and glucosuria incidence was estimated using Uropiece S (Fujisawa

Pharmaceutical, Osaka, Japan). Twenty-one days after the initial administration, these mice were fasted 8 h before sampling blood for metabolic characteristic analysis, and tissues for RNA isolation, lipid measurements and immuno-blot analysis. Plasma glucose, triglyceride (TG) and free fatty acid (FFA) levels were measured enzymatically using glucose CII Wako, the TG G test Wako, the NEFA C Wako (Wako), respectively. Plasma insulin, leptin (Morinaga Institute of Biological Science, Yokohama, Japan) and adiponectin (R&D Systems, Minneapolis, MN, USA) were measured with an enzyme-linked immunosorbent assay kit in accordance with the manufacturer's instructions. Surrogate indexes for estimating insulin resistance were calculated from fasting blood glucose and plasma insulin concentrations as follows: homeostasis model assessment–insulin resistance (HOMA-IR) = (G0 × I0) / 22.5, where I0 indicates insulin (μ U/ml) and G0 indicates glucose (mmol/l) [22]; quantitative insulin-sensitivity check index (QUICKI) = $1 / [\log(I0) + \log(G0)]$, with insulin concentration expressed in μ U/ml and glucose concentration expressed in mg/dl [23]. The animal care procedures and methods were approved by the Animal Care Committee of Kyoto University.

2.3. Measurement of oxygen consumption and RER

Ten or 11 days after the initial administration, the oxygen consumption and RER of mice under the fed condition were measured using an indirect calorimetric system (Oxymax; Columbus Instruments, Columbus, OH, USA) every 8 min for 20 h. These experiments were started at 4:00 PM and finished at 12:00 PM (the dark and light phases were 12 and 8 h, respectively).

2.4. Sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

SDS-PAGE was performed in accordance with the standard Laemmli's method [24]. A sample buffer (Bio-Rad Laboratories, Hercules, CA, USA) with (reducing) or without (nonreducing) 5% 2-mercaptoethanol was added to protein samples. For heat denaturation, samples were heated at 95°C for 5 min, unless indicated. Protein samples were subjected to SDS-PAGE on a 5%–15% gradient gel (Bio-Rad Laboratories).

For immunoblotting, proteins separated by SDS-PAGE were transferred electrophoretically to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked with phosphate-buffered saline containing 0.1% Tween-20 and 5% skim milk. Then they were incubated with a specific antibody diluted in phosphate-buffered saline containing 0.1% Tween-20 and 5% skim milk overnight at 4°C. After washing, the membranes were incubated with a 1:5000 diluted horseradish peroxidase-conjugated anti-rabbit immunoglobulin G antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h at room temperature and then washed thoroughly. Each protein was detected using an ECL Western blotting detection reagent (GE Healthcare, Piscataway, NJ, USA).

2.5. Analysis of multimerization of plasma adiponectin

Multimerization analysis of plasma adiponectin was performed as described previously [25,26]. In brief, 1.2 μ l plasma harvested from mice was diluted in a nonreducing sample buffer and subjected to SDS-PAGE under nonreducing and nonheat-denaturing conditions. Adiponectin was detected using 1:200 diluted rabbit polyclonal antiadiponectin antibodies for mouse adiponectin (Thermo Fisher Scientific Inc., Rockford, IL, USA). After the detection, NIH-Image J was used for band quantification.

2.6. Phosphorylation analysis of AMP-activated protein kinase (AMPK) and acetyl-CoA carboxylase (ACC)

The AMPK pathway was analyzed as described in Yamauchi et al [27]. Briefly, harvested liver and skeletal muscle (gastrocnemius) from mice were homogenized in the extraction buffer (0.125 M Tris-HCl, 4% SDS, 10% sucrose) containing protease and phosphatase inhibitor cocktails (Roche Diagnostics, Mannheim, Germany) using a Polytron tissue grinder (Kinematica AG, Luzern, Switzerland), followed by the sonication. After heat denaturation, the homogenate was centrifuged at 15,000 × g for 30 min at 4°C, and the resulting supernatant was recovered. The protein concentration of samples was determined using a protein assay kit (Bio-Rad Laboratories), and 30 μ g protein samples were subjected to SDS-PAGE. Total AMPK, phosphorylated AMPK (P-AMPK), total ACC and phosphorylated ACC were visualized using their specific antibodies (Cell Signaling Technology, Beverly, MA, USA). After the detection, NIH-Image J was used for band quantification.

2.7. Lipid analysis

To quantify hepatic and muscular TG amounts, harvested liver and skeletal muscle (gastrocnemius) samples from mice were homogenized in an extraction solution (hexane–isopropyl alcohol (3:2, vol/vol)) using a Polytron tissue grinder. After centrifugation, the resulting supernatant was evaporated under reduced pressure. Samples were resuspended in 10% Triton X-100 in isopropyl alcohol, and TG contents were measured enzymatically using TG G test Wako.

