Biotechnol Lett (2012) 34:1607–1616
DOI 10.1007/s10529-012-0944-1

ORIGINAL RESEARCH PAPER

Heat-processed *Gynostemma pentaphyllum* extract improves obesity in *ob*/*ob* mice by activating AMP-activated protein kinase

Rehman Gauhar · Seung-Lark Hwang · Si-Sung Jeong · Ji-Eun Kim · Hebok Song · Dong Chan Park · Kyung-Sik Song · Tae Young Kim · Won Keun Oh · Tae-Lin Huh

Received: 8 February 2012 / Accepted: 25 April 2012 / Published online: 11 May 2012

Abstract *Gynostemma pentaphyllum* is widely used in Asian countries as a herbal medicine to treat dyslipidemia, type 2 diabetes and inflammation. An ethanol extract of *G. pentaphyllum* lessened obesity by activating AMP-activated protein kinase (AMPK). The levels of damulins A and B, components responsible for AMPK activation in the extract, were increased by autoclaving in a time-dependent manner. Heat-processed *G. pentaphyllum* extract, actiponin containing damulins A (0.93 %, w/w) and B (0.68 %, w/w), significantly stimulated fat oxidation and glucose uptake via AMPK activation in L6 myotube cells. Oral administration of actiponin to *ob*/*ob* mice for 8 weeks decreased body weight gain, liver weight, and blood cholesterol levels with AMPK activation in the soleus muscle. Our results demonstrate the beneficial effect of *G. pentaphyllum* on improving obesity and have elucidated the underlying molecular mechanisms.

Keywords AMP-activated protein kinase · Damulins · Fat oxidation · *Gynostemma pentaphyllum* · Obesity

Rehman Gauhar and Seung-Lark Hwang contributed equally to this study.

Electronic supplementary material The online version of this article (doi:10.1007/s10529-012-0944-1) contains supplementary material, which is available to authorized users.

R. Gauhar · S.-S. Jeong · T.-L. Huh (✉)
School of Life Science and Biotechnology, Kyungpook National University, Daegu 702-701, Republic of Korea
e-mail: tlhuh@knu.ac.kr

S.-L. Hwang · J.-E. Kim · H. Song · D. C. Park · T.-L. Huh
TG Biotech Research Institute, Kyungpook National University, Techno Building, Daegu 702-701, Republic of Korea

K.-S. Song
College of Pharmacy, Kyungpook National University, Daegu 702-701, Republic of Korea

T. Y. Kim
BTC, Technology Development Center, Gyeonggi Technopark, Ansan, Gyeonggido 426-901, Republic of Korea

W. K. Oh
College of Pharmacy, Chosun University, Gwangju 501-759, Republic of Korea

T.-L. Huh
Korea Basic Science Institute Daegu Center, Kyungpook National University, Daegu 702-701, Republic of Korea

T.-L. Huh
Kyungpook National University Hospital, Daegu 700-721, Republic of Korea

Introduction

Obesity is a global epidemic, resulting in significant morbidity and mortality due to weight-related disorder and reduced quality of life. Therefore, prevention or treatment of obesity is important for reducing the incidence of co-morbidities, including type 2 diabetes, hyperlipidemia, heart disease, and cancer (Guh et al. 2009).

AMP-activated protein kinase (AMPK) is an evolutionarily conserved intracellular energy sensor and regulates whole-body and cellular energy balance in responses to energy demand and supply. It has been implicated in metabolic diseases, such as obesity, type 2 diabetes, and dyslipidemia as its activation stimulates β-oxidation and glucose uptake in skeletal muscle while inhibiting hepatic fat and cholesterol synthesis (Zhang et al. 2009). AMPK is a heterotrimeric serine/threonine kinase composed of catalytic α and regulatory β and γ subunits. Its activation is mainly achieved via phosphorylation of Thr^{172} in the α subunit by LKB1 kinase or Ca^{2+}/calmodulin-dependent protein kinase kinase (Towler and Hardie 2007; Xiao et al. 2011). AMPK phosphorylates downstream targets and switches off ATP-consuming pathways, including fatty acid and cholesterol synthesis, and switches on ATP-generating pathways, including fatty acid oxidation and glycolysis (Fogarty and Hardie 2010). AMPK also inactivates acetyl-CoA carboxylase (ACC) by direct Ser^{79} phosphorylation, thereby inhibiting fat synthesis by reducing malonyl-CoA production from acetyl-CoA (Cool et al. 2006). AMPK also directly inactivates 3-hydroxy-3-methylglutaryl-CoA reductase by increasing its phosphorylation, thereby inhibiting cholesterol synthesis (Davies et al. 1992). Moreover, AMPK decreases blood glucose levels by stimulating translocation of glucose transporter 4 (GluT4) to cell membrane and regulating GluT4 expression independent of insulin signaling (Burcelin et al. 2003). These beneficial effects of AMPK activation on glucose and lipid metabolism highlight AMPK as an emerging drug target for treating metabolic syndrome including obesity and type 2 diabetes (Fogarty and Hardie 2010).

Gynostemma pentaphyllum, a member of the Cucurbitaceae family, has been widely used in Asian countries as an herbal medicine. Extracts or saponins from this plant have a wide range of beneficial effects such as anti-hyperlipidemic, hypoglycemic, anti-inflammatory, and anti-tumor activities (Razmovski-Naumovski et al. 2005). Mechanisms underlying these diverse effects are poorly understood but are likely associated with the diverse downstream effects of AMPK activation (Fogarty and Hardie 2010).

To explore the mechanisms underlying the beneficial effects of *G. pentaphyllum*, we recently characterized two novel dammarane-type saponins, damulins A and B, from *G. pentaphyllum* extract as potent AMPK activators (Nguyen et al. 2011). In the present study, we found that damulins A and B levels in the extract were increased by autoclaving and correlated with the ability of the extract to activate AMPK. Treatment with *G. pentaphyllum* extract enriched in damulins (actiponin) activated AMPK in L6 myotubes with stimulation with stimulating fatty acid oxidation and glucose uptake. In *ob*/*ob* mice, oral treatment with actiponin reduced body weight gain, liver weight and blood cholesterol levels with AMPK activation in the soleus muscle, demonstrating for the first time the ability of *G. pentaphyllum* extracts on improving obesity by stimulating AMPK activation.

Materials and methods

Reagents

Compound C, insulin and wortmannin were purchased from Calbiochem. 5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) and bovine serum albumin were purchased from Sigma Chemical Co. Antibodies against phospho-AMPKα (Thr^{172}), total AMPK, phospho-ACC (Ser^{79}), total ACC, adiponectin, integrin-α1, carnitine palmitoyltransferase 1 (CPT1), peroxisome proliferator-activated receptor-γ (PPARγ), CCAAT/enhancer binding protein-α (C/EBP1α), and sterol regulatory element binding protein-1c (SREBP1c) antibodies were purchased from Cell Signaling Technology. Antibodies against GluT4 and β-actin antibodies were from Santa Cruz Biotechnology, Inc. Fetal bovine serum (FBS), trypsin/EDTA, and penicillin/streptomycin were obtained from Gibco. 2-Deoxy-[^{3}H]D-glucose (2-DG) was from Perkin-Elmer Life Sciences.

