AMPK activation with glabridin ameliorates adiposity and lipid dysregulation in obesity[s]

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Abstract In this study, we demonstrate that activation of AMP-activated protein kinase (AMPK) with glabridin alleviates adiposity and hyperlipidemia in obesity. In several obese rodent models, glabridin decreased body weight and adiposity with a concomitant reduction in fat cell size. Further, glabridin ameliorated fatty liver and plasma levels of triglyceride and cholesterol. In accordance with these findings, glabridin suppressed the expression of lipogenic genes such as sterol regulatory element binding transcription factor (SREBP)-1c, fatty acid synthase (FAS), acetyl-CoA carboxylase (ACC), and stearoyl-CoA desaturase (SCD)-1 in white adipose tissues and liver, whereas it elevated the expression of fatty acid oxidation genes such as carnitine palmitoyl transferase (CPT)1, acyl-CoA oxidase (ACO), and peroxisome proliferator-activated receptor (PPAR)α in muscle. Moreover, glabridin enhanced phosphorylation of AMPK in muscle and liver and promoted fatty acid oxidation by modulating mitochondrial activity.[Jlr] Together, these data suggest that glabridin is a novel AMPK activator that would exert therapeutic effects in obesity-related metabolic disorders.—Lee, J.-W., S. S. Choe, H. Jang, J. Kim, H. W. Jeong, H. Jo, K.-H. Jeong, S. Tadi, M. G. Park, T. H. Kwak,. J. M. Kim, D.-H. Hyun, and J. B. Kim. **AMPK activation with glabridin ameliorates adiposity and lipid dysregulation in obesity.** *J. Lipid Res.* **2012.** 53: 1277–1286.

Supplementary key words AMP-activated protein kinase • fatty acid oxidation • fatty liver

Obesity is characterized by excessive accumulation of lipid metabolites in adipose tissues as well as in nonadipose tissues, such as liver, muscle, and pancreas (1). Because obesity is considered as a major risk factor for most metabolic diseases, including hyperlipidemia, hypercholesterolemia, type 2 diabetes, hypertension, and cardiovascular disease (2–4), numerous investigations have been conducted to elucidate the key components in energy homeostasis, which is crucial to develop therapeutic agents for obesity and obesity-associated metabolic disorders.

AMP-activated protein kinase (AMPK) is a master energy sensor that integrates nutrients, hormones, and stress signals to maintain whole-body energy homeostasis (5, 6). Under various stress conditions, AMPK is activated by allosteric stimulation in response to an increased AMP/ATP ratio or mitochondria activity change. AMPK activation also is induced by upstream stress kinases, such as liver kinase B1 (LKB1) or calcium/calmodulin-dependent protein kinase kinase (CaMKK) (7–10). Activated AMPK is involved in the regulation of diverse metabolic pathways. For example, in skeletal and cardiac muscle, AMPK activation leads to phosphorylation of acetyl-CoA carboxylase (ACC), subsequently leading to reduction of malonyl-CoA,

an allosteric inhibitor of carnitine palmitoyltransferase (CPT); this, in turn, results in fatty acid oxidation by accelerating CPT-mediated fatty acid translocation into mitochondria (11–13). Moreover, activated AMPK increases mitochondrial activity and biogenesis in muscle (14–17). In accordance with these data, whole-body and liver-specific AMPKα_2 knockout mice exhibit increased plasma triglyceride levels (18, 19). Recently, it has been reported that AMPK is involved in NAD-dependent deacetylase sirtuin-1 (SIRT1)-dependent metabolic regulation (20–23). Thus, it has been proposed that AMPK is a key target for treating obesity and metabolic disorders (6).

Recent studies have demonstrated that various bioactive chemicals, such as metformin, phenformin, thiazolidinediones, quercetin, resveratrol, and berberine, act as AMPK activators and exhibit beneficial effects on metabolic disorders, including obesity, hyperlipidemia, and insulin resistance (24–30). In this study, we have investigated whether glabridin, a key chemical of major isoflavans from licorice extracts, might regulate AMPK activity, leading to anti-obesity or antidiabetic effects in obese animals. Although it has been previously reported that licorice extracts show metabolically beneficial effects, including anti-atherosclerosis and body weight control, its underlying mechanisms are not clearly understood (31–37). Moreover, it is unknown which single chemical in licorice extracts would mediate such effects. In several obese rodent models, we observed that glabridin decreases adiposity and ameliorates lipid dysregulation as well as insulin resistance. More importantly, we discovered that glabridin stimulates AMPK activity through the regulation of mitochondrial activity. Taken together, these results suggest that glabridin would be an effective AMPK activator having therapeutic potency for obesity-related metabolic diseases.

MATERIALS AND METHODS

Animal care and experimental protocol

Mice (C57BL/6J strain) were maintained according to the guidelines of the Seoul National University Animal Experiment Ethics Committee. They were housed in solid-bottom cages with wood shavings for bedding in a room maintained at 25°C with a 12:12 h light:dark cycle (lights on at 0700 h). The mice were fed a nonpurified diet until they were assigned to an experimental protocol. To induce obesity, mice were given a high-fat diet (HFD; 45% of calories derived from fat; Research Diets Inc., New Brunswick, NJ) for 12 weeks, and HFD-induced obese mice were then orally administered with vehicle (0.1% sodium lauryl sulfate) or glabridin (Mazence Inc, Suwon, Korea) at a dose of 150 mg/kg/day for 4 weeks. Body weight and food intake were measured daily during the experimental protocol. At the end of the experimental period, one mouse from each group was anesthetized, and the distribution of body fat was examined by magnetic resonance imaging (MRI). All other mice were euthanized, and dissected tissue specimens were immediately stored at −80°C until analysis.

For the intraperitoneal glucose tolerance test (IPGTT), fasted mice were injected with glucose (2 g/kg). Blood glucose levels were measured at the indicated time points with a freestyle blood glucose meter (Therasense; Uppsala, Sweden).

A subset of mice from each group (n = 6/group) was subjected to cold exposure at 4°C, and rectal temperature was monitored for 4 h to determine changes in the core body temperature.

Biochemical analysis

The levels of plasma triglycerides, free fatty acids, and total cholesterol were measured using Infinity reagents (Thermo, Melbourne, Australia). LDL cholesterol levels were determined as previously described (30). Plasma insulin (Mercodia, Uppsala, Sweden) and adiponectin (Adipogen, Incheon, Korea) levels were quantified by an ELISA kit, according to the manufacturer's instructions. Hepatotoxicity was determined by measuring plasma alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels (30).

