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Eucommia ulmoides Extract Stimulates Glucose Uptake through PI 3-kinase Mediated Pathway in L6 Rat Skeletal Muscle Cells

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Abstract

Eucommia ulmoides **(Duchung) is commonly used for treatment of diabetes in Korean traditional medicine. However, the exact mechanism of its anti-diabetic effect has not yet been fully elucidated. In this study, the effect of *E. ulmoides* extract on glucose uptake was investigated in L6 rat skeletal muscle cells. *E. ulmoides* extract stimulated the activity of phosphatidylinositol (PI) 3-kinase that is a major regulatory molecule in glucose uptake pathway. Protein kinase B (PKB) and protein kinase C-ζ (PKC-ζ), downstream mediators of PI 3-kinase, were also activated by *E. ulmoides* extract. We assessed the activity of AMP-activated protein kinase (AMPK), another regulatory molecule in glucose uptake pathway. Phosphorylation level of AMPK did not change with treatment of *E. ulmoides* extract. Phosphorylations of p38 mitogen activated protein kinase (p38 MAPK) and acetyl-CoA carboxylase (ACC), downstream mediators of AMPK, were not significantly different. Taken together, our results suggest that *E. ulmoides* may stimulate glucose uptake through PI 3-kinase but not AMPK in L6 skeletal muscle cells.**

Keywords: *Eucommia ulmoides*, Glucose uptake, Phosphatidylinositol (PI) 3-kinase, Protein kinase B, Protein kinase C-ζ

Eucommia ulmoides Oliver (Duchung) belongs to a single species genus in the plant family Eucommiaceae. The bark of this plant has been used as a tonic to strengthen the liver and kidney, and its leaves have been used as food and tea[1]. Twenty two known compounds, consisting of eight iridoids, seven flavonoids, five phenolic derivatives, and two triterpenoids, were identified from the roasted leaves of *E. ulmoides*[2]. Recent pharmacological researches on the leaves have shown their hypotensive effects in humans and spontaneous hypertensive rats (SHR)[3]. In addition, the extract of *E. ulmoides* leaves may have recuperative effects for hypercholesterolemia and fatty liver[4]. Hsieh and Yen[5] reported that the water extract of *E. ulmoides* leaves exhibits antioxidant activity with a good correlation between polyphenol contents and antioxidant activity. Recently, Lee *et al*.[6] reported that *E. ulmoides* leaves improve hyperglycemia and enhance the function of pancreatic β-cell in streptozotocin-induced type 1 diabetic rat model. In addition, *E. ulmoides* leaves also showed hypoglycemic effect in C57BL/KsJ-db/db mice[7].

Maintenance of glucose homeostasis is critical for normal physiology, and alteration of glucose level directly affects various insulin actions. In recent, many studies on insulin-responsive glucose transport have been reported using the cultured adipocyte model[8]. Both adipocyte and skeletal muscle cells exhibit glucose transporter 4 (GLUT4) translocation and glucose uptake, which is dependent on phosphatidylinositol (PI) 3-kinase activity. However, enhanced glucose transport in skeletal muscle cells is also induced by the insulin-independent mechanism that is activated by muscle contractions and hypoxia[9,10], which is thought to be mediated via activation of 5′-AMP-activated protein kinase (AMPK)[11,12]. Although hypoglycemic effect of *E. ulmoides* extract has been reported, the exact mechanism of its hypoglycemic effect is yet to be fully elucidated. Therefore, we investigated the effect of *E. ulmoides* leaves extract on glucose uptake