Plant materials

Gynostemma pentaphyllum was purchased from Wonkwang Food Co. (Geochang, Republic of Korea) and identified by Professor Jae-Hong Park, Department of Biology, Kyungpook National University. A voucher specimen (TLH 1001) was deposited in the herbarium of Kyungpook National University (KNU).

Preparation of *G. pentaphyllum* extract and actiponin

Dried *G. pentaphyllum* leaves (1 kg) were extracted using ethanol, concentrated under reduced pressure, and then subjected to physical modification by autoclaving at 121 °C for 1–4 h. After 4 h of autoclaving, the actiponin yield was 18.7 % (w/w).

HPLC analysis

Analytical HPLC was performed with a C18 column (250 × 4.6 mm, 5 μm) using a stepwise gradient of acetonitrile/water at 0–20 % (v/v) for 2 min, 20–60 % for 31 min, 60–100 % for 6 min, 100 % for 9 min, 100–20 % for 5 min, 20 % for 7 min) at 0.8 ml/min. The eluate was measured at 205 nm.

Cell culture

L6 Cells, 3T3 L1 preadipocyte cells and HepG2 cells were obtained from ATCC. L6 cells and 3T3 L1 preadipocyte cells were cultured and differentiated into myotube and adipocyte cells, respectively, as previously described (Hwang et al. 2008; Koh et al. 2004). HepG2 cells were cultured in Dulbecco's modified eagle media containing 10 % (v/v) FBS, 100 U penicillin/ml and 100 μg streptomycin/ml in 5 % CO_2 at 37 °C.

Flow cytometry

Flow cytometric analysis with excitation wavelength of 495 nm and emission spectrum of 521 nm was performed using FACS Calibur flow cytometer (Becton–Dickinson) with CellQuest software. For labeling GluT4 protein in plasma membranes, L6 myotube cells (10^4 cells) were incubated with rabbit anti-GluT4 antibody at 1:1,000 and subsequently labeled with FITC-conjugated anti-rabbit IgG (Santa Cruz Biotechnology) secondary antibody at 1:2,000.

Measurement of glucose uptake and β-oxidation

L6 myotubes were treated with actiponin, insulin, and AICAR with or without pretreatment of compound C for 30 min at 37 °C and subjected to glucose uptake assay using radiolabelled 2-deoxy-D-[1-^{3}H]glucose (2-DG) (Hwang et al. 2008). Changes of β-oxidation were analyzed as previously described (Cabrero et al. 2001).

Western blot analysis

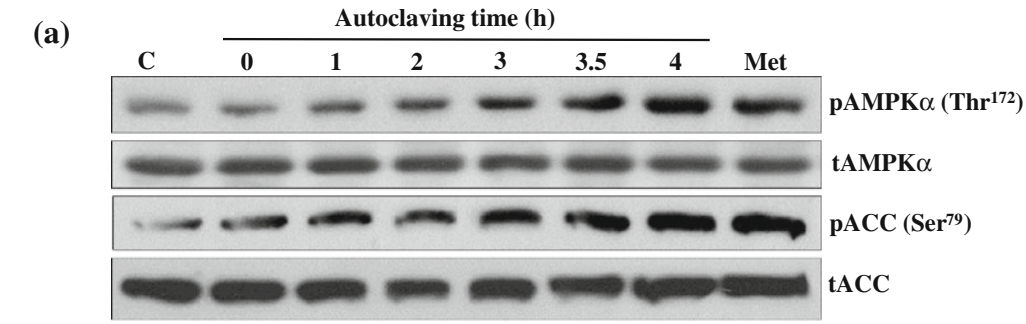
Total cell lysates were prepared as previously described (Hwang et al. 2008). Isolated mouse soleus muscle tissues were homogenized in HEPES buffer (20 mM HEPES, 250 mM sucrose, 1 mM EDTA, pH 7.4) containing a protease inhibitor cocktail (Roche Molecular Biochemicals). Plasma membrane fractions of cultured L6 myotubes were prepared as previously described by Nishiumi and Ashida (2007). Protein samples were subjected to Western blot analysis according to the standard procedure using respective antibodies.