Histological analysis

Tissue sections were prepared as described previously (29). Briefly, dissected samples were mounted on glass slides and fixed. The epididymal WAT and liver samples were then stained with perilipin and hematoxylin and eosin (H and E), respectively. Images were taken at the magnification indicated in the figures.

Culture and adenoviral infection

Myocytes (C_2C_{12}) and hepatoma cells (FAO) were grown in DMEM (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 mg/ml streptomycin. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2. The C_2C_{12} cells were differentiated as described previously (38). Briefly, confluent C_2C_{12} cells were incubated with DMEM supplemented with 2% horse serum for 6–7 days. The medium was changed daily during myocyte differentiation.

For viral infection, differentiated C_2C_{12} cells were incubated with adenovirus expressing null or dominant negative-AMPKα2 (*T172A*) (10 plaque formation units/cell) in serum-free DMEM for 3 h. The medium was then replaced with fresh DMEM containing 10% FBS for 16 h. Cells were then subjected to experimental treatments.

Quantitative real-time RT-PCR analysis

Total RNA was isolated from liver, muscle, and epididymal fat tissues as described previously (39), and cDNA was synthesized using the M-MuLV reverse transcriptase kit (Fermentas, Glen Burnie, MD). The primers used for the real-time PCR analyses were produced by Bioneer (Korea), and the primer sequences are provided in supplementary Table I.

Western blotting

Western blotting was performed as previously described, with minor modifications (40). An equal amount of protein from each sample was separated on 8% or 10% SDS-PAGE gels and transferred to nitrocellulose membranes. Blots were blocked with 5% nonfat milk in TBS containing 0.1% Tween-20 (TBST) at room temperature for 1 h, and then incubated overnight at 4°C with specific antibodies as follows: AMPK (Cell Signaling Danver, MA), phosphoAMPK (Thr[172]) (Cell Signaling), acetyl-CoA carboxylase (Upstate), phosphoACC (Ser[79]) (Upstate), and GAPDH (Ab Frontier, Korea). After washing three times with TBST, the blots were hybridized with secondary antibodies conjugated to horseradish peroxidase. Immunoreactive signals were detected using a LuminoImager (LAS-3000) and Science Lab 2001 Image Gauge software (Fuji Photo Film, Tokyo, Japan).

Fatty acid oxidation

Fatty acid oxidation was measured as previously described, with minor modifications (38). C_2C_{12} cells and FAO cells were

incubated with medium containing 0.1 mmol/l palmitate (9,10-[^{3}H]palmitate, 5 μCi/ml; PerkinElmer Life, Boston, MA) and 1% BSA. After 24 h of incubation, the medium was precipitated with an equal volume of 10% trichloroacetic acid (Sigma), vigorously mixed, and centrifuged at 4°C for 10 min. The supernatants were transferred to capless 1.5 ml microcentrifuge tubes placed in a scintillation vial containing 0.5 ml unlabeled water, and incubated at 55°C overnight. The tubes were then removed, and the ^{3}H$_2$O content was measured in a scintillation counter.

Measurement of AMPK activity

AMPK activity was measured by detection of phosphorylation of SAMS peptide (HMRSAMSGLHLVKRR) as previously described, with minor modifications (24). Cell lysates were prepared with digitonin buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.25% sucrose, 0.4 mg/ml digitonin, 1.5 mM PMSF), followed by precipitation with 35% saturated ammonium sulfate. Samples were suspended with assay buffer, and the reaction was carried out at 30°C for 30 min with following mixture [200 μM AMP, 200 μM ATP (0.5 μCi [γ-^{32}P]ATP), 50 μM SAMS peptide in assay buffer]. The reactions were terminated by spotting on P-81 phosphocellulose paper, washing three times in 1% phosphoric acid and once with acetone, and air-drying. AMPK activity was determined by measuring the incorporation of ^{32}P using scintillation counting.

Mitochondrial ATP production rate and activities of complexes I and II

Mitochondria were isolated from the cells, and ATP production rate (APR) was determined as previously described, with minor modifications (41). Isolated mitochondria (10 μg) were suspended in reaction buffer A (0.1% BSA, 150 mM KCl, 0.1 mM MgCl$_2$, 25 mM Tris-HCl, 10 mM potassium phosphate, pH 7.4) containing 160 μM diadenosine pentaphosphate, 1 mM pyruvate, 100 μM ADP, and either 5 mM glutamate/malate or 5 mM succinate. To determine APR, luciferase assays were performed by addition of buffer B (500 mM Tris-acetate, pH 7.75) containing 20 μg/ml luciferase (Invitrogen) and 0.8 mM D-luciferin (Invitrogen). The light emission was monitored using a luminometer (20/20^n; Turner Biosystems, Sunnyvale, CA) at 10 s intervals for 5 min. A standard curve for luminescence was obtained using different concentrations of ATP solution.

Mitochondrial complex I and II activities were assessed using decylubiquinone and dichloroindophenol (DCIP), as described previously (42). Briefly, 10 μg of the isolated mitochondrial fractions was preincubated in the appropriate reaction buffer [complex I buffer (70 μM decylubiquinone, 60 μM DCIP, 1 μM antimycin A, 0.35% BSA, 25 mM potassium phosphate, pH 7.4); complex II buffer (70 μM decylubiquinone, 60 μM DCIP, 1 μM antimycin A, 50 μM rotenone, 500 μM EDTA, 200 μM ATP, 0.1% BSA, 80 mM potassium phosphate, pH 7.4)] at 37°C. The reaction was initiated by addition of substrates (complex I: 5 mM glutamate/malate, and complex II: 5 mM succinate and 300 μM potassium cyanide) to the mixture, and absorbance was read at 600 nm for 5 min at 20 s intervals. For measurement of the activity of complex I, rotenone (1 μM) was added to the reaction mixture, and absorbance was read at 20 s intervals for 5 min.

