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Molecular Mechanism of Action for the Geranyl Flavonoid to Counter Dyslipidemia in Diabetic Milieu

Hai Niu[1,2,*], Ke Li[1,3], Limei Ma[1], Weihong Gong[1,4], Wen Huang[1,3,*]

[1]Laboratory of Ethnopharmacology, Institute for Nanobiomedical Technology and Membrane Biology, Regenerative Medicine Research Center, West China Hospital, West China Medical School, Sichuan University, Chengdu, Sichuan 610041, China
[2]College of Mathematics, Sichuan University, Chengdu 610064, P.R. China
[3]College of Light Industry, Textile and Food Engineering, Sichuan University, Chengdu, Sichuan 610065, PR China
[4]School of Basic Clinical Medical, Beijing University of Traditional Chinese Medicine, Beijing 100029, China
*Corresponding author: niuhai@scu.edu.cn; huangwen@scu.edu.cn

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Abstract This work was to examine the effect of 3'-methyl-4', 7-dihydroxyflavanone, a geranyl flavonoid (GF), on hepatocellular AMPK activity and lipid levels in HepG2 cells and diabetic mice, and identify molecular mechanism of the GF action on remedying dyslipidemia. The AMPK activation and lipid-lowering effect of the GF in diabetic mice and in HepG2 cells paralleled observations. The GF activated AMPK in HepG2 cells treated with high glucose, and enhance phosphorylation of ACC1 and ACC2, two isoforms of ACC, resulting in decrease in ACC activity and hepatic lipids. As demonstrated in cells overexpressing a dominant-negative AMPK mutant, the effect of GF was shown to be mediated by the activation of AMPK. The AMPK was activated relatively rapidly by GF and well before any potential change in adenosine triphosphate (ATP) level was detected. Thus, both in vivo and in vitro inhibition of AMPK, activation of ACC, and hepatocellular lipid accumulation caused by sustained high glucose levels was effectively counteracted by activating AMPK with treatment of GF. It can be conclude that GF lower lipids both in vivo and in vitro by activating AMPK and inactivating ACC, and consequently down-regulating fatty acid synthesis. The work provides a strong evidence for GF as a new therapeutic agent to definitively remedy dyslipidemia in diabetic milieu.

Keywords: *geranyl flavonoid, AMPK, ACC, diabetic milieu, HepG2 cells, dyslipidemia*

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1. Introduction

Diabetes mellitus, characterized mainly by dyslipidemia, is a progressively debilitating metabolic disorder of epidemic proportions and is estimated to afflict 5-7% of the population [1]. This process leads to long−term damage, dysfunction, and failure of various organs, especially the eyes, kidneys, nerves, heart, and blood vessels, and creates a huge economic burden related to the management of diabetic complications [2]. Given the alarming increase in the worldwide diabetic population, there is a need for novel therapies which are effective with minimal adverse events [3].

Significantly, for effective control of diabetes, combination therapy is being considered because no single drug is able to target diabetes and its associated complications. This necessitates the identification of novel drugs which might function in a mechanistically distinct fashion to the existing drug targets [4]. Hence, the search for a definitive cure for diabetes mellitus is being pursued vigorously by the scientific community.

Geranyl flavonoid (GF), belonging to polyphenolic flavonoids, was one of the main active compounds present in *Artocarpus altilis* named bread tree, which is used as food and medicine in Chinese and Indonesian for the treatment of liver cirrhosis, hypertension, and diabetes [5,6]. The flavonoids from this and other species have also been shown to have anti-inflammatory [5], anti-oxidative [7], hemostatic [8], anti-hypertensive [9], anti-hypercholesterolemic [10] and anti-atherosclerotic activities [11].

Long-term treatment with Adenosine monophosphate-activated protein kinase (AMPK) activator like metformin has prevented the development of diabetes in animal models [12]. AMPK is a metabolite-sensing protein kinase that shares amino acid sequence homology with yeast sucrose nonfermenting 1 [13]. AMPK is known to play a major role in energy homeostasis by coordinating a number of adaptive responses in adenosine triphosphate-depleting metabolic states such as ischemia/reperfusion, hypoxia, heat shock, oxidative stress, and exercise [14]. In order to find a novel substance that has the therapeutic potential of oral agent for treatment of dyslipidemia in diabetes, we have assessed GFs from *Artocarpus altilis* and used metformin as positive control in high fat diet and

STZ-co-induced diabetic rats. We found that the GF as 3'-methyl-4',7-dihydroxyflavanone (Figure 1), which can significantly improve diabetic hyperlipidemia as metformin do.

In the study, we further examine the effect of GF on hepatocellular AMPK activity and lipid levels in HepG2 cells, as well as in diabetic mice, and identify molecular mechanism of action for GF on regulating dyslipidemia.

