

# Hispidin rescues palmitate-induced insulin resistance in C2C12 myotubes

JONG SEOK LEE<sup>1\*</sup>, JUN MYOUNG PARK<sup>2\*</sup>, SARAH LEE<sup>1</sup>, HYE JIN LEE<sup>1</sup>, HEE-SUN YANG<sup>1</sup>, JOOHONG YEO<sup>1</sup>, KI RIM LEE<sup>2</sup>, BYUNG HYUN CHOI<sup>2</sup> and EOCK KEE HONG<sup>2</sup>

<sup>1</sup>National Institute of Biological Resources, Incheon, Gyeonggi 22689; <sup>2</sup>Department of Bioengineering and Technology, Kangwon National University, Chuncheon, Gangwon 24341, Republic of Korea

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**Abstract.** Skeletal muscle serves an important role in the utilization of glucose during insulin-stimulated conditions. Excessive saturated fatty acids are considered to be a major contributing factor to insulin resistance in skeletal muscle cells. The present study investigated the effects of hispidin on palmitate-induced insulin resistance in C2C12 skeletal muscle myotubes via an MTT assay, glucose uptake assay, Oil-Red-O staining and western blot analysis. Hispidin reversed the palmitate-induced inhibition of glucose uptake, and inhibited palmitate-induced intracellular lipid accumulation. Hispidin suppressed insulin receptor substrate-1 Ser307 phosphorylation, and significantly promoted the activation of phosphatidylinositol-3-kinase and Akt, via inhibition of protein kinase C theta. Furthermore, hispidin treatment of C2C12 muscle cells increased glucose uptake via activation of adenosine monophosphate-activated protein kinase. These findings indicated that hispidin may improve palmitate-induced insulin resistance in skeletal muscle myotubes, and therefore hispidin treatment may be beneficial for patients with diabetes.

## Introduction

Diabetes mellitus is a group of metabolic diseases characterized by high glucose levels resulting from defects in insulin secretion and action (1). There are two types of diabetes mellitus: Type 1 or insulin-dependent diabetes, which results from the destruction of pancreatic beta cells causing insulin deficiency, and type 2 or non-insulin-dependent diabetes, which is caused by the non-responsiveness of cells

to insulin (2-4). Over 90-95% of diabetes cases are type 2, which is largely associated with diet, lifestyle and excess body weight (5,6). In contrast, type 1 accounts for only 3-5% of diabetes cases. Insulin resistance is recognized as a major risk factor for the development of type 2 diabetes mellitus, and it is characterized by the reduced ability of insulin to regulate glucose homeostasis in target tissues, including muscle, liver and adipose (7). Among these target tissues, skeletal muscle is responsible for >75% of glucose disposal in response to insulin in the post-prandial state (8,9). Therefore, skeletal muscle insulin resistance is commonly viewed as a critical component of whole-body insulin resistance.

Numerous reports have demonstrated that obesity-associated insulin resistance is a major risk factor for skeletal muscle insulin resistance (10-12). It is believed that free fatty acid (FFA) levels become elevated prior to the onset of hyperglycemia in overweight and obese individuals, and this elevation is associated with impaired glucose tolerance (13,14). Furthermore, excess lipids in obese individuals raises circulating FFA levels and contributes to the development of type 2 diabetes (15). High FFA levels induce insulin resistance by inhibiting the insulin-signaling pathway in muscle cells. Skeletal muscle insulin resistance resulting from high FFA levels may be explained by different underlying mechanisms, including a direct effect on the function of proteins involved in the insulin signaling pathway, and the Randle hypothesis (16,17). Insulin signaling is initiated by insulin binding to the insulin receptor, which results in insulin receptor substrate-1 (IRS-1) phosphorylation, during which the receptor undergoes rapid tyrosine phosphorylation, and then forms a stable complex with the 85 kDa subunit of phosphatidylinositol-3-kinase (PI3K) (18). PI3K serves a major role in insulin signaling, predominantly via activation of protein kinase B (Akt), which activates glucose transporter 4 (GLUT4) (19). GLUT4 subsequently translocates to the membrane and imports glucose into the cell. Notably, it has been reported that an increase in intracellular FFA metabolites leads to phosphorylation of serine/threonine sites on IRS-1 (20). Serine/threonine phosphorylation of IRS-1 impairs its ability to associate with the insulin receptor, which inhibits subsequent insulin-stimulated tyrosine phosphorylation (21). Furthermore, serine phosphorylation of IRS-1 may result in inhibition of insulin-stimulated tyrosine phosphorylation of

*Correspondence to:* Dr Eock Kee Hong, Department of Bioengineering and Technology, Kangwon National University, 192-1 Hyoja-2-dong, Chuncheon, Gangwon 24341, Republic of Korea  
E-mail: ekhong@kangwon.ac.kr

\*Contributed equally

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the insulin receptor. This process leads to decreased PI3K activation, resulting in reduced downstream signaling and glucose transporter activity. As a result, elevated FFA inhibits glucose uptake (22,23).