Adenine nucleotide extraction and measurement

ATP and AMP contents in FAO cells and liver tissues were measured by using high-performance liquid chromatography (HPLC) as previously described, with minor modifications (43). Briefly, FAO cells and liver tissues were lysed with 10% percholic acid and neutralized with 5 N KOH. HPLC analysis was performed by DIONEX Ultimate 3000 HPLC system (Thermo, CA)

with Inno C18 column (5.0 μm, 4.6 × 150 mm). The mobile phase consisted of 0.02 M KH$_2$PO$_4$ (pH 6.0) in water (A) and 0.02 M KH$_2$PO$_4$ in 10% methanol (B). The optimal mobile phase was set as follows: 0–15 min, 100% A; 15–18 min, gradient to A/B = 5:95; 18–30 min, 100% B; 30–40 min 100% A; the flow rate and the wavelength were set at 0.7 ml/min and 254 nm. ATP and AMP appeared at 11.3 and 24.3 min, respectively.

Statistical analysis

Error bars represent standard errors, and P values are calculated from Student t-test. $P < 0.05$ was interpreted as statistically significant.

RESULTS

Glabridin reduces body weight in obese animals

To investigate the effects of glabridin on body weight change, glabridin was administered to high-fat diet (HFD)-fed obese mice (**Fig. 1A**). Glabridin gradually reduced body weight in HFD-fed obese mice (Fig. 1B, C). Glabridin also decreased body weight in leptin-deficient Lep^{ob}/Lep^{ob} mice (supplementary Fig. I-A). Interestingly, glabridin
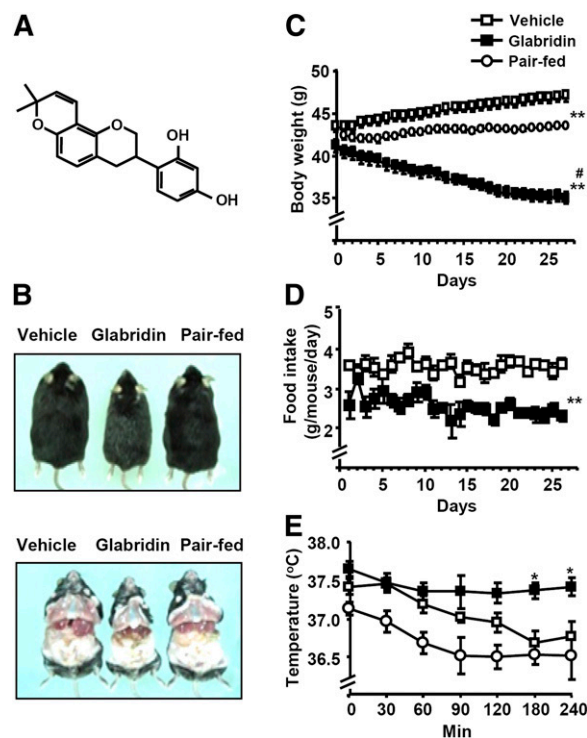
Fig. 1. Glabridin decreases body weight and food intake in obese mice. A: Structure of glabridin. Glabridin is one of the major flavonoids in the hydrophobic fraction of *G. glabra* extract. B–E: HFD-fed obese mice (n = 7–8 per group) were orally administered with vehicle or glabridin (150 mg/kg) or used for pair-feeding, for four weeks. B: Gross images of whole body (top) and abdominal fat (bottom) of mice in each group. C, D: Body weight and food consumption were measured every day throughout the experimental period. E: Body temperature during 4 h of cold exposure (n = 6 per group) was measured at indicated time points. Data represent mean ± SE. *$P < 0.05$ versus vehicle; **$P < 0.01$ versus vehicle; #$P < 0.05$ versus pair-feeding (Student t-test). Each experiment was independently performed at least twice.

suppressed food intake (Fig. 1D and supplementary Fig. I-B), which directly contributes to body weight loss. To examine whether the glabridin-induced anti-obesity effect mainly resulted from decreased food intake, pair-feeding tests were conducted. As shown in Fig. 1C, glabridin-treated mice clearly showed a greater reduction in body weight than their pair-fed counterparts. In accordance with these data, glabridin slightly, but substantially, elevated body temperature (Fig. 1E), implying that glabridin might stimulate energy expenditure in obese animals. These results suggest that glabridin exerts an anti-obesity effect by enhancing energy expenditure and decreasing food intake.

## Glabridin attenuates adiposity, inflammation, and fatty liver in obese mice

In obese mice, glabridin greatly decreased the size of epididymal and retroperitoneal adipose tissue masses (**Fig. 2A**). As shown in coronal and transverse MRI sections, the fat contents of visceral and subcutaneous adipose tissues were evidently diminished in glabridin-treated obese animals (Fig. 2B). However, glabridin did not cause significant changes in the weights of the other organs, such as heart, muscle, lung, kidney, and spleen (supplementary Fig. II). Consistent with fat mass reduction, glabridin

decreased fat cell size determined by perilipin staining, whereas there was no significant alteration in adipogenesis and expression of adipogenic genes (Fig. 2C, D and supplementary Fig. III). Hypertrophic adipocytes are closely associated with increased chronic and low-grade inflammatory responses in obesity (44). To test the idea that glabridin-dependant reduction in fat mass might affect proinflammatory responses in the fat tissue of obese animals, gene expression was analyzed. As shown in Fig. 2E, glabridin suppressed the expression of proinflammatory genes, such as *mcp-1* and *inos*, in adipose tissues of obese animals, which is correlated with decreased hypertrophic adiposity. In accordance with these results, we also observed that glabridin repressed tumor necrosis factor (TNF)α-mediated proinflammatory responses in 3T3-L1 adipocytes (supplementary Fig. IV).