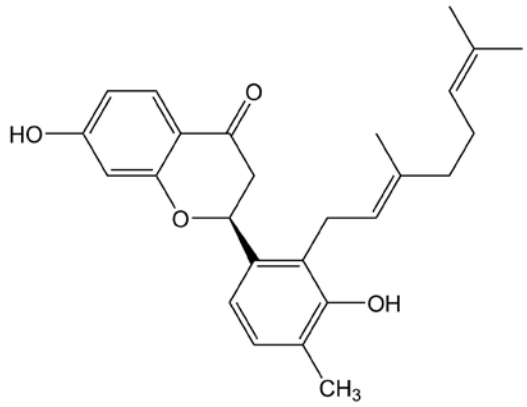

Figure 1. Molecular structure of 3'-methyl-4',7-dihydroxyflavanone.

2. Materials and Methods

2.1. Materials

3'-methyl-4',7-dihydroxyflavanone from *Artocarpus altilis* was separated by pilot-scale plant, Beijing University of Traditional Chinese Medicine, China. The GF was identified by Chemistry Dept. Peking University, China. The purity of the compounds is 95%. The purified robinelinidol-flavon was stored at 4 °C for further investigation. STZ, metformin (1,1-dimethylbiguanide), insulin, MTT, AMP, Nonidet P-40, aprotinin, leupeptin, and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma (Beijing, China). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), and Dulbecco's PBS were from Invitrogen (Shanghai, China). Rabbit polyclonal pan-AMPKα antibody and phospho-AMPKα (Thr-172) antibody were purchased from Beijing Luqiao Bio-tech Company (Beijing, China). Rabbit polyclonal anti-AMPKα subunit antibodies (the peptide sequences used to generate and immunopurify the antibodies) were from Saier Bio-tech Company (Tianjing,China). Rabbit polyclonal anti-phospho-Ser-79 ACC1 (Ser-221 ACC2) antibody, SAMS peptide, and P81 phosphocellular paper were purchased from Hushing Biotechnology (Shanghai, China). Mouse monoclonal anti-myc antibody was from Bio-Protea Test-Tech Company (Beijing, China). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies and protein A/G plus agarose were obtained from Cell Signaling Technology Inc. (Danvers, MA, USA). Adenopure kits for adenovirus purification were purchased from Puresyn (Malvern, PA, USA).

Enzymatic lipid assay kits were from Thermo Fisher Scientific (Shanghai, China). ATPLite for measuring intracellular ATP and [γ-32P] ATP were from PerkinElmer Life and Analytical Sciences Inc. (Waltham, MA, USA). All other reagents were of analytical grade.

2.2. Animal Protocols and Diets

Male homozygous LDL receptor-deficient (LDLR-/-) mice (6 weeks of age) with C57BL/6 genetic background were created by homologous recombination (Life Sicence Dept PKU, Beijing, China) [15]. The mice were maintained on normal mouse chow and given free access to both food and water in a temperature- and light-controlled animal facility with a light/dark cycle of 6 A.M.to 6 P.M. After 1 week of acclimatization, diabetes was induced by intraperitoneal injection of STZ (100 mg kg^{-1} day^{-1}) dissolved in citrate buffer (0.05 mol/L, pH 4.5) for 5 consecutive days. Glucose levels were measured in tail blood by a FreeStyle blood glucose monitoring system (Beijing YiCheng Bio-Tech, Beijing, China). Hyperglycemia was confirmed by nonfasting blood glucose >200 mg/dL (11 mmol/L) 1 week after initial STZ injection. The diabetic LDLR-/- mice were randomly divided into two groups: untreated and GF-treated mice. For GF treatment, 2 g of customized chow diet (Pharma Dept PKU, Beijing, China) contained GF in a dose of 150 mg/kg/day/mice was fed to mice. After the diet with GF was eat completely, the animals were allowed free access to chow diet without GF. The untreated mice were fed normal mouse chow containing 5% fat freely.

After 6 weeks the mice were killed under isoflurane anesthesia, and tissues were taken and frozen immediately in liquid nitrogen or fixed. Blood samples for serum lipids were collected from the vena cava. Animal Center and use committee of Peking University approved the protocol. Serum cholesterol (TC) and triglycerides (TG) were measured enzymatically, using Infinity reagents from Thermo according to the manufacturer's instructions. Hepatic total TC and TG concentrations were determined and normalized to protein concentrations, and they were expressed as milligrams of lipid per gram of tissue protein [16].