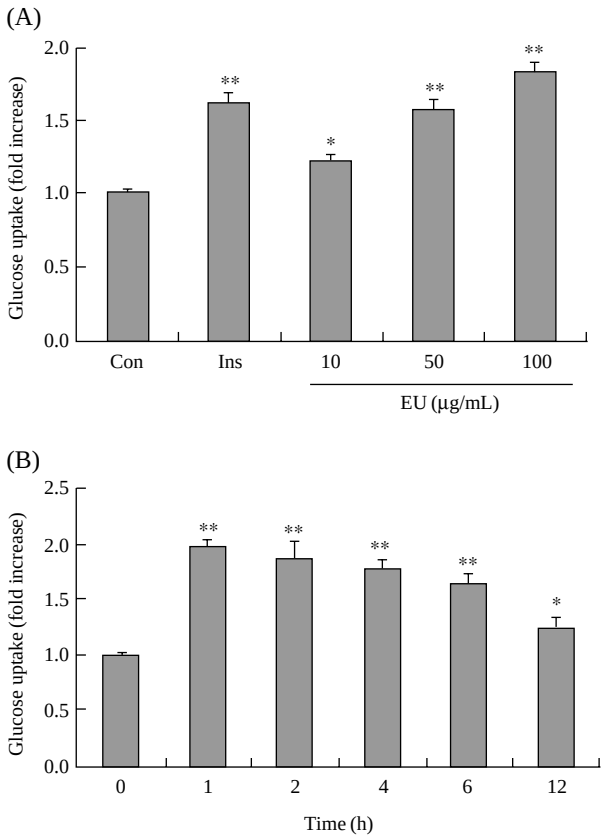

Figure 1. *E. ulmoides* extract stimulates glucose uptake in L6 skeletal muscle cells. A: Cells were incubated in serum free media for 2 h, and maintained for 30 min with various concentrations of *E. ulmoides* extract. The 2-[^{3}H] deoxy-d-glucose uptake was measured using a scintillation counter. Insulin (100 nM) was used as a positive control. B: Cells were incubated with 100 μg/mL *E. ulmoides* extract at different time periods up to 6 h. Data are the mean ± S.E.M. of at least five experiments. Con, control; Ins, insulin; EU, *E. ulmoides* extract, $^{**}P < 0.01$, $^{*}P < 0.05$ vs. control.

in skeletal muscle cells by assessing the activities of PI 3-kinase and AMPK, two crucial mediators in the glucose uptake pathway.

E. ulmoides Extract Stimulates Glucose Uptake in Skeletal Muscle Cells

We examined the dose- and time-dependent effects of *E. ulmoides* extract on glucose uptake using 2-[^{3}H] deoxy-d-glucose in L6 skeletal muscle cells. For treatment with *E. ulmoides* extract, cells were incubated in serum-free media for 2 h, and then with the indicated concentrations of *E. ulmoides* extract for 30 min (Figure 1A). Glucose uptake was induced in a concentration dependent manner, showing a maximum ~2 -fold induction at 100 μg/mL (Figure 1A). *E. ulmoides* extract did not influence the viability of L6 skeletal

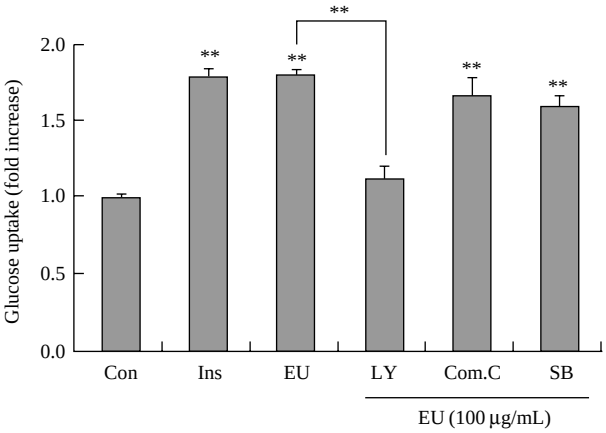

Figure 2. *E. ulmoides* extract-stimulated glucose uptake is blocked by LY294002, a PI 3-kinase inhibitor. L6 skeletal muscle cells were pretreated with 10 μM compound C (AMPK inhibitor), 20 μM SB202190 (p38 MAPK inhibitor), and 20 μM LY294002 (PI 3-kinase inhibitor) for 30 min, and then treated with 100 μg/mL *E. ulmoides* extract for 1 h. Data are the mean ± S.E.M. of at least five experiments. Ins, insulin; EU, *E. ulmoides* extract; PI-3-P, phosphatidylinositol-3-phosphate; LY, LY294002; Com.C, compound C; SB, SB202190. $^{**}P < 0.01$ vs. control or between two groups as indicated.