Because obesity is often accompanied by the development of fatty liver, we examined the liver histology of obese mice in the absence or presence of glabridin administration. Glabridin clearly alleviated fatty liver, as evaluated by macroscopic and microscopic examinations, accompanied with decrease in liver tissue weight (**Fig. 3A**, B and supplementary Fig. II). Furthermore, glabridin significantly decreased the levels of plasma triglycerides, total cholesterol, and LDL cholesterol (Fig. 3C), which are closely associated
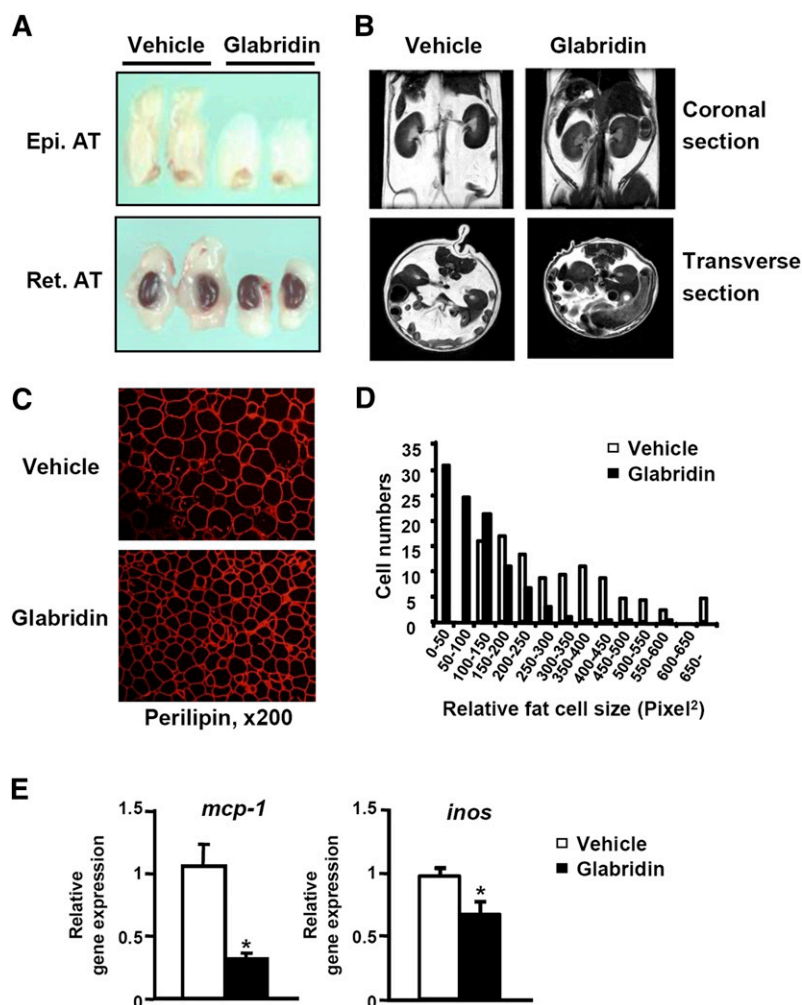
Fig. 2. Glabridin ameliorates adiposity and fat tissue inflammation in obese mice. A, B: Appearance of epididymal (upper panel) and retroperitoneal (lower panel) white adipose tissue (WAT) and coronal (upper panel) and transverse (lower panel) MRI sections of whole body from vehicle- or glabridin-treated obese mice. C, D: Histological analysis of epididymal WAT from vehicle- or glabridin-treated obese mice and adipocyte size was quantified. E: Total RNA was isolated from WAT of obese mice. Relative mRNA levels of inflammatory genes, such as *mcp-1* and *inos*, from WAT were determined using qRT-PCR and normalized with cyclophilin mRNA. Data represent mean ± SE of triplicates. *$P < 0.05$ versus vehicle (Student t-test). Epi. AT, epididymal adipose tissue; Ret. AT, retroperitoneal adipose tissue.
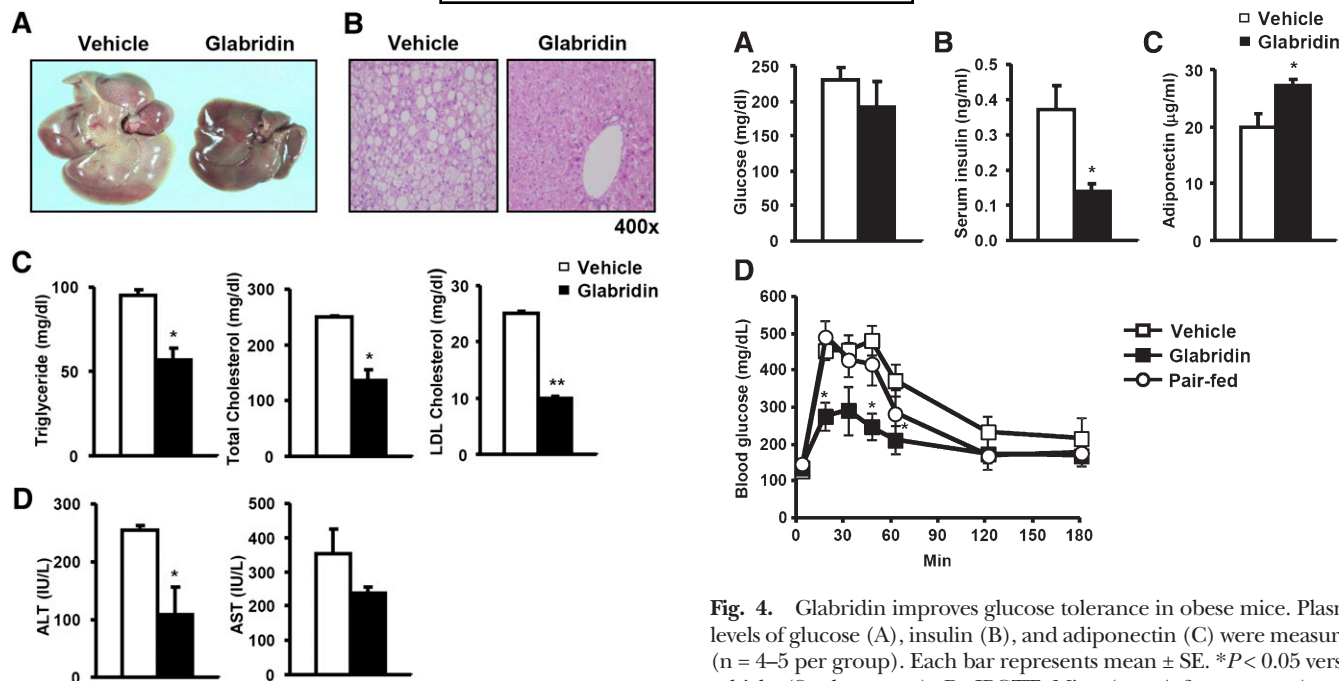
Fig. 3. Glabridin alleviates fatty liver and hyperlipidemia. A, B: Gross appearance and histological (H and E staining) analysis of liver from vehicle- or glabridin-treated obese mice. C: Plasma levels of triglyceride, total cholesterol, and LDL cholesterol (n = 4–5 per group) were determined as described in Materials and Methods. D: Hepatotoxicity was assessed by measuring plasma levels of ALT and AST (n = 4–5 per group). Each bar represents mean ± SE. *$P < 0.05$ versus vehicle, **$P < 0.01$ versus vehicle (Student t-test).