2.3. Cell Culture and Treatments

The cultured human hepatoma HepG2 cell line was grown in DMEM containing normal glucose (5.5 mmol/L D-glucose) supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/mL streptomycin. Cells were incubated in a humidified atmosphere of 5% CO_2 at 37 °C. HepG2 cells were cultured in complete medium with 10% FBS to 80% cell confluence and subjected to assays after overnight serum depletion. GF dissolved in DMSO were added to the medium. The final concentration of DMSO did not exceed 0.1%, which did not affect cell viability or AMPK phosphorylation. A cell model for high-glucose-induced accumulation of hepatic lipids was used by exposing HepG2 cells to a high concentration of glucose (30 mmol/L) for 24 h. Briefly, HepG2 cells were quiesced in serum-free DMEM overnight and incubated in DMEM containing either a normal (5.5 mmol/L) or high (30 mmol/L) concentration of D-glucose. Normal glucose refers to medium containing 5.5 mmol/L D-glucose, and high glucose refers to medium supplemented with 30 mmol/L D-glucose. For the luminescence ATP detection assay, HepG2 cells (2.0×10^4 per well) were cultured in 96-well microplates and treated with GF as indicated. Intracellular ATP levels were measured using ATP Lite, an ATP monitoring system based on firefly (Neogen Corporation, Lansing, MI, USA) luciferase according to

the manufacturer's instructions. A microplate reader (Maxwell Sensors Inc, Santa Fe Springs, CA, USA) was used to measure the luminescence [17]. For cellular toxicity, the MTT assay was performed according to the manufacturer's protocol (Sigma). HepG2 cells were seeded on a 96 well plate and grown to 70% confluence. Cells were treated for 24 h with increasing concentrations of GF in 100 μL of DMEM without phenol red and serum in quadruplicate for each condition, and they were subsequently incubated with 10 μL of the MTT solution (5 mg/mL in PBS) at 37°C for another 3 h, followed by incubation in 100 μL of 10% Triton X-100 and 0.1 N HCl in isopropanol for 10 min. The optical density at 570 nm was measured using a plate reader (Microplate spectrophotometer, BioTek Instruments, Winooski, VT, USA). Cell viability was calculated from the optical density readings of GF treatment, using control cells as 100%. Treatment with GF (2.5-30 μmol/L) had no detectable effect on cell viability. Intracellular TG and total TC contents were measured in HepG2 cell lysates and expressed as micrograms of lipid per milligram of cellular protein [18].

2.4. Immunoblotting Analysis

Immunoblotting analysis was conducted as described previously by Zang et al [19]. HepG2 cells were washed with PBS and lysed at 4°C in lysis buffer (20 mmol/L Tris-HCl, pH 8.0, 1% Nonidet P-40 (v/v), 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L sodium orthovanadate, 1 mmol/L dithiothreitol, 1 mmol/L phenylmethylsulfonyl fluoride, 2 μg/mL aprotinin, 2 μg/mL leupeptin, and 1 μg/mL pepstatin), followed by centrifugation at 15,000 g for 10 min at 4 ^{o}C. Protein concentrations in cell lysates were measured using a Bio-Rad protein assay kit. The cell lysates (20-50 μg protein) were combined with the appropriate amount of 6 SDS sample buffer (0.32 mol/L Tris-HCl, pH 6.8, 30% glycerol (v/v), 12% SDS (w/v), 5% β-BME (v/v), and BPB and then heated at 95°C for 5 min.

Samples were subjected to 8% SDS-PAGE and electrophoretically transferred to PVDF by wet transfer at 30 V overnight. The membranes were blocked with 5% (w/v) non-fat dry milk in Tris-buffered saline with Tween (TBST) buffer (20 mmol/L Tris-HCl, pH 7.6, 0.14 mol/L NaCl, and 0.1% Tween 20 (v/v)) and subsequently blotted with the appropriate antibodies in TBST containing 1% BSA (w/v). Antibodies were used at the following conditions: anti-phospho-AMPK (1:500 dilutions) overnight at 4°C and anti-phospho-ACC antibody (1:5,000), anti- AMPKα1 or α2 antibodies (1:4,000), anti-myc antibodies (1:1,000), and anti-β-actin antibody (1:5,000) for 2 h at room temperature. The membranes were incubated with the secondary antibodies at a 1:10,000 dilution in TBST containing 5% non-fat dry milk (w/v) for 1 h, and the bound antibodies were visualized by an enhanced chemiluminescence (CL) system. Phosphorylated AMPK was quantified using a GS-700 Imaging Densitometer (Bio-Rad) and normalized to the levels of endogenous AMPK protein. Unless stated otherwise, phosphorylation of ACC was expressed as the ratio of the sum of ACC1 and ACC2 phosphorylation to the level of endogenous AMPK expression. In some cases, ACC1 and ACC2 bands were individually assessed by densitometry. Phosphorylation intensity of AMPK and ACC was expressed relative to the basal or control level.