*Phellinus linteus* (*P. linteus*) is a fungus that is used in Asian countries as a traditional medicine for the treatment of various diseases, including inflammation, neurodegenerative disorders and various cancers (24,25). A previous study demonstrated that phenolic compounds isolated from *P. linteus* may have antioxidant, antitumor and antimutagenic activities (26). *P. linteus* produces the yellow antioxidant pigments hispidin and hispidin derivatives. Hispidin has been indicated to possess strong antioxidative, protein kinase C inhibitory and anticancer activities (27,28). However, there have been no reports on the effects of hispidin on palmitate-induced insulin resistance in skeletal muscle cells. Therefore, the present study investigated the effect of hispidin on palmitate-induced insulin resistance in C2C12 skeletal muscle cells *in vitro*.

## Materials and methods

**Materials.** Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin and trypsin-EDTA were obtained from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Hispidin, fatty acid free bovine serum albumin (BSA) and palmitate were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Antibodies against IRS-1 (dilution 1:1,000; catalog no. 2390), phospho-IRS-1 Ser307 (dilution 1:1,000; catalog no. 2381), PI3K (dilution 1:1,000; catalog no. 4292), Akt (dilution 1:1,000; catalog no. 9272), phospho-Akt (dilution 1:1,000; catalog no. 9271), protein kinase C theta (PKC  $\theta$ , dilution 1:1,000; catalog no. 13,643), adenosine monophosphate-activated protein kinase (AMPK, dilution 1:1,000; catalog no. 2603), phospho-AMPK (dilution 1:1,000; catalog no. 2531), acetyl CoA carboxylase (ACC, dilution 1:1,000; catalog no. 3662), phospho-ACC (dilution 1:1,000; catalog no. 3661) and horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin (Ig)-G (dilution 1:1,000; catalog no. 7,074) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibodies against  $\beta$ -actin (dilution 1:1,000; catalog no. sc-47778) and HRP-conjugated goat anti-mouse IgG (dilution 1:10,000; catalog no. sc-2005) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). All other chemicals were analytical grade.

**Culture conditions and cell differentiation.** C2C12 myoblasts were derived from a mouse skeletal myoblast cell line. C2C12 myoblasts were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and grown in High Glucose DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml) and streptomycin (100  $\mu$ g/ml) in a humidified 5% CO<sub>2</sub> incubator at 37°C. The cells were cultured to ~60% confluence, harvested with 0.25% trypsin-EDTA, and subcultured for an additional 48 h. C2C12 myotubes were obtained by culturing myoblasts in DMEM containing 10% heat-inactivated horse serum (Gibco; Thermo Fisher Scientific, Inc.). Myotube formation was achieved following 4 days of incubation. To investigate palmitate-induced myotube atrophy and loss, cells

were fixed for 10 min with 4% formaldehyde, stained with 1% crystal violet solution for 15 min, and washed three times in water. Images were taken under a microscope. Differentiated cells were used for subsequent experiments.

**Preparation of palmitate-BSA complex and treatment.** The BSA-conjugated palmitate complex was prepared as previously described (29). In brief, palmitate was dissolved in 100 mM NaOH by heating at 70°C. After filtration, the solution was diluted with 10% fatty acid-free BSA and stored at -20°C. BSA-conjugated palmitate was used for subsequent experiments. Cells were treated with various concentrations of BSA-conjugated palmitate for 16 h. In all experiments, 2 h prior to treatment with fatty acids, the culture medium was replaced with serum-free DMEM. Control BSA was prepared by mixing 1 ml of 0.1 M NaOH with 9 ml of cell culture medium containing 3.3 mM of fatty acid-free BSA.

**Glucose uptake.** Glucose uptake assays were performed using the glucose analog 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NBDG, Thermo Fisher Scientific, Inc.), a fluorescent indicator of direct glucose uptake. To investigate the effect of palmitate, BSA-conjugated palmitate or hispidin on insulin-mediated glucose uptake, C2C12 myotubes were incubated with control BSA or various concentrations of palmitate and BSA-conjugated palmitate for 16 h and/or various concentrations and exposure times of hispidin, prior to measuring insulin-induced glucose uptake. Cells were pre-incubated in glucose-free DMEM containing Krebs Ringer Bicarbonate buffer (115 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl<sub>2</sub>, 1.2 mM MgSO<sub>4</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 20 mM NaHCO<sub>3</sub> and 10 mM HEPES at pH 7.4) with 2% fatty acid-free BSA at 37°C for 30 min. Cells were subsequently incubated in the presence or absence of insulin (100 nM) for 30 min and then further incubated with insulin and 50  $\mu$ M 2-NBDG for 2 h at 37°C in glucose-free Krebs Ringer bicarbonate buffer containing 2% BSA. The cells were subsequently washed with phosphate-buffered saline (PBS), and the relative fluorescence intensity was assessed at excitation and emission wavelengths of 485 and 535 nm, respectively, using a microplate fluorometer.