Fig. 4. Glabridin improves glucose tolerance in obese mice. Plasma levels of glucose (A), insulin (B), and adiponectin (C) were measured (n = 4–5 per group). Each bar represents mean ± SE. *$P < 0.05$ versus vehicle (Student t-test). D: IPGTT. Mice (n = 4–6 per group) were fasted and injected with glucose as described in Materials and Methods. Blood glucose levels were measured at indicated time points. Data represent mean ± SE. *$P < 0.05$ versus vehicle (Student t-test).
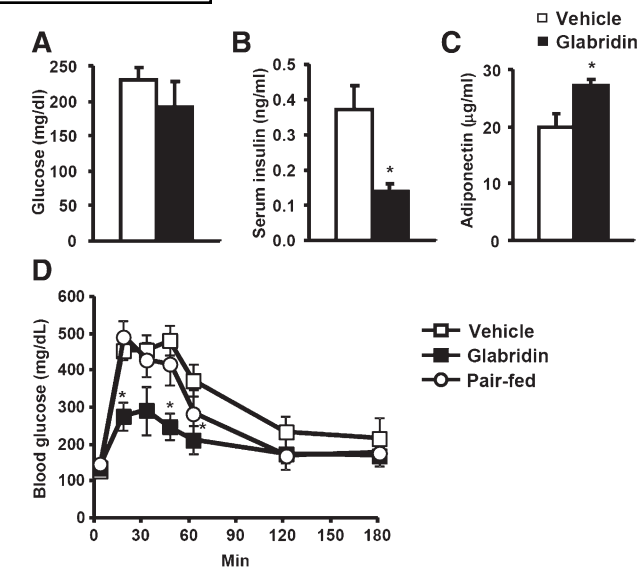
with fatty liver in obesity. Simultaneously, glabridin exhibited a tendency to reduce plasma levels of ALT and AST in HFD-fed obese mice (Fig. 3D), implying that hepatic dysfunction in obesity would be relieved by glabridin.

Glabridin improves hyperinsulinemia and glucose metabolism

As obesity is one of the key risk factors for insulin resistance as well as type 2 diabetes, the effects of glabridin on glucose and insulin sensitivity were tested. In HFD-fed obese mice, glabridin barely changed basal glucose levels (**Fig. 4A**). However, in the presence of glabridin, the plasma level of insulin markedly decreased, whereas adiponectin levels increased (Fig. 4B, C), which seemed to be associated with reduced adiposity and plasma lipid metabolites. Moreover, glabridin significantly relieved glucose intolerance and insulin sensitivity in HFD-fed obese mice (Fig. 4D). Consistently, in obese Zucker *fa/fa* rats, glabridin ameliorated glucose intolerance and insulin sensitivity (supplementary Fig. V-A, B). Together, these results clearly indicate that glabridin could improve insulin sensitivity and glucose metabolism in obese and/or diabetic animals.

Glabridin regulates metabolic gene expression in fat, liver, and muscle

To better understand the molecular mechanism(s) by which glabridin mediates the beneficial effects on obese animals described above, the expression of genes involved in energy metabolism was investigated. Consistent with the

reduced hepatic and plasma lipid content upon glabridin administration in obese animals, expression of lipogenic genes (*srebp-1c, fas, acc,* and *scd-1*) in adipose tissue and liver was downregulated by glabridin (**Fig. 5A**, B). Conversely, in muscle, the expression of genes for fatty acid oxidation (i.e., *cpt1* and *aco*) and mitochondrial biogenesis (i.e., *nrf* and *cytc*) was stimulated by glabridin (Fig. 5C). Moreover, glabridin showed a tendency to suppress expression of gluconeogenic genes (*pepck* and *g6pase*) in the liver of obese animals (Fig. 5D). These data indicate that glabridin might alleviate HFD-induced metabolic abnormalities, at least in part, through regulating the expression of a certain set of metabolic genes.

Glabridin stimulates AMPK phosphorylation

AMPK is able to dissipate excessively stored energy by stimulating fatty acid oxidation. To test whether glabridin could potentiate AMPK activity, the levels of AMPK and ACC phosphorylation were examined. In the muscle, liver, and adipose tissue of obese mice, glabridin promoted phosphorylation of AMPK and ACC (**Fig. 6A**–C). Next, to confirm whether glabridin could indeed activate AMPK, glabridin was tested in various cell lines. In adipocytes (3T3-L1), hepatoma cells (FAO), myotubes (C_2C_{12}), and human embryonic kidney cells (HEK 293), glabridin stimulated AMPK phosphorylation in a dose- and time-dependent manner (Fig. 6D, E and supplementary Fig. VI). These data clearly indicate that glabridin is a potent activator of AMPK, which results in its beneficial effects on glucose and lipid metabolism.

Glabridin promotes fatty acid oxidation through AMPK activation

It has been well established that activated AMPK stimulates fatty acid oxidation and fat assimilation (11–13). To
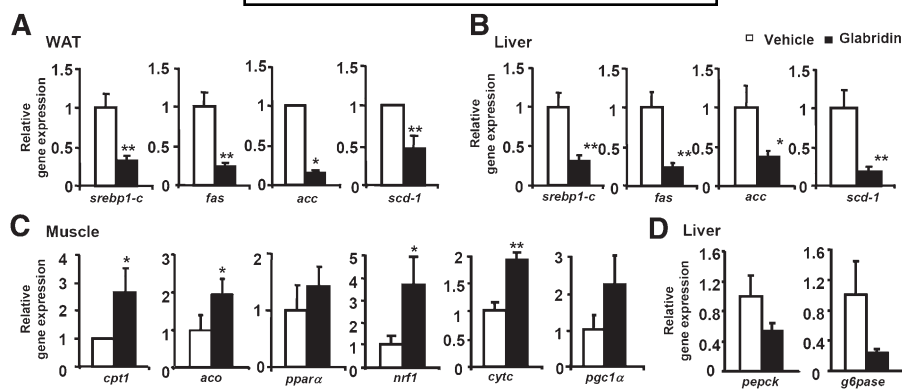
Fig. 5. Glabridin regulates expression of genes involved in lipid metabolism. Total RNA was isolated from epididymal WAT, liver, and muscle of vehicle- or glabridin-treated obese mice. Relative mRNA levels of metabolic genes: (A) WAT and (B) liver lipogenic genes; (C) muscle fatty acid oxidation and mitochondrial biogenesis genes; and (D) liver gluconeogenic genes were determined using qRT-PCR. The gene expression levels were normalized by the level of cyclophilin mRNA. Experiments were independently performed at least three times. Data represent mean ± SE. *$P < 0.05$ versus vehicle; **$P < 0.01$ versus vehicle (Student t test).

