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# [6]-Gingerol Affects Glucose Metabolism by Dual Regulation via the AMPKα2-Mediated AS160–Rab5 Pathway and AMPK-Mediated Insulin Sensitizing Effects

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## ABSTRACT

[6]-Gingerol has been used to control diabetes and dyslipidemia; however, its metabolic role is poorly understood. In this study, [6]-gingerol increased adenosine monophosphate (AMP)-activated protein kinase (AMPK) phosphorylation in mouse skeletal muscle C2C12 cells. Stimulation of glucose uptake by [6]-gingerol was dependent on AMPKα2. Moreover, both Inhibition and knockdown of AMPKα2 blocked [6]-gingerol-induced glucose uptake. [6]-Gingerol significantly decreased the activity of protein phosphatase 2A (PP2A). Inhibition of PP2A activity with okadaic acid enhanced the phosphorylation of AMPKα2. Moreover, the interaction between AMPKα2 and PP2A was increased by [6]-gingerol, suggesting that PP2A mediates the effect of [6]-gingerol on AMPK phosphorylation. In addition, [6]-gingerol increased the phosphorylation of Akt-substrate 160 (AS160), which is a Rab GTPase-activating protein. Inhibition of AMPKα2 blocked [6]-gingerol-induced AS160 phosphorylation. [6]-gingerol increased the Rab5, and AMPKα2 knockdown blocked [6]-gingerol-induced expression of Rab5, indicating AMPK play as an upstream of Rab5. It also increased glucose transporter 4 (GLUT4) mRNA and protein expression and stimulated GLUT4 translocation. Furthermore, insulin-mediated glucose uptake and Akt phosphorylation were further potentiated by [6]-gingerol treatment. This potentiation was not observed in the presence of AMPK inhibitor compound C. In summary, our results suggest that [6]-gingerol plays an important role in glucose metabolism via the AMPKα2-mediated AS160–Rab5 pathway and through potentiation of insulin-mediated glucose regulation. J. Cell. Biochem. 116: 1401–1410, 2015. 

**KEY WORDS:** AMPK; [6]-GINGEROL; DIABETES; INSULIN SENSITIZING; SIGNAL TRANSDUCTION

*Zingiber officinale* Roscoe (Zingiberaceae), commonly known as ginger, is one of the most widely consumed spices worldwide. It has a long history of use as an herbal medicine to treat a variety of ailments, including vomiting, pain, indigestion, and cold-induced symptoms [Wang and Wang, 2005; White, 2007]. [6]-Gingerol ((S)-5-hydroxy-1-(4-hydroxy-3methoxyphenol)-3-decanone) is an aromatic polyphenol that is a major constituent of ginger. Recent reports have shown that [6]-gingerol also exhibits anticancer, anticlotting, anti-inflammatory, and analgesic activities [Chrubasik et al., 2005; Ali et al., 2008]. Furthermore, long-term treatment with ginger has been reported to not only reduce blood glucose levels but also to decrease serum triglyceride and total cholesterol levels, and to increase insulin levels and prevent body, liver, and kidney weight loss in type 1 diabetic animals [Akhani et al., 2004; Al-Amin et al., 2006; Abdulrazaq et al., 2011].

Adenosine monophosphate (AMP)-activated protein kinase (AMPK) is an enzyme that plays an important role in cellular energy homeostasis. AMPK is activated upon phosphorylation at Thr172 of its alpha subunit by AMPK kinase. Activation of the catalytic activity of AMPK accelerates ATP-generating catabolic pathways, including glycolysis, while simultaneously suppressing ATP-consuming anabolic pathways, such as cholesterol synthesis [Henin et al., 1995]. In addition, the activation of AMPK enhances glucose uptake through translocation of glucose transporter 4 (GLUT4) to the cell membrane and by regulating GLUT4 gene expression [Holmes et al., 1999; Nishino et al., 2004; Song et al., 2010]. In addition, our previous findings showed that homocysteine sulfinic acid and curcumin possess antidiabetic properties that can improve glucose uptake through the AMPK pathway [Kim et al., 2010a,b, 2011].

Abbreviations used: ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidazole-4-carboxy-amide-1-D-ribofuranoside; AMPK, adenosine monophosphate (AMP)-activated protein kinase; AS160, Akt-substrate 160; GLUT4, glucose transporter 4; PP2, protein phosphatase 2.

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Several studies have reported on the anti-diabetic and hypolipidaemic properties of [6]-gingerol in streptozotocin-treated diabetic rats and in rats fed high-fat diet [Al-Amin et al., 2006; Ojewole, 2006; Nammi et al., 2009], and on the mechanisms behind the antidiabetic effects of [6]-gingerol in cultured cells and obese diabetic model mice [Son et al., 2014; Rani et al., 2012], showing that [6]-gingerol stimulates glucose uptake and GLUT4 translocation in L6 myotube cells. Furthermore, another in vitro study showed that the activation of AMPK is associated with [6]-gingerol-mediated glucose uptake [Li et al., 2013]. However, only a few studies have been conducted to examine the mechanism underlying the downstream effects of AMPK or the relationship between AMPK and insulin-mediated signaling. In the current study, we examined the types of molecules associated with [6]-gingerol-mediated signaling and also examined whether [6]-gingerol affects insulin-mediated glucose regulation.

## MATERIALS AND METHODS

### REAGENTS

Antibodies against AMPKα2 (rabbit polyclonal, cat. # 07-363), acetyl-CoA carboxylase (ACC; rabbit polyclonal, cat. # 04-322), protein phosphatase 2 (PP2A; mouse monoclonal, cat. # 05-545), Akt-substrate 160 (AS160; rabbit polyclonal, cat. # 07-741), and phospho-ACC (Ser79) (rabbit polyclonal, cat. # 07-303) were purchased from Merck Millipore (Darmstadt, Germany). Antibodies against phospho-PP2A (Tyr307) (mouse monoclonal, cat. # ab32104), GLUT4 (mouse monoclonal, cat. # ab35826), and phospho-AMPKα2 (Thr172) (rabbit polyclonal, cat. # ab133448) were purchased from Abcam (MA). Antibodies against Akt (rabbit polyclonal, cat. # 9272), p-AS160 (Thr642) (rabbit polyclonal, cat. # 4288), p-Akt (Ser473) (rabbit polyclonal, cat. # 9271), and Rab5 (rabbit polyclonal, cat. # 2143) were purchased from Cell Signaling Technology (Danvers, MA). Antibodies against β-actin (cat. # F3165) and FLAG (cat. # A5316) were purchased from Sigma–Aldrich (St. Louis, MO). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (cat. # ADI-SAB-300) and goat anti-mouse (cat. # ADI-SAB-100) secondary antibodies were purchased from Enzo Life Sciences (Farmingdale, NY). [6]-Gingerol (cat. # 076-05901) was obtained from Wako Pure Chemicals (Osaka, Japan). Okadaic acid 9,10-deepithio-9,10-didehydroacanthifolicin (cat. # 1 355 554 001) was purchased from Roche Diagnostics. Insulin (cat. # I-6634) was purchased from Sigma-Aldrich. Compound C (6-[4-(2-piperidin-1-yl-ethoxy)-phenyl)]-3-pyridin-4-yl-pyrazolo[1,5-a]pyrimidine (cat. # 171260), a potent and selective small-molecule AMP kinase inhibitor, was obtained from Calbiochem (San Diego, CA). 5-Aminoimidazole-4-carboxy-amide-1-D-ribofuranoside (AICAR) (cat. # 9944) was obtained from Cell Signaling Technology (Danvers, MA). Protein A-agarose beads (cat. # 17-5138-03) were obtained from GE Healthcare (Piscataway, NJ). 2-Deoxy-D [$^3$H] glucose was obtained from ARC (St. Louis, MO).