**Oil-Red-O staining.** To measure cellular lipid accumulation, C2C12 myotubes were stained using the Oil-Red-O method as described previously (30). Briefly, C2C12 cells were plated at a density of 1x10<sup>5</sup> cells/well in a 6-well plate. After differentiation, the C2C12 myotubes were treated with various concentrations of hispidin for 4 h, followed by 0.5 mM BSA-conjugated palmitate per well for 16 h. Cells were washed three times with PBS and fixed with 3.7% formaldehyde for 30 min at room temperature. After fixation, the cells were washed and stained with Oil-Red-O solution (stock solution, 3 mg/ml in isopropanol; working solution, 60% Oil-Red-O stock solution and 40% distilled water) for 60 min at room temperature, and then washed with water to remove unbound dye. The cells were air-dried on a clean bench, neat isopropanol was added, and the cells were agitated at room temperature for 30 min to elute the bound dye. Absorbance was determined at a wavelength of 520 nm using an ELISA microplate reader (Model 550; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

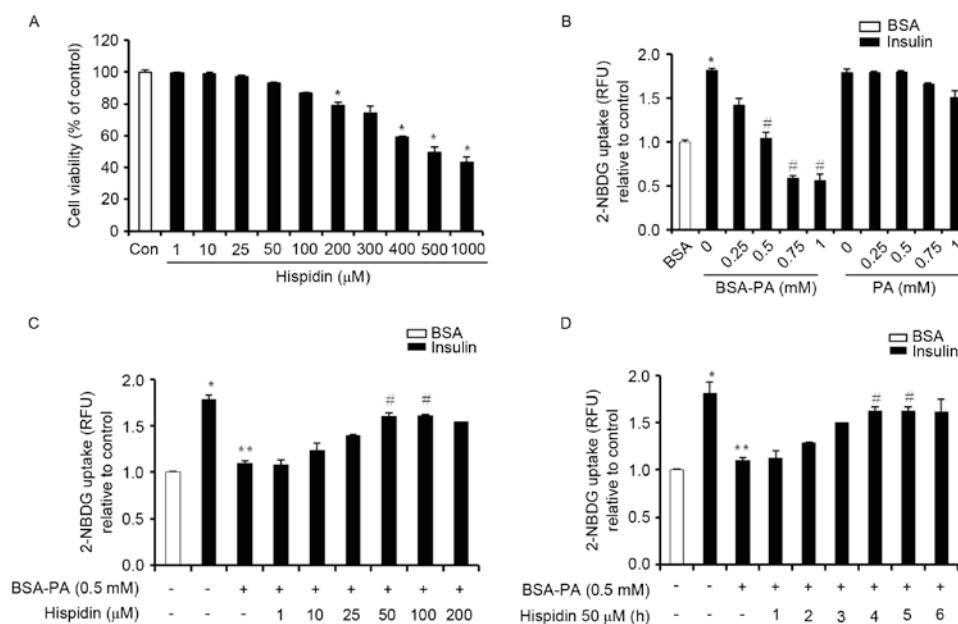


Figure 1. Effect of hispidin on insulin-stimulated glucose uptake in PA-treated C2C12 myotubes. (A) Cell viability of C2C12 myotubes treated with various concentrations of hispidin. \* $P < 0.05$  vs. control. (B) Effect of BSA-PA on insulin-induced glucose uptake in C2C12 myotubes. \* $P < 0.05$  vs. BSA control, \* $P < 0.05$  vs. 0 mM BSA-PA-treated. (C) Effect of hispidin on insulin-stimulated glucose uptake in PA-treated C2C12 myotubes. \* $P < 0.05$  vs. BSA control, \*\* $P < 0.01$  vs. insulin only-treated and \* $P < 0.05$  vs. 0.5 mM BSA-PA with insulin. (D) Longitudinal effect of hispidin on insulin-stimulated glucose uptake in palmitate-treated C2C12 myotubes. \* $P < 0.05$  vs. BSA, \*\* $P < 0.01$  vs. insulin only-treated and \* $P < 0.05$  vs. 0.5 mM BSA-PA with insulin. Data are presented as the mean  $\pm$  standard error of three independent experiments. BSA, bovine serum albumin; PA, palmitate; RFU, relative fluorescence units; 2-NBDG, 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose.

**Cell viability assay.** To assess the cytotoxic effects of hispidin on C2C12 myotubes, cell viability was assessed using the (3-[4,5-dimethyl thiazol-2-yl]-2,5-diphenyl tetrazolium) bromide (MTT) assay, which relies on the ability of viable cells to metabolically reduce the tetrazolium salt MTT to a purple formazan product, which can be quantified colorimetrically. Briefly, C2C12 cells were seeded at a density of  $5 \times 10^4$  cells/well in a 12-well plate and incubated for 48 h at 37°C in a humidified 5% CO<sub>2</sub> incubator. After differentiation, cells were incubated with hispidin (1-1,000  $\mu$ M) for 24 h. Following hispidin exposure, 50  $\mu$ l MTT stock solution (5 mg/ml) in serum-free medium was added to each well, and the mixture was incubated for 2 h at 37°C. The cells were subsequently washed with PBS and the supernatant was aspirated. The formazan crystals in each well were dissolved in isopropyl alcohol, and the absorbance was determined at a wavelength 570 nm using an ELISA microplate reader (Model 550; Bio-Rad Laboratories, Inc.).