test whether glabridin promotes fat burning via AMPK, the effect of glabridin on fatty acid oxidation was examined. As expected, glabridin significantly enhanced fatty acid oxidation in both C_2C_{12} and FAO cells (**Fig. 7A**, B). To verify whether the increased fatty acid oxidation with glabridin requires AMPK, AMPK activity was repressed by either ComC, a well-known AMPK inhibitor, or dominant negative (DN) AMPK. In C_2C_{12} cells, glabridin-induced AMPK activation, ACC phosphorylation, and fatty acid oxidation were attenuated by either ComC or DN-AMPK (Fig. 7C–F). These data suggest that glabridin can promote fatty acid oxidation through AMPK activation, leading to the dissipation of excessively stored energy sources in obese animals.

Glabridin affects mitochondrial functions and cellular AMP/ATP ratio

Consistent with the results for AMPK activation, glabridin significantly increased AMPK activity in FAO cells (**Fig. 8A**). However, glabridin failed to directly stimulate AMPK activity, unlike AMP (supplementary Fig. VIII-A). Also, it is likely that CaMKKβ and LKB were not key players in glabridin-induced AMPK phosphorylation (supplementary Fig. VIII-B, C). To decipher the mechanism(s) underlying AMPK activation by glabridin, we tested mitochondrial activities in the absence or presence of glabridin. As shown in Fig. 8B, C, glabridin decreased the ATP production rate by about 15% in the presence of glutamate/malate or succinate, which are the best-known electron donors in mitochondria. As a positive control, berberine, another well-characterized AMPK activator, was used to validate the change in mitochondrial function (45). In addition, in comparison with the vehicle, glabridin significantly reduced the activities of mitochondrial electron transport chain (ETC) complexes I and II (Fig. 8D, E). To verify whether mitochondrial function attenuated by glabridin is associated with change of AMP/ATP ratio, cellular adenine nucleotide contents were measured in FAO cells with or without glabridin or berberine. Similar

to berberine, glabridin treatment for 12 h significantly elevated cellular AMP level, leading to increase in AMP/ATP ratio by 52.6% (Fig. 8F). Consistently, AMP/ATP ratio in the liver of glabridin-treated obese mice was also slightly, but substantially, increased (Fig. 8G). These data suggest that glabridin is able to activate AMPK by reducing mitochondrial ATP production like other AMPK activators, including metformin and berberine.

DISCUSSION

Various phytochemicals used to treat metabolic diseases are often able to stimulate AMPK activity (24–30). In this study, we present data indicating that glabridin could alleviate body weight, adiposity, fatty liver, and hyperlipidemia by activation of AMPK, which would lead to the improvement of glucose and lipid homeostasis. Previous studies have demonstrated that licorice flavonoid oil containing glabridin decreases visceral adiposity, hyperglycemia, and hepatic lipogenesis (34–37). However, biological pathways, as well as molecular mechanisms to mediate the beneficial effects of glabridin on whole-body energy homeostasis, have not been elucidated.

In peripheral tissues, AMPK activation facilitates lipid dissipation via stimulation of fatty acid oxidation (6). Upon activation of AMPK, fatty acid oxidation is augmented by rapid ACC phosphorylation and is further sustained by expression of certain genes involved in fatty acid oxidation. In this study, we observed that glabridin promoted AMPK activation and stimulated fatty acid oxidation, suggesting that upregulation of fatty acid oxidation via activation of AMPK is the key mechanism for the beneficial effects of glabridin. This speculation was further supported by the observations that ComC treatment or DN-AMPK overexpression decreased the effects of glabridin on fatty acid oxidation. For sustainable and chronic fat burning, glabridin also affects lipid homeostasis by regulation of gene expression. In muscle, glabridin stimulated the expression of a set of genes associated with fatty acid
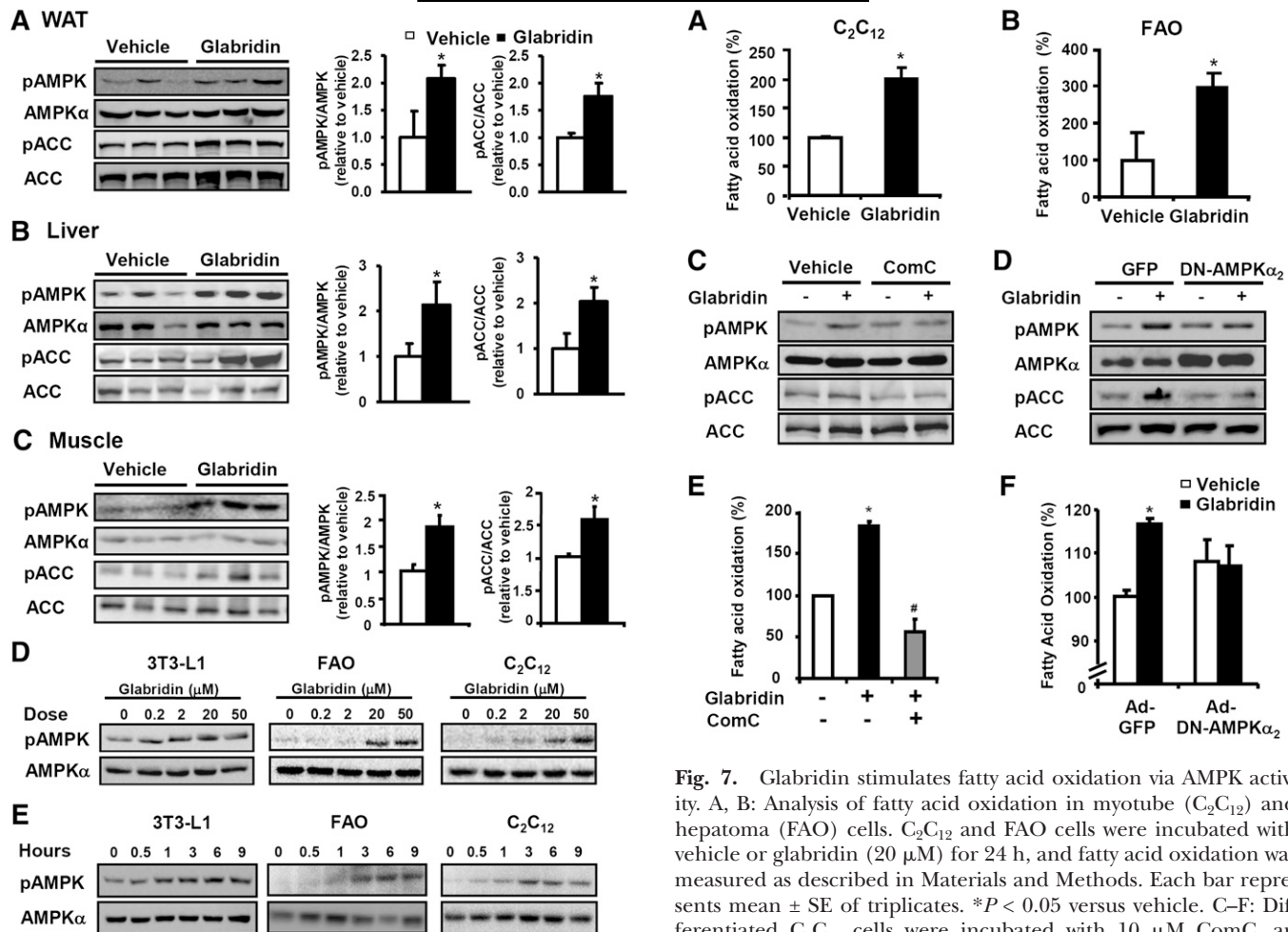
Fig. 6. Glabridin stimulates AMPK in vivo and in vitro. A–C: Total lysates from epididymal WAT (A), liver (B), or muscle (C) from vehicle- or glabridin-treated obese mice were subjected to Western blot analysis using specific antibodies (pAMPK, total AMPK, pACC, and total ACC). The pAMPK/AMPK and pACC/ACC ratios were quantified. More than three independent experiments were performed. D, E: Differentiated adipocytes (3T3-L1), hepatoma (FAO) cells, and differentiated myotube (C_2C_{12}) cells were treated with various concentrations (0, 0.2, 2, 20, and 50 μM) of glabridin for 30 min (D) or were incubated with glabridin (20 μM) for different time points as indicated (E). Cells were lysed and total cell lysates were subjected to Western blot analysis. Each experiment was independently performed more than twice.

