Polyphenol Extracted from *Ecklonia cava* Increases Insulin-mediated Glucose Uptake in 3T3-L1 Cells and Reduces Fasting Blood Glucose Levels in C57BL/KsJ db/db Mice

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Abstract Previous study suggested that polyphenol-enriched extracts from *Ecklonia cava* (PREC, Seapolynol) have an inhibitory effect on lipid accumulation *in vitro* and *in vivo*. Based on these results, we evaluated the effect of PREC on insulin-mediated glucose uptake in 3T3-L1 cells and in male diabetic C57BL/KsJ-db/db mice. The mice were divided into four groups, including db/db, Rosiglitazone 10mg/kg/day, PREC 60mg/kg/day and PREC 150mg/kg/day. Treatment with PREC upregulated glucose uptake-associated gene expression and improved glucose uptake in fully differentiated 3T3-L1 adipocytes. Additionally, α-glucosidase (α-carbohydrate-hydrolase) was inhibited by PREC in a dose-dependent manner. Moreover, PREC effectively improved GLUT4-associated gene expression and suppressed fasting blood glucose levels. These results suggest that PREC may have a mitigating effect on hyperglycaemia and could help to improve blood glucose levels in diabetes patients.

Keywords: 3T3-L1, Seapolynol, diabetes, insulin sensitivity

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1. Introduction

Diabetes is one of the major public health problems in the world [1]. It is associated with obesity, depression, impaired cognitive performance [2,3,4], and exhibited insulin resistance following the appearance of hyperglycaemia and hyperinsulinemia [5,6]. Insulin resistance is also associated with an increased risk of metabolic syndrome [7] and impairs insulin sensitivity in peripheral tissues such as skeletal muscle, adipocytes and liver [8]. In particular, the liver, a vital organ, is a key player in regulating glucose homeostasis in the bloodstream.

Numerous studies have reported that insulin stimulates glucose transporter 4 (GLUT4)-mediated glucose uptake into peripheral tissues through the regulation of insulin receptor tyrosine kinase (IRS1), phosphatidylinositol 3 kinase (PI3K), and protein kinase B (PKB, AKT) [9,10], which in turn leads to decrease blood glucose levels.

Dietary bioactive compounds are present in vegetables, fruits, herbs, and seaweed [11,12,13]. It is well known that such compounds have preventive and therapeutic properties in metabolic disorders [14,15,16]. For example, phenolic compound-rich seaweed extracts reduce weight gain and blood glucose levels in db/db mice [17,18,19]. However, the beneficial effects of polyphenol-enriched extracts from *Ecklonia cava* (PREC, Seapolynol), marine brown algae, remain unclear. PREC consist of several bioactive components, including eckol, bieckol, dieckol, and phloroglucinol. Recent studies revealed that PREC and their components have an inhibitory effect on hyperlipidemia [20], and these results suggest that PREC are a potential treatment for hyperglycaemia. However, a mechanistic understanding of the effect of PREC on glucose homeostasis is lacking. In this study, PREC were evaluated for their potential effect on glucose uptake, including evaluation of the their mechanistic pathways *in vitro* and of anti-diabetic properties *in vivo*.

2. Materials and Methods

2.1. Materials

Ecklonia cava were harvested in Jeju Island. Botamedi, Inc. (Jeju, Korea) produce *Ecklonia cava* extracts. The *Ecklonia cava* extracts (Seapolynol) were kindly supplied by Botamedi, Inc. (Jeju, Korea). Dulbecco's modified Eagle's medium (DMEM), bovine calf serum (BCS), fetal bovine serum (FBS), penicillin/streptomycin, and trypsin-ethylenediaminetetraacetic acid (EDTA) were purchased from Gibco (Gaithersburg, MD). Dexamethasone (DEX), 3-isobutyl-1-methylxanthine (IBMX), insulin, Phosphatase

inhibitor cocktails II and III, α-Glucosidase from Bacillus stearothermophilus, and 2-Deoxy-D-glucose were purchased from Sigma-Aldrich (St. Louis, MO). Rosiglitazone (Rosi) was purchased from selleckchem.com (Houston, TX). Phosphate-buffered saline (PBS) was purchased from iNtRON Biotechnology (Gyeonggi, Korea). Antibodies against AKT, p-AKT (Ser473), PI3K, pPI3K p85α (Tyr508) were purchased from Cell Signalling Technology (Danvers, MA). Antibodies against IRS-1 (C-20), p-IRS-1 (Tyr632), GLUT4 (H-61), and β-actin (N-21) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

2.2. Cell Culture

3T3-L1 preadipocytes were obtained from the American Type Culture Collection (CL-173, ATCC, Manassas, VA). The preadipocytes were cultured in DMEM with 3.7g/L bicarbonate, 10% BCS and 1% penicillin-streptomycin solution. Two days after the cells reached confluence, the preadipocytes were differentiated using MDI and DMEM containing 10% FBS. MDI is a differentiation inducer that consists of 0.5mM IBMX, 1.0μM DEX, and 2μM insulin. PREC concentrations of 0, 25, 50 and 100μg/mL were applied to the cells. To assess the effects of PREC, cells were grown in the presence or absence of PREC during adipocyte differentiation. The growth medium was refreshed with DMEM containing 10% FBS, 2μM insulin, 2μM Rosi, and with or without PREC every two days until the adipocytes were fully differentiated.

