Effect of Ecklonia Cava on glucose consumption in rat skeletal muscle cells

Jian Guan, Deok Bae Park*

Department of Histology, Jeju National University School of Medicine, Jeju, Korea

Abstract

Ecklonia cava is a kind of brown sea algae and its extract has known to be anti-adipogenic, anti-inflammatory, and so on. However, It less has been known about the action mechanism in regulating glucose metabolism in skeletal muscle cells. The present study investigated the effect of the ethanol extract of Ecklonia cava (E-EC) on glucose consumption and its related signaling properties in L6 rat skeletal muscle cells.

E-EC stimulated glucose consumption as well as the translocation of glucose transporter 4 (Glut4) from cytosol to plasma membrane in L6 muscle cells. E-EC also stimulated PI3 kinase (PI3K)-dependent Akt activity, one of crucial signaling cascades for glucose uptake. Inhibition of PI3K suppresed E-EC-stimuated glucose consumption and Glut4 translocation. However, E-EC failed to stimulate AMPK. These results suggest that the extract of Ecklonia cava stimulates glucose consumption regardless insulin activity in skeletal muscle cells via PI3K-Akt pathway through stimulating Glut4 translocation to the plasma membrane. (J Med Life Sci 2012;9:44-48)

Key Words : Ecklonia cava, glucose, PI3 kinase, muscle

Introduction

Type 2 diabetes, called non-insulin-dependent diabetes mellitus, is a metabolic disorder that is characterized by high glucose in the context of insulin resistance or relative insulin deficiency. Diabetes mellitus can cause many complications, including acute (ketoacidosis, etc.) as well as serious long-term (cardiovascular disease, chronic renal failure, retinal damage, nerve damage, etc.) problems. The insulin resistance is a physiological condition where the natural hormone insulin becomes less effective at lowering blood sugars. These lead to increasing in blood glucose levels and cause adverse health effects, depending on dietary conditions. Certain cell types such as fat and muscle cells require insulin to absorb glucose. When these cells fail to respond adequately to circulating insulin, blood glucose levels rise. The liver and muscle regulate glucose levels by absorbing the glucose in the presence of insulin.

Insulin signaling is mediated by a complex network linking to a variety of different processes. Briefly, in the presence of insulin, the insulin receptor phosphorylates insulin receptor substrate (IRS) proteins, which are linked to the phosphatidylinositol 3 kinase (PI3K)-dependent pathway, at present, known as an essential role in insulin-stimulated translocation of glucose transporter 4 (Glut4) from cytosol to plasma membrane.

AMP-activated protein kinase (AMPK), a phylogenetically conserved serine/threonine protein kinase, promotes ATP-producing and inhibits ATP-consuming pathways in various tissues[1]. AMPK stimulates oxidation of fatty acids and inhibits their synthesis, and it is also involved in promoting glucose uptake[2]. AMPK seems to be responsible in part for this exercise-induced glucose uptake[3]. But the exact mechanisms are unclear. Recent evidences indicate that pharmacological activation of AMPK improves blood glucose homeostasis, lipid profile and blood pressure in insulin-resistant rodents, make this protein kinase a novel therapeutic target in the treatment of type 2 diabetes[4].

Many natural products have long been used as therapeutics for diabetes in complementary medicine. As an example, Curcuma longa has been used for the treatment of diabetes by ayurvedic physicians in India[5]. A study on curcumin (an active principle of rhizome of C. longa) has shown that tetrahydrocurcumin (THC) inhibits the formation of advanced glycation and products in streptozotocin (STZ)-induced diabetic rats[6]. The therapeutic potential of other polyphenols has been also demonstrated from the studies of an acute or chronic administration of polyphenols

Address for correspondence : Deok Bae Park
Department of Internal Medicine, Jeju National University School of Medicine, 102 Jejudaehakno, 690-756, Jeju, Korea
E-mail : parkdb@jejunu.ac.kr

(EGCG from green tea, resveratrol from grape skin) to diabetes mellitus SD rats[7-8]. The Ecklonia cava is a brown algae found in the ocean of Korea and Japan. In Korea, brown algae have long been used as a food remedy to promote maternal health after parturition. Recently, several evidences have demonstrated that Ecklonia cava(E.cava) have various biological activities, such as anti-adipogenic[9], anti-inflammatory[10], anti-oxidant[11], and anti-bacterial activities[12]. However precise action mechanism underlying its anti-diabetic effect remains poorly understood. In the present study, we examined the effect of E.cava ethanol extract on glucose consumption by cultured rat skeletal muscle cells.