muscle cells in dose ranges between 0 and 100 μg/mL, as assessed by MTT assay, but severe toxicity was observed at high concentration (data not shown). Therefore, concentration of 100 μg/mL was used in subsequent experiments. We next determined the time-dependent effect, and cells were maintained in serum-free media with *E. ulmoides* extract (100 μg/mL) for the indicated times (Figure 1B). Activation induced by *E. ulmoides* extract was reached a peak after 1 h and gradually decreased until 6 h.

PI 3-kinase Inhibitor Blocks *E. ulmoides* Extract-stimulated Glucose Uptake

To determine whether PI 3-kinase or AMPK is involved in the effect of *E. ulmoides* extract on glucose uptake, we investigated experiments on inhibition of PI 3-kinase, AMPK, and p38 mitogen activated protein kinase (p38 MAPK; AMPK down streamer). LY294002, a PI 3-kinase inhibitor, significantly reduced *E. ulmoides* extract-stimulated glucose uptake ($P < 0.01$). However, compound C (an AMPK inhibitor) and SB202190 (a p38 MAPK inhibitor) did not reduced uptake (Figure 2). These results indicate that PI 3-kinase has a crucial role in *E. ulmoides* extract-stimulated glucose uptake.

E. ulmoides Extract-stimulated Glucose Uptake is Mediated by PI 3-kinase

PI 3-kinase plays a critical role in insulin-stimulated

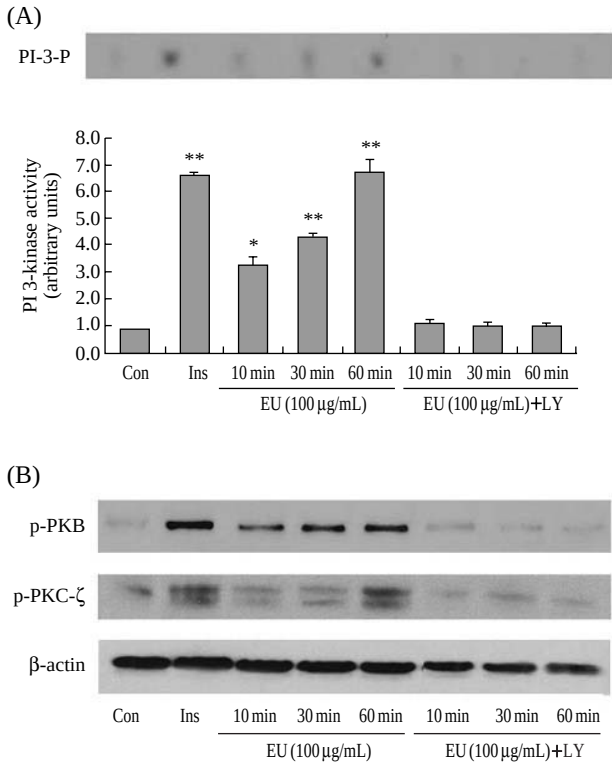

Figure 3. *E. ulmoides* extract increases phosphatidylinositol 3-kinase (PI 3-kinase), protein kinase B (PKB), and protein kinase C-ζ (PKC-ζ) activities. A: PI 3-kinase activity was measured in anti-phosphotyrosine immunocomplex. Cells were treated with 100 μg/mL *E. ulmoides* extract. Insulin (100 nM) was used as a positive control. B: Phosphorylations of PKC-ζ and PKB were analyzed by immunoblotting. Cells were treated with 100 μg/mL *E. ulmoides* extract with 20 μM LY294002 (PI 3-kinase inhibitor). β-actin was used as an internal control. Con, control; Ins, insulin; EU, *E. ulmoides* extract; PI-3-P, phosphatidylinositol-3-phosphate; LY, LY294002; p-PKB, phospho-protein kinase B; p-PKC-ζ, phospho-protein kinase C-ζ. $*P < 0.05$, $**P < 0.01$ vs. control.