### CELL CULTURE

Mouse skeletal muscle cells, C2C12 myoblasts, and rat skeletal muscle cells, L6 myoblasts, were obtained from the American Type Culture Collection and were maintained in DMEM supplemented with 10% heat inactivated fetal bovine serum (FBS) and 1% antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) at 37°C in a humidified atmosphere with 5% $CO_2$. For differentiation into myotubes, rat myoblast L6 cells were reseeded into 12-well plates (for glucose uptake) at a density of $2 \times 10^4$ cells/ml. After 24 h (over 80% confluence), the medium was changed to DMEM containing 2% (v/v) FBS and was replaced after 2, 4, and 6 days of culture. Experiments were initiated on day 7 when myotube differentiation was complete.

### IMMUNOBLOT ANALYSIS

L6 and C2C12 cells were grown in 6-well plates and were subjected to serum starvation for 12 h prior to treatment. Following experimental manipulations, the medium was removed and the cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed with 100 μl of lysis buffer (50 mM Tri–HCl, pH 7.4, 1% TritonX-100, 0.25% sodium deoxycholate, 150 mM EDTA, 1 mM sodium orthovanadate ($Na_3VO_4$), 1 mM NaF, 1 mM PMSF (phenylmethylsulfonyl fluoride)). The samples were sonicated for 3 min and centrifuged for 30 min at 13,200 r.p.m. (16,000*g*) at 4°C to remove cellular debris. The protein concentration of the cell lysates was measured using Bio-Rad Protein Assay Reagent (Bio-Rad, CA). The proteins were resolved on 10% SDS–PAGE gels and then transferred to nitrocellulose membranes. Uniform transfer was confirmed by Ponceau S staining. Membranes were blocked in Tris-buffered saline with 0.1% Tween 20 (TBS-T buffer) and 5% dry milk (w/v) for 1 h at room temperature and washed three times in TBS-T. Membranes were then incubated overnight at 4°C with primary antibodies diluted appropriately in TBS-T with 5% dry milk and then with a secondary donkey anti-rabbit or anti-mouse IgG antibody conjugated with horseradish peroxidase (Amersham International plc, Buckinghamshire, UK) for 1 h at room temperature. The blots were washed and visualized via chemiluminescence using the Amersham Biosciences ECL Western Blotting Detection System (Amersham International plc) on blue light-sensitive film (Fujifilm Corporation, Tokyo, Japan). Densitometric analysis was performed with ImageJ software (National Institutes of Health, Bethesda, MD).

### siRNA KNOCKDOWN OF AMPKα2

C2C12 cells were seeded into 6-well plates and grown to 70% confluence over 24 h. Transient transfections were performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Briefly, 5 μl of siRNA for mouse AMPKα2 (UGCUUUAGCUCGUUGAUUA, CCAGAUGAACGCUAAGAUA, GUUUAGAUGUUGUUGGAAA, and ACGAGAACAUGAAUGGUUU; NM_178143, Dharmacon, Lafayette, CO, USA) and 5 μl of Lipofectamine 2000 were each diluted with 95 μl of reduced serum medium (Opti-MEM; Invitrogen) and then combined. The mixtures were incubated for 15 min at room temperature before being added dropwise to a culture well containing 800 μl of Opti-MEM for a final siRNA concentration of 100 nM. After 4 h, the medium was replaced with fresh DMEM, and the cells were cultivated for 48 h before being harvested for immunoblot analysis of AMPKα2.

### REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION (RT–PCR)

To analyze the mRNA expression of genes, total RNA was extracted from C2C12 cells with the QIAzol Lysis reagent (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The mRNA

was eluted in 20 μl of DEPC water (Qiagen) and quantified with a NanoDrop ND-100 device (Thermo Fisher Scientific, Waltham, MA). One microgram of total RNA from each sample was subjected to cDNA synthesis using AMV reverse transcriptase and Oligo (dT) (Promega, Madison, WI). cDNA synthesis was performed according to the manufacturer's recommendations by mixing 1 μg/μl mRNA, 4 μl 5× RT buffer, 1 μl 500 nM Oligo(dT), 1 μl 10 mM dNTP, 0.5 μl RNasein, 1 μl AMV reverse transcriptase, and 11.5 μl $dH_2O$ in PCR tubes. The mixture was then incubated at 37°C for 1 h. cDNA was diluted with 20 μl $dH_2O$ and stored at −80°C until use. Primers were designed using primer3 version 0.4.0 (http://primer3.ut.ee/). The following primers were used for amplification: mGLUT4 (NM_009204), 5′-CAG CCT ACG CCA CCA TAG TAC-3′ (sense) and 5′-TTC CAG CAG CAG CAG AG-3′ (antisense); mRab5 (NM 025887), 5′-ATT GGG GCT GCC TTT CTA AC-3′ (sense) and 5′-GCT GAG TTT GCA CCA GGA TT-3′ (antisense); and β-actin (NM_007393), 5′-ATT TGG TCG TAT TGG GCG CCT GGT CAC C-3′ (sense) and 5′-GAA GAT GGT GAT GGG ATT TC-3′ (antisense). The RT–PCR program was as follows: 95°C for 10 min, followed by 35 cycles at 95°C for 15 s, 60°C for 15 s, and 72°C for 45 s. Next, 10 μl of product from each RT–PCR reaction was analyzed by agarose gel electrophoresis. Bands were stained with ethidium bromide, and band intensity was quantified using a gel documentation system (Gene Genius, Syngene, Cambridge, UK).

### IMMUNODETECTION OF GLUT4myc

Cell surface expression of GLUT4myc was quantified using an antibody-coupled colorimetric absorbance assay. Briefly, following stimulation with insulin or [6]-gingerol, cells stably expressing L6-GLUT4myc were incubated with polyclonal anti-myc antibody (1:1,000) for 60 min, fixed with 4% paraformaldehyde (PFA) in PBS for 10 min, and incubated with HRP-conjugated goat anti-rabbit IgG (1:1,000) for 1 h. Cells were washed six times in PBS, and incubated in 1 ml of OPD reagent (0.4 mg/ml *o*-phenylenediamine dihydrochloride and 0.4 mg/ml urea hydrogen peroxide) for 30 min at room temperature. The reaction was stopped with 0.25 ml of 3 N HCl. Optical absorbance of the supernatant was measured at 492 nm. Background, as measured in samples incubated in peroxidase-conjugated anti-rabbit IgG alone (without primary antibody), was subtracted from all values. Each experimental condition was tested in triplicate within each experiment, and each experiment was repeated the number times indicated in the figure legends, typically on different dates.

### 2-DEOXYGLUCOSE UPTAKE

Glucose uptake activity was analyzed by measuring the uptake of 2-deoxy-D [$^3$H] glucose in differentiated L6 myotubes. Two days after the myoblasts achieved confluence, differentiation to myotubes was induced by incubation for 6–7 days in DMEM with 2% FBS, which was changed every 2 days. Cells were rinsed twice with warm PBS (37°C), and then starved in serum-free DMEM for 3 h. After [6]-gingerol treatment, the cells were incubated in KRH buffer (20 mM HEPES, pH 7.4, 130 mM NaCl, 1.4 mM KCl, 1 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM $KH_2PO_4$) containing 0.5 μCi of 2-deoxy-D [$^3$H] glucose for 15 min at 37°C. The reaction was terminated by placing the plates on ice and washing twice with ice-cold PBS. Cells were lysed in 0.5% SDS and 0.5 N NaOH. A scintillation counter was used to measure the radioactivity in lysates extracted in 0.5% SDS and 0.5N NaOH, whereas the total protein content in lysates extracted in 1N NaOH was determined by the Bradford procedure (Bio-Rad Laboratories).