2.8. RNA analysis

Total RNA was prepared from the mouse liver and skeletal muscle (gastrocnemius) using Qiazol lysis reagent (QIAGEN, Valencia, CA, USA) and an RNeasy Mini kit (QIAGEN) in accordance with the manufacturer's protocol. Total RNA was reverse-transcribed using M-MLV reverse transcriptase (Promega, Madison, WI, USA) in accordance with the manufacturer's instructions using a thermal cycler (Takara PCR Thermal Cycler SP; Takara, Shiga, Japan). To quantify messenger RNA (mRNA) expression, real-time reverse transcriptase polymerase chain reaction (PCR) was performed with a LightCycler system (Roche Diagnostics) using SYBR Green fluorescent signals (TOYOBO, Osaka, Japan) as described previously [28–30]. The oligonucleotide primers used were designed using a PCR primer selection program in the Web site of the Virtual Genomic Center from the GenBank database. The oligonucleotide primer set used to measure the expression levels of 36B4 was previously described [26]. The primers used for measurements of mRNA expression levels of other genes (upstream and downstream, respectively) were 5'-ACGTTGGA-GAGTCATCCCCG-3' and 5'-CTCTGTGTGGATGCGGAAGAT-3' for AdipoR1 mRNA, 5'-TCCCAGGAAGATGAAGGTTTAT-3' and 5'-TTCAATTCGTCGATAGCATGA-3' for AdipoR2 mRNA, 5'-TCAGGGTACCACTACGAGT-3' and 5'-CTGGCATTCTTCAAAGCG-3' for PPAR α mRNA, 5'-GCACCAATGCCATTCGATACA-3' and 5'-CCACTGCTGTGAGAA-TAGCCGT-3' for ACO mRNA, 5'-CACTTTCCTCTGGATACCGC-3' and 5'-GATCCCTTCTCTCGTCACT-3' for UCP2 mRNA, and 5'-CTGTAGGCCTCAACACC-GAAC-3' and 5'-CTGTATGGCTAGGCTGTACAT-3' for carnitine palmitoyltransferase 1 (CPT1) mRNA. To compare mRNA expression level among the samples, the copy numbers of all the transcripts were divided by that of mouse 36B4 showing a constant expression level in the liver and skeletal muscle. All the mRNA expression levels were presented as the ratio relative to that of the control in each experiment.

2.9. Statistical analysis

The data, presented as means \pm S.E.M., were statistically analyzed using the analysis of variance followed by the Benferroni's test and the Student's *t* test. Differences were considered significant when $P < .05$.

3. Results

3.1. Tiliroside administration did not affect body weight gain and oxygen consumption, but significantly decreased RER

Because a previous report has shown that tiliroside, a glycosidic flavonoid, has an antiobesity effect in nonobese mice after fasting [16], we first asked whether tiliroside can ameliorate obesity and obesity-associated metabolic disorders in the obese model, KK-*A^y* mice fed an HFD. Contrary to our expectation, there was no significant difference in body weight gain between KK-*A^y* mice treated with vehicle control and those treated with tiliroside (100 mg/kg body weight/day) throughout the experimental period (21 days) (Fig. 1A). Tiliroside treatment did not affect food intake (Fig. 1B). After the anatomical analysis, it was revealed that tiliroside treatment did not affect white adipose tissue (WAT) accumulation, whereas liver weight was significantly decreased in mice treated with tiliroside (Table 1). These findings indicate that in KK-*A^y* mice, tiliroside does not have the antiobesity effect, but it suppresses obesity-induced hepatomegaly. To further deepen our understanding of tiliroside effects on energy metabolism in obese mice, 10 or 11 days after the initial administration, we assessed oxygen consumption and RER using an indirect calorimetric system. As expected, tiliroside treatment did not change oxygen consumption in both the light and dark phases (Fig. 1C). However, interestingly, RER was significantly lower in tiliroside-treated mice than in control mice (Fig. 1D). Because these mice were fed HFD, RER was always low during the measurements, but tiliroside treatment slightly but significantly decreased RER in both the light and dark phases. These findings suggest that tiliroside treatment enhances FA oxidation, although it does not change the energy expenditure.

3.2. Tiliroside administration improved insulin resistance and dyslipidemia

Next, we examined whether tiliroside affects plasma metabolic parameters in KK-*A^y* mice fed HFD. After 21 days of administration,

obesity-induced hyperinsulinemia was significantly improved in mice treated with tiliroside (Fig. 2A), although plasma glucose level was slightly but not significantly lower in the tiliroside-treated group (Fig. 2B; $P = .096$). In the tiliroside-treated group, plasma insulin level was decreased by 43% compared with that in the control group. On day 19, glucosuria incidence in the control mice rose to 57%, whereas only 14% of tiliroside-treated mice showed glucosuria (Fig. 2C). To assess the effect of tiliroside on obesity-associated insulin resistance, we calculated simple surrogate indexes for insulin resistance, HOMA-IR and QUICKI. In tiliroside-treated mice, the HOMA-IR value was higher and the QUICKI value was lower than those in control mice (Fig. 2D, E), suggesting that tiliroside improved obesity-associated insulin resistance. Moreover, both plasma FFA and TG levels were significantly decreased by tiliroside treatment (Fig. 2F, G). Tiliroside treatment led to 24% and 26% decreases in plasma FFA and TG levels, respectively. These findings indicate that tiliroside ameliorates the dysfunctions of both glucose and lipid metabolism in obese mice.