glucose uptake. We performed the PI 3-kinase assay using [γ-^{32}P] ATP. In Figure 3A, an autoradiogram of a TLC plate shows the incorporation of ^{32}P into the 3′ position of PI. PI-3-phosphate (PI-3-P), which is formed by PI 3-kinase activity, markedly was increased by 100 nM insulin administration for 30 min (~7-fold compared to control). Insulin was used as a positive control[13]. Levels of PI-3-P were increased by *E. ulmoides* extract for 10, 30, and 60 min, compared to control. Densitometry analysis showed that activities of PI 3-kinase stimulated by *E. ulmoides* extract for 10, 30, and 60 min were 3.2, 4.3, and 6.7-fold higher as compared to those of control, respectively. The *E. ulmoides* extract-stimulated PI 3-kinase activity was completely blocked by treatment of LY294002 (Figure 3A). Protein kinase (PK) B and PKC-ζ are downstream molecules of PI 3-kinase. As shown in Figure 3B, phosphorylation levels of PKB and PKC-ζ by treatment of *E. ulmoides* extract were increased in a time-dependent manner. LY294002 almost inhibited the *E. ulmoides* extract-stimulated phosphorylation of PKB and PKC-ζ. These results conform that PI 3-kinase is a key molecule in the *E. ulmoides* extract-stimulated glucose uptake.

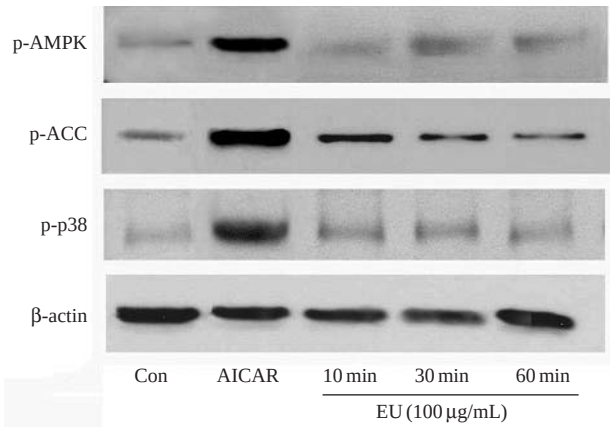

Figure 4. *E. ulmoides* extract shows no effect on phosphorylations of AMP-activated protein kinase (AMPK), acetyl-CoA carboxylase (ACC), and p38 mitogen activated protein kinase (p38 MAPK). Phosphorylations of AMPK, ACC, and p38 MAPK were analyzed by immunoblotting. Cells were treated with 100 μg/mL *E. ulmoides* extract. AICAR (500 μM) was used as a positive control. β-actin was used as an internal control. EU, *E. ulmoides* extract; AICAR, 5-aminoimidazole-4-carboxamide-ribonucleoside; p-AMPK, phospho-AMP-activated protein kinase; p-ACC, phospho-acetyl-CoA carboxylase; p-p38 MAPK, phospho-p38 mitogen activated protein kinase.

E. ulmoides Extract-stimulated Glucose Uptake is not Mediated by AMPK

Next, we investigated the effect of the extract on AMPK and its downstream molecules, acetyl-CoA carboxylase (ACC) and p38 MAPK. The 5-aminoimidazole-4-carboxamide-ribonucleoside (AICAR, 500 μM)[14], an AMPK agonist, as a positive control was used. In Figure 4, AICAR increased phosphorylations of AMPK, ACC, and p38 MAPK, compared to control. *E. ulmoides* extract did not affect AMPK, ACC, and p38 MAPK activations for 10, 30, and 60 min (Figure 4). These results suggest that the *E. ulmoides* extract-stimulated glucose uptake is not mediated by AMPK pathway.