### IMMUNOPRECIPITATION

The amount of protein in C2C12 cells or FLAG-AMPKα2-transfected HEK293 cells was determined using the Bradford method. Cellular protein (1,000 μg) was mixed with 1 μg of anti-FLAG (monoclonal antibody) or anti-PP2A (monoclonal antibody) and incubated at 4°C for 2 h. Immune complexes were captured with Protein A-sepharose (Amersham, Uppsala, Sweden) by adding Protein A-sepharose and incubating for an additional 3 h at 4°C. Precipitated immune complexes were washed three times with wash buffer (25 mM HEPES, 5 mM EDTA, 1% Triton X-100, 50 mM NaF, 150 mM NaCl, 10 mM phenylmethylsulfonyl fluoride, 1 μM leupeptin, 1 μM pepstatin, and 1 μM aprotinin [pH 7.2]). The washed sample was resuspended in SDS sample buffer (125 mM Tris–HCl [pH 6.8], 20% [v/v] glycerol, 4% [w/v] SDS, 100 mM dithiothreitol, and 0.1% [w/v] bromophenol blue) and heated at 100°C for 5 min before electrophoresis.

### DATA ANALYSIS

Statistical analyses were conducted using SigmaStat (SPSS Inc., Chicago). Data are expressed as mean ± SEM values. $P$ values $< 0.05$ were considered statistically significant.

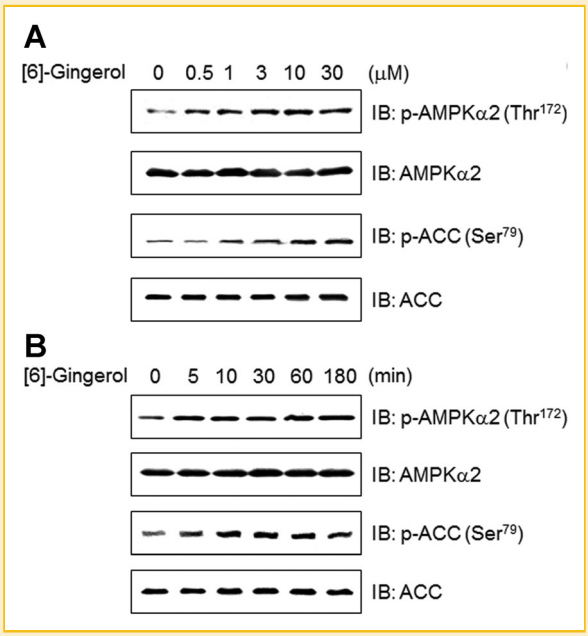


Fig. 1. [6]-Gingerol activates AMPKα2 and its downstream target ACC in myoblasts. A: The phosphorylation of AMPKα2 and ACC after [6]-gingerol treatment. Cells were stimulated for 60 min at several concentrations of [6]-gingerol. Cell lysates (30 μg) were analyzed by Western blotting using antibodies against phospho-AMPKα2 (Thr172) and phospho-ACC (Ser79), while AMPK and ACC served as controls. B: Time-dependent phosphorylation of AMPKα2 and ACC after [6]-gingerol treatment. Cells were incubated with 10 μM [6]-gingerol for the indicated times. Cell lysates were analyzed by Western blotting using antibodies against phospho-AMPKα2 (Thr172) and phospho-ACC (Ser79), while AMPK and ACC served as controls.

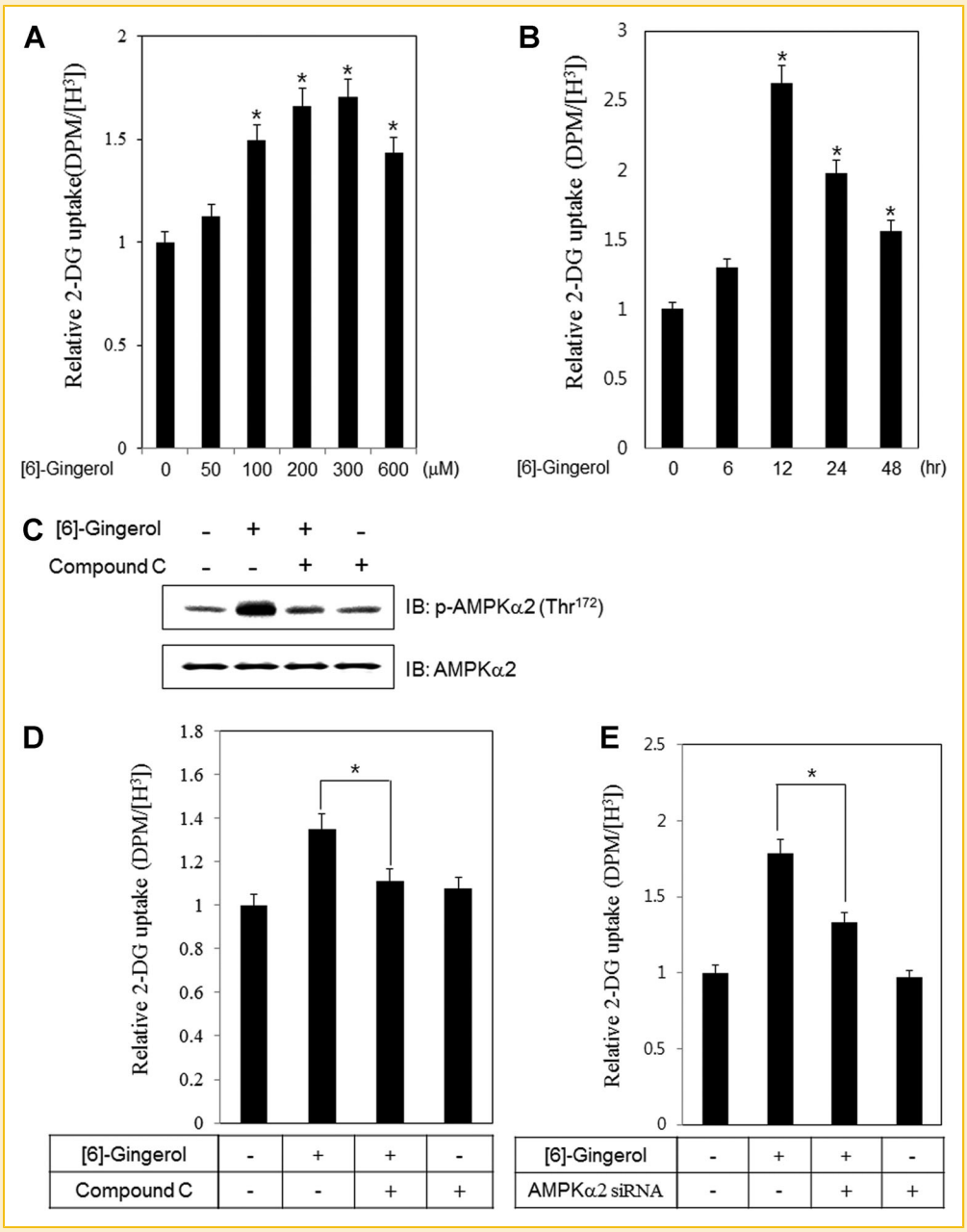


**Fig. 2.** [6]-Gingerol stimulates glucose uptake through activation of the AMPKα2 pathway in differentiated muscle cells. A: Glucose uptake by [6]-gingerol treatment. L6 myotube cells were incubated with [6]-gingerol at various concentrations for 12 h, and then assayed for glucose uptake. $^{*}P < 0.05$ versus basal value. B: Time-dependent uptake of glucose by [6]-gingerol treatment. L6 myotube cells were incubated with 100 μM [6]-gingerol for the indicated times and then assayed for glucose uptake. $^{*}P < 0.05$ versus basal value. C: Pharmacological inhibition of AMPK blocks [6]-gingerol-induced phosphorylation of AMPK. Cells were incubated for 1 h with 10 μM [6]-gingerol in the presence of the AMPK inhibitor compound C (5 μM) and cell lysates were analyzed by Western blotting using antibodies against phospho-AMPKα2 (Thr172), while AMPK served as controls. D: Pharmacological inhibition of AMPK blocks [6]-gingerol-induced glucose uptake. L6 myotube cells were incubated for 12 h with 100 μM [6]-gingerol in the presence of the AMPK inhibitor compound C (5 μM) and then assayed for glucose uptake. $^{*}P < 0.05$ versus [6]-gingerol-treated condition. E: Knockdown of AMPKα2 blocks 100 μM [6]-gingerol-induced glucose uptake. L6 myotube cells were transiently transfected with AMPKα2 siRNA (100 nM) for 48 h and then incubated with 100 μM [6]-gingerol for 12 h before assaying for glucose uptake. The results are displayed as mean ± SD for three independent experiments. $^{*}P < 0.05$ versus [6]-gingerol-treated condition.