Fig. 7. Glabridin stimulates fatty acid oxidation via AMPK activity. A, B: Analysis of fatty acid oxidation in myotube (C_2C_{12}) and hepatoma (FAO) cells. C_2C_{12} and FAO cells were incubated with vehicle or glabridin (20 μM) for 24 h, and fatty acid oxidation was measured as described in Materials and Methods. Each bar represents mean ± SE of triplicates. *$P < 0.05$ versus vehicle. C–F: Differentiated C_2C_{12} cells were incubated with 10 μM ComC, an antagonist of AMPK, for 3 h in prior to glabridin (20 μM) treatment for 30 min (C, E), and differentiated C_2C_{12} cells were infected with Ad-GFP or Ad-DN-AMPKα$_2$ (D, F) as described in Materials and Methods. Then cells were treated with vehicle or glabridin (20 μM) for 30 min. Total cell lysates were subjected to Western blot analysis (C, D). Fatty acid oxidation assays (E, F) were conducted as described in Materials and Methods. Each experiment was independently performed at least four times. Each bar represents mean ± SE. *$P < 0.05$ versus vehicle; #$P < 0.05$ versus glabridin.

oxidation. Conversely, glabridin reduced expression of lipogenic genes in liver and adipose tissue, eventually leading to the suppression of unnecessary de novo lipogenesis. Although details of the mechanism by which glabridin influences expression of these genes remain to be elucidated, previous reports have shown that AMPK activation upon treatment with metformin, berberine, or AICAR, which are well-characterized AMPK activators, also controls expression of those genes (15–17, 24, 29, 30). Recently, several reports have shed light on the connection between AMPK and SIRT1 in the regulation of energy homeostasis (20–23, 46, 47). For instance, in liver, SIRT1 activates AMPK activity and reduces expression of *fas,* leading to diminished lipid accumulation (46, 47). On the other

hand, as a metabolic adaptation to fasting in muscle, AMPK promotes SIRT1 activity by increasing the cellular levels of NAD$^+$ and triggers SIRT1-dependent deacetylation of PCG1α and FOXO1, resulting in induction of expression of the genes involved in mitochondrial and fatty acid metabolism (20–23, 48, 49). Thus, it is plausible that glabridin-stimulated AMPK activation alters the expression of genes involved in lipid metabolism, which might chronically ameliorate lipid dysregulation in obese animals.