3.3. Tiliroside administration affected plasma adipocytokine profiles

Recently, it has been reported that adipose tissues secrete bioactive molecules called adipocytokines, which are related to the regulation of whole-body energy metabolism [31,32]. Because tiliroside administration decreased RER, we investigated the effects of tiliroside on the secretion of adipocytokines involved in FA oxidation, leptin [33] and adiponectin [27]. In tiliroside-treated mice, plasma leptin level tended to be higher than that in control mice, but this difference was not significant statistically ($P = .053$; Fig. 3A). On the other hand, tiliroside treatment resulted in a 1.4-fold

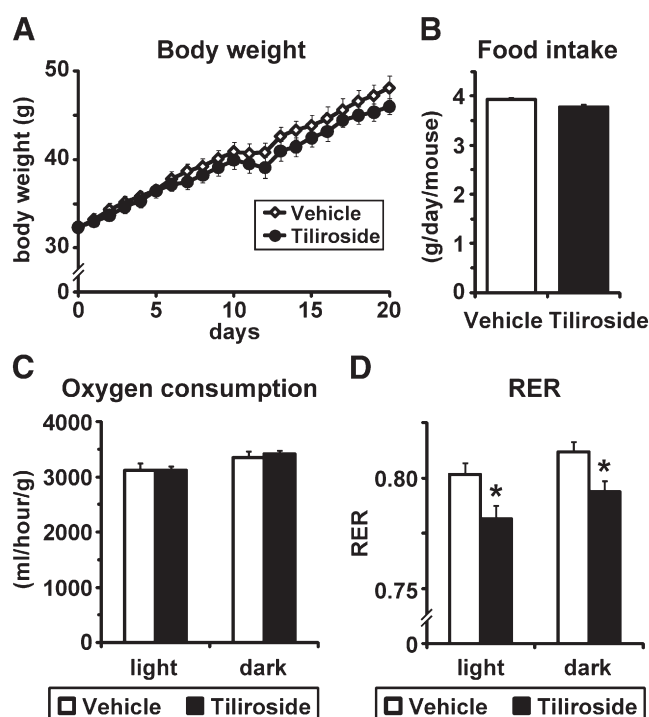


Fig. 1. Effects of tiliroside administration on whole-body energy metabolism in obese KK-*A^y* mice. Seven-week-old female KK-*A^y* mice fed an HFD were treated with vehicle control or tiliroside (100 mg/kg/day) for 21 days. (A) Body weight change. (B) Food intake. (C, D) On day 10 or 11, oxygen consumption (C) and RER (D) were measured using an indirect calorimetric system for 20 h (the light and dark phases were 8 and 12 h, respectively). Data are means \pm S.E.M. of six to seven animals per group. * $P < .05$ compared with vehicle-control-treated group.

Table 1

Effects of tiliroside treatment for 3 weeks on weight of body and several tissues and food intake in KK-*A^y* mice fed an HFD

	Vehicle (0.5% MC)	Tiliroside (100 mg/kg/day)
Body weight (g)	46.48±1.33	44.65±0.93
Epididymal WAT (g)	5.417±0.263	5.466±0.224
Perirenal WAT (g)	1.091±0.057	1.170±0.067
Mesenteric WAT (g)	1.684±0.093	1.694±0.068
Intraperitoneal WAT (g)	8.193±0.330	8.331±0.327
Interscapular BAT (g)	0.216±0.012	0.199±0.006
Liver (g)	1.615±0.057	1.376±0.061 **
Gastrocnemial muscle (g)	0.256±0.008	0.247±0.004
Kidney (g)	0.329±0.005	0.327±0.007
Spleen (g)	0.118±0.008	0.114±0.003
Food intake (g/day)	3.92±0.05	3.77±0.05

Values are means±S.E.M. of seven mice per group. MC, methylcellulose; BAT, brown adipose tissue.

** $P<0.01$ compared with vehicle-control-treated group.

increase in plasma adiponectin level (Fig. 3B). Adiponectin in human or mouse serum forms a wide range of multimers from trimers and hexamers to high-molecular-weight (HMW) multimers, and HMW multimers have more potent insulin-sensitizing effects than trimers and hexamers [32]. Next, we examined whether tiliroside administration affected the multimerization of adiponectin. As shown in Fig. 3 (C, D), the ratio of the amount of HMW multimers to the total amount of adiponectin was increased by the tiliroside treatment. These findings indicate that tiliroside affects adipocytokines secretion and increases plasma adiponectin level and the ratio of the amount of HMW multimers of adiponectin to the total amount of adiponectin.

3.4. Tiliroside administration affected adiponectin receptor (AdipoR) mRNA expression levels in liver and skeletal muscle

Next, we studied whether tiliroside affects the mRNA expression levels of AdipoRs in the liver and skeletal muscle. In the liver, both

AdipoR1 and AdipoR2 mRNA expression levels were significantly increased by tiliroside treatment (Fig. 4A, B). Tiliroside administration induced a 1.5-fold increase in both AdipoR1 and AdipoR2 mRNA expression levels in the liver. In the skeletal muscle, tiliroside treatment led to a 2.1-fold increase in AdipoR1 mRNA expression level, whereas it had no effect on AdipoR2 mRNA expression level (Fig. 4C, D). These findings suggest that tiliroside changes mRNA expression levels of AdipoRs in the liver and skeletal muscle.