2.5. Immunoprecipitation and Kinase Activity of AMPK

AMPK activity was measured after immunoprecipitation with polyclonal rabbit AMPKα antibodies raised against synthetic peptides of AMPKα. For immunoprecipitation [20], 200 μg of protein from HepG2 cell lysates was incubated with 25 μL of protein A/G plus agarose as well as isoform-specific AMPKα antibody or the antibody that was pre-incubated with competing peptide at 4°C overnight. The immunoprecipitates were washed once with lysis buffer, twice with lysis buffer containing 0.5 mol/L NaCl, and twice with kinase buffer (50 mmol/L HEPES, 80 mmol/L NaCl, and 1 mmol/L DTT, pH 7.4). AMPK activity in α1or α2 immunocomplexes was measured using the SAMS peptide phosphorylation assay [21]. Briefly, the kinase assay was performed in 40 μl kinase buffer containing 8 μL SAMS peptide (2.2 mg/mL), 200 μmol/L AMP, 5 mmol/L MgCl2, 100 μmol/L ATP, and 10 μCi (γ-32 P) ATP (specific activity 2,000 cpm/pmol) at 30 ^{o}C for 15 min with continuous shaking. Aliquots of the reaction mixture were spotted on 2 cm × 2 cm squares of P81 phosphocellular paper. The P81 paper was washed three times for 15 min in 0.38% phosphoric acid (v/v) with gentle stirring to remove free ATP and then dried to quantify 32P-labeled incorporation into SAMS peptide by scintillation counting. Control immunoprecipitation that contained no AMPK antibody was performed to ensure that SAMS peptide phosphorylation was dependent on specific immunoprecipitation of AMPK. AMPK activity was calculated as picomoles per minute per milligram of protein.

2.6. Statistical Analyses

Data were analyzed by one-way analysis of variance (ANOVA) and expressed as means ± S.D. Inter-group difference was detected by Dunnett's test using SPSS 19.0 software. Comparison between two groups was done by using Student's t-test. Comparison between three groups was done by using one way ANOVA test. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. The Effect of GF on Serum and Hepatic Lipid Levels and the Phosphorylation of AMPK and ACC in the Liver of STZ-induced Diabetic LDLR-/- mice

As shown in Table 1, at the end of the experiment, blood glucose level in STZ-injected mice was significantly higher than that in the non-diabetic control mice, treatment with the GF (150 mg/kg/day) had no effect on the level of blood glucose in diabetic mice compared with diabetic control. Even though the body weight and heart weight were significantly lower in diabetic control compared with non-diabetic control mice, there was no significant change in heart weight-to-body weight ratio between non-diabetic control and diabetic

mice. There was also no significant effect of treatment with GF on heart weight or body weight in diabetic mice.

As shown in Figure 2, GF remarkably decreased serum TC and TG levels in diabetic LDLR-/- mice (Figure 2A). The contents of TG and TC in the liver were increased more than 3.7-fold and 1.7-fold in the diabetic mice, respectively and treatment with GF significantly lowered their levels by 44% and 25% , respectively (Figure 3B). Thus, these results indicate that GF prevents hepatic lipid accumulation.

Table 1. Body composition and blood glucose in STZ−induced diabetic LDLR−/− mice treated with GF [a]

Parameter	Non-diabetic control (n=10)	Diabetic control (n=12)	Diabetes + GF (n=12)
Body weight, g	25.6±0.8	19.3±0.5*	22.0±0.9*
Heart weight, mg	108.3±2.3	81.2±2.2*	91.4±2.7*
Heart weight /body weight	4.2±0.1	4.2±0.1	4.2±0.2
Blood Glucose, mg/dl	129.2±5.2	444.9±14.2*	429.5±18.9*

[a] Diabetes was induced in LDLR-/- mice at 8 weeks of age by injection of STZ for 5 days. Data represent the mean ± SD. *$P < 0.05$, compared with untreated non-diabetic mice.