## RESULTS

### [6]-GINGEROL ACTIVATES AMPKα2 AND ITS DOWNSTREAM TARGET ACC IN MYOBLASTS

To assess whether [6]-gingerol plays a role in AMPKα2 regulation, the extent of AMPKα2 phosphorylation in [6]-gingerol-treated C2C12 cells was determined. Using phosphorylation-specific antibodies for AMPKα2 and its downstream molecule, ACC, We showed the phosphorylation of AMPKα2 and ACC was increased in [6]-gingerol (0–30μM)-treated cells compared to control cells, were dose-dependently higher in [6]-gingerol-treated cells than in control cells, with a maximal increase occurring at 10 μM [6]-gingerol (Fig. 1A). In addition, [6]-gingerol induced a time-dependent increase in AMPKα2 and ACC phosphorylation (Fig. 1B). These results demonstrate that [6]-gingerol activates AMPKα2 and its downstream target ACC in C2C12 cells.

### [6]-GINGEROL STIMULATES GLUCOSE UPTAKE BY ACTIVATING THE AMPKα2 PATHWAY IN DIFFERENTIATED MUSCLE CELLS

AMPKα2 activation plays an important role in regulating glucose uptake during exercise [Li et al., 2013]. Therefore, we hypothesized that AMPKα2 activation by [6]-gingerol could trigger glucose uptake, so we examined the effect of [6]-gingerol treatment on glucose uptake in differentiated L6 cells. Treatment with [6]-gingerol resulted in increases in glucose uptake. Glucose uptake was induced at a concentration of 50 μM [6]-gingerol, with a maximal increase occurring at 300 μM [6]-gingerol for 12 h, reaching maximally to a 1.7-fold increase, followed by decrease at the higher concentration (Fig. 2A, B). Inhibition of AMPK with compound c, AMPK inhibitor, blocked [6]-gingerol-mediated phosphorylation of AMPK (Fig. 2C). To verify the role of AMPKα2 in [6]-gingerol-induced glucose uptake, we pretreated cells with compound C (5 μM), an AMPK inhibitor, and found that it prevented [6]-gingerol-induced glucose uptake (Fig. 2D). To confirm that the effects of [6]-gingerol on glucose uptake were due to AMPKα2 activation, AMPKα2 expression was knocked down using AMPKα2 siRNA. [6]-Gingerol-induced glucose uptake was blocked in AMPKα2-knocked down cells (Fig. 2E), indicating that [6]-gingerol stimulates glucose uptake through the AMPKα2 pathway.

### [6]-GINGEROL ACTIVATES AMPKα2 THROUGH PP2A

Protein phosphatase 2A (PP2A) is a ubiquitously expressed cytoplasmic serine/threonine phosphatase that plays an important role in the regulation of a diverse set of cellular proteins, including

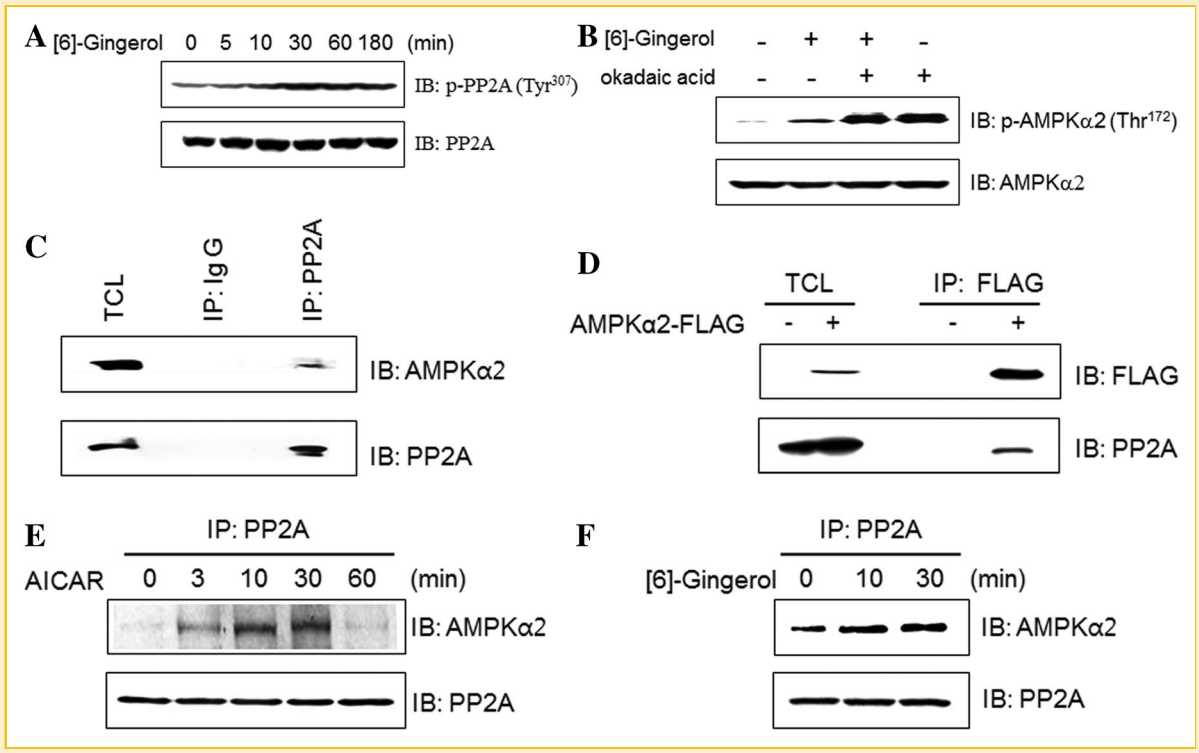


**Fig. 3.** [6]-Gingerol activates AMPKα2 through down regulation of PP2A in myoblasts. A: PP2A is inhibited by [6]-gingerol treatment. Cells were incubated with 10 μM [6]-gingerol, and lysates were analyzed by Western blotting using an antibody against phospho-PP2A, while PP2A served as a control. B: Cells were pretreated with okadaic acid (5 μM) and then incubated with 10 μM [6]-gingerol for 60 min. Cell lysates (30 μg) were analyzed by Western blotting using an antibody against phospho-AMPKα2, while AMPKα2 served as a control. C: Cells were immunoprecipitated with an anti-PP2A antibody, followed by Western blotting using AMPKα2 and PP2A antibodies. D: Western blot of anti-PP2A immunoprecipitates from 293 cells transiently transfected with FLAG-AMPKα2. E: Cells were treated with 1 mM AICAR for the indicated times before lysis and lysates were immunoprecipitated (IP) with PP2A antibody, followed by Western blotting with anti-PP2A and AMPKα2 antibodies. (F) Cells were treated with 10 μM [6]-gingerol for the indicated times before lysis and lysates were immunoprecipitated (IP) with PP2A antibody, followed by Western blotting with anti-AMPKα2 and PP2A antibodies. The results are representative of three independent experiments. TCL, total cell lysates.