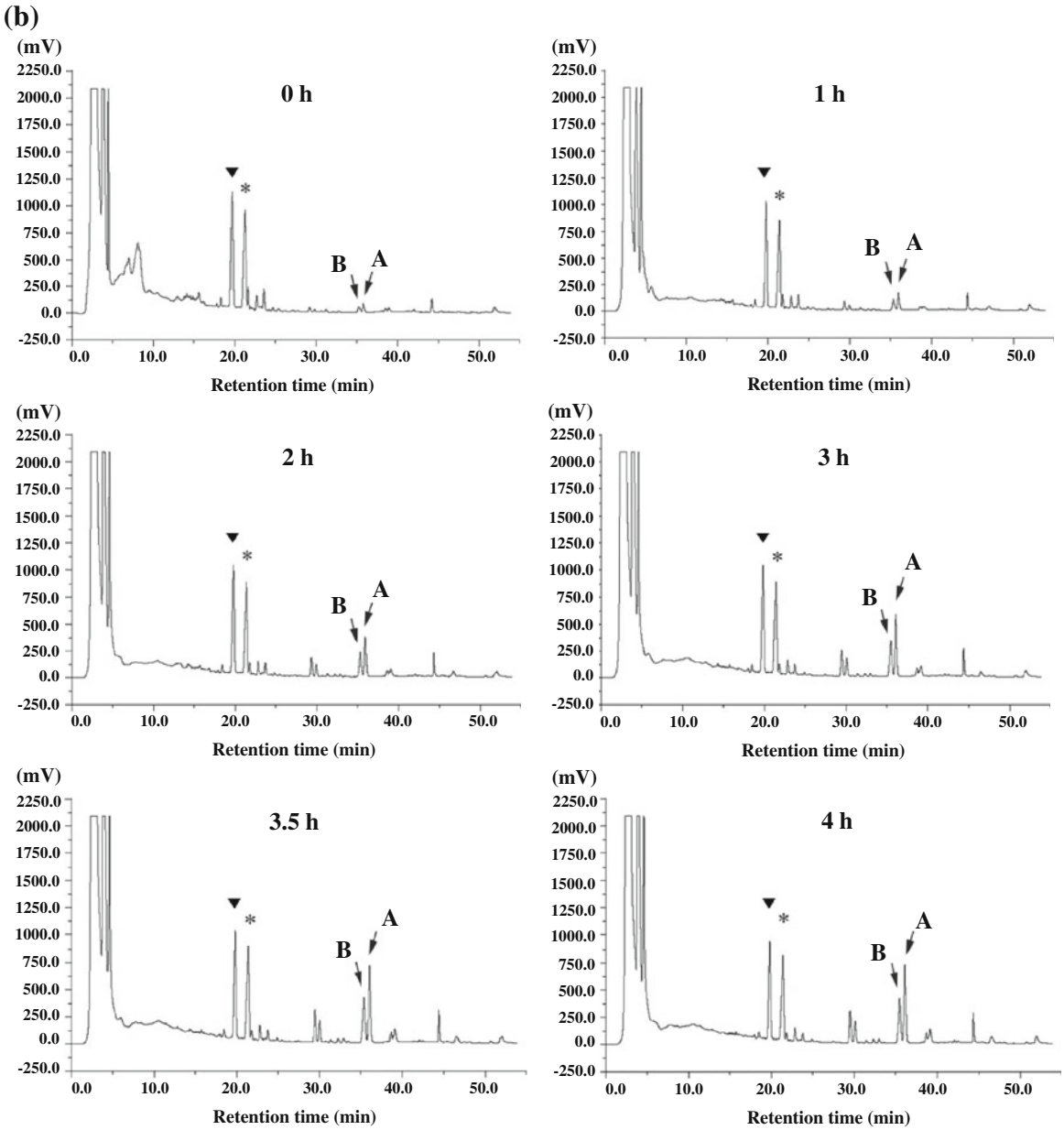
Animal experiments

Male *ob*/*ob* mice with a C57BL/6 background (5 weeks old) were purchased from Jackson Laboratory and fed a standard rodent diet with water ad libitum. Mice were kept individually housed in standard cages with controlled temperature (22 ± 2 °C), humidity (55 ± 5 %) and a 12-h light/dark cycle. After acclimation, 6- or 9-week-old mice were used for the experiments. The mice were orally administered vehicle (saline), actiponin (150, 200 and 300 mg/kg/day) or sibutramine (30 mg/kg/day) for 8 weeks. Food intake, body weight and lipid levels were measured weekly. The experiments were performed in accordance with the principles outlined by the Institutional Animal Care and Use Committee for the care and use of laboratory animals.

Analysis of plasma lipid levels

Mice were fasted for 5 h and blood was collected by retro-orbital venous plexus puncture. Plasma was


(a)
Autoclaving time (h)
C
0
1
2
3
3.5
4
Met
pAMPKα (Thr172)
tAMPKα
pACC (Ser79)
tACC


(b)
(mV)
0 h
1 h
2 h
3 h
3.5 h
4 h
B
A
Retention time (min)

◀**Fig. 1** Positive correlation between autoclaving time and ability of *G. pentaphyllum* extract to increase AMPK and ACC phosphorylation. **a** L6 myotubes were treated with actiponin (60 μg/ml) for 2 h. As a positive control, 2 mM metformin (Met) was treated for 1 h. Cell lysates (80 μg protein) were subjected to Western blotting. **b** Damulins A (peak *A*) and B (peak *B*) levels were increased in *G. pentaphyllum* extracts by autoclaving in a time-dependent manner. To analyze damulins A and B contents, heat-processed *G. pentaphyllum* extract (2 mg) was dissolved in 60 % MeOH and subjected to HPLC analysis. Gypenosides XLVI and LXXVII are indicated by an *asterisk* and *closed triangle*, respectively

separated by centrifugation and frozen at −80 °C. Total cholesterol and triglyceride levels were measured by using kits (Cliniqa Inc.), according to the manufacturer's instructions.

Statistical analysis

One-way analysis of variance was used to measure differences between multiple groups. When the differences were statistically significant, a Scheffe test was used for post hoc analysis. For comparison between two groups, Student's *t* test was used. All data are expressed as the mean ± SE. P values <0.05 were considered statistically significant. All statistical analyses were performed using SPSS version 19.0 (SPSS, Inc., Chicago, IL).

Results and discussion

Activity of *G. pentaphyllum* extract on AMPK activation is increased by autoclaving

Modification of ethanol extract of *G. pentaphyllum* ethanol extract by autoclaving at 121 °C increased AMPK(Thr^{172}) and ACC (Ser^{79}) phosphorylations in L6 cells in a time-dependent manner (Fig. 1a). This was positively correlated with elevated levels of AMPK activators, damulins A and B (Nguyen et al. 2011), in the extract (Fig. 1b; Table 1). damulins A and B enrichment might be accomplished by heat-induced modification of other saponins in *G. pentaphyllum* similar to ginsenosides F(4), Rg(3) and Rg(5), in heat-processed ginseng (Kim et al. 2000). Heat-processed *G. pentaphyllum* extract containing damulins A (0.93 %, w/w) and B (0.68 %, w/w), obtained by autoclaving for 4 h (Fig. 1b; Table 1), was called "actiponin", and used for in vitro and in vivo studies. Along with damulins A and B, two other saponins in actiponin (Fig. 1b) were also purified and their chemical structures were determined with 1D (including ^{1}H and ^{13}C) and 2D (including gHSQC, COSY, HMBC and NOESY) NMR spectra as well as mass data analyses as previously described (Nguyen et al. 2011). These two saponins were characterized as gypenosides XLVI (Takemoto et al. 1984) and LXXVII (Yoshikawa et al. 1987). In contrast to damulins A and B, the two known gypenosides did not activate AMPK in cultured L6 myotubes (data not shown).

Actiponin phosphorylated AMPK and ACC in a dose- and time-dependent manner in L6 myotubes (Fig. 2a, b). In 3T3 L1 adipocytes, actiponin increased the expression of adiponectin, an adipocyte-derived hormone that increases insulin sensitivity and energy homeostasis (Fogarty and Hardie 2010), similar to rosiglitazone (Fig. 2c). This finding suggests that increased adiponectin levels might contribute to the hypoglycemic effect of *G. pentaphyllum* extract previously observed in *db/db* diabetic mice (Yeo et al. 2008). Compound C, an AMPK inhibitor, abrogated AMPK and ACC phosphorylation in L6 myotubes (Fig. 2d), indicating that AMPK was activated by actiponin.

Actiponin regulates the expression of key factors of adipogenesis and fat oxidation

The effect of actiponin on regulation of lipid metabolism in cultured HepG2 and L6 myotubes was

Table 1 Increased Damulin A B levels in *G. pentaphyllum* extract by autoclaving

	0 h	1 h	2 h	3 h	3.5 h	4 h
Damulin A	0.10 ± 0.01	0.20 ± 0.01	0.46 ± 0.02	0.73 ± 0.04	0.89 ± 0.05	0.93 ± 0.05
Damulin B	0.09 ± 0.01	0.16 ± 0.01	0.36 ± 0.02	0.55 ± 0.03	0.63 ± 0.04	0.68 ± 0.04

Levels of Damulins A and B in Actiponin (2 mg) were determined by HPLC analysis using purified Damulin A and B as standards. All data (%, w/w) are expressed as the mean ± SD of three experiments

analyzed. The expression of CPT1, a rate-limiting enzyme for mitochondrial β-oxidation, while the expression of lipogenic transcription factors (Lee et al. 2006), including PPARγ, C/EBP1α, and SREBP1c in both liver and muscle cells (Fig. 2e, f) was decreased. Additionally, expression of PPARγ coactivator-1α, a key regulator of several mitochondrial genes involved in adaptive thermogenesis (Lee et al. 2006), was increased by actiponin in a dose-dependent manner (Fig. 2e, f). These findings suggest that actiponin stimulates fat oxidation by increasing CPT1 expression but inhibits adipogenesis by decreasing expression of PPARγ, C/EBP1α, and SREBP1c.