2.3. 2-deoxyglucose (2-DG) Uptake Assay

The 2-deoxyglucose (2-DG) uptake assay was performed according to the standard method [21], with slight modifications. The preadipocytes were grown on 12-wells flatted cell culture plates. Four days after the induction of differentiation, the cells were maintained for 10days in PREC and 2μM Rosi without insulin. Differentiated cells were then washed twice and starved in serum-free medium containing 0.1% (w/v) BSA for 3h. To test the effect of PREC on insulin resistance, 100nM insulin, PREC and 2μM Rosi were added to the medium for 30min, and the cells were then incubated in KRPH buffer (4.7mM KCl, 136mM, NaCl, 1mM CaCl$_2$, 5mM KH$_2$PO$_4$, 1mM MgSO$_4$, 20mM HEPES, pH7.4, 0.1% BSA) and 1mM 2-DG for 15min. The adipocytes were rapidly washed with ice-cold KRBH, incubated in 0.1N NaCl for 45min at -80ºC, and then incubated again at 85ºC for 40min. Next, 0.1N HCL and 150mM TEA buffer were added to the cells. The samples were transferred to 96-well plates and then combined with the assay cocktail (50mM TEA, pH8.1), 150μM NADP+, 4mM MgCl$_2$, 0.02% BSA, 3.0units/mL G6PDH, 5.5units/mL hexokinase, and 670μM ATP). The radioactivity levels were determined using an ELISA reader, the Wallac 140 Victor 2 plate reader, (Perkin-Elmer, Boston, MA) at 412nm.

2.4. α-glucosidase Assay

The α-glucosidase inhibitory activity was assessed by the standard method [22], with slight modifications. Briefly, a volume of 25μL of PREC was mixed with 50μL of α-glucosidase enzyme (0.1U/mL in 0.1M potassium phosphate buffer solution, pH6.9) in 96-well plates and incubated at 37°C for 20min. After pre-incubation, 25μL of 5mM pNPG in 0.1M phosphate buffer were added to each well and incubated at 37ºC for another 20min. The reaction was stopped by adding 30μL of 0.1M NaCO$_3$ for 20min. The α-glucosidase activity was determined by measuring the release of p-nitrophenol from p-nitrophenyl α-D-glucopyranoside using an ELISA reader, the Wallac 140 Victor 2 plate reader, (Perkin-Elmer, Boston, MA) at 405nm. The glucosidase inhibitory activity was determined as a percentage of the control without the inhibitors:

Inhibition activity (%) = (Absorbance of sample / Absorbance of control) x 100

2.5. Animal Husbandry and Experimental Design

All experimental mice were housed in a specific pathogen-free facility at the Korea Food Research Institute, Seongnam, Korea. The project was approved by the Institutional Animal Care and Use Committee of CHA University (IACUC140054). Male C57BL/KsJ-db/db mice were purchased from Joong-Ang Experimental Animal Co. (Seoul, Korea) and were housed at 21 ± 2.0°C with 50 ± 5% relative humidity under 12-h light/dark cycles. The commercial chow (normal diet) was a purified diet based on the Purina Laboratory Rodent Diet 38057 (Dyets Inc., Bethlehem, PA). All of the mice were fed a commercial chow diet and tap water ad libitum for 1week prior to dividing into the following experimental groups (n=8/group): db/db, Rosi 10mg/kg/day, PREC 60mg/kg/day and PREC 150mg/kg/day. The mice were grown for 6weeks. Administration of PREC and Rosi, which proceeded by oral injection, and measurements of body weights were recorded routinely. After the end of the experiment, 12-h fasted animals were anesthetized with ether. For sample analysis, blood plasma was drawn from the tail vein into an EDTA-coated tube. The plasma and liver samples were collected and stored at -80°C.

2.6. Western Blotting

Tissue was harvested using lysis buffer containing protease inhibitors and phosphatase inhibitor cocktails II and III. Protein extracts (20μg) were separated via SDS-PAGE and transferred to a PVDF membrane. The membranes were blocked with 5% skim milk and immunoblotted overnight with primary antibodies specific for the indicated proteins. Secondary antibodies conjugated with horseradish peroxidase (1:5000) were applied for 4h. The bands were visualized by enhanced chemiluminescence, and proteins were detected with LAS Image Analysis software (Fuji, New York, NY).

2.7. Fasting Blood Glucose Test

Blood glucose concentrations were determined by a glucometer (Abbott Laboratories, MA) weekly, following a 12-h fast.

2.8. Statistical Analysis

All values are expressed as the means ± standard deviation values. SAS 9.0 software (SAS Institute, NC)

was used for statistical analysis. One-way analysis of variance was used for comparisons among groups. Significant differences between the means were assessed using Duncan's test, and p-values < 0.05 were considered statistically significant.

3. Results

3.1. Effect of PREC on Glucose Uptake in 3T3-L1 adipocytes

Four days after adipocytes differentiation, the 3T3-L1 cells were cultured with PREC or rosiglitazone without insulin for 10days. As shown in Figure 1A, 2DG uptake levels were increased by PREC in a dose-dependent manner. In addition, PREC significantly increases the rosiglitazone effect on 2DG in a nearly additive fashion.

To test glucose uptake-associated signaling elements, we examined the expression of PI3K, IRS, AKT, and GLUT4 by western blot analysis. As shown in Figure 1B, PREC increased the phosphorylation of IRS, PI3K, and AKT. We also observed that GLUT4 protein expression was significantly increased compared to the control, by 260, 456, and 462% at PREC concentrations of 25, 50, and 100μg/mL, respectively. These data suggested that PREC increases glucose uptake via regulation of the GLUT4-associated signaling pathway in 3T3-L1 cells.

3.2. Effect of PREC on α-glucosidase Activity *in vitro*

The effect of PREC on α-glucosidase activity were measured by α-glucosidase inhibition assay as described in materials and methods. Figure 1C shows that inhibition activity was increased in a dose-dependent manner. This result suggested that PREC has α-glucosidase inhibitory properties. Thus, PREC may impede the digestion and absorption of glucose and thus suppress rapid rises in blood glucose levels.