Discussion

The present study shows that *E. ulmoides* stimulates

glucose uptake by activating PI 3-kinase in L6 skeletal muscle cells. Despite of reports on the hypoglycemic effect of *E. ulmoides*, the exact mechanism of its anti-diabetic effect has not been fully elucidated. To our knowledge, this is first report demonstrating the anti-diabetic effect of *E. ulmoides* in glucose uptake through PI 3-kinase pathway in L6 skeletal muscle cells.

Skeletal muscle is known as a major tissue in glucose metabolism, accounting for about 75% of whole -body insulin-stimulated glucose uptake[15]. In skeletal muscle, glucose uptake occurs through two major pathways. One is the PI 3-kinase pathway. PI 3-kinase is a key molecule that mediates glucose transport by insulin[16]. Activation of PKB can lead to the translocation of GLUT4[17]. PKC participates in the insulin-stimulated glucose transport in skeletal muscle[18]. Therefore, we investigated PI 3-kinase, PKB, and PKC -ζ activities to identify signaling molecules involved in the *E. ulmoides*-stimulated glucose uptake.

First of all, *E. ulmoides* increased glucose uptake, and blocked by LY294002, a PI 3-kinase inhibitor (Figures 1 and 2). In Figure 3A, *E. ulmoides* stimulated PI 3-kinase with an increase in time (10, 30, and 60 min). PI 3-kinase activity stimulated by *E. ulmoides* was decreased to basal line by LY294002. In Figure 3B, PKB and PKC-ζ, downstream mediators of PI 3-kinase, were also activated when the extract was treated. These data suggest that PI 3-kinase is involved in the *E. ulmoides*-stimulated glucose uptake in L6 skeletal muscle cells.

AMPK is another regulatory molecule in glucose uptake. It is activated by various stimuli such as exercise, hypoxia, and hyperosmolarity[18]. AMPK stimulates glucose transport, as an independent PI 3-kinase pathway[19]. Activation of AMPK leads to activation of p38 MAPK, a downstream molecule of AMPK, resulting in the translocation and activation of GLUT4[20,21]. In this study, we found that *E. ulmoides* did not change phosphorylation levels of AMPK at Thr 172 and p38 MAPK. There was no significant difference in the phosphorylation level of ACC at Ser79, a downstream target of AMPK[22] (Figure 4). This result was confirmed by data, showing that *E. ulmoides*-stimulated glucose uptake did not inhibit by compound C, an AMPK inhibitor, and SB202190, a p38 MAPK inhibitor (Figure 2). From these results, we suggest that *E. ulmoides* does not stimulate glucose uptake by the AMPK pathway in skeletal muscle cells.

In conclusion, we found that *E. ulmoides* significantly stimulated glucose uptake in L6 skeletal muscle cells. An interesting result was that glucose uptake by *E. ulmoides* might be mediated via PI 3-kinase, but not via AMPK.

Materials and Methods

Extract Preparation

E. ulmoides leaves were purchased from the Kyungdong market (Seoul, Korea) and authenticated by College of Oriental Medicine, Semyung University. Methanol extract from *E. ulmoides* (yield: 19.7% of dry weight) was obtained by 48 h maceration at room temperature, and the extract was filtered through a 0.45 μM filter (Osmonics, Minnetonka, MN, USA), lyophilized, and kept at 4˚C.

Materials

All chemicals were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA), unless otherwise indicated. Fetal bovine serum was purchased from Invitrogen (Carlsbad, CA, USA). Dulbecco's modified Eagle's medium (DMEM) and other culture products were purchased from GIBCO BRL (San Diego, CA, USA). [γ-^{32}P] ATP (6,000 Ci/mM) and 2-[^{3}H] deoxy-d-glucose (6.0 Ci/mM) were purchased from PerkinElmer Life And Analytical Sciences, Inc. (Boston, MA, USA). The anti-phospho specific antibodies of AMPK, ACC, p38 MAPK, PKB, and PKC-ζ were obtained from Upstate (Charlottesville, VA, USA) and Cell Signaling Technology (Beverly, MA, USA).

Cell Culture

L6 skeletal muscle cells was incubated in growth media containing DMEM with 0.045 g/mL glucose, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum. Cells were maintained in a humidified 37˚C incubator with ambient oxygen and 5% CO_2.