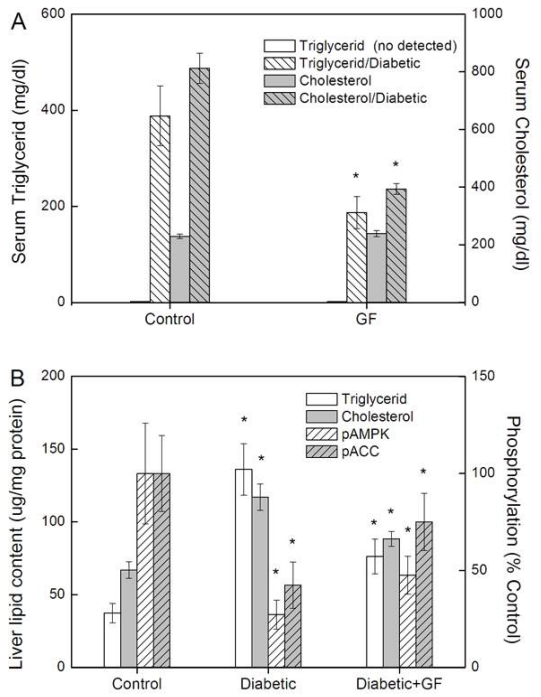

Figure 2. Treatment with GF lowers serum and hepatic lipid levels as well as stimulates the phosphorylation of AMPK and ACC in the liver of STZ-induced diabetic LDLR-/- mice. A, GF reduces serum TC and TG levels in diabetic LDLR-/- mice. Serum lipid levels were expressed as the means ± SD (n = 10) in diabetic LDLR-/- mice treated or not treated with GF (150 mg/kg/day for 6 weeks). *$P < 0.05$ compared between two groups as indicated. B, GF decreases the lipid content in diabetic mouse livers. Liver lipids were expressed as milligrams of lipid per gram protein. Data are the means ± SD (n = 5). Quantitative analysis of phosphorylated AMPK and ACC is expressed as the means ± SD (n =5) in the livers. *$P < 0.05$ versus untreated diabetic LDLR-/- mice. pACC, phosphor-ACC; pAMPK, phosphor-AMPKα

Compared with the control, phosphorylation of AMPK was dramatically decreased in diabetic LDLR-/- mice livers by 73% (Figure 2B). In diabetic LDLR-/- mice the GF (150 mg/kg/day) could increase phosphorylation of AMPK and ACC approximately twofold (Figure 2B). These results indicate that geranyl flavonoids prevent inhibition of AMPK activity and ACC phosphorylation in the livers of diabetic LDLR-/- mice.

3.2. The Effect of GF on AMPK and ACC Phosphorylation in Human HepG2 Hepatocytes

To demonstrate molecular mechanism of the lipid-lowering effect of GF in the livers of diabetic LDLR-/- mice we investigate effect of GF on the AMPK and ACC in cultures of HepG2 cells. As evident in Figure 4 the concentrations as low as 2.5 μmol/L GF significantly increase AMPK and ACC phosphorylation by 1.5-fold and 2.3-fold in 1 h, respectively. Two- to three- fold stimulation of AMPK and ACC phosphorylation occurred at higher concentrations of GF (10-30 μmol/L) (Figure 3). GF increases phosphorylation of AMPK and ACC in a concentration-dependent manner in HepG2 cells which is similar in diabetic mice. The increased phosphorylation level of AMPK caused by GF closely correlated with the increase in ACC phosphorylation in HepG2 cells.

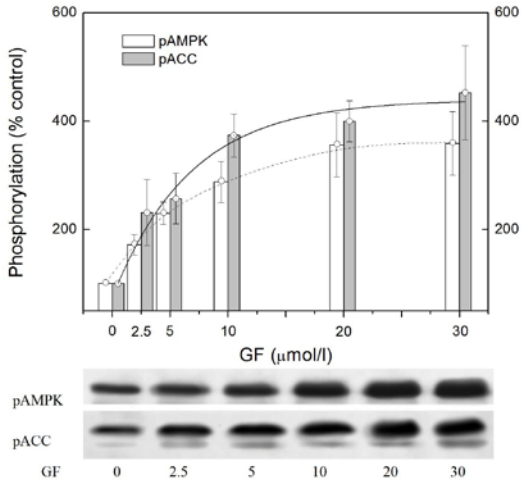

Figure 3. GF increases phosphorylation of AMPK and ACC in a concentration-dependent manner in HepG2 cells. Phosphorylation levels of AMPK and ACC are expressed as fold stimulation over the basal phosphorylation level, mean ± SD (n=3)

As shown in Figure 4A, metformin, a known activator of AMPK, significantly stimulated AMPK phosphorylation by 2-fold over the basal level (Figure 4A). AMPK activation by GF (10 μmol/L) was further confirmed by enhanced phosphorylation of both ACC1 and ACC2 in HepG2 cells comparable to that of metformin (2 mmol/L). Moreover, the phosphorylation of AMPK and ACC occurred very rapidly, rising to near maximal levels within 15 min, and was sustained for 24 h (Figure 4B).

To elucidate the mechanisms responsible for GF-induced activation of AMPK, we determined whether GF decrease the concentration of cellular ATP, which would result in an increase in the AMP-to-ATP ratio. After treating cells with GF (10 μmol/L), no change in ATP level was evident up to 30 min, but there was a significant decrease at 1 h (Figure 5A). Treatment with GF (5 μmol/L) for 1 h had no effect on ATP levels (Figure 5B). This indicates that the rapid activation of AMPK by GF within 30 min is likely to be independent of ATP hydrolysis.