metabolic enzymes, hormone receptors, kinase cascade members, and cell growth regulators [Millward et al., 1999; Sontag, 2001]. The catalytic subunit of PP2A is inactivated by the phosphorylation of its Tyr307 residue by receptor and nonreceptor protein tyrosine kinases. Okadaic acid, an inhibitor of PP2A, activated glucose transport and GLUT4 translocation [Standaert et al., 1999], and down regulating PP2A activity was known to increase the phosphorylation of AMPK [Wu et al., 2007; Yu et al., 2013]. We thus examined whether [6]-gingerol regulates PP2A activity. The phosphorylation of PP2A was increased in [6]-gingerol-treated cells, indicating that [6]-gingerol inhibited PP2A activity (Fig. 3A). To verify the role of PP2A in the phosphorylation of AMPK, we assessed the effects of the PP2A inhibitor okadaic acid on AMPKα2 phosphorylation. Inhibition of PP2A activity increased phosphorylation of AMPKα2, suggesting that PP2A is involved in [6]-gingerol-mediated AMPKα2 phosphorylation (Fig. 3B). To elucidate the mechanism underlying [6]-gingerol-mediated AMPK phosphorylation, we examined the interaction between AMPKα2 and PP2A. We found that PP2A interacted with AMPKα2 in C2C12 cells (Fig. 3C). FLAG-tagged AMPKα2 was associated with endogenous PP2A (Fig. 3D). The effect of AICAR, an AMPK activator, and [6]-gingerol on the AMPKα2–PP2A interaction was examined by immunoprecipitation. AICAR and [6]-gingerol treatments increased this interaction (Fig. 3E, F). These results indicate that [6]-gingerol stimulates AMPKα2 phosphorylation by regulating PP2A activity.

### [6]-GINGEROL STIMULATES AS160 PHOSPHORYLATION

Akt substrate of 160 kDa (AS160) is a Rab GTPase-activating protein (GAP) that modulates GLUT4 translocation [Lansey et al., 2013]. Distinct signals regulate AS160 phosphorylation in response to insulin, AICAR, and contraction in mouse skeletal muscle [Kramer et al., 2006]. To determine whether AS160 is involved in [6]-gingerol-mediated glucose uptake, we examined the effects of [6]-gingerol on the activity of AS160, a protein with a Rab-GTPase activating protein (Rab-GAP) domain involved in regulating GLUT4 translocation [Lansey et al., 2013]. Levels of phosphorylation of AS160 were determined based on the immunodetection of phosphorylated AS160. [6]-Gingerol (10 μM) treatment resulted in a time-dependent increase in AS160 phosphorylation (Fig. 4A). The presence of the AMPK inhibitor abrogated the observed [6]-gingerol-induced increase in AS160 phosphorylation (Fig. 4B). These results demonstrate that AS160 acts as a downstream molecule in the [6]-gingerol-mediated AMPK pathway.

### [6]-GINGEROL INDUCES Rab5 mRNA AND PROTEIN EXPRESSION

To gain insight into the role of AMPKα2 in GLUT4 translocation, we evaluated the effect of [6]-gingerol on the expression of Rab5, a member of the Ras superfamily of monomeric G proteins. Rab regulates several steps involved in membrane trafficking, including vesicle formation, movement, and fusion [Huang et al., 2001]. The administration of [6]-gingerol induced an increase in Rab5 messenger RNA expression (Fig. 5A). The level of Rab5 mRNA began to increase within 3h after [6]-gingerol treatment and was sustained for up to 6h, reaching maximally to a 2.9-fold increase. Consistent with the increase in mRNA, the level of Rab5 protein increased after [6]-gingerol treatment, reaching maximally to 2.3-fold increase (Fig. 5B). To determine the hierarchy between AMPKα2 and Rab5, we investigated the levels of Rab5 protein in cells pretreated with

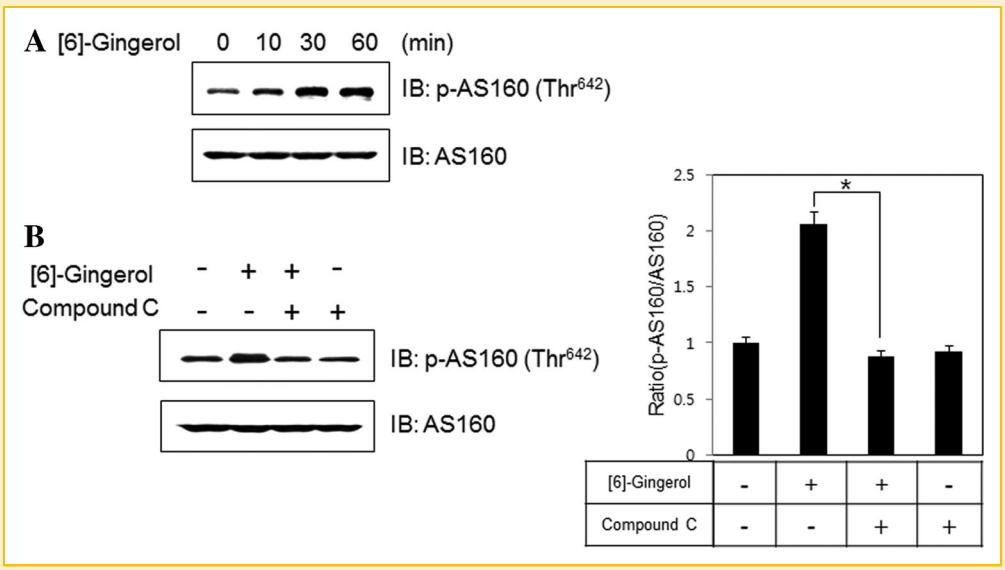


Fig. 4. [6]-Gingerol stimulates the phosphorylation of AS160. A: AS160 is activated by [6]-gingerol treatment. Cells were incubated with 10 μM [6]-gingerol and lysates were analyzed by Western blotting using an antibody against phospho-AS160, while AS160 served as a control. B: Cells were pretreated with compound C (5 μM) and then incubated with 10 μM [6]-gingerol for 60 min. Cell lysates (30 μg) were analyzed by Western blotting using an antibody against phospho-AS160, while AS160 served as a control. The results are representative three independent Western blot experiments. $^{*}P < 0.05$ versus [6]-gingerol-treated condition.

AMPKα2 siRNA. Expression levels of Rab5 protein were down regulated in cells treated with AMPKα2 siRNA (Fig. 5C). These results suggest that AMPK may be involved in [6]-gingerol-induced Rab5 expression.