**Western blot analysis.** Differentiated C2C12 myotubes were treated with various concentrations of hispidin for 4 h, followed by 0.5 mM BSA-conjugated palmitate per well for 16 h. Cells were subsequently incubated in the presence or absence of 100 nM insulin for 30 min. Cells were washed with PBS and lysed in lysis buffer [10 mM Tris-HCl (pH 7.5), 10 mM NaH<sub>2</sub>PO<sub>4</sub>/NaHPO<sub>4</sub> (pH 7.5), 130 mM NaCl, 1% Triton X-100, 10 mM sodium pyrophosphate, 1 mM phenyl methyl sulfonyl fluoride, 2  $\mu$ g/ml pepstatin A] for 30 min on ice. Lysates were centrifuged at 12,000  $\times$  g for 20 min at 4°C. The supernatant was collected, and the protein content of the supernatant was measured using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Inc.). A total of 50  $\mu$ g protein was added to each

lane of 10-15% gels, separated by SDS-PAGE and transferred onto 0.2  $\mu$ m nitrocellulose membranes (Bio-Rad Laboratories, Inc.). Membranes were blocked with 5% nonfat powdered milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for 1 h, and then incubated with primary antibodies at 4°C overnight. Finally, the membranes were incubated with HRP-conjugated secondary antibodies for 1 h at 4°C. The membranes were washed with TBS-T following each antibody incubation. Detection was performed using an Enhanced Chemiluminescence kit (Merck KGaA).

**Statistical analysis.** Data were reported as the mean  $\pm$  standard error. Experiments were performed in triplicate, and independently repeated at least three times. Data were compared using Student's t-test or a one-way analysis of variance followed by Duncan's multiple range test, using SAS software version 9.1 (SAS Institute, Inc., Cary, NC, USA).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Hispidin reverses palmitate-induced insulin resistance.** Concentration-response experiments were carried out to determine the highest non-toxic concentration of hispidin. MTT assays indicated that there was no significant alteration in cell viability following treatment with hispidin at concentrations from 1 to 1,000  $\mu$ M. At 200  $\mu$ M hispidin, cell viability was ~80% (Fig. 1A). Therefore, subsequent experiments were conducted using  $\leq 200$   $\mu$ M hispidin. To examine whether palmitate has an impact on insulin-mediated glucose uptake, C2C12 myotubes were incubated with various concentrations of palmitate and BSA-conjugated palmitate for 16 h,

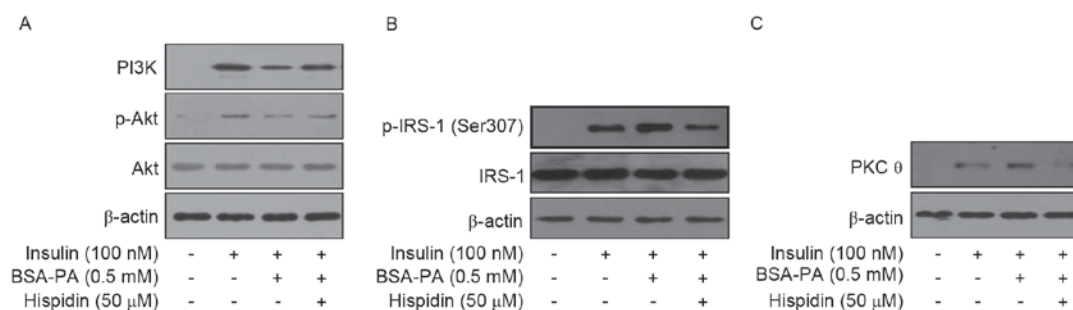


Figure 2. Effect of hispidin on insulin signaling molecules in PA-treated C2C12 myotubes. Representative western blot images of (A) PI3K p-Akt and Akt, (B) p-IRS-1 and IRS-1 and (C) PKC  $\theta$  protein expression levels.  $\beta$ -actin served as an internal control. BSA, bovine serum albumin; PA, palmitate; PI3K, phosphatidylinositol-3-kinase; IRS-1, insulin receptor substrate 1, p, phosphorylated; PKC  $\theta$ , protein kinase C  $\theta$ ; Akt, protein kinase B.