Actiponin stimulates glucose uptake and fatty acid oxidation

Increased glucose uptake is a well-known downstream effect of AMPK activation (Kurth-Kraczek et al. 1999). In L6 myotubes, 2-DG uptake was significantly increased (1.7-fold) by actiponin; this was comparable to that achieved with 1 mM AICAR or 100 nM insulin (Fig. 3a). However, increased 2-DG uptake was abrogated by pretreatment of an AMPK inhibitor, compound C (10 μM), confirming that actiponin increases 2-DG uptake through AMPK activation (Fig. 3b). Along with increased 2-DG uptake,

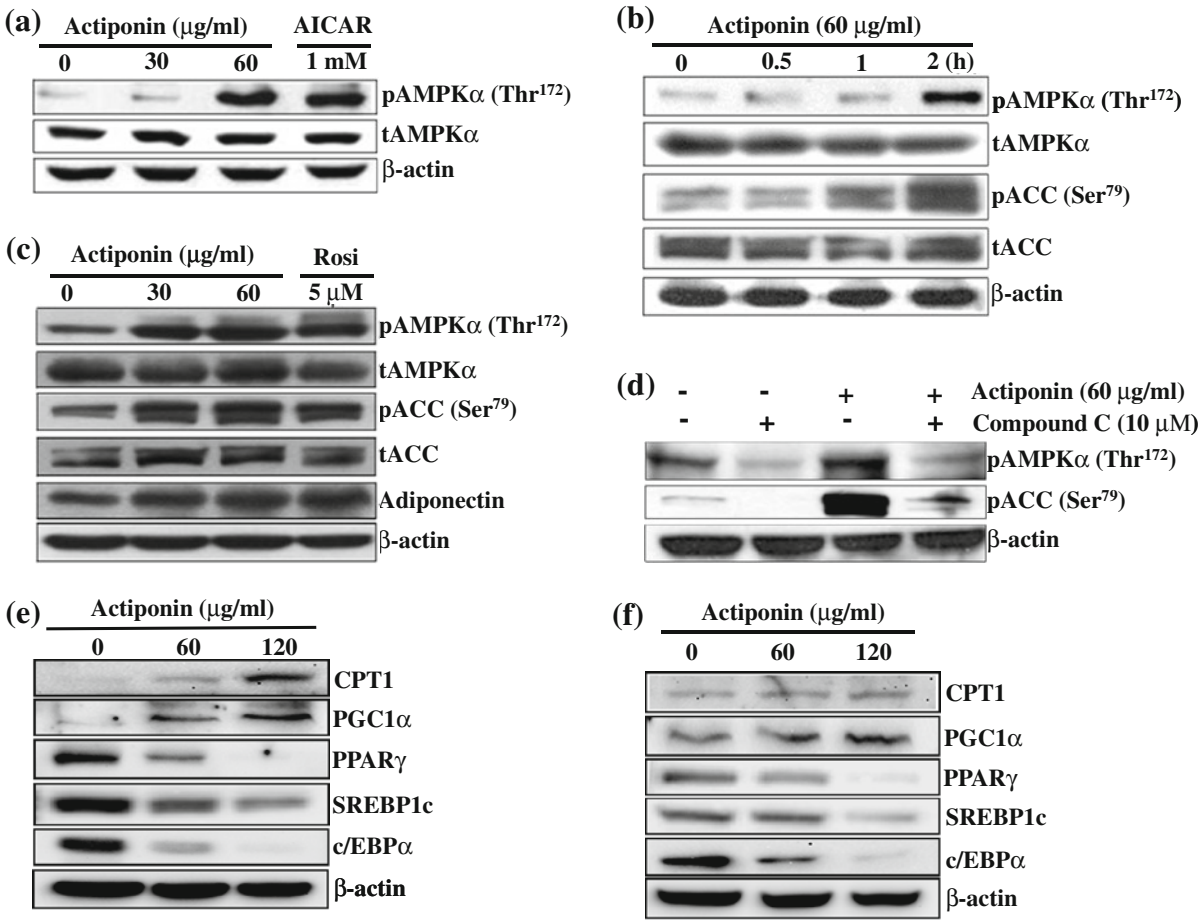


Fig. 2 Effect of actiponin on AMPK phosphorylation and expression of key factors in lipid metabolism. **a** L6 myotubes were treated with actiponin for 2 h or AICAR for 1 h. **b** L6 myotube cells were treated with actiponin (60 μg/ml) for different times. **c** 3T3 L1 adipocytes were treated with actiponin or rosiglitazone (Rosi) for 2 h. **d** AMPK activation by actiponin in L6 myotubes was abrogated by pretreatment (10 min) with compound C, an AMPK inhibitor. Cell lysates (80 μg protein) were subjected to Western blotting using β-actin as a loading control. Cultured HepG2 cells (**e**) and L6 myotubes (**f**) were treated with actiponin for 2 h and changes in protein expression of various key factors that regulate lipid metabolism were analyzed. Cell lysates (100 μg protein) were subjected to Western blotting