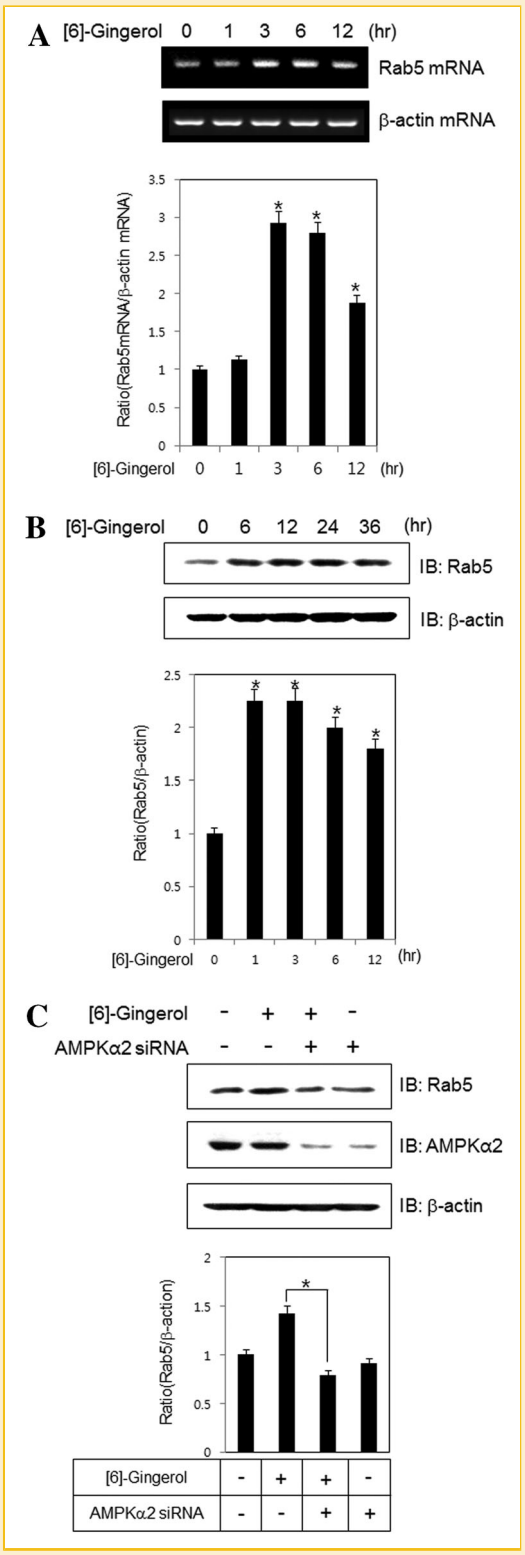


Fig. 5. [6]-Gingerol induces Rab5 mRNA and protein expression in C2C12 cells. A: Total RNA was prepared after [6]-gingerol treatment, and RT-PCR was performed using Rab5-specific primers. $^{*}P < 0.05$ versus [6]-gingerol-treated condition. B: Cells were incubated with 10 μM [6]-gingerol for the indicated times. Cell lysates (30 μg) were analyzed by Western blotting using an antibody against Rab5. $^{*}P < 0.05$ versus [6]-gingerol-treated condition. C: Cells were transiently transfected with AMPKα2 siRNA for 48 h prior to 10 μM [6]-gingerol treatment for 30 min. Cell lysates (30 μg) were analyzed by Western blotting using antibodies against Rab5 and AMPKα2. $^{*}P < 0.05$ versus [6]-gingerol-treated condition. The results are representative three independent Western blot experiments.

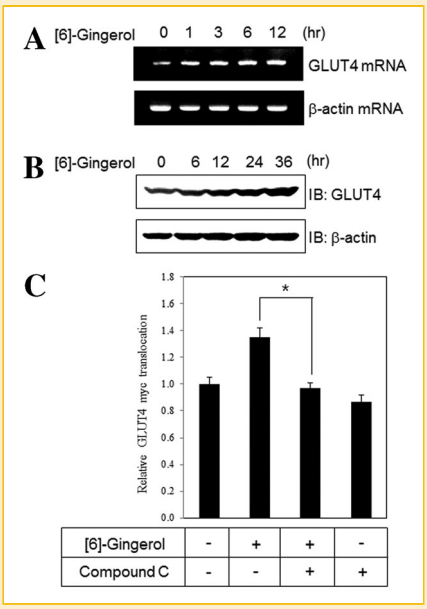


Fig. 6. [6]-Gingerol stimulates GLUT4 translocation by increasing GLUT4 mRNA and protein expression. A: Total RNA from C2C12 cells was prepared after [6]-gingerol treatment, and RT-PCR was performed using GLUT4-specific primers. B: Cells were incubated with 10 μM [6]-gingerol for the indicated times. Cell lysates (20 μg) were analyzed by Western blotting using an antibody against GLUT4. C: Confluent monolayers of myotubes were pretreated with compound C (5 μM) and then incubated with 10 μM [6]-gingerol for 5 h. Cell surface expression of GLUT4myc was detected using an antibody-coupled colorimetric absorbance assay. $^{*}P < 0.05$ versus [6]-gingerol treated condition.

## [6]-GINGEROL STIMULATES GLUT4 TRANSLOCATION AND INCREASES GLUT4 mRNA AND PROTEIN EXPRESSION

Based on a previous study reporting that AMPKα2 regulates GLUT4 expression [Fisher et al., 2002], the effect of [6]-gingerol on GLUT4 was evaluated. [6]-Gingerol treatment increased the levels of GLUT4 mRNA. The level of GLUT4 mRNA began to increase within 1 h after [6]-gingerol treatment and was sustained for up to 6 h, reaching maximally to a 1.9-fold increase (Fig. 6A). Consistent with the increase in mRNA, the level of GLUT4 protein increased after [6]-gingerol treatment, reaching maximally to 1.6-fold increase (Fig. 6B). A colorimetric assay was used to measure cell surface localization of GLUT4myc. An increase in plasma membrane GLUT4myc following treatment with [6]-gingerol was not observed in the presence of

compound C (Fig. 6C). The results indicate that [6]-gingerol enhances glucose uptake via translocation of GLUT through the AMPKα2 pathway.

### [6]-GINGEROL INCREASES INSULIN SENSITIVITY

Akt is a serine/threonine protein kinase that leads to insulin-sensitive glucose uptake [Alessi and Cohen, 1998]. To determine whether the basal activity of Akt is involved in [6]-gingerol induced glucose metabolism, we first examined whether [6]-gingerol activates Akt phosphorylation. [6]-Gingerol increased the phosphorylation of Akt in a time-dependent manner (Fig. 7A). Next, we examined whether [6]-gingerol has am insulin-sensitizing effect. To this end, C2C12 cells were treated with 10 nM insulin in the presence or absence of [6]-gingerol. When [6]-gingerol was co-administered with insulin, the phosphorylation of Akt further increased (Fig. 7B). To confirm this observation, we performed a glucose uptake experiment. When [6]-gingerol was co-administered with insulin, glucose uptake was further increased also (Fig. 7C). Inhibition of AMPK with AMPK inhibitor blocked both [6]-gingerol and insulin-mediated phosphorylation of AMPKα2 (Fig. 7D). [6]-Gingerol potentiated insulin-mediated glucose uptake, an effect that was not observed with compound C, indicating that [6]-gingerol has an insulin-sensitizing effect acting through AMPKα2 (Fig. 7E). These results demonstrate that [6]-gingerol has a role in regulating glucose through its insulin-sensitizing role via AMPKα2.

## DISCUSSION

In the present study, we found that [6]-gingerol stimulated glucose uptake in myoblasts and potentiated insulin-mediated glucose