and insulin-induced glucose uptake was assessed. Treatment with 0.5 mM BSA-conjugated palmitate for 16 h lowered the stimulatory effect of insulin on glucose uptake by ~44%, compared with 0 mM BSA-PA-treated cells. The concentration of BSA-conjugated palmitate necessary to induce insulin resistance was 0.5 mM, this level of palmitate demonstrated a glucose uptake ability similar to that in the BSA-only control, which did not contain insulin (Fig. 1B). Unconjugated palmitate did not induce insulin resistance. To investigate the role of hispidin on BSA-conjugated palmitate-induced insulin resistance, C2C12 myotubes were incubated with 0.5 mM BSA-conjugated palmitate for 16 h and then treated with various concentrations of hispidin for 4 h. Hispidin treatment of C2C12 myotubes increased glucose uptake in a concentration- and time-dependent manner (Fig. 1C). Hispidin at concentrations of 50 and 100  $\mu$ M increased glucose uptake by 31 and 32%, respectively, compared with the 0.5 mM BSA-PA only-treated group. Furthermore, 50  $\mu$ M hispidin increased the glucose uptake in BSA-conjugated palmitate-treated C2C12 myotubes in a time-dependent manner (Fig. 1D). These results indicated that hispidin treatment may inhibit palmitate-induced insulin resistance in C2C12 myotubes.

**Effect of hispidin on insulin-dependent signaling.** The PI3K-Akt signaling pathway is involved in insulin-stimulated glucose uptake, and therefore its activation was assessed by western blot analysis. Insulin treatment stimulated Akt phosphorylation and expression levels of PI3K, which was reduced by treatment with 0.5 mM BSA-conjugated palmitate; however, treatment with hispidin slightly rescued insulin-induced Akt phosphorylation and expression levels of PI3K (Fig. 2A). Insulin signaling may be attenuated as a result of phosphorylation on IRS-1 serine residues. IRS-1 contains pleckstrin homology and phosphotyrosine domains, which provide a docking site for PI3K when phosphorylated, and serves a critical role in the insulin signaling pathway (31,32). To investigate the effects of hispidin on BSA-conjugated palmitate-induced IRS-1 Ser307 phosphorylation, C2C12 myotubes were incubated with 0.5 mM BSA-conjugated palmitate for 16 h. The cells were subsequently treated with 50  $\mu$ M hispidin for 4 h. Treatment with BSA-conjugated palmitate increased IRS-1 Ser307 phosphorylation in the presence of insulin, and this phosphorylation was slightly reduced by 50  $\mu$ M hispidin (Fig. 2B). These data indicated that hispidin exerts a recovery effect on insulin-dependent

signaling against palmitate-induced insulin resistance. IRS-1 phosphorylation on Ser307 following exposure of skeletal muscle cells to palmitate is facilitated by the diacylglycerol (DAG)-mediated activation of PKC  $\theta$ , therefore the ability of hispidin to modulate PKC  $\theta$  was investigated. Incubation of cells exposed to BSA-conjugated palmitate increased the expression of PKC  $\theta$ , however, this was inhibited by 50  $\mu$ M hispidin (Fig. 2C). These findings indicate that hispidin may reverse palmitate-induced insulin resistance, and this may occur via the insulin-signaling pathway, by preventing PKC  $\theta$  activation.

**Hispidin reduces lipid accumulation and increases glucose uptake in C2C12 myotubes.** Free fatty acids are converted into DAG and ceramide, and this induces lipid accumulation (13-15). Accumulated lipids stimulate insulin resistance by activating protein kinase C isoforms. To assess intracellular lipid accumulation, lipid content was measured by Oil-Red-O staining. A significant increase in lipid content in C2C12 myotubes was observed following treatment with palmitate for 16 h (Fig. 3A). However, treatment with various concentrations of hispidin reduced palmitate-induced lipid accumulation in C2C12 myotubes. To examine whether hispidin directly increases glucose uptake in C2C12 myotubes in the absence of palmitate, glucose uptake was measured in cells pretreated with various concentrations of hispidin for 4 h, or with 100 nM insulin for 30 min. After incubation with hispidin for 4 h, glucose uptake was enhanced by approximately 23% compared with that in the control (Fig. 3B).

**Effect of hispidin on AMPK signaling.** To investigate whether the hispidin-induced increase in glucose uptake was due to AMPK activation, AMPK and ACC phosphorylation were studied by western blot analysis. Hispidin exposure increased phosphorylation of AMPK and its downstream mediator ACC in a concentration-dependent manner, compared with that in the control (Fig. 4). These results indicated that hispidin may exert a beneficial effect on glucose uptake in C2C12 myotubes, via activation of the AMPK-ACC pathway.

## Discussion

Insulin resistance is commonly associated with obesity and obesity-associated features, including elevated plasma