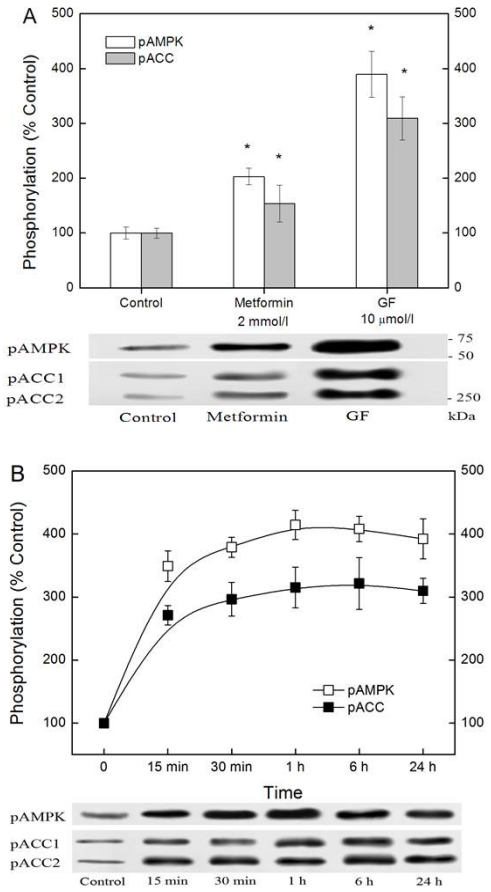

Figure 4. GF or known activator increases phosphorylation of both AMPK and ACC in HepG2 cells and the effect of GF in time course. A, GF (10μmol/L) mimics the effect of metformin (2 mmol/L) on AMPK and ACC phosphorylation. B, Effects of GF on AMPK and ACC phosphorylation in time course. Results are representative of 3 independent experiments (n=3)

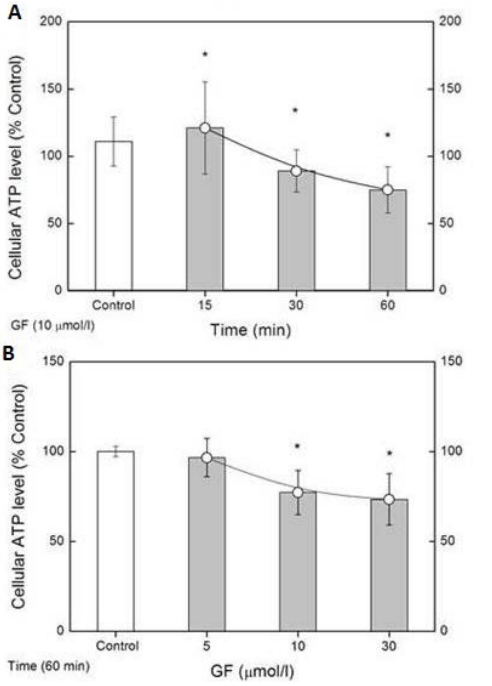

Figure 5. GF stimulate isoform-specific AMPK-α activity in HepG2 cells. A, B, Effect of GF on intracellular ATP levels. The ATP concentrations in HepG2 cells treated with GF as indicated were measured by a luciferase-based assay and expressed as a percentage of that under control conditions. Data are the means ± SD (n =4). **P* < 0.05 compared with vehicle control

3.3. GF Prevent High-Glucose-induced Lipid Accumulation in HepG2 cells

As shown in Figure 6A and B, inhibition of AMPK and ACC phosphorylation occurred very rapidly at 1 h after exposing the cells to high glucose, reducing the levels to 40% of control, and was sustained up to 24 h. Stimulation of AMPK and ACC phosphorylation by pre-incubation with GF (10 μmol/L, 1 h) countered the decrease in AMPK and ACC phosphorylation caused by high glucose and maintained their levels at the same levels as those observed in cells incubated in normal glucose throughout 24 h.