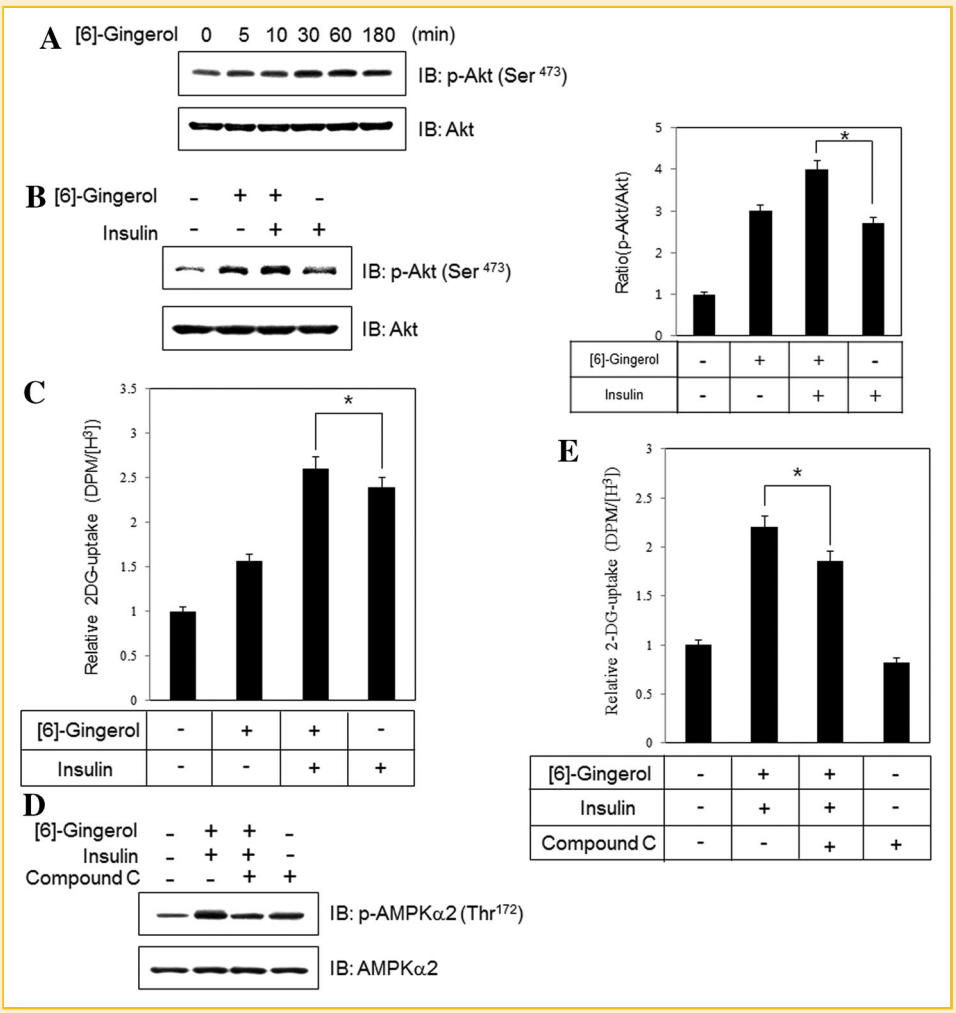


**Fig. 7.** [6]-Gingerol improves insulin sensitivity by activating the AKT pathway. A: Cells were stimulated for the indicated times. Cell lysates (30 μg) were analyzed by Western blotting using an antibody against phospho-Akt, while Akt served as a control. B: Cells were treated with insulin (10 nM, 30 min) in the presence or absence of [6]-gingerol. $^{*}P < 0.05$ versus co-administration with [6]-gingerol and insulin. C: Differentiated L6 cells were treated with insulin (10 nM, 30 min) in the presence or absence of [6]-gingerol and then assayed for glucose uptake as described in MATERIALS AND METHODS. $^{*}P < 0.05$ versus co-administration with [6]-gingerol and insulin. D: Cells were co-treated with insulin and [6]-gingerol in the presence or absence of compound c. $^{*}P < 0.05$ versus co-administration with [6]-gingerol and insulin. E: Differentiated L6 cells were co-treated with insulin and [6]-gingerol in the presence or absence of compound C and then assayed for glucose uptake as described in MATERIALS AND METHODS. $^{*}P < 0.05$ versus co-administration with [6]-gingerol and insulin.

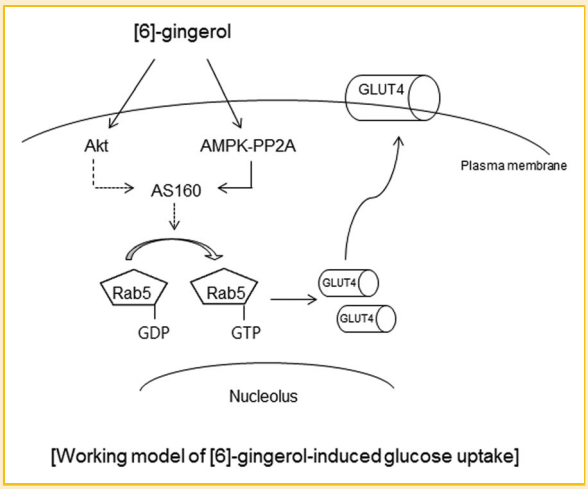


**Fig. 8.** Schematic diagram of the mechanism of [6]-Gingerol. [6]-Gingerol triggers the activation of AMPK pathway, which in turn activates AS160. [6]-gingerol also triggers the activation of Akt–AS160 pathway.

uptake. Treatment of cells with [6]-gingerol increased the phosphorylation of AMPK and AS160, the AMPK downstream effector, and also elevated Rab5 and Glut4 levels. As well, administration of [6]-gingerol increased insulin-mediated glucose uptake, but this increase was inhibited by treatment with an AMPK inhibitor. Therefore, these results suggest that the hypoglycemic effect of [6]-gingerol occurs through dual regulation: one mechanism is via a direct effect on the AMPK–PP2A mediated AS160, while the other is through potentiation of Akt-mediated AS160 pathway (Fig. 8). Rab5 plays as a convergent pathway for [6]-gingerol-mediated GLUT4 translocation.

Because [6]-gingerol has shown to play metabolic roles in animal models, several studies have focused on the characterization of its mechanisms of action. Recently, the function of [6]-gingerol was reported to exert a hypoglycemic effect through AMPK [Li et al., 2013]. That study found that elevated intracellular calcium levels were associated with [6]-gingerol-mediated AMPKα1 phosphorylation. Additionally, knockdown of AMPKα1 completely eliminated [6]-gingerol-mediated glucose uptake, indicating that [6]-gingerol stimulated glucose uptake via AMPKα1 rather than via AMPKα2. In the present study, we demonstrated that [6]-gingerol stimulates glucose uptake via the phosphorylation of AMPKα2. There is some controversy surrounding the exact mechanism underlying the effect of [6]-gingerol on AMPKα isozymes, specifically, whether [6]-gingerol effects AMPKα1 or AMPKα2. It was previously found that AMPKα2 is a major isozyme in skeletal muscles [Wang and Zhang, 2010]. Other previous studies have shown that AMPKα1 is also expressed, albeit weakly, in skeletal muscles [Quentin et al., 2011]. Thus, it has been very difficult to determine the exact contribution of AMPK isozymes in [6]-gingerol function. The results of our current study, in combination with those from other studies, suggest that both alpha isozymes are involved in [6]-gingerol-mediated glucose regulation. The exact role of each isozyme or the degree of each isozyme′s contribution in glucose regulation should be examined in future studies. The metabolic role of [6]-gingerol has been determined in myoblasts but not in AMPK-related animal models, such as an AMPKα1 knockout or AMPKα2 knockout model. Because insulin-mediated glucose uptake and Akt phosphorylation were potentiated by treatment with [6]-gingerol, the metabolic role of [6]-gingerol could be attributed to a factor other than AMPKα2. Activation of AMPK is known to increase insulin sensitivity [O′Neill, 2013]. This indicates that the [6]-gingerol-mediated increase in insulin sensitivity is due to the activation of AMPK. This hypothesis is supported by results showing that [6]-gingerol-induced increase in insulin sensitivity was not observed in the presence of AMPK inhibition. Moreover, [6]-gingerol alone increased the phosphorylation of Akt, implying that the [6]-gingerol-mediated increase in insulin sensitivity was caused by [6]-gingerol-induced Akt phosphorylation. Future studies should focus on characterizing the relationship between gingerol and insulin sensitivity in order to fully elucidate the molecular mechanisms underlying [6]-gingerol function.

In conclusion, we have demonstrated that [6]-gingerol plays metabolic roles via mechanisms in the skeletal muscle system: the first may be through AMPK and its related pathway, such as the AS160–Rab5 pathway, while the second may be through AMPKα2-mediated insulin sensitivity. These findings increase understanding of the molecular mechanisms underlying the hypoglycemic functions of [6]-gingerol, and suggest potential applications of [6]-gingerol in the development of medications for treatment of diabetes.