3.5. Tiliroside administration activated AMPK pathway in the liver and skeletal muscle

Because a previous report has shown that adiponectin stimulated FA oxidation via the activation of AMPK [27], we determined whether tiliroside treatment affects AMPK activity in the liver and skeletal muscle. As shown in Fig. 5A and B, the ratio of the amount of P-AMPK to the amount of total AMPK significantly increased by 120% in the liver of mice treated with tiliroside. Moreover, tiliroside treatment also enhanced the phosphorylation of ACC, which is phosphorylated by AMPK, by up to 157% in the liver compared with vehicle treatment (Fig. 5A, C). Similar results were obtained in the skeletal muscle. In the skeletal muscle, tiliroside treatment led to 48% and 75% increase in phosphorylation levels of AMPK and ACC, respectively (Fig. 5D–F). These findings indicate that tiliroside treatment activates the AMPK pathway in the liver and skeletal muscle.

3.6. Tiliroside administration activated peroxisome proliferator-activated receptor α (PPAR α) pathway in liver but not in the skeletal muscle

Adiponectin also activates the PPAR α pathway and then enhances FA oxidation [34]. Therefore, we next investigated whether tiliroside treatment affects the PPAR α pathway in the liver and skeletal muscle. In the liver, tiliroside treatment increased the mRNA expression level of PPAR α by 1.5-fold compared with vehicle treatment (Fig. 6A). Furthermore, PPAR α target genes involved in FA oxidation, such as genes encoding acyl-CoA oxidase (ACO), uncoupling protein 2 (UCP2)

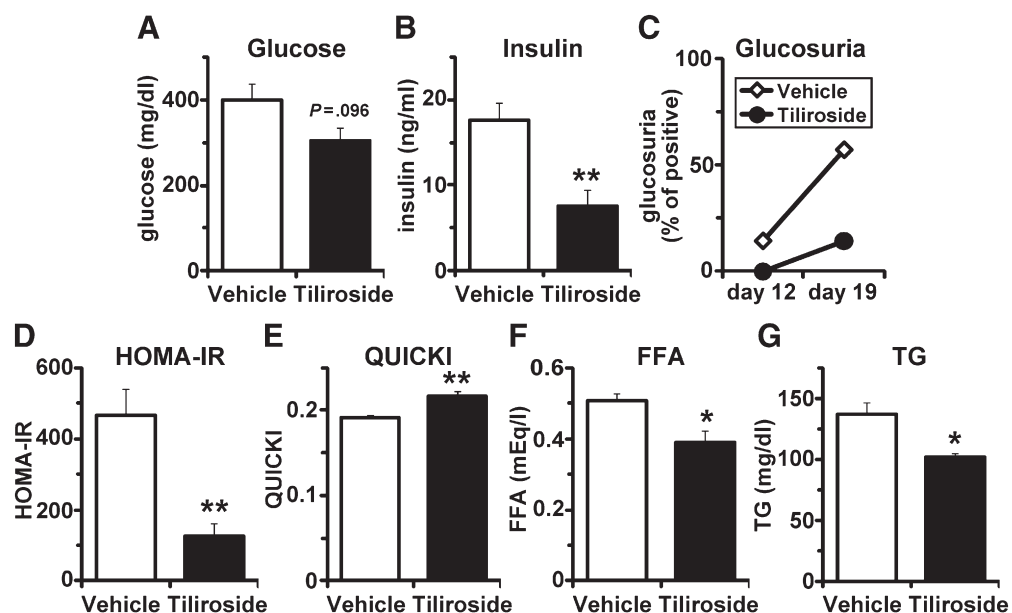


Fig. 2. Effects of tiliroside administration on glucose and lipid metabolism in obese KK-*A^y* mice. Plasma and urine samples were obtained from mice treated with or without tiliroside as mentioned in Fig. 1. (A–C, F, G) Plasma glucose (A), insulin (B), FFA (F) and TG (G) levels and glucosuria incidence (over 100 mg/dl) (C) were determined as described in “Materials and Methods.” (D, E) HOMA-IR (D) and QUICKI (E) were calculated as described in “Materials and Methods.” Data are means±S.E.M. of five to seven animals per group. * $P<0.05$, ** $P<0.01$ compared with vehicle-control-treated group.