Materials and Methods

Materials

The brown seaweed Ecklonia cava was collected along the coast of Jeju island in Korea. The samples were carefully rinsed with fresh water and freeze-dried. 70% ethanol extract of E. cava (E-EC) was prepared as described previously[13].

Cell culture

L6 myocytes were obtained from Korean Cell Line Bank (KCLB, Seoul, Korea). Dulbecco' s minimal essential medium (DMEM) was from Sigma (St. Louis, USA) and fetal bovine serum (FBS) was from PAA (Etobicoke Ontario, Canada). Penicillin-streptomycin was from GIBCO (N.Y., USA) and Lactate dehydrogenenase (LDH) cytotoxicity kit was from TAKARA (Otsu, Shiga Japan). Other reagents were from Sigma. L6 myocytes cells were grown in DMEM containing 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin in an atmosphere of 5% CO_2 at 37°C. To differentiate into myotubes, L6 myocytes were incubated in DMEM containing 2% horse serum for 3-4 days and used for experiments.

Cell viability assay

Lactate dehydrogenase (LDH) leakage is a means of measuring membrane integrity as a function of the amount of cytoplasmic LDH released from the cytosol into the medium. Briefly, cell-free culture medium (50 μl) was collected and then incubated with 50 μl of the reaction mixture of cytotoxicity detection reagents (Takara) in a 96-well microwell plate for 30 min at room temperature. The optical density at 490 nm wavelength was then measured by using the ELISA plate reader (Sunrise, TECAN Austria). MTT assay is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form a dark blue formazan crystals which is largely impermeable to cell membrane, thus resulting in its accumulation within healthy cells. Briefly, 500 μl MTT solution (1 mg/ml) was added into each well and cultured for 30 min at 37℃. The supernatant was removed and 2-propanol (500 μl) was added to each well. The optical density at 570 nm wavelength was measured by the ELISA plate reader (Sunrise, TECAN, Austria).

Glucose assay

The cell-free culture medium (5 μl) was collected and then mix with 150 μl glucose assay reagent (Asan, Korea) in a 96-well plate for 3 min at room temperature. The optical density at 490 nm wavelength was measured with the ELISA plate reader (Sunrise, TECAN Austria).

Western blot analysis

Cells were preincubated in serum-free medium for overnight and then treated with various compounds for purposes. Cells were collected by scrapping and washed with D-PBS. Collected cell pellets were lysed in a lysis buffer (RIPA, Millipore, Billerica, USA) supplemented with inhibitors for various proteases and phosphatases (Sigma, Louis, USA) and kept on ice for 15 min. Lysates were centrifuged at 15,000 rpm at 4℃ for 15 min and the supernatant was stored at -20℃ until use. Protein concentration was determined with BCA protein assay reagent (Pierce, USA). Aliquots of the lysates (15 ?g protein) were separated on a 4-12% Tris-Bis gel (Invitrogen, Carlsland, USA) and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, USA) with transfer buffer (Invitrogen, Carlsland, USA) . After blocking the nonspecific site with 5% non-fat dry milk (Santa cruz, CA, USA) in TBS-T buffer. The membrane was incubated with specific primary antibodies at 4℃ for overnight. Primary antibodies against p-AMPK were from Millipore (Bedford, USA), and antibodies against phosphor-Akt and Glut4 were from Santa cruz (CA, USA). The membranes were further incubated with secondary antibodies. The bands of immunoactive protein was visualized with western lightning Plus-ECL reageants (Perkin Elmer, MA, USA) and exposed onto a x-ray film.

Preparation of plasma membrane fraction

L6 myotube cells grown in 100 mm plates. Cells were scrapped in 500 μl buffer. Collected cells were placed in 1.5 ml tube and mechanically homogenized and kept on ice for 20 min. After centrifugation at 8,000 rpm for 5 min at 4℃, the supernatant was transferred into a fresh labeled tube. The supernatant was centrifuged again at 100,000g for 1 h at 4℃ and the precipitate was collected (the membrane fraction).

Statistical analysis

Student's t-test was used to determined the statistical significance of difference between for a variety of experimental and control groups. P-values less than 0.05 were considered statistically significant.

Results and Discussion

First, it was examined whether the ethanol extract of Ecklonia cava (E-EC) is cytotoxic to differentiated L6 rat skeletal muscle cells. Exposure of cells to E-EC (62.5~500 μg/mℓ) for 3 days did not significantly affect LDH cytotoxicity or reduce MTT cell viability, showing no significant toxic adverse effects compared with the untreated control (Fig. 1).