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## REFERENCES

Abdulrazaq NB, Cho MM, Win NN, Zaman R, Rahman MT. 2011. Beneficial effects of ginger (*Zingiber officinale*) on carbohydrate metabolism in streptozotocin-induced diabetic rats. Br J Nutr 108:1194–1201.

Akhani SP, Vishwakarma SL, Goyal RK. 2004. Anti-diabetic activity of *Zingiber officinale* in streptozotocin-induced type I diabetic rats. J Pharm Pharmacol 56:101–105.

Al-Amin ZM, Thomson M, Al-Qattan KK, Ali M. 2006. Anti-diabetic and hypolipidaemic properties of ginger (*Zingiber officinale*) in streptozotocin-induced diabetic rats. Br J Nutr 96:660–666.

Alessi DR, Cohen P. 1998. Mechanism of activation and function of protein kinase B Curr Opin Genet Dev 8:55–62.

Ali BH, Blunden G, Tanira MO, Nemmar A. 2008. Some phytochemical, pharmacological and toxicological properties of ginger (*Zingiber officinale* Roscoe): A review of recent research. Food Chem Toxicol 46:409–420.

Chrubasik S, Pittler MH, Roufogalis BD. 2005. Zingiberis rhizoma: A comprehensive review on the ginger effect and efficacy profiles. Phytomedicine 12:684–701.

Fisher JS, Gao J, Han DH, Holloszy JO, Nolte LA. 2002. Activation of AMP kinase enhances sensitivity of muscle glucose transport to insulin. Am J Physiol Endocrinol Metab 282:E18–E23.

Henin N, Vincent MF, Gruber HE, Van den Berghe G. 1995. Inhibition of fatty acid and cholesterol synthesis by stimulation of AMP-activated protein kinase. FASEB J 9:541–546.

Holmes BF, Kurth-Kraczek EJ, Winder WW. 1999. Chronic activation of 5′-AMP-activated protein kinase increases GLUT-4, hexokinase, and glycogen in muscle. J Appl Physiol 87:1990–1995.

Huang J, Imamura T, Olefsky JM. 2001. Insulin can regulate GLUT4 internalization by signaling to Rab5 and the motor protein dynein Proc Natl Acad Sci U S A 98:13084–13089.

Kim JH, Lee JO, Lee SK, Jung JH, You GY, Park SH, Park M, Kim SD, Kim HS. 2010a Clozapine activates AMP-activated protein kinase (AMPK) in C2C12 myotube cells and stimulates glucose uptake. Life Sci 87:42–48.

Kim JH, Lee JO, Lee SK, Moon JW, You GY, Kim SJ, Park SH, Park JM, Lim SY, Suh PG, Uhm KO, Song MS, Kim HS. 2011. The glutamate agonist homocysteine sulfinic acid stimulates glucose uptake through the calcium-dependent AMPK-p38 MAPK-protein kinase C zeta pathway in skeletal muscle cells. J Biol Chem 286:7567–7576.

Kim JH, Park JM, Kim EK, Lee JO, Lee SK, Jung JH, You GY, Park SH, Suh PG, Kim HS. 2010b Curcumin stimulates glucose uptake through AMPK-p38 MAPK pathways in L6 myotube cells. J Cell Physiol 1223:771–778.

Kramer HF, Witczak CA, Fujii N, Jessen N, Taylor EB, Arnolds DE, Sakamoto K, Hirshman MF, Goodyear LJ. 2006. Distinct signals regulate AS160 phosphorylation in response to insulin, AICAR, and contraction in mouse skeletal muscle. Diabetes 55:2067–2076.

Lansey MN, Walker NN, Hargett SR, Stevens JR, Keller SR. 2013. Deletion of Rab GAP AS160 modifies glucose uptake and GLUT4 translocation in primary skeletal muscles and adipocytes and impairs glucose homeostasis. Am J Physiol Endocrinol Metab 303:E1273–E1286.

Li Y1, Tran VH, Koolaji N, Duke C, Roufogalis BD. 2013. (S)-[6]-Gingerol enhances glucose uptake in L6 myotubes by activation of AMPK in response to [Ca2+]. J Pharm Pharm Sci 16:304–312.

Nammi S, Sreemantula S, Roufogalis BD. 2009. Protective effects of ethanolic extract of *Zingiber officinale* rhizome on the development of metabolic syndrome in high-fat diet-fed rats. J Basic Clin Pharmacol Toxicol 104:366–373.

Nishino Y, Miura T, Miki T, Sakamoto J, Nakamura Y, Ikeda Y, Kobayashi H, Shimamoto K. 2004. Ischemic preconditioning activates AMPK in a PKC-dependent manner and induces GLUT4 up-regulation in the late phase of cardioprotection. Cardiovasc Res 61:610–619.

Millward, TA, Zolnierowicz, S, Hemmings, BA. 1999. Regulation of protein kinase cascades by protein phosphatase2A. Trends Biochem Sci 24:186–191.

Ojewole JA. 2006. Analgesic, antiinflammatory and hypoglycaemic effects of ethanol extract of *Zingiber officinale* (Roscoe) rhizomes (Zingiberaceae) in mice and rats. Phytother Res 20:764–772.

O′Neill HM. 2013. AMPK and exercise: glucose uptake and insulin sensitivity. Diabetes Metab J 37:1–21.

Quentin T, Kitz J, Steinmetz M, Poppe A, Bär K, Krätzner R. 2011. Different expression of the catalytic alpha subunits of the AMP activated protein kinase-an immunohistochemical study in human tissue. Histol Histopathol 26:589–596.

Rani MP, Krishna MS, Padmakumari KP, Raghu KG, Sundaresan A. 2012. *Zingiber officinale* extract exhibits antidiabetic potential via modulating glucose uptake, protein glycation and inhibiting adipocyte differentiation: An in vitro study. J Sci Food Agric 92:1948–1955.

Sontag, E. 2001. Protein phosphatase 2A: The Trojan horse of cellular signaling. Cell Signal 13:7–16.

Son MJ, Miura Y, Yagasaki K. 2014. Mechanisms for antidiabetic effect of gingerol in cultured cells and obese diabetic model mice. Cytotechnology May 4. [Epub ahead of print].

Song H, Guan Y, Zhang L, Li K, Dong C. 2010. SPARC interacts with AMPK and regulates GLUT4 expression. Biochem Biophys Res Commun 396:961–966.

Standaert, ML, Bandyopadhyay, G, Sajan, MP, Cong, L, Quon, MJ, Farese, RV. 1999. Okadaic acid activates atypical protein kinase C (zeta/lambda) in rat and 3T3/L1 adipocytes. An apparent requirement for activation of Glut4 translocation and glucose transport. J Biol Chem 274:14074–14078.

Wang Y, Zhang Y. 2010. Effect of bouts of swimming exercise on AMPKa2 expression in mouse skeletal muscle. J Exerc Sci Fit 8:11–16.

Wang WH, Wang ZM. 2005. Studies of commonly used traditional medicine-ginger. Zhongguo Zhong Yao Za Zhi 30:1569–1573.

White B., 2007. Ginger: an overview. Am Fam Physician 75:1689–1691.

Wu Y, Song P, Xu J, Zhang M, Zou MH. 2007. Activation of protein phosphatase 2A by palmitate inhibits AMP-activated protein kinase. J Biol Chem 282:9777–9788.

Yu HC, Lin CS, Tai WT, Liu CY, Shiau CW, Chen KF. 2013. Nilotinib induces autophagy in hepatocellular carcinoma through AMPK activation. J Biol Chem 288:18249–18259.

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