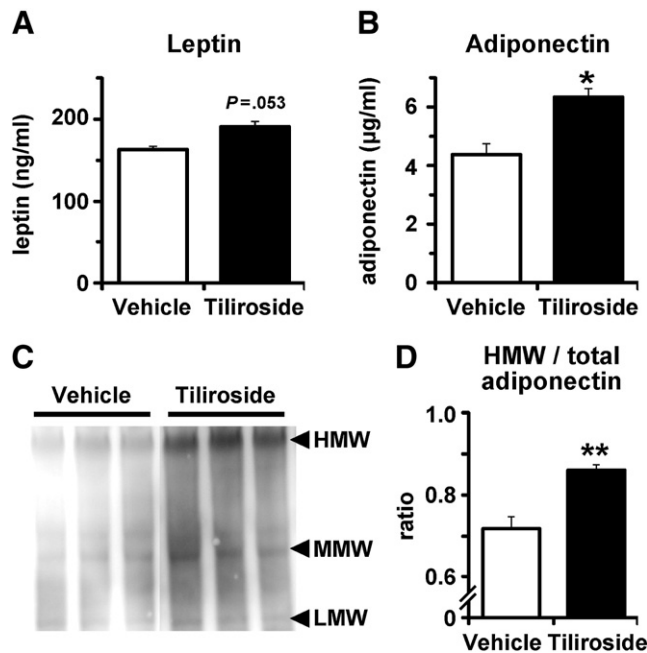


Fig. 3. Effects of tiliroside administration on plasma adipocytokine profile in obese KK-A^y mice. Plasma was harvested from mice treated with or without tiliroside as mentioned in Fig. 1. (A, B) Plasma leptin (A) and adiponectin (B) levels were measured by enzyme-linked immunosorbent assay. (C, D) Plasma samples were subjected to SDS-PAGE under nonreducing and nonheat-denaturing conditions. Multimer forms of adiponectin (HMW; or high molecular weight), MMW (middle molecular weight) and LMW (low molecular weight) were immunodetected using an antibody specific for adiponectin (C). (D) Quantitative results of SDS-PAGE. The values are means \pm S.E.M. of four to six tests. * $P < 0.05$, ** $P < 0.01$ compared with vehicle-control-treated group.

and CPT1, were also significantly up-regulated (2.0- and 1.6-fold increases in ACO and UCP2 mRNA expression levels, respectively) or tended to be up-regulated (CPT1, $P=0.078$) (Fig. 6B–D). However, these up-regulations of PPAR α -related gene expression detected in the liver were not observed in the skeletal muscle (Fig. 6E–H). These findings suggest that the PPAR α pathway is activated by tiliroside treatment in the liver but not in the skeletal muscle.

3.7. Tiliroside administration suppressed obesity-induced hepatic and skeletal muscular TG accumulation

Finally, to investigate the effects of tiliroside on obesity-induced lipid accumulation in the liver and skeletal muscle, we measured TG contents in the liver and skeletal muscle. In both organs, tiliroside treatment significantly decreased TG contents (34% and 47% reductions in the liver and skeletal muscle, respectively; Fig. 7A, B). These findings indicate that tiliroside suppresses obesity-induced hepatic and skeletal muscular lipid accumulation.

4. Discussion

Obesity and its associated disorders are the major noncommunicable public health problems of the 21st century. Studies indicate that high levels of body fat are associated with an increased risk of developing numerous adverse health conditions [1,2]. Thus, effective therapeutic approaches to obesity and obesity-induced metabolic syndrome are currently of general interest. Previous reports have raised the possibility that several flavonoids, such as catechins and anthocyanins, are useful dietary compounds for managing obesity and obesity-induced metabolic syndrome [10–14]. In the present study, we examined the effects of a glycosidic flavonoid, tiliroside, on

obesity and obesity-induced metabolic syndrome using obese mice. Tiliroside treatment enhanced whole-body FA oxidation via the enhancement of adiponectin signaling and ameliorated obesity-induced metabolic disorders, such as hyperinsulinemia and hyperlipidemia, in spite of having no effects on body weight gain and visceral fat accumulation in obese-diabetic model mice. Thus, tiliroside may be a novel dietary flavonoid useful for suppressing the development of obesity-induced metabolic diseases.

Adiponectin is an adipocytokine that is secreted from adipocytes, and it is present in plasma at relatively high concentrations [31,32]. Unlike the concentrations of most adipocytokines, such as tumor necrosis factor α , interleukin 6 and monocyte chemoattractant protein 1, those of adiponectin are low in patients with obesity and type 2 diabetes and are inversely associated with measures of overall adiposity [31,32]. Adiponectin has been reported to have antidiabetic, anti-insulin resistance and antiatherosclerotic effects [31–35]. Several flavonoids have been reported to enhance adiponectin expression and secretion in isolated adipocytes and cultured adipocytes [36,37]. Thus, the increase in circular adiponectin levels in tiliroside-treated mice might be caused by the direct effects of tiliroside on adipocytes. Moreover, tiliroside treatment increased not only circular total adiponectin levels but also the ratio of the amount of HMW multimers to the total amount of adiponectin. A mutation study of human adiponectin has shown that the Gly84Arg and Gly90Ser mutants are capable of assembling into low-molecular-weight and middle-molecular-weight multimers but not into HMW multimers [25], which are clinically associated with diabetes [38]. Recently, it has also been reported that an increase in the ratio of the amount of HMW forms to the total amount of adiponectin correlates with an improvement in insulin sensitivity induced by an insulin sensitizer