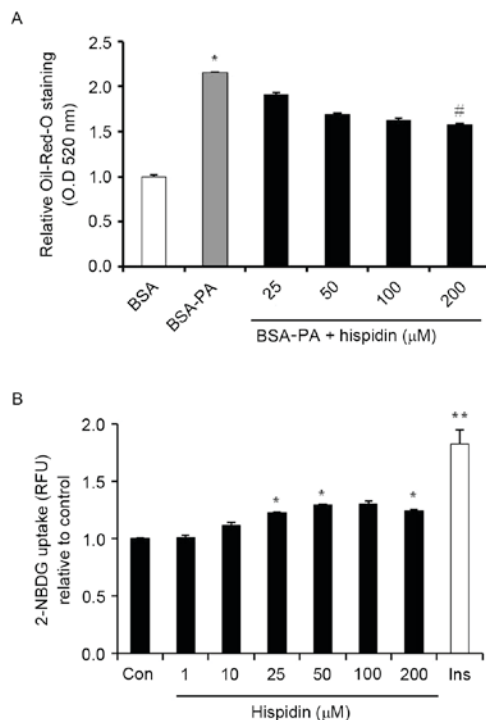


Figure 3. Effect of hispidin on lipid accumulation and glucose uptake in C2C12 myotubes. (A) Quantitative analysis of Oil-Red-O staining on BSA-PA-treated C2C12 myotubes. \* $P < 0.05$  vs. BSA control and # $P < 0.05$  vs. BSA-PA-only treatment. (B) Effect of hispidin on glucose uptake in C2C12 myotubes. \* $P < 0.05$  vs. control and \*\* $P < 0.01$  vs. control. Data are presented as the mean  $\pm$  standard error of three independent experiments. BSA, bovine serum albumin; PA, palmitate; Ins, insulin; OD, optical density; RFU, relative fluorescence units; 2-NBDG, 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose.

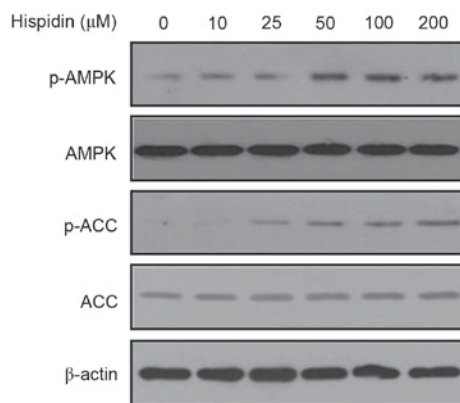


Figure 4. Effect of hispidin on AMPK phosphorylation in C2C12 myotubes. Representative western blot images of protein expression levels of p-AMPK (Thr172), AMPK, p-ACC (Ser79) and ACC.  $\beta$ -actin served as an internal control. AMPK, adenosine monophosphate-activated protein kinase; ACC, acetyl CoA carboxylase; p, phosphorylated.

FFA levels (11). In particular, because skeletal muscle is the main target of insulin action for glucose disposal, studies investigating FFA-induced insulin resistance in muscle cells are crucial to improve knowledge of this process. Previous studies have demonstrated the ability of various natural compounds to rescue skeletal muscle C2C12 myotubes from palmitate-induced insulin resistance, which may be due to higher stability and lower side effects (33-35). It is well

known that certain mushrooms, including *Phellinus linteus* and *Inonotus hispidus*, produce hispidin and its derivatives. Previous research has investigated the antioxidant activity of hispidin in palmitate-treated cells (36); however, currently there is no research investigating the effects of hispidin on palmitate-induced insulin resistance. The present study examined the effects of hispidin on palmitate-induced insulin resistance in muscle cells, at the level of insulin-dependent signaling and AMPK activation.

Incubation of skeletal muscle cells with saturated fatty acid results in the dysfunction of glucose uptake, and this has been implicated in the promotion of IRS-1 serine phosphorylation (37,38). Amongst these saturated fatty acids, palmitate is responsible for more than 25% of circulating FFAs in blood plasma. Exposure to high palmitate levels results in the activation of PKC $\theta$ , which phosphorylates IRS-1 at Ser307 (39). This phosphorylation results in inhibition of insulin signaling, which downregulates the PI3K-Akt signaling pathway and reduces insulin-stimulated glucose uptake. Consistent with these reports, the present study revealed that excess BSA-conjugated palmitate impaired glucose uptake and induced lipid accumulation; however, unconjugated palmitate did not induce insulin resistance. Palmitate is the most prominent saturated fatty acid utilized in the body. However, the utilization of palmitate in cell-based assays is challenging due to its low solubility in aqueous solutions. BSA has been used as a carrier and stabilizing agent for insoluble fatty acids. As palmitate conjugated to BSA creates an aqueous-soluble reagent that is absorbed and utilized by cells, it is appropriate for cell-based assays (40). This was accompanied by increased serine phosphorylation of IRS-1 and phosphorylation of PKC $\theta$ , and decreased activation of PI3K and Akt. Skeletal muscle cells exposed to BSA-conjugated palmitate demonstrated enhanced phosphorylation of IRS-1. In agreement with previous studies, the present study revealed that BSA-conjugated palmitate increased the levels of phosphorylation of IRS-1 (41,42). However, these effects were mostly rescued by treatment with hispidin. However, this was not observed in the present study. Two distinct signaling pathways with two distinct mechanisms are critical for glucose uptake: The insulin signaling pathway and AMPK activation. AMPK induces glucose uptake in an insulin-independent manner, and therefore, it may be an important alternative target for treatment of type 2 diabetes (43). Furthermore, AMPK inhibits lipid accumulation via the phosphorylation of ACC, which has been used as a biological research tool for the insulin signaling blockade (44). The present study demonstrated that hispidin increases AMPK and ACC phosphorylation in a concentration-dependent manner.