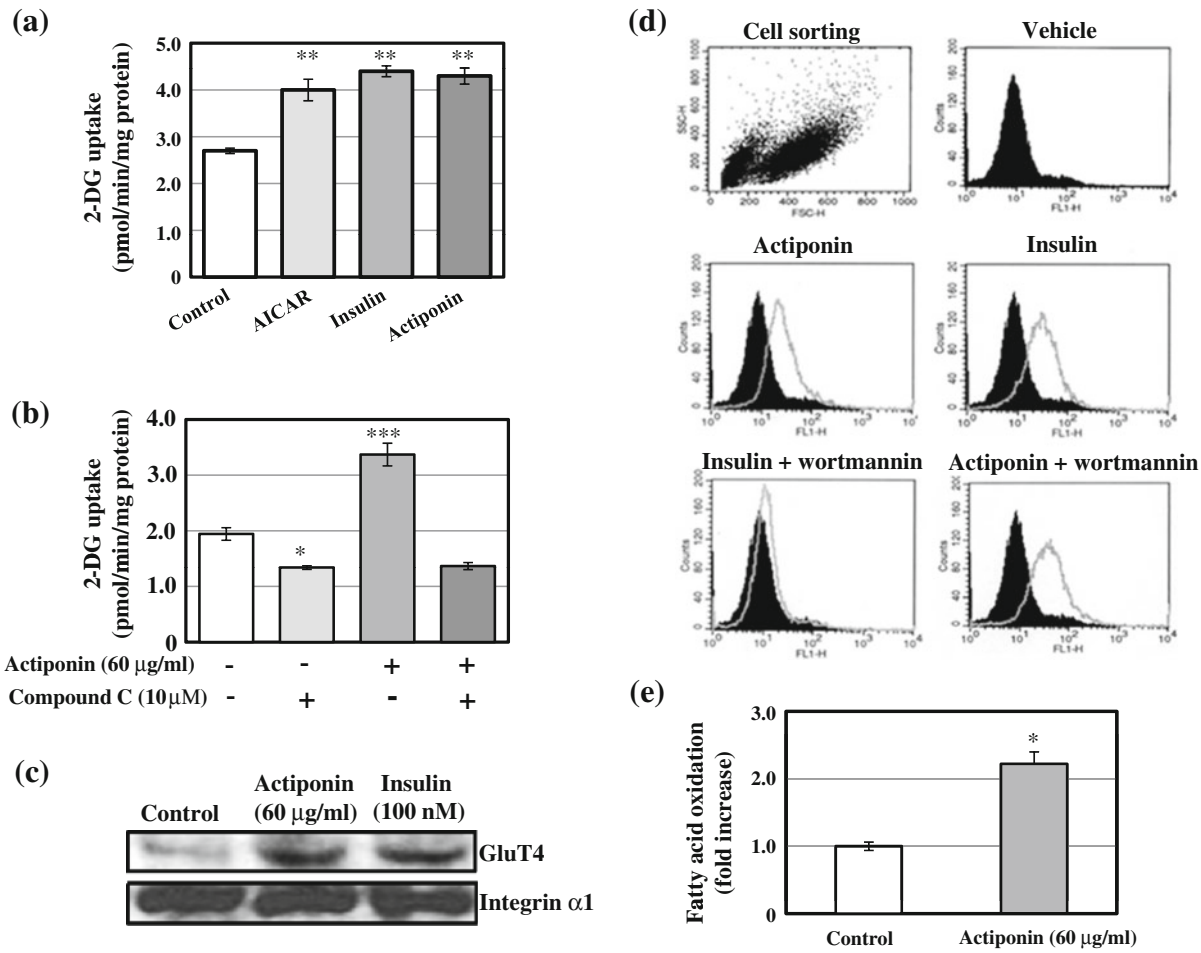
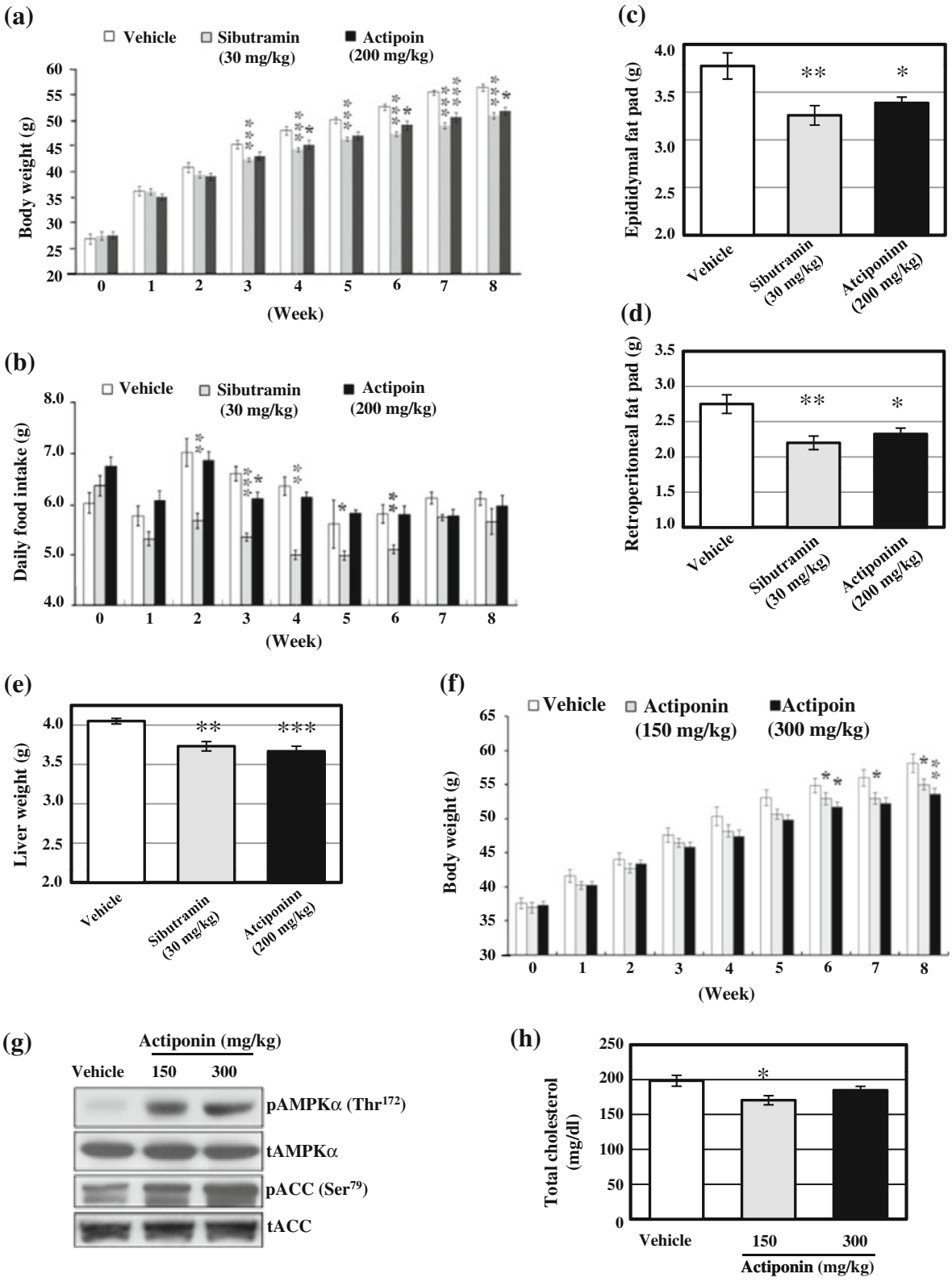


Fig. 3 Effect of actiponin on AMPK activation and GluT4 translocation in L6 myotubes. **a** Stimulation of 2-DG uptake in L6 myotubes by actiponin (60 μg/ml) treatment for 2 h. Cells were also treated with AICAR (1 mM) or insulin (100 nM) for 1 h and 10 min, respectively. **b** Increase in 2-DG uptake by L6 myotubes treated with actiponin (60 μg/ml) was blocked by pretreatment with compound C (10 μM) for 10 min. **c** Translocation of GluT4 to plasma membranes by actiponin and insulin. The plasma membrane fraction of cell lysates (30 μg) was subjected to Western blotting with respective antibodies. **d** FACS analysis of GluT4 translocation to the cell membrane. Cells were treated with actiponin (60 μg/ml, 2 h) and insulin (100 nM, 10 min) in the presence or absence of wortmanin (100 nM) pretreatment for 30 min. **e** Fatty acid oxidation increased by actiponin treatment for 2 h in L6 myotubes. Control cells were treated with DMSO. **a**, **b**, **e** Data were obtained for three independent experiments. $*P < 0.05$; $**P < 0.01$; $***P < 0.005$ compared to the control

actiponin triggered GluT4 translocation to plasma membranes similar to insulin-treated cells (Fig. 3c). Flow cytometry analysis (Fig. 3d) revealed that wortmannin, a phosphoinositide 3-kinase inhibitor (Okada et al. 1994), did not abrogate increased GluT4 translocalization on the cell surface of actiponin-treated L6 myotubes, whereas it blocked nsulin-mediated GluT4 translocation. These findings suggest that actiponin-stimulated glucose uptake is independent of insulin signaling and demonstrates how *G. pentaphyllum* extracts lower blood glucose levels in *db*/*db* diabetic mice as seen in our previous report (Yeo et al. 2008). Concurrent with increased CPT1 expression (Fig. 2f), actiponin increased fatty acid oxidation (2.2-fold) in L6 myotubes (Fig. 3e). Collectively, these results indicate that actiponin stimulates glucose uptake and fatty acid oxidation via AMPK activation.