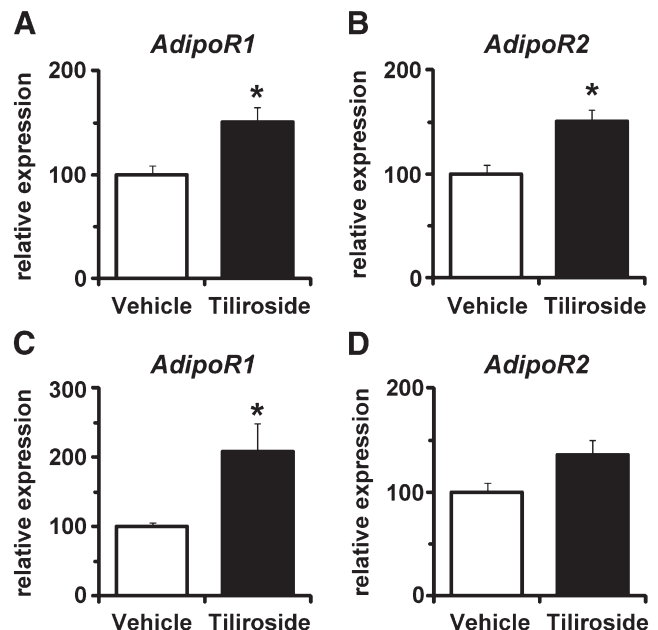


Fig. 4. Effects of tiliroside administration on mRNA expression levels of AdipoRs in liver and skeletal muscle in obese KK-A^y mice. The liver and skeletal muscle were harvested from mice treated with or without tiliroside as mentioned in Fig. 1. (A, B) mRNA expression levels of AdipoR1 (A) and AdipoR2 (B) in liver. (C, D) mRNA expression levels of AdipoR1 (C) and AdipoR2 (D) in skeletal muscle. mRNA expression levels of genes were measured by real-time monitoring reverse transcriptase PCR and normalized to the mRNA expression level of the ribosomal 36B4 gene. The expression levels of the vehicle-control-treated group are set at 100%, and relative expression levels are presented as fold inductions over the vehicle-control-treated group. Results are shown as means \pm S.E.M. normalized to the mRNA expression level of the 36B4 gene of four to five animals per group. * $P < 0.05$ compared with vehicle-control-treated group.

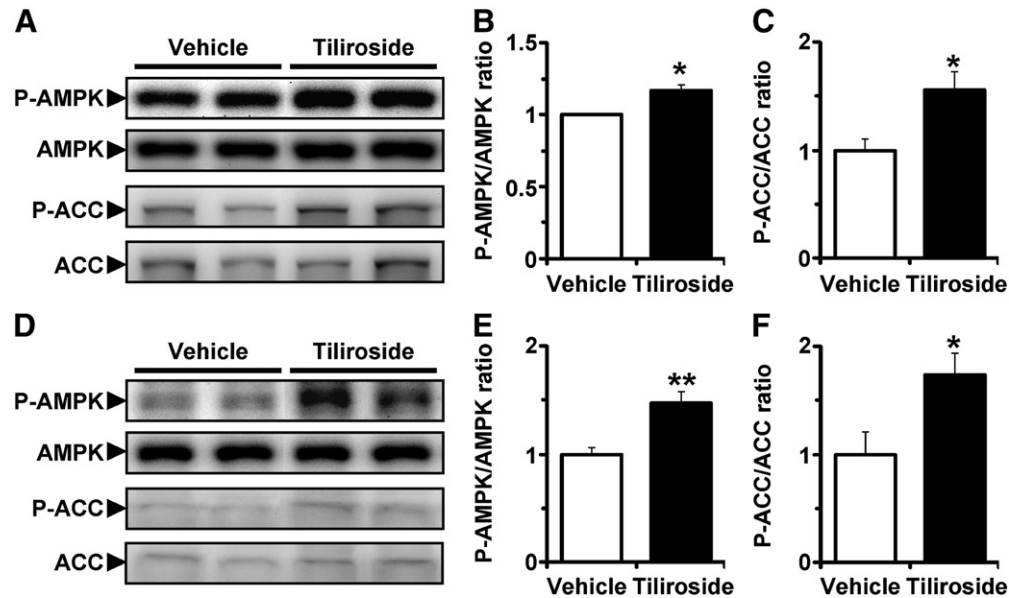


Fig. 5. Effects of tiliroside administration on AMPK pathway in liver and skeletal muscle in obese KK-*Ay* mice. The liver and skeletal muscle were harvested from mice treated with or without tiliroside as mentioned in Fig. 1. Protein samples extracted from the liver (A–C) and skeletal muscle (D–F) were subjected to SDS-PAGE under reducing and heat-denaturing conditions. (A, D) P-AMPK, total AMPK, P-ACC and total ACC were immunodetected using antibodies specific for these proteins. The ratio of the amount of P-AMPK to that of total AMPK (B, E) and the ratio of the amount of P-ACC to that of ACC (C, F) were calculated from the results of band quantification. Relative phosphorylated protein levels are presented as fold inductions over the vehicle-control-treated group. The values are means \pm S.E.M. of four to six tests. * $P < .05$, ** $P < .01$ compared with vehicle-control-treated group.

drug, thiazolidinedione [39]. Therefore, the effect of tiliroside on the multimer formation of adiponectin might be important for its antidiabetic effect.

The metabolic effects of adiponectin are mediated by two putative receptors: AdipoR1 and AdipoR2 [40,41]. The mRNA expression levels of both AdipoR1 and AdipoR2 are decreased in obese mice, and the adenovirus-mediated overexpression of these receptors in the liver reverses obesity-induced insulin resistance [41]. Simultaneous disruption of both AdipoR1 and R2 abolishes adiponectin binding and

actions, resulting in an increased tissue TG content, insulin resistance and marked glucose intolerance [41]. AdipoR1 is reportedly linked with AMPK activation and AdipoR2 with PPAR α activation [41]. Tiliroside treatment stimulated both the AdipoR1-AMPK and AdipoR2-PPAR α pathways in the liver, whereas only the AdipoR1-AMPK pathway was enhanced in the skeletal muscle by this treatment. The reason for these differing results in the liver and skeletal muscle is not clear, but the expression patterns of AdipoRs and their effectors in these tissues might be related. AdipoR1 is ubiquitously