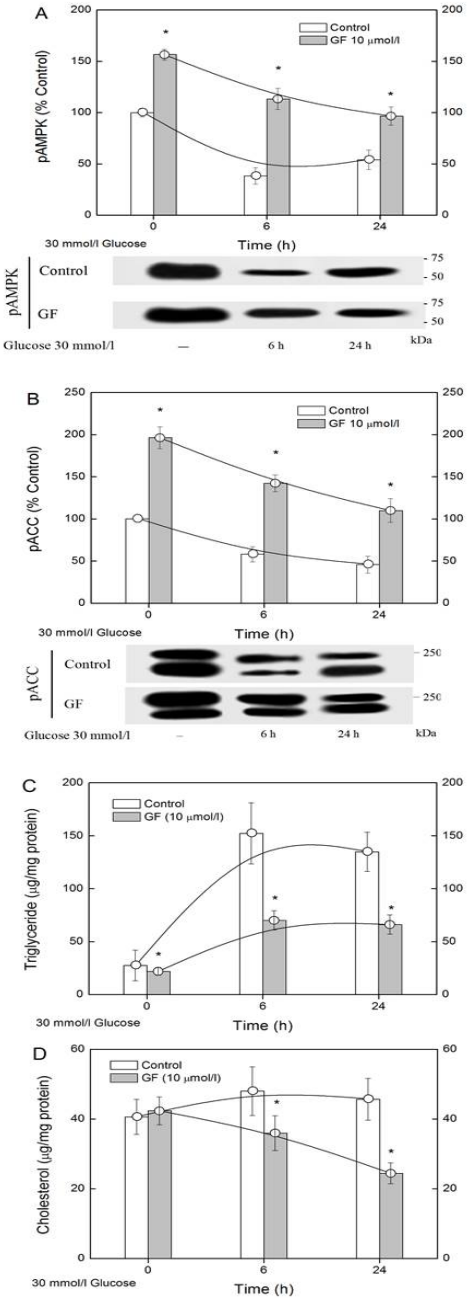

Figure 6. GF prevents the inhibition of AMPK and accumulation of lipids caused by high glucose in HepG2 cells. A and B, the phosphorylation of AMPK and ACC was expressed as a percentage of that under control conditions. Data were shown by the means ± SD (n= 3). **P* < 0.05 compared with normal glucose control. C and D, GF lowers lipid contents and protects against high-glucose-induced lipid accumulation in HepG2 cells. Intracellular TG and TC contents in cell lysates were expressed as micrograms lipid per milligram protein. Data are the means ± SD (n = 4). **P* < 0.05 compared between two groups as indicated. pACC, phosphor-ACC; pAMPK, phosphor-AMPKα

Intracellular level of TG was increased in HepG2 cells as early as 6 h after incubation in high glucose (Figure 6C). GF (10 μmol/L) largely prevented the increase in hepatocellular TG at 6 and 24 h. GF (10 μmol/L) also decreased the TC content of HepG2 cells incubated in high glucose for 6 or 24 h (Figure 6D).

4. Discussion

In diabetic patients, dyslipidemia and its clinical complications are dramatically accelerated. Naturally occurring flavonoids have been found to have beneficial effects on health, and these compounds have drawn attention because of their relative safety and accumulated evidence of anti-obesity and anti-diabetic effects in animals and humans [22]. GF, one of polyphenolic flavonoids, have been found to possess widespread biological functions and health benefits [6,7].

In recent research, we found that GF as 3'-methyl-4',7-dihydroxyflavanone can significantly improve diabetic hyperlipidemia in diabetic mice. We tested its effects on AMPK activity in human HepG2 hepatocytes. AMPK is a key metabolic regulator in liver, skeletal muscle, and heart that responds to increased cellular AMP-to-ATP ratio and upstream signaling pathways stimulated by cellular stress [23]. In turn, AMPK regulates fatty acid oxidation and lipid synthesis, two important determinants of tissue lipids and hyperlipidemia in diabetes [24]. Although information about action mechanism of the GF on lowering lipid were rarely reported previously to activate AMPK, other lipid-lowering mechanisms that are known to be stimulated by flavonoids could potentially involve AMPK. For instance, soy isoflavones exert antidiabetic and hypolipidemic effects through PPAR pathways in obese Zucker rats and murine RAW 264.7 cells [25]. The PPAR agonist troglitazone, which has beneficial effects on lipids in diabetic patients, was shown to increase AMPK phosphorylation and to lower lipid levels in heart [26].

Based on these observations, we assessed the activation state of total AMPK by determining phosphorylation of AMPK and its best-characterized downstream substrate, ACC, because Thr-172 phosphorylation of the activation loop of the AMPK catalytic domain is essential for activation of both the α1 and α2 subunits of AMPK [27], AMPK phosphorylates and inactivates ACC1 and ACC2 [28], which in turn downregulates lipid biosynthesis and up-regulates fatty acid oxidation [29]. We discovered that hyperglycemia inhibits AMPK and ACC phosphorylation in the livers of diabetic LDLR-/- mice, mimicking the effects of high glucose on AMPK activity and phosphorylation in HepG2 cells in vitro. The effect of GF was significantly higher than that of metformin. Furthermore, our results indicate that activation of AMPK by treatment with GF has effects on lipid accumulation in the liver in vivo that are similar to those observed in HepG2 cells, which require AMPK for the lipid-lowering effect of GF.