(a)
Vehicle
Sibutramin (30 mg/kg)
Actipoin (200 mg/kg)
Body weight (g)
(Week)
(b)
Daily food intake (g)
(c)
Epididymal fat pad (g)
Vehicle
Sibutramin (30 mg/kg)
Atciponinn (200 mg/kg)
(d)
Retroperitoneal fat pad (g)
(e)
Liver weight (g)
(f)
Actiponin (150 mg/kg)
Actipoin (300 mg/kg)
(g)
Actiponin (mg/kg)
Vehicle
150
300
pAMPKα (Thr172)
tAMPKα
pACC (Ser79)
tACC
(h)
Total cholesterol (mg/dl)

◀**Fig. 4** Effect of actiponin on body weight, body fat and total plasma cholesterol levels in *ob*/*ob* mice. **a–e** Actiponin (200 mg/kg, $n = 8$), sibutramine (30 mg/kg, $n = 8$) and vehicle (saline, $n = 8$) were orally administered to 6 week-old pre-obese mice for 8 weeks. Body weights (**a**), food intake (**b**), epididymal fat pad weight (**c**), retroperitoneal fat weight (**d**), and liver weight (**e**) were measured. **f–h** Actiponin (150 mg/kg, $n = 8$; 300 mg/kg, $n = 8$) or vehicle (saline, $n = 8$) was orally administrated to 9-week-old obese mice for 8 weeks. **f** Changes in body weight. **g** Western blot for AMPK and ACC phosphorylation in the soleus muscle (60 μg protein). **h** Change in total plasma cholesterol levels. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$ compared to the vehicle group

Actiponin reduces body weight and plasma total cholesterol level in *ob*/*ob* mice

Daily oral administration of actiponin (200 mg/kg) and sibutramine (30 mg/kg) to 6-week-old pre-obese *ob*/*ob* male mice (~27 g body weight) for 8 weeks reduced body weight gain by 8.1 % (51.8 ± 0.7 g, $n = 8$) and 10 % (50.9 ± 0.6 g, $n = 8$), respectively, compared to the vehicle group (56.4 ± 0.6 g, $n = 8$; Fig. 4a). In contrast to mice treated with sibutramine, no significant difference in food intake was observed in the actiponin and vehicle groups (Fig. 4b), suggesting that decreased body weight gain in the actiponin group was independent of food intake. Decrease in body weight gain by actiponin was accompanied by reduced epididymal (10.3 %) and retroperitoneal (15.5 %) fat pad mass as well as liver weight (18.8 %; Fig. 4c–e), suggesting that decreased fat contents in these organs most likely accounted for decreased body weight.

We also tested whether actiponin can reduce body weight of older obese mice. Two doses of actiponin (150 and 300 mg/kg) were orally administered to 9-week-old *ob*/*ob* male mice (~37 g body weight). After 8 weeks, the body weight of mice treated with actiponin at 150 and 300 mg/kg was decreased 5.7 % (53.6 ± 1.2 g, $n = 8$) and 7.7 % (55.0 ± 1.1 g, $n = 8$), respectively, compared to the vehicle group (58.1 ± 1.3 g, $n = 8$; Fig. 4f), without changes in daily food intake (data not shown). In the soleus muscle of the two actiponin-treated groups, AMPK and ACC phosphorylation was greatly increased (Fig. 4g), suggesting that reduced body weight and fat mass in the actiponin groups was most likely caused by AMPK activation. In mice treated with actiponin at 150 and 300 mg/kg, 14.2 and 7.1 % reductions in total plasma total cholesterol levels were observed, respectively, compared to control group (Fig. 4h). However, no significant decreased in plasma triglyceride levels were seen in the actiponin groups (data not shown).

Fasting increases AMPK activity in multiple hypothalamic regions, leading to increased food intake, whereas inhibition of hypothalamic AMPK activity promotes hypophagia and weight loss (Lage et al. 2008). Food intake is increased in animals following intracerebroventricular injection of the AMPK activator AICAR or α-lipoic acid (Kim et al. 2004). In contrast, actiponin that activated AMPK did not increase food intake in *ob*/*ob* mice, indicating that AMPK activators including damulins in actiponin may be inefficiently delivered to the brain. A lack of increased food intake likely promotes the beneficial effect of actiponin for treating obesity.

To prevent or improve obesity, AMPK activation in both skeletal muscle and liver is important since these two organs are the major sites of fat oxidation and lipid synthesis, respectively. Oral treatment with actiponin (300 mg/kg) for 9 days significantly increased AMPK phosphorylation in the soleus muscle and liver of C57BL/6 mice (Supplementary Fig. 1). Our results suggest that the damulins in actiponin were successfully delivered to the skeletal muscle and liver, and prevent or improve obesity by stimulating fatty acid oxidation and activating AMPK in these organs.

Damulins A and B are likely to be the major compounds in *G. pentaphyllum* responsible for AMPK activation (Nguyen et al. 2011). However, we cannot ignore the possibility that other uncharacterized dammarane saponins, namely gypenosides and/or panaxadiol-type ginsenosides also identified in *G. pentaphyllum* (Razmovski-Naumovski et al. 2005), may promote the beneficial effects of actiponin exerted via AMPK activation.

Conclusions

The current study identified a molecular basis for the beneficial effects of *G. pentaphyllum* on improving obesity. Further investigations are needed to characterize other AMPK activators and mechanism that increases levels of damulins in *G. pentaphyllum*. Our results may be used to prepare *G. pentaphyllum* extracts with more potent effects on AMPK activation, and help elucidate the molecular mechanism underlying *G. pentaphyllum* action for treating abnormal lipid metabolism and other inflammatory diseases.

Acknowledgments This work was supported by a grant (PF06212-00) from the Plant Diversity Research Center of the 21st Century Frontier Research Program funded by the Ministry of Science and Technology, and by a grant (A111345) from the Korean Health Technology R&D Project from the Ministry of Health and Welfare, Republic of Korea.