To determine whether E-EC can increase the glucose consumption in L6 myotube cells, we measured the concentration of glucose in culture medium before and after treatments. The differences of medium glucose between two groups were regarded as the amount of glucose consumed by the myotube cells. Treatment of L6 myotubes cells with E-EC significantly ($p<0.05$) increased the basal glucose consumption (Fig. 2).

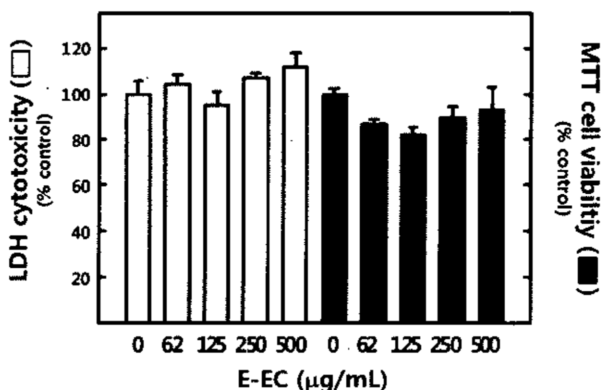

Figure 1. Effect of ethanol extract of E. cava on cell viability. Differentiated L6 skeletal muscle cells were preincubated in a serum-free medium for 4h and then treated with different concentrations of E. cava ethanol extract. Assays for LDH and MTT were performed after the treatment of cells for 3 days.
E-EC, 70% ethanol extract of E. cava. Assays for LDH and MTT were preformed as described in "Materials and Methods".

The stimulation of glucose consumption by 125 μg/mℓ E-EC was comparable to that by 10 nM insulin. Because glucose consumption is known to be stimulated by different signaling components including PI3K and AMPK, it was tested whether E-EC can stimulate PI3K-Akt signaling cascades or AMPK activity (Fig. 3A). When L6 myotube cells were treated by 125 μg/mℓ E-EC for 2 h, E-EC treatment stimulated the phosphorylation of Akt, but not of AMPK. It suggests that AMPK does not play any significant role in E-EC-induced glucose consumption. Instead, PI3K-Akt signaling is likely to mediate the E-EC-induced glucose consumption. To prove it, the effect of the PI3K inhibitor (wortmannin) on the E-EC-induced glucose consumption was evaluated. E-EC-induced glucose consumption was significantly suppressed by the inhibition of PI3K, showing the essential role of PI3K in this process (Fig. 3B).

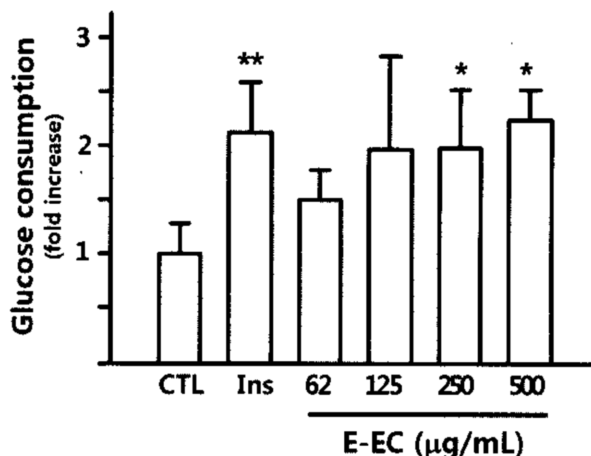

Figure 2. Effect of ethanol extract of E. cava on glucose consumption. Differentiated L6 skeletal muscle cells were preincubated in a serum-free medium for 4h and further treated with different concentrations of E. cava ethanol extract for 5h.
$P<0.05$*, $p<0.01$** compared to the control.
CTL, control; E-EC, 70% ethanol extract of Ecklonia cava; Ins, insulin (10 nM)

In muscle cells, Glut4 plays a key role in glucose uptake. Thus, we tested whether E-EC can stimulate Glut4