In conclusion, hispidin exhibits a notable capacity to improve palmitate-induced insulin resistance, via inhibition of lipid accumulation and regulation of the insulin signaling pathway. Hispidin may have a potent anti-diabetic effect, and may represent a useful agent for the treatment of type 2 diabetes in the future.

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## References

- Alberti KG and Zimmet PZ: Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: Diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. *Diabet Med* 15: 539-553, 1998.
- Mathis D, Vence L and Benoist C: beta-Cell death during progression to diabetes. *Nature* 414: 792-798, 2001.
- Kahn SE: The relative contributions of insulin resistance and beta-cell dysfunction to the pathophysiology of type 2 diabetes. *Diabetologia* 46: 3-19, 2003.
- Weyer C, Bogardus C, Mott DM and Pratley RE: The natural history of insulin secretory dysfunction and insulin resistance in the pathogenesis of type 2 diabetes mellitus. *J Clin Invest* 104: 787-794, 1999.
- Zimmet PZ: Diabetes epidemiology as a tool to trigger diabetes research and care. *Diabetologia* 42: 499-518, 1999.
- Zimmet P, Alberti KG and Shaw J: Global and societal implications of the diabetes epidemic. *Nature* 414: 782-787, 2001.
- Caro JF, Dohm LG, Pories WJ and Sinha MK: Cellular alterations in liver, skeletal muscle, and adipose tissue responsible for insulin resistance in obesity and type II diabetes. *Diabetes Metab Rev* 5: 665-689, 1989.
- Björnholm M and Zierath JR: Insulin signal transduction in human skeletal muscle: Identifying the defects in type II diabetes. *Biochem Soc Trans* 33: 354-357, 2005.
- Zierath JR, Krook A and Wallberg-Henriksson H: Insulin action and insulin resistance in human skeletal muscle. *Diabetologia* 43: 821-835, 2000.
- Kahn SE, Hull RL and Utzschneider KM: Mechanisms linking obesity to insulin resistance and type 2 diabetes. *Nature* 444: 840-846, 2006.
- Kahn BB and Flier JS: Obesity and insulin resistance. *J Clin Invest* 106: 473-481, 2000.
- Qatanani M and Lazar MA: Mechanisms of obesity-associated insulin resistance: Many choices on the menu. *Genes Dev* 21: 1443-1455, 2007.
- Yalow RS and Berson SA: Plasma insulin concentrations in nondiabetic and early diabetic subjects. Determinations by a new sensitive immuno-assay technic. *Diabetes* 9: 254-260, 1960.
- Shulman GI: Cellular mechanisms of insulin resistance. *J Clin Invest* 106: 171-176, 2000.
- Olefsky JM and Kolterman OG: Mechanisms of insulin resistance in obesity and noninsulin-dependent (type II) diabetes. *Am J Med* 70: 151-168, 1981.
- Ogawa W, Matozaki T and Kasuga M: Role of binding proteins to IRS-1 in insulin signaling. *Mol Cell Biochem* 182: 13-22, 1998.
- Kriauciunas KM, Myers MG Jr and Kahn CR: Cellular compartmentalization in insulin action: Altered signaling by a lipid-modified IRS-1. *Mol Cell Biol* 20: 6849-6859, 2000.
- Knight ZA, Gonzalez B, Feldman ME, Zunder ER, Goldenberg DD, Williams O, Loewith R, Stokoe D, Balla A, Toth B, *et al*: A pharmacological map of the PI3-K family defines a role for p110alpha in insulin signaling. *Cell* 125: 733-747, 2006.
- Kohn AD, Summers SA, Birnbaum MJ and Roth RA: Expression of a constitutively active Akt Ser/Thr kinase in 3T3-L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. *J Biol Chem* 271: 31372-31378, 1996.
- Saltiel AR and Kahn CR: Insulin signalling and the regulation of glucose and lipid metabolism. *Nature* 414: 799-806, 2001.
- Rui L, Aquirre V, Kim JK, Shulman GI, Lee A, Corbould A, Dunaif A and White MF: Insulin/IGF-1 and TNF-alpha stimulate phosphorylation of IRS-1 at inhibitory Ser307 via distinct pathways. *J Clin Invest* 107: 181-189, 2001.
- Homko CJ, Cheung P and Boden G: Effects of free fatty acids on glucose uptake and utilization in healthy women. *Diabetes* 52: 487-491, 2003.
- Bloch-Damti A and Bashan A: Proposed mechanisms for the induction of insulin resistance by oxidative stress. *Antioxid Redox Signal* 7: 1553-1567, 2005.
- Kim BC, Choi JW, Hong HY, Lee SA, Hong S, Park EH, Kim SJ and Lim CJ: Heme oxygenase-1 mediates the anti-inflammatory effect of mushroom *Phellinus linteus* in LPS-stimulated RAW264.7 macrophages. *J Ethnopharmacol* 106: 364-371, 2006.