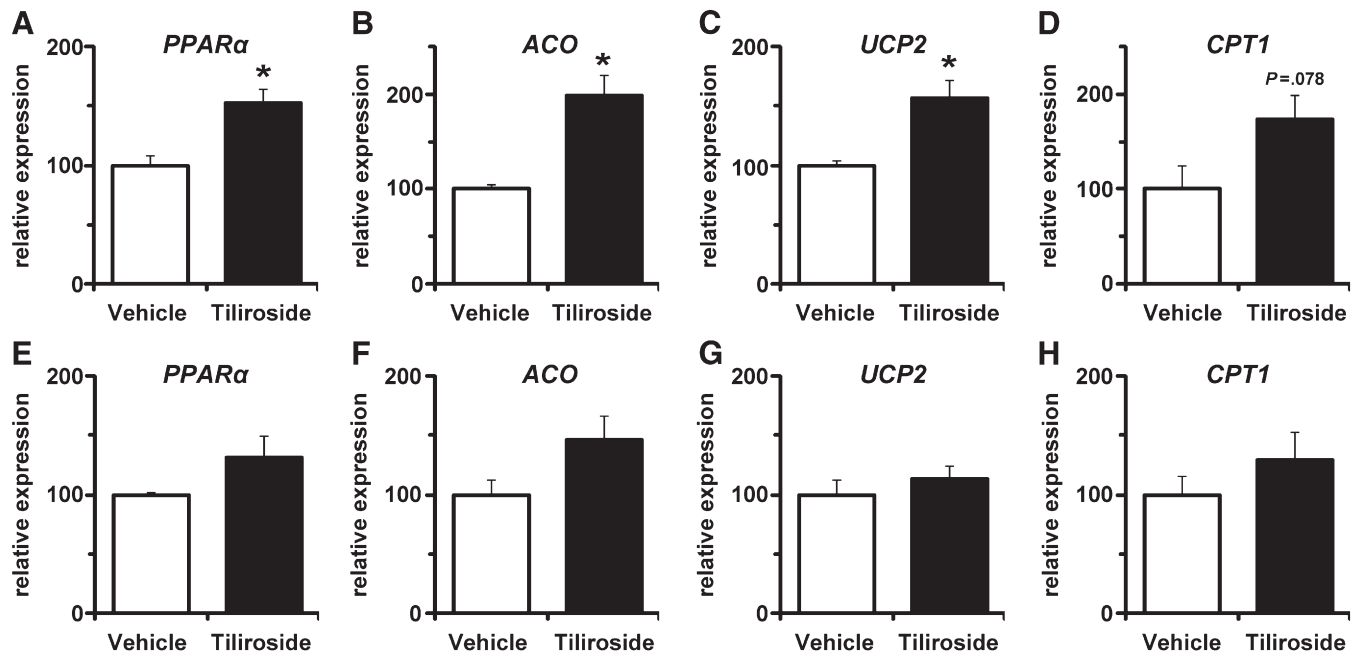


Fig. 6. Effects of tiliroside administration on PPAR α pathway in liver and skeletal muscle in obese KK-*Ay* mice. The liver and skeletal muscle were harvested from mice treated with or without tiliroside as mentioned in Fig. 1. (A–D) mRNA expression levels of PPAR α (A), ACO (B), UCP2 (C) and CPT1 (D) in liver. (E–H) mRNA expression levels of PPAR α (E), ACO (F), UCP2 (G) and CPT1 (H) in skeletal muscle. mRNA expression levels of each gene were measured as described in Fig. 4. Results are shown as means \pm S.E.M. normalized to the mRNA expression level of 36B4 gene of three to five animals per group. * $P < .05$ compared with vehicle-control-treated group.

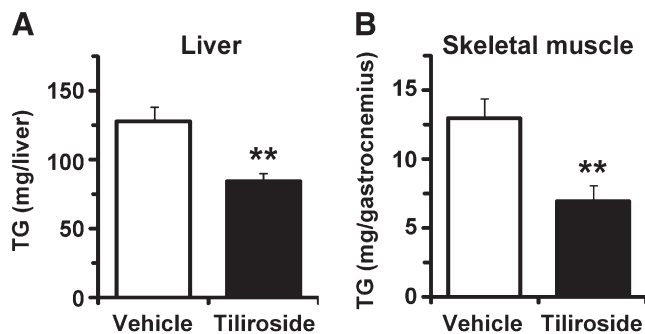


Fig. 7. Effects of tiliroside administration on hepatic and skeletal muscular TG accumulation in obese KK-*A^y* mice. The liver and skeletal muscle were harvested from mice treated with or without tiliroside as mentioned in Fig. 1. After the extraction of lipids, hepatic (A) or skeletal muscular (B) TG levels were measured by enzymatic colorimetric assay. Data are means \pm S.E.M. of five to six animals per group. ** $P < .01$ compared with vehicle-control-treated group.

and most abundantly expressed in the skeletal muscle, whereas AdipoR2 was most abundantly expressed in the liver but slightly expressed in the skeletal muscle [40]. Moreover, PPAR α mRNA expression levels in the liver are much higher than those in the skeletal muscle [42,43]. It has also been indicated that the mRNA expression level of AdipoR1 but not that of AdipoR2 in the skeletal muscle cells correlates with adiponectin effects *in vivo* [44,45] and that exercise training up-regulates AdipoR1 expression but not AdipoR2 expression in the skeletal muscle of KK-*A^y* mice [46]. These reports indicated that the skeletal muscular AdipoR1-AMPK pathway more flexibly changes than the AdipoR2-PPAR α pathway and that the AdipoR1-AMPK pathway is the major adiponectin effector in the skeletal muscle.