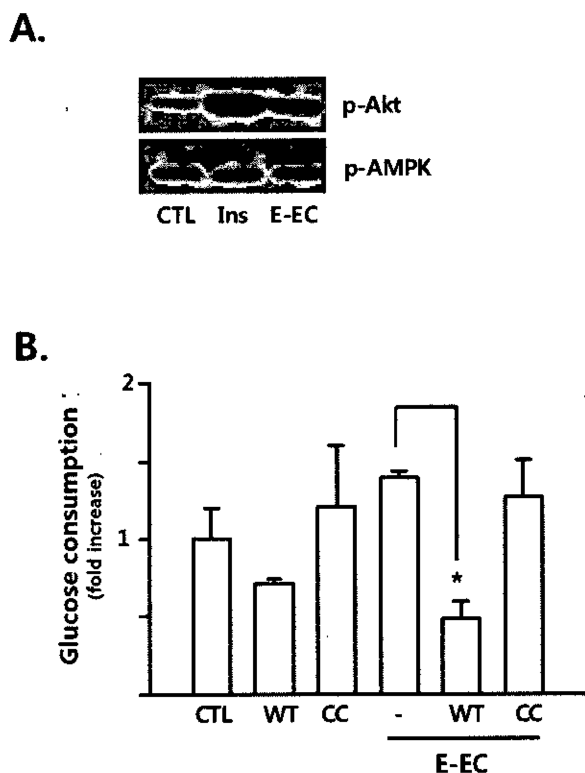

Figure 3. Role of PI3 kinase or AMPK in the E. cava-stimulated glucose consumption. Differentiated L6 skeletal muscle cells were preincubated in a serum-free medium for 4h, pretreated with wortmannin (100 μM) or compound C (10 μM) for 30 min before treatment with E. cava ethanol extract (125 μg/ml) for 2h. $P<0.05$* compared to E-EC.
E-EC, 70% ethanol extract of E. cava; WT, wortmannin CC, compound C

translocation from the cytosol to the plasma membrane. E-EC increased the amount of Glut4 in the plasma membrane compared to the control (Fig. 4A). In addition, pretreatment of cells with wortmannin decreased the amount of Glut4 in the plasma membrane increased by E-EC (Fig. 4B) whereas pretreatment with compound-c could not affect at all. These results suggest that E-EC can stimulate glucose consumption through the PI3K-Akt pathway but not AMPK pathway in L6 myotube cells.

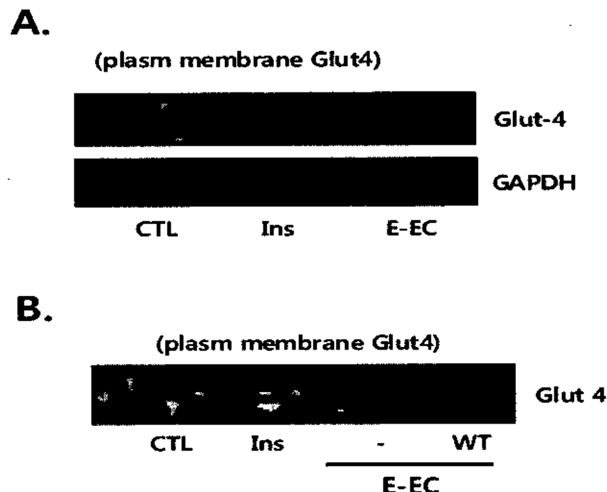

Figure 4. E. cava-stimulated glucose consumption is mediated by the translocation of Glut4 to the plasma membrane. Differentiated L6 skeletal muscle cells were preincubated in a serum-free medium for 4h, pretreated with wortmannin (100 μM)(B) for 30 min before treatment with E. cava ethanol extract (125 μg/ml) or insulin (10 nM) for 30 min.
E-EC, 70% ethanol extract of E. cava; WT, wortmannin

In the present study, we examined the effects of the ethanol extract of Ecklonia cava (E-EC) on glucose consumption in cultured rat skeletal muscle cells. Our study showed that E-EC stimulates glucose consumption through PI3K-Akt pathway but not AMPK pathway in muscle cells. Extract of Ecklonia cava contains seven phlorotannin derivatives (eckol, phloroglucinol, fucodiphloroethol G, phlorofucofuroeckol A, dieckol, 7-phloroeckol, and 6,6'-bieckol), along with three common sterols (fucosterol, cholesterol, and ergosterol)[14]. Dieckol has known to be the strongest antioxidant among the seven phlorotannins of Ecklonia cava from various in vitro assays. PI3K-Akt signaling pathway and AMPK pathway play important roles in glucose metabolism. In the present study, E-EC stimulated PI3K-Akt signaling that is sensitive to the inhibition of PI3K in L6 myotube cells. These results suggest that E-EC increases the glucose consumption via PI3 kinase pathway in muscle cells, not via AMPK activity. In other studies, E-EC activates both AMPK/ACC and PI3K/Akt signaling in C2C12 skeletal muscle cells and ECP can recover the diabetic pancreatic function[15]. It means that the E-EC has a therapeutic potential on diabetes mellitus.

Taken together, our study showed that E-EC can increase glucose consumption in L6 skeletal muscle cells via PI3 Kinase pathway but not AMPK pathway.

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