- Guo J, Zhu T, Collins L, Xiao ZX, Kim SH and Chen CY: Modulation of lung cancer growth arrest and apoptosis by *Phellinus linteus*. *Mol Carcinog* 46: 144-154, 2007.
- Lee IK and Yun BS: Hispidin analogs from the mushroom *Inonotus xeranticus* and their free radical scavenging activity. *Bioorg Med Chem Lett* 16: 2376-2379, 2006.
- Jang JS, Lee JS, Lee JH, Kwon DS, Lee KE, Lee SY and Hong EK: Hispidin produced from *Phellinus linteus* protects pancreatic beta-cells from damage by hydrogen peroxide. *Arch Pharm Res* 33: 853-861, 2010.
- Chen W, Feng L, Huang Z and Su H: Hispidin produced from *Phellinus linteus* protects against peroxynitrite-mediated DNA damage and hydroxyl radical generation. *Chem Biol Interact* 199: 137-142, 2012.
- Koshkin V, Dai FF, Robson-Doucette CA, Chan CB and Wheeler MB: Limited mitochondrial permeabilization is an early manifestation of palmitate-induced lipotoxicity in pancreatic beta-cells. *J Biol Chem* 283: 7936-7948, 2008.
- Lim S, Rashid MA, Jang M, Kim Y, Won H, Lee J, Woo JT, Kim YS, Murphy MP, Ali L, *et al*: Mitochondria-targeted antioxidants protect pancreatic  $\beta$ -cells against oxidative stress and improve insulin secretion in glucotoxicity and glucolipotoxicity. *Cell Physiol Biochem* 28: 873-886, 2011.
- Evans JL, Maddux BA and Goldfine ID: The molecular basis for oxidative stress-induced insulin resistance. *Antioxid Redox Signal* 7: 1040-1052, 2005.
- Gual P, Le Marchand-Brustel Y and Tanti JF: Positive and negative regulation of insulin of insulin signaling through IRS-1 phosphorylation. *Biochimie* 87: 99-109, 2005.
- Zhao M, Zhang ZF, Ding Y, Wang JB and Li Y: Astragalus polysaccharide improves palmitate-induced insulin resistance by inhibiting PTP1B and NF- $\kappa$ B in C2C12 myotubes. *Molecules* 17: 7083-7092, 2012.
- Zhou QG, Hou FF, Guo ZJ, Liang M, Wang GB and Zhang X: 1,25-Dihydroxyvitamin D improved the free fatty-acid-induced insulin resistance in cultured C2C12 cells. *Diabetes Metab Res Rev* 24: 459-464, 2008.
- Deng YT, Chang TW, Lee MS and Lin JK: Suppression of free fatty acid-induced insulin resistance by phytopolyphenols in C2C12 mouse skeletal muscle cells. *J Agric Food Chem* 60: 1059-1066, 2012.
- Park JM, Lee JS, Song JE, Sim YC, Ha SJ and Hong EK: Cytoprotective effect of hispidin against palmitate-induced lipotoxicity in C2C12 myotubes. *Molecules* 20: 5456-5467, 2015.
- Schmitz-Peiffer C: Signalling aspects of insulin resistance in skeletal muscle: Mechanisms induced by lipid oversupply. *Cell Signal* 12: 583-594, 2000.
- Coll T, Eyre E, Rodríguez-Calvo R, Palomer X, Sánchez RM, Merlos M, Laguna JC and Vázquez-Carrera M: Oleate reverses palmitate-induced insulin resistance and inflammation in skeletal muscle cells. *J Biol Chem* 283: 11107-11116, 2008.
- Schmitz-Peiffer C: Protein kinase C and lipid-induced insulin resistance in skeletal muscle. *Ann NY Acad Sci* 967: 146-157, 2002.
- Ferrick DA, Neilson A and Beeson C: Advances in measuring cellular bioenergetics using extracellular flux. *Drug Discov Today* 13: 268-274, 2008.
- Coll T, Alvarez-Guardia D, Barroso E, Gómez-Foix AM, Palomer X, Laguna JC and Vázquez-Carrera M: Activation of peroxisome proliferator-activated receptor- $\delta$  by GW501516 prevents fatty acid-induced nuclear factor- $\kappa$ B activation and insulin resistance in skeletal muscle cells. *Endocrinology* 151: 1560-1569, 2010.
- Wang X, Yu W, Nawaz A, Guan F, Sun S and Wang C: Palmitate induced insulin resistance by PKC $\theta$ -dependent activation of mTOR/S6K pathway in C2C12 myotubes. *Exp Clin Endocrinol Diabetes* 118: 657-661, 2010.
- Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doebber T, Fujii N, *et al*: Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest* 108: 1167-1174, 2001.
- Kim MS, Hur HJ, Kwon DY and Hwang JT: Tangeretin stimulates glucose uptake via regulation of AMPK signaling pathways in C2C12 myotubes and improves glucose tolerance in high-fat diet-induced obese mice. *Mol Cell Endocrinol* 358: 127-134, 2012.