AMPK acts as a fuel sensor [30] so it is not surprising that in vivo or in vitro exposure to high glucose decreases hepatic AMPK phosphorylation and activity and its energy-conserving effects on downstream signaling targets. Inhibition of AMPK was also observed in pancreatic cell lines exposed to elevated glucose (30 mmol/L) [31], in cultured hepatocytes exposed to ethanol (100 mmol/L), in the fatty livers of mice fed with ethanol [32], in cultured human skeletal muscle of obese type 2 diabetic patients [33]. Inhibition of AMPK increases fatty acid synthesis by decreasing phosphorylation and increasing activity of ACC. In addition, increased production of malonyl-CoA decreases fatty acid oxidation by inhibiting carnitine palmitoyl transferase-1-mediated uptake of fatty acids into mitochondria [34]. Moreover, inhibition of AMPK increases the activity of sterol regulatory element-binding protein 1 and thereby increases expression of its target enzymes involved in fatty acid and triglyceride biosynthesis [35]. Our data showing that GF protect inhibition of the activity of AMPKα in HepG2 cells of exposure to high glucose, the major isoform in liver, and enhance phosphorylation of ACC1 and ACC2, resulting in decrease in ACC activity and hepatic lipids, suggests that activation of AMPK in hepatocytes is responsible for decrease in the high-glucose-induced lipid accumulation.

ACC catalyzes the carboxylation of acetyl-CoA to form malonyl-CoA, an intermediate metabolite that plays a key role in the regulation of fatty acid metabolism [36]. In mammals, there are two isoforms of ACC, ACC1 (265 kDa) and ACC2 (280 kDa). ACC1 is abundant in lipogenic tissues, such as liver and adipose tissues, where malonyl-CoA is the C2 unit donor for de novo synthesis of long−chain fatty acids and for chain elongation of fatty acids to very-long-chain fatty acids. ACC2 is highly expressed in liver, skeletal muscle, and heart, where malonyl-CoA regulates fatty acid oxidation through inhibition of carnitine palmitoyltransferase I. Studies with ACC1 or ACC2 knock-out mice demonstrate that malonyl-CoA also exists in two different pools: the cytosolic pool, which is used as the precursor of fatty acid synthesis, and the mitochondrial pool, which regulates fatty acid oxidation [36], because ACC1 and ACC2 are located in the cytosol or mitochondrial membrane, respectively. Both isoforms of ACC are phosphorylated and inactivated by AMPK [29]. In the study, phosphorylation of both ACC1 and ACC2 was significantly increased in HepG2 cells and in the liver of diabetic mice by treatment with GF. This is consistent with the observation that adiponectin increased phosphorylation of ACC1 and ACC2 in cultured primary hepatocytes and in mouse liver [37].

In addition, activation of AMPK by metformin in vivo downregulates lipid synthesis and increases fatty acid oxidation [38]. For instance, overexpression of constitutively active AMPK in the liver or treatment with metformin inhibits ACC activity or decreases expression of sterol regulatory element-binding protein 1 and its target genes in mouse liver [39]. As demonstrated in cells overexpressing a dominant-negative AMPK mutant, the effects of GF was shown to be mediated by the activation of AMPK. Like the effect of the known AMPK activator as metformin, the lipid-lowering effect of GF may be attributable to activation of AMPK and inactivation of ACC1 and ACC2 and consequently the effects to downregulate fatty acid synthesis and upregulate fatty acid oxidation.

Our data showed that GF promote AMPK α1 and α2 activity as well as prevent the ability of high glucose on inhibition of AMPK activity, consistent with its ability to

increase phosphorylation of AMPK and ACC. The activation of AMPK by GF occurred relatively rapidly and well before any potential change in ATP level was detected. Flavonoids have long been postulated to lower lipids through multiple mechanisms that have been implicated in the beneficial effects of tea and red wine on diabetic cardiovascular disease [40]. This novel finding was made most evident in HepG2 cells exposed to high glucose, in which GF prevented the decrease in AMPK activity and ACC phosphorylation. Furthermore, GF decreased lipid accumulation caused by exposure to high glucose to the same extent. The AMPK activation and lipid-lowering effects of GF in HepG2 cells paralleled observations in vivo where GF increased AMPK and ACC phosphorylation and decreased lipid content in the liver of diabetic LDLR-/- mice. Thus, both in vivo and in vitro inhibition of AMPK, activation of ACC, and hepatocellular lipid accumulation caused by sustained high glucose levels was effectively opposed by activating AMPK with GF.

It is very likely that the effects of GF on serum lipids in diabetic LDLR-/- mice, is attributable to the same mechanisms by which we have shown that GF regulates AMPK activity and lipids in HepG2 cells. This is made more likely in this study by the fact that we used LDLR-/- mice. Because the major lipoprotein disposition mechanism is eliminated in these mice [15], serum levels of lipids largely reflect hepatic lipid synthesis. Although other mechanisms may have contributed, our results suggest that GF exhibits its lipid-lowering effect, which is associated with its ability to stimulate hepatic AMPK activation.

In this study, the dramatic activation of AMPK by GF both in vitro and in vivo under conditions of high glucose and the demonstration that AMPK is required for its lipid lowering effects in HepG2 cells make it likely that the effects of GF on serum lipids are also mediated by AMPK. Thus, activation of AMPK may help to explain some of the anti-hyperlipidemic effects of GF and provide a strategy for ameliorating metabolic disorder related to dyslipidemia in diabetes.

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