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# Effects of Icariin on insulin resistance via the activation of AMPK pathway in C2C12 mouse muscle cells

Q1 Yunkyung Han [a], Hyo Won Jung [a,b], Yong-Ki Park [a,b,*]

[a] Korean Medicine R&D Center, College of Korean Medicine, Dongguk University, Gyeongju 780-714, Republic of Korea
[b] Department of Herbology, College of Korean Medicine, Dongguk University, Gyeongju 780-714, Republic of Korea

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ABSTRACT

Insulin resistance in skeletal muscle is a major risk factor for the development of type 2 diabetes (T2D). In this study, we investigated the effect of icariin on insulin resistance in C2C12 mouse skeletal muscle cells. C2C12 myoblasts were differentiated into myotubes for five days, then treated with icariin (50 and 100 μM) or metformin (1 mM) in the presence of 100 nM insulin for 24 h. Adiponectin production was measured in culture media by ELISA, and AMP-activated protein kinase (AMPK)/insulin signaling pathway activation was assessed by the western blot analysis. Icariin significantly increased adiponectin production in C2C12 myotubes. Moreover, icariin markedly promoted the phosphorylation of AMPK and acetyl-CoA carboxylase (ACC). Icariin up-regulated the expression of phosphatidylinositol 3-kinase (PI3K) and the phosphorylation of insulin receptor substrate-1 (IRS-1) in C2C12 myotubes. These results suggest that icariin has therapeutic potential for the treatment of T2D via the regulation of insulin resistance in skeletal muscle.

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## 1. Introduction

Modern life style characterized by over-nutrition and lack of exercise has resulted in an increase in obesity and associated metabolic disorders such as type 2 diabetes (T2D) (Chellan et al., 2012). In treating diabetes, controlling the blood glucose level as close as possible to normal is important (Kimura et al., 2014). There is a considerable need for safe therapeutic agents that can stabilize blood glucose.

Insulin resistance is a major risk factor in T2D, which is characterized by a reduced ability of insulin action to regulate Q4 blood glucose concentrations (Deng et al., 2012; Lee et al., 2012). Due to its mass (>40% of total body weight), skeletal muscle becomes the primary site of insulin resistance during T2D development and is important in postprandial glucose homeostasis (Kimura et al., 2014; Aguer and Harper, 2012). Among the insulin signaling pathways associated with improvement in insulin resistance, the PI3K pathway is a key pathway of glucose uptake and glucose transport systems (Lee et al., 2012). Normally, the phosphorylation of insulin receptor substrate 1 (IRS-1) stimulates phosphatidylinositol 3-kinase (PI3K), an important step for stimulating insulin-induced glucose transport (Han et al., 2013). In an insulin-resistant state, tyrosine phosphorylation of IRS-1 is inhibited by c-Jun N-terminal protein kinase (JNK)-dependent serine phosphorylation of IRS-1, resulting in inhibition of insulin signaling (Mor et al., 2011). AMP-activated protein kinase (AMPK) is the downstream component of a protein kinase cascade that is crucial in the regulation of energy balance at both the cellular and whole-body level (Bijland et al., 2013). The activation of AMPK induces glucose uptake in muscle via an insulin-independent mechanism in T2D. AMPK and its signaling pathway are thus potential molecular targets in the development of drugs for the treatment of T2D such as metformin (Shen et al., 2014; Zhang et al., 2009). Metformin is widely used for the treatment of T2D by activating AMPK, reducing acetyl-CoA carboxylase (ACC) activity and inducing fatty acid oxidation (Zhou et al., 2001).

Icariin has a variety of pharmacological and biological activities (Chen et al., 2014), including cardiac protective effects (Pan et al., 2013), anti-tumor effects (Song et al., 2011), anti-osteoporotic effects (Zhou et al., 2011) and improved sexual function (Ji et al., 2009). Icariin is efficacious in treatment of renal damage in rats with streptozotocin-induced diabetic nephropathy (Qi et al., 2011). However, the potential mechanism underlying the anti-diabetic mechanism remains incompletely understood.

Q3 * Corresponding author at: Department of Herbology, College of Korean Medicine, Dongguk University, Gyeongju 780-714, Republic of Korea. Tel.: +82 54 770 2661; fax: +82 54 770 2647.
*E-mail addresses:* hanyk1028@hanmail.net (Y. Han), tenzing2@hanmail.net (H.W. Jung), yongki@dongguk.ac.kr (Y.-K. Park).

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Therefore, we investigated the anti-diabetic effects of icariin on insulin resistance in C2C12 mouse skeletal muscle cells, and its action mechanism in insulin-dependent or AMPK signaling pathway.

## 2. Materials and methods

### 2.1. Cell culture and differentiation

C2C12 mouse skeletal muscle cells (ATCC, Manassas, VA) were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; WelGENE biopharmaceuticals, Daegu, Korea) at 37 °C under a 5% $CO_2$ atmosphere. For differentiation into myotubes, C2C12 myoblasts (called C2C12 cells in following sentences) were cultured in DMEM and supplemented with 2% horse serum, which was changed every other day (Mor et al., 2011). Myotubes formation was achieved (Veliça and Bunce, 2011) after 5 days of incubation, and the cells were used for subsequent experiments. Icariin (from Sigma-Aldrich (St. Louis, MO)) was added during the last 24 h at the indicated concentrations in the presence of 100 nM insulin.