Glucose Uptake Assay

Glucose uptake was determined as previously described[23]. In brief, cells were washed with Krebs-Ringer phosphate buffer (KRB) (25 mM HEPES, 118 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.3 mM $MgSO_4$, and 5 mM $NaHCO_3$) containing 0.07 % bovine serum albumin and 5.5 mM glucose, and then incubated in KRB buffer for 60 min. Cells were treated with 100 nM insulin, or 10, 50, and 100 μg/mL of *E. ulmoides* leaves extract for 30 min. Glucose uptake was measured by adding 20 μL glucose mixture (5 mM 2-deoxyglucose and 0.5 μCi 2-[^{3}H] deoxy -d-glucose) to 980 μL KRB for 20 min. After washing with ice-cold phosphate-buffered saline (PBS), cells were lysed with 0.5 M NaOH solution containing 0.1% SDS. Nonspecific glucose uptake was also measured by treatment of 10 μM cytochalasin B, which blocks the transporter-mediated glucose uptake. Radioactivi-

ty was measured using a liquid scintillation counter (PerkinElmer Life and Analytical Sciences, Inc., Boston, MA, USA).

Immunoprecipitation

Immunoprecipitation was determined as previously described[24]. In brief, cells were solubilized by 1 mL lysis buffer (50 mM Tris HCl, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM sodium orthovanadate, 100 mM NaF, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 5 μg/mL leupeptin, and 1 mM phenylmethylsulfonyl fluoride) on ice. The cell lysates were then centrifuged at 15,000 × g for 15 min at 4˚C, and the supernatant was harvested. For immunoprecipitation, 20 μL anti-phosphotyrosine antibody agarose beads was incubated with supernatant containing 500 μg protein for 1 h at 4˚C. Immunoprecipitates were washed three times with PBS containing 1% Nonidet P-40, three times with 100 mM Tris HCl containing 500 mM $LiCl_2$, and finally twice with 25 mM Tris HCl containing 100 mM NaCl and 1 mM EDTA.

PI 3-kinase Assay

Immunoprecipitates were resuspended in 100 μL kinase assay buffer (20 mM Tris HCl, 75 mM NaCl, 10 mM $MgCl_2$, 200 μg/mL phosphatidylinositol, 1 mM EGTA, 20 μM ATP, and 10 μCi [γ-^{32}P] ATP), and incubated for 30 min at room temperature. PI 3-kinase activity was measured by the phosphorylation of PI. The reaction was stopped by addition of 100 μL 1 M HCl, and the reaction products were extracted with 200 μL chloroform : methanol (1 : 1) mixture. The samples were centrifuged, and the lower organic phase was harvested and applied to a silica gel thin layer chromatography (TLC) plate (Merck, Aichach, Germany) coated with 1% potassium oxalate. TLC plates were developed in chloroform : methanol : ammonium hydroxide : water (60 : 47 : 11.3 : 2) mixture, dried, and visualized by autoradiography.

Immunoblotting

Cell lysates were centrifuged for 15 min at 12,000 × g at 4˚C, and the supernatant was collected. Proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane (Schleicher & Schuell, Middlesex, UK). After transfer, the membrane was blocked with 5% nonfat milk in Tris buffered saline plus 0.1% Tween 20 (TBS-T), and then incubated with 1 : 1,000 diluted phospho-AMPK, phospho-ACC, phospho-p38 MAPK, phospho-PKB, phospho-PKC-ζ, and β-actin antibodies. The membrane was incubated with a horseradish peroxidase-conjugated secondary antibody (1 : 2,000). The immunoreactive bands were detected by an enhanced chemiluminescence kit (Amersham Pharmacia, Uppsala, Sweden).

Statistical Analysis

Statistical analysis was performed using Student's t-test. The accepted level of significance was preset as $P < 0.05$. Data are presented as mean ± S.E.M. All calculations were performed using SPSS software (SPSS, Inc., Chicago, IL, USA).

Acknowledgements

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