Accumulating evidence has demonstrated the critical roles of hypothalamic AMPK in the regulation of feeding behavior and energy homeostasis (30, 50–52). In the hypothalamus, decreased phosphorylation of AMPK and ACC induces anorexic effects (51, 52). Interestingly, this hypothalamic AMPK regulation is also transmitted via neuronal signals to peripheral tissues, such as skeletal muscle, and thereby rapidly potentiates fatty acid oxidation, leading to dissipation of excess lipid (30, 53, 54). In this study, we
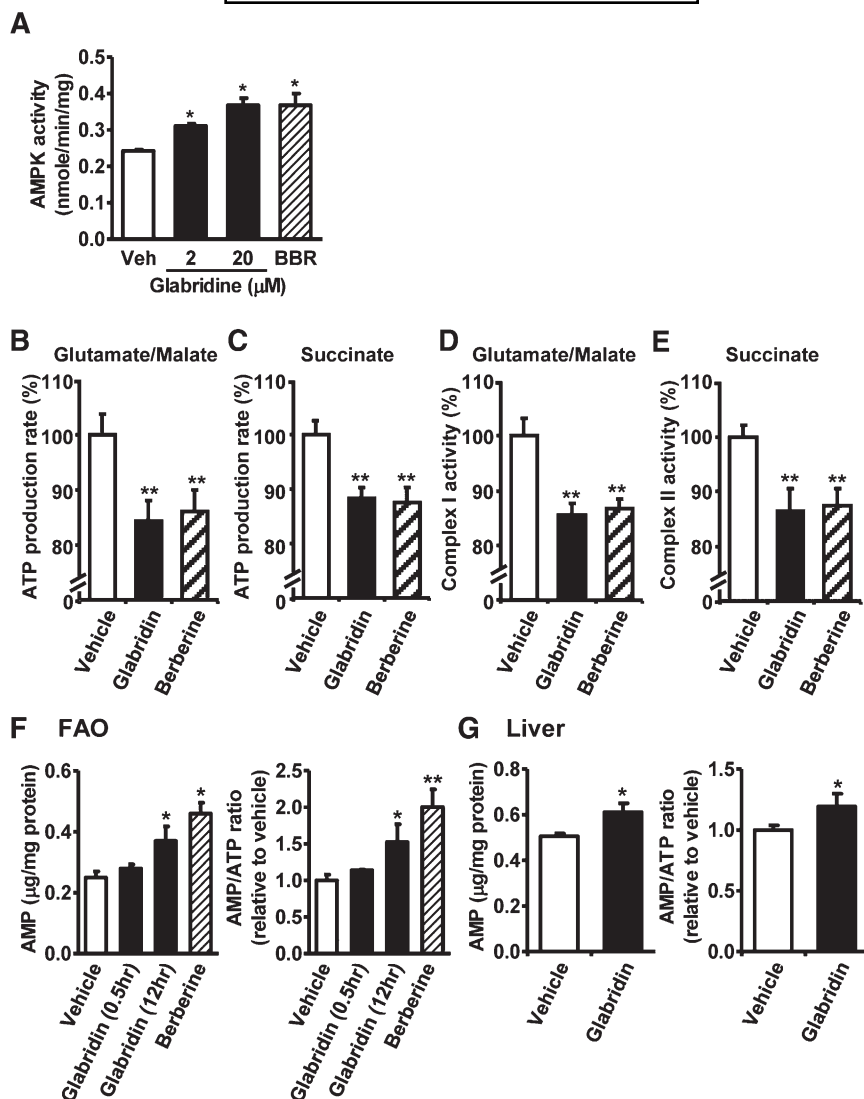
Fig. 8. Glabridin affects mitochondrial functions and cellular AMP/ATP ratio. A: AMPK activity was determined with SAMS peptide. FAO cells were treated with 2 or 20 μM glabridine or 10 μM berberine (BBR) for 1 h. Then AMPK activity was determined by measurement of phosphorylation of SAMS peptides as described in Materials and Methods. Two independent experiments were performed. Each bar represents mean ± SE. * $P < 0.05$ versus vehicle; ** $P < 0.01$ versus vehicle. B–E: ATP production rates (B, C) and activities of mitochondrial complexes I and II (D, E) in FAO cells. Mitochondria were isolated from FAO cells treated with glabridin or berberine and then challenged with 5 mM glutamate/malate (B, D) or 5 mM succinate (C, E). Each experiment was independently performed more than twice. Each bar represents mean ± SE. ** $P < 0.01$ versus vehicle. F, G: Cellular AMP contents and AMP/ATP ratio were determined in (F) FAO cells treated with 20 μM glabridine or 10 μM berberine for 30 min or 12 h and (G) liver from vehicle- or glabridin-treated obese mice. Each bar represents mean ± SE. * $P < 0.01$ versus vehicle; ** $P < 0.01$ versus vehicle.

observed that glabridin exhibited an anorexic effect and promoted fatty acid oxidation. Although it is currently unresolved whether glabridin might affect the central nervous system to regulate whole-body energy homeostasis, we cannot rule out the possibility that glabridin may modulate hypothalamic AMPK activity, which might influence food intake and peripheral fatty acid oxidation.

Depending on the signaling cues, AMPK activation is mediated by several different mechanisms, such as changes of AMP/ATP ratio, intracellular Ca^{2+}, and activities of AMPK upstream kinases (45). For instance, AICAR, a well-known AMPK activator, directly binds to AMPK after intracellular

conversion into ZMP, an AMP mimetic (55). On the other hand, several antidiabetic drugs, including thiazolidine-diones, metformin, and berberine, activate AMPK by changing mitochondrial activity (45, 56–58). Although the mechanism(s) by which glabridin might change LKB1 or CAMKKβ needs to be further investigated, we obtained some clues that glabridin would activate AMPK independent of CAMKKβ- or LKB-activation. Rather, we observed that glabridin evidently attenuated mitochondrial ATP production and suppressed ETC complex I and II activities, accompanied with elevation of cellular AMP contents and AMP/ATP ratio. Thus, these data imply that increase

in cellular AMP/ATP ratio by glabridin would be the major molecular mechanism responsible for AMPK activation.

Glabridin efficiently reduces adipose mass and adipocyte hypertrophy in obese animals. Although it has been demonstrated that AMPK activation with berberine potently inhibits adipogenesis and lipogenesis (29), glabridin did not significantly affect adipogenesis. Consistently, in adipose tissue, expression of adipogenic genes, including *ppar*γ and *aP*2, was not changed by glabridin. However, the expression of lipogenic genes was potently suppressed in adipose tissue of glabridin-treated obese mice, which is similar to the findings obtained for berberine or metformin (24, 29, 30). Thus, it is very likely that glabridin reduces lipogenesis, but not adipogenesis, via AMPK activation, which might contribute to reduced adipose tissue mass and adipocyte hypertrophy.

In macrophages, AMPK activation relieves lipopolysaccharide-induced inflammation (59). Similarly, glabridin decreased proinflammatory gene expression in adipose tissues. In differentiated adipocytes, glabridin stimulated AMPK phosphorylation and decreased expression of proinflammatory genes in the presence of TNFα, a potent inflammatory inducer. Consistent with these data, glabridin exhibits anti-inflammatory responses in colitic subjects (60). Therefore, it appears that glabridin-induced AMPK might also contribute to the suppression of inflammatory responses, which would lead to improvements in metabolic disorders, such as insulin resistance in obesity.

In conclusion, glabridin, a novel AMPK activator, ameliorates adiposity and metabolic disorders in obese animals. These effects of glabridin are produced through strong and sustained changes in metabolic regulation that facilitate catabolism of fuel storage, implying that glabridin would be a potential candidate for treatment of obesity and metabolic disorders.∎

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