Obesity is associated with lipid accumulation not only in adipose tissues but also in nonadipose tissues, such as the liver and skeletal muscle [3,4]. The latter is also known as ectopic lipid accumulation and may be a possible link between obesity and its comorbidities such as insulin resistance, type 2 diabetes mellitus and cardiovascular disease. In tiliroside-treated mice, hepatic and skeletal muscular TG accumulation was significantly inhibited. Previous reports have indicated that an impaired ability to utilize fat as a fuel source because of a reduced activity of enzymes involved in oxidative metabolism, and FA utilization could also result in increases in the levels of cytosolic lipids [47–51]. Thus, the lipid level-lowering effects of tiliroside seem to be caused by the enhancement of FA oxidation, at least partially, via the activation of adiponectin signaling in the liver and skeletal muscle. Both the liver and skeletal muscle are organs with high rates of FA oxidation, and the enhancement of FA oxidation in these organs ameliorates tissue lipid accumulation [52–54]. Thus, it is suggested that FA oxidation stimulated by tiliroside followed by the amelioration of ectopic lipid accumulation is important for the useful effect of tiliroside.

In general, phenolic compounds including flavonoids have antioxidant activity, and tiliroside has been reported to possess antioxidant activity [18,20]. Recently, oxidative stress has been found to correlate with insulin resistance [55]. Therefore, the antioxidative effect of tiliroside might contribute to the anti-insulin resistant effect observed in the present study. However, the antioxidant activity (1,1-diphenyl-2-picryl hydrazyl [DPPH] radical and superoxide anion scavenging activities) of tiliroside is almost the same as that of kaempferol [20], a moiety of tiliroside, whereas the antiadiposity effect after fasting in nonobese mice is observed in tiliroside-treated mice but not in kaempferol-treated mice [16]. Matsuda et al. [15] have shown that the kaempferol 3-*O*- β -D-glucopyranoside moiety is essential for, and *p*-coumaroyl moiety-enhanced hepatoprotective activity, of tiliroside. Therefore, tiliroside seems to possess specific

activities for metabolic regulations. It was generally considered that flavonoid glycosides have to be hydrolyzed to their aglycones before being absorbed [56–58]. However, recent studies showed that some flavonoid glycosides can be detected in human or rat plasma in its intact form [59,60], indicating that flavonoid glycosides can be absorbed before being hydrolyzed. Although the absorption mechanism of tiliroside has not yet been elucidated, in our preliminary experiment, unchanged tiliroside in peripheral blood was nearly undetectable after oral tiliroside administration. This result indicated that specific bioactivities of tiliroside are exerted in the gastrointestinal tract, or metabolites derived from tiliroside play an important role in tiliroside function. Recently, it has been reported that the plasma concentration of quercetin, a flavonol, after oral administration of several quercetin glucosides, such as α -monoglucosyl rutin and isoquercitrin, is higher than that of quercetin [61,62]. Thus, oral tiliroside administration may enhance the bioavailability of its aglycone, kaempferol. Interestingly, da Silva et al. [63] reported that kaempferol up-regulates the expression levels of genes related to FA oxidation and mitochondrial biogenesis in normal human skeletal muscle myoblasts. Therefore, other than the activation of adiponectin signaling, tiliroside and/or its metabolites directly activate FA oxidation in the skeletal muscle and liver. To clarify these issues, it is necessary to perform further pharmacokinetic studies on tiliroside.

In this study, we could not observe the antiadiposity effect of tiliroside, although tiliroside was administered at a much higher dosage (100 mg/kg/day) than that in the previous report (\sim 10 mg/kg/day) [16]. The reason for this difference in the findings is unclear, but it is possible that the difference may be due to the use of a different model mouse and diets and the different experimental conditions. Ninomiya et al. [16] reported the antiadiposity effect of tiliroside using nonobese ddY mice fed a standard diet. In that report, they showed that tiliroside administration at 10 mg/kg/day reduced body weight rebound after fasting. On the other hand, in this study, to investigate the effects of tiliroside on obesity and obesity-induced metabolic disorders, we used KK-*A^y* mice fed HFD, which has been widely used in obesity research [64]. Further studies, for example, experiments using another obese model, are needed to better understand of the antiadiposity effect of tiliroside, but we consider that tiliroside administration (100 mg/kg/day) does not have any effect on obesity-induced body weight gain and visceral fat accumulation in KK-*A^y* mice.

In conclusion, the present study indicates that tiliroside, a glycosidic flavonoid, ameliorates obesity-induced metabolic disorders via the activation of adiponectin signaling followed by enhancement of FA oxidation in the liver and skeletal muscle in obese KK-*A^y* mice. These findings suggest that tiliroside can be used as a novel functional naturally occurring compound to regulate adiponectin signaling for the management of metabolic syndrome.

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