References

Burcelin R, Crivelli V, Perrin C, Da Costa A, Mu J, Kahn BB, Birnbaum MJ, Kahn CR, Vollenweider P, Thorens B (2003) GLUT4, AMP kinase, but not the insulin receptor, are required for hepatoportal glucose sensor-stimulated muscle glucose utilization. J Clin Invest 111:1555–1562

Cabrero A, Alegret M, Sánchez RM, Adzet T, Laguna JC, Vázquez M (2001) Bezafibrate reduces mRNA levels of adipocyte markers and increases fatty acid oxidation in primary culture of adipocytes. Diabetes 50:1883–1890

Cool B, Zinker B, Chiou W, Kifle L, Cao N, Perham M, Dickinson R, Adler A, Gagne G, Lyengar R, Zhao G, Marsh K, Kym P, Jung P, Camp HS, Frevert E (2006) Identification and characterization of a small molecule AMPK activator that treats key components of type 2 diabetes and the metabolic syndrome. Cell Metab 3:403–416

Davies SP, Carling D, Munday MR, Hardie DG (1992) Diurnal rhythm of phosphorylation of rat liver acetyl-CoA carboxylase by the AMP-activated protein kinase, demonstrated using freeze-clamping. Effects of high fat diets. Eur J Biochem 203:615–623

Fogarty S, Hardie DG (2010) Development of protein kinase activators: AMPK as a target in metabolic disorders and cancer. Biochim Biophys Acta 1804:581–591

Guh DP, Zhang W, Bansback N, Amarsi Z, Birmingham CL, Anis AH (2009) The incidence of co-morbidities related to obesity and overweight: a systematic review and meta-analysis. BMC Public Health 9:88

Hwang SL, Kim HN, Jung HH, Kim JE, Choi DK, Hur JM, Lee JY, Song H, Song KS, Huh TL (2008) Beneficial effects of β-sitosterol on glucose and lipid metabolism in L6 myotube cells are mediated by AMP-activated protein kinase. Biochem Biophys Res Commun 377:1253–1258

Kim WY, Kim JM, Han SB, Lee SK, Kim ND, Park MK, Kim CK, Park JH (2000) Steaming of ginseng at high temperature enhances biological activity. J Nat Prod 63:1702–1704

Kim MS, Park JY, Namkoong C, Jang PG, Ryu JW, Song HS, Yun JY, Namgoong IS, Ha J, Park IS, Lee IK, Viollet B, Youn JH, Lee HK, Lee KU (2004) Anti-obesity effects of α-lipoic acid mediated by suppression of hypothalamic AMP-activated protein kinase. Nat Med 10:727–733

Koh HJ, Lee SM, Son BG, Lee SH, Ryoo ZY, Chang KT, Park JW, Park DC, Song BJ, Veech RL, Song H, Huh TL (2004) Cytosolic $NADP^+$-dependent isocitrate dehydrogenase plays a key role in lipid metabolism. J Biol Chem 9:9968–39974

Kurth-Kraczek EJ, Hirshman MF, Goodyear LJ, Winder WW (1999) 5′ AMP-activated protein kinase activation causes GLUT4 translocation in skeletal muscle. Diabetes 48: 1667–1671

Lage R, Diéguez C, Vidal-Puig A, López M (2008) AMPK: a metabolic gauge regulating whole-body energy homeostasis. Trends Mol Med 14:539–549

Lee YS, Kim WS, Kim KH, Yoon MJ, Cho HJ, Shen Y, Ye JM, Lee CH, Oh WK, Kim CT, Hohnen-Behrens C, Gosby A, Kraegen EW, James DE, Kim JB (2006) Berberine, a natural plant product, activates AMP-activated protein kinase with beneficial metabolic effects in diabetic and insulin-resistant states. Diabetes 55:2256–2264

Nguyen PH, Gauhar R, Hwang SL, Dao TT, Park DC, Kim JE, Song H, Huh TL, Oh WK (2011) New dammarane-type glucosides as potential activators of AMP-activated protein kinase (AMPK) from *Gynostemma pentaphyllum*. Bioorg Med Chem 19:6254–6260

Nishiumi S, Ashida H (2007) Rapid preparation of a plasma membrane fraction from adipocytes and muscle cells: application to detection of translocated glucose transporter 4 on the plasma membrane. Biosci Biotechnol Biochem 71:2343–2346

Okada T, Kawano Y, Sakakibara T, Hazeki O, Ui M (1994) Essential role of phosphatidylinositol 3-kinase in insulin-induced glucose transport and antilipolysis in rat adipocytes. Studies with a selective inhibitor wortmannin. J Biol Chem 269:3568–3573

Razmovski-Naumovski V, Huang TH, Tran VH, Li GQ, Duke CC, Roufogalis BD (2005) Chemistry and pharmacology of *Gynostemma pentaphyllum*. Phytochem Rev 4:197–219

Takemoto T, Arihara S, Yoshikawa K, Kawasaki J, Nakajima T, Okuhira M (1984) Studies on the constituents of cucurbitaceae plants. XI. On the saponin constituents of *Gynostemma pentaphyllum* Makino (7). Yakugaku Zasshi 104:1043–1049

Towler MC, Hardie DG (2007) AMP-activated protein kinase in metabolic control and insulin signaling. Circ Res 100: 328–341

Xiao B, Sanders MJ, Underwood E, Heath R, Mayer FV, Carmena D, Jing C, Walker PA, Eccleston JF, Haire LF, Saiu P, Howell SA, Aasland R, Martin SR, Carling D, Gamblin SJ (2011) Structure of mammalian AMPK and its regulation by ADP. Nature 472:230–233

Yeo J, Kang YJ, Jeon SM, Jung UJ, Lee MK, Song H, Choi MS (2008) Potential hypoglycemic effect of an ethanol extract of *Gynostemma pentaphyllum* in C57BL/KsJ-*db*/*db* mice. J Med Food 11:709–716

Yoshikawa K, Arimitsu M, Kishi K, Takemoto T, Arihara S (1987) Studies on the constituents of Cucurbitaceae plants. XVIII. On the saponin constituents of *Gynostemma pentaphyllum* Makino. (13). Yakugaku Zasshi 107:361–366

Zhang BB, Zhou G, Li C (2009) AMPK: an emerging drug target for diabetes and the metabolic syndrome. Cell Metab 9:407–416

Supplementary Data

A single-dose oral toxicity study of Actiponin in Sprague-Dawley rats

This study was carried out to evaluate the single-dose oral toxicity of Actiponin in Sprague-Dawley rats and to determine the minimum lethal dose. The test doses (2,000 and 5,000 mg/kg) were administered to male and female rats. Vehicle control groups were treated with 0.5% CMC-Na. Each group included 5 rats of each gender. Mortality, clinical signs, and body weight changes were monitored for 14 days. At the end of the 14-day observation period, all animals were sacrificed necropsies were observed. Followings are the summary of the results.

1. No animalsdied during the study period, but diarrhea and soiled perineal region were observed in males and females treated with 2,000 and 5,000 mg/kg on the day of administration.

2. No abnormal body weight changes were observed during the study.

3. No abnormal necropsy findings were observed in any animals.

The minimal lethal dose (MLD) of Actiponin in rats is higher than 5,000 mg/kg.