### 2.2. Cell viability assay

Cell viability was measured using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT; Roche, Mannheim, Germany). The cells were cultured in DMEM containing 10% FBS and various concentrations of icariin for 24 h. The MTT working solution was added to each well and incubated for 4 h at 37 °C (final concentration, 0.5 mg/ml). After the MTT solution was removed, the formazan formed inside the cells was dissolved in 100 μl of DMSO. The absorbance was measured at 550 nm on a microplate reader (GENios, TEKAN Instruments, Inc., Austria).

### 2.3. Quantification of adiponectin

Adiponectin concentrations were measured in culture media by a commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instruction (R&D systems, Inc., Minneapolis, USA). The concentration of adiponectin was calculated with the equation obtained from a standard curve using the standard solution in the ELISA kit.

### 2.4. Western blot

C2C12 cells were lysed with lysis buffer (10 mM Tris–HCl, pH 7.9, 10 mM NaCl, 3 mM $MgCl_2$, and 1% NP-40) and the cell lysates were centrifuged at 14,000 rpm for 20 min at 4 °C, and the supernatants were collected as whole cell extracts. For western blotting, equal amounts of total cellular protein (30 μg) were subjected to SDS-PAGE gels and transferred onto nitrocellulose membranes. Incubation with primary and secondary antibodies was overnight at 4 °C. The antibodies used in this study were an anti-IRS-1 (1:1000, Cell Signaling Technology, Beverly, MA), anti-phospho-IRS-1 (1:1000, Cell Signaling Technology), anti-PI3K (1:1000, Cell Signaling Technology), anti-AMPK (1:1000, Cell Signaling Technology), anti-phospho-AMPK (1:1000, Cell signaling Technology), anti-ACC (1:1000, Cell signaling Technology), anti-phospho-ACC (1:1000, Cell signaling Technology), anti-β-actin (1:1000, Sigma-Aldrich) and horseradish peroxidase (HRP)-labeled anti-rabbit or mouse IgG (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA).

### 2.5. Statistical analysis

Continuous data from all experiments are expressed as the mean ± standard deviation (S.D.) computed from at least three independent experiments. Statistical analysis was carried out by one-way ANOVA with a post-hoc test using GraphPad Prism 5.0 statistical analysis software (GraphPad Software, Inc., San Diego, CA). Null hypotheses of no difference were rejected if *P*-values were less than.05.

## 3. Results

### 3.1. Effect of icariin on the production of adiponectin

We investigated the effect of icariin on the levels of adiponectin which plays an important role on insulin sensitivity in C2C12 cells. As shown in Fig. 1, treatment with icariin in C2C12 cells, especially at a concentration of 100 μM, significantly increased the production of adiponectin compared with control cells. This increasing effect of icariin on adiponectin production was similar to that of metformin-treated cells.

### 3.2. Effect of icariin on the phosphorylation of AMPK and ACC

In this study, we investigated the effect of icariin on the phosphorylation of AMPK and ACC by the western blotting analysis. As shown in Fig. 2, treatment of C2C12 cells with icariin at a concentration of 100 μM significantly increased the phosphorylation of AMPK and its substrate, ACC, compared with control group. Metformin also slightly increased the phosphorylation of AMPK and ACC, but the expression levels were lower than those in icariin treated cells.

### 3.3. Effect of icariin on insulin signaling pathway

To investigate the effect of icariin on insulin signaling pathway, we examined the expression of the IRS-1 and PI3K in C2C12 cells. As shown in Fig. 3, the IRS-1 phosphorylation and PI3K activation increased in icariin-treated cells compared with those in control cells. Metformin also increased the IRS-1 phosphorylation and PI3K activation compared with control group, but the expression levels were lower than those in icariin-treated cells.

## 4. Discussion

The present study provides the evidence that the icariin activated the insulin signaling pathway by increasing PI3K/IRS-1 phosphorylation and AMPK in C2C12 skeletal muscle cells. Our results suggest that icariin has anti-diabetic activity through activation of the insulin signaling pathway.

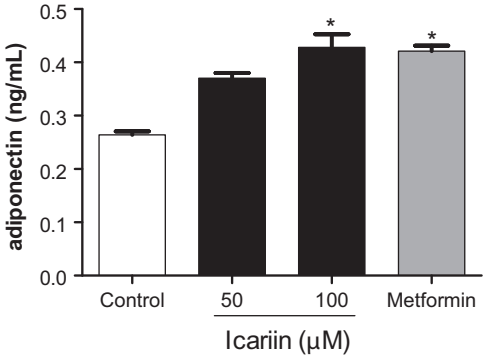


**Fig. 1.** Effect of icariin on the production of adiponectin in C2C12 cells. The cells were differentiated from myoblasts to myotubes for 5 days with 2% horse serum, and treated with icariin (50 and 100 μM) or metformin (1 mM) in the presence of 100 nM insulin for 24 h. Adiponectin was measured in culture media by ELISA. Each value represents the mean ± S.D. ($n=3$). $^*P<0.05$ versus control.

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