A 13-week repeated dose oral toxicity study of Actiponin in Sprague-Dawley rats

The present study was carried out to evaluate the toxicity of Actiponin in Sprague-Dawleyrats after repeated oral administration over 13 weeks, and to identify the no observed adverse effect level(NOAEL) and target organs. Actiponin was administered daily (2,000 and 5,000 mg/kg) by oral intubation to 20 animals per group (10 males and 10 females each) for 13 weeks. Clinical signs, mortality, body weight, body weight gain, food consumption, water consumption, ophthalmic examination, urinalysis, hematology, serum biochemistry, organ weights, necropsy, and histopathological findings were compared to those of the vehicle control groups. Followings are summariesof the results.

1. No animals died during the study period, but continuous diarrhea and soiled perineal region were observed in rats treated with 2,000 mg/kg/day.

2. No treatment-related changes in body weight, food, and water consumption, ophthalmology test results, urinalysisindings, bloodanalyses, organ weights, or necropsy and histopathological findings were observed.

Results of this study indicated that a 13-week repeated oral administration of Actiponin had adverse effects as it resulted in continuous diarrhea and soiled perineal region in the 2,000 mg/kg/daygroup. However this was not toxicologically significant and it did not produce any indication to be related to the treatment in other items.

Under the present experimental conditions, NOEL and NOAEL was 1,000 mg/kg/day, and 2,000 mg/kg/day, respectively. On the other hand, the target organ was identified.

Bacterial reverse mutation test of Actiponin

The ability of Actiponin to induce reverse mutation in histidine auxotroph strains of *Salmonella typhimurium* TA100, TA1535, TA98, TA1537 strains, and tryptophan auxotroph *Escherichia coli* WP2 *uvrA*strain was evaluated. Actiponin was dissolved in sterile distilled water and serial dilutions were made. The tests were conducted in the presence (+S) or absence (-S) of a metabolic activation system.

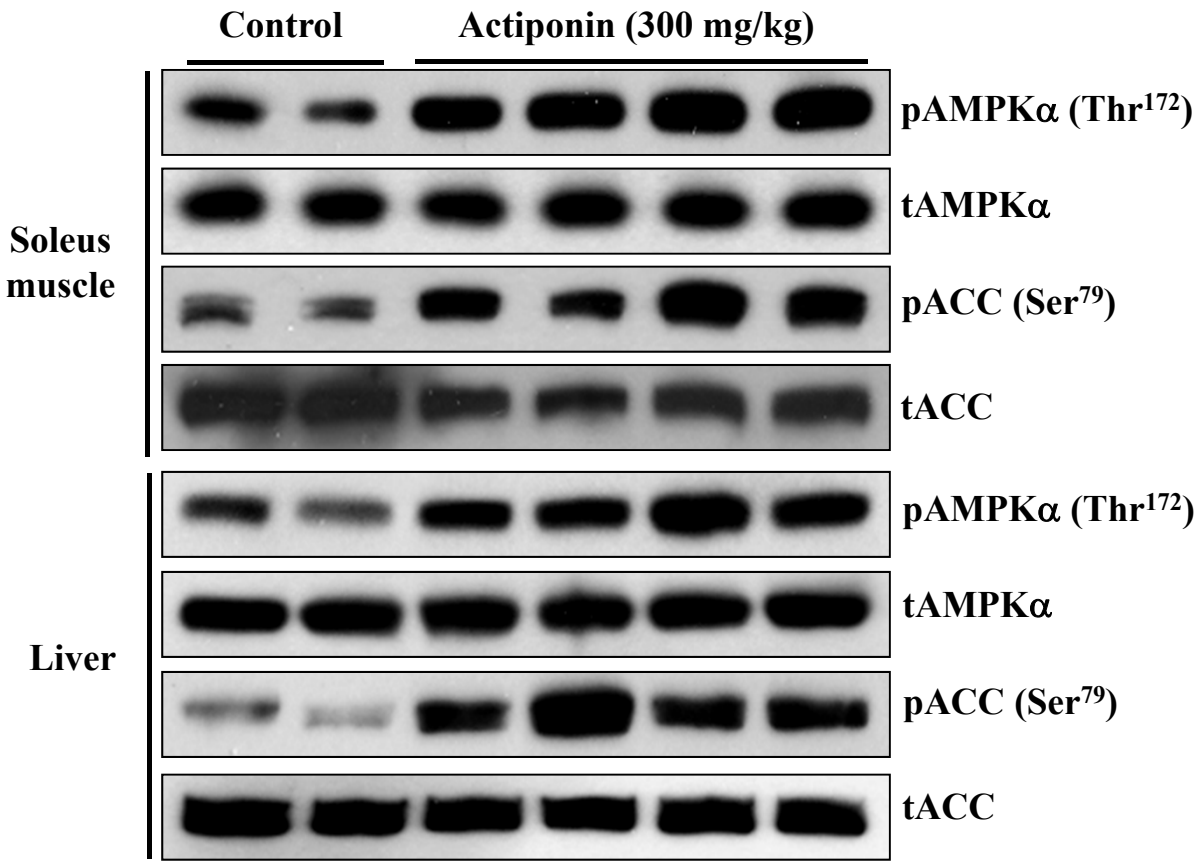
The bacteria were treated with 185, 556, 1,667, 5,000, and 10,000 μg Actiponin/plate with negative and positive controls. Triplicate plates were used for each dose. Increased numbers of revertant colonies at the highest dose of

Actiponin in TA98 (+S) and TA1537 (+S) were observed in the first experiment, but no increase was observed in the other strains. A second experiment was done with the TA98 and TA1537 tester strains at the same dose ranges to confirm the results of the first experiment. Increased numbers of ofrevertant colonies were again observed in TA98 (+S/-S) treated with the highest doses. Colonies from the highest dose treatment were streaked onto the biotin plates to confirm that they were true revertants. As result, true revertants were increased to 1.6- (+S) and 1.3-fold (-S) at the highest doses. Thus, the increases were not attributed to reverse mutation. In the positive control groups, the number of revertants clearly increased compared to the negative control.It was concluded that Actiponin did not induce reverse mutation in the tester strains.

Micronucleus test of Actiponin in bone marrow cells of male ICR mice

A micronucleus test of Actiponin was conducted using specific-pathogen-free (SPF) 8-week-oldmale ICR mice. Actiponin (0, 1,250, 2,500 and 5,000 mg/kg)was administered via oral gavage daily for 2 consecutive days. Six animals were assigned to each treatment group. All animals were sacrificed about 24 h after the second administration and bone marrow preparations were made. Micronucleated polychromatic erythrocytes (MNPCEs) were counted among 2,000 polychromatic erythrocytes(PCEs) per animal.

Actiponin did not significantly increase the frequency of MNPCEs at any dose compared to the vehicle control group. No significant difference was observed in the ratio of PCEs to total erythrocytes (PCE/PCE+NCE) with any dose of Actiponin compared to the vehicle control.These findings demonstrate that Actiponin is not capable of inducing micronuclei formation.



Supplementary Fig. 1. Oral treatment with Actiponin increased AMPK and ACC phosphorylation in the soleus muscle and liver of mice. C57BL/6 male mice were treated with saline or Actiponin (300 mg/kg) for 9 days, and AMPK and ACC phosphorylation in the soleus muscle (60 μg protein) and liver tissues (80 μg protein) were analyzed by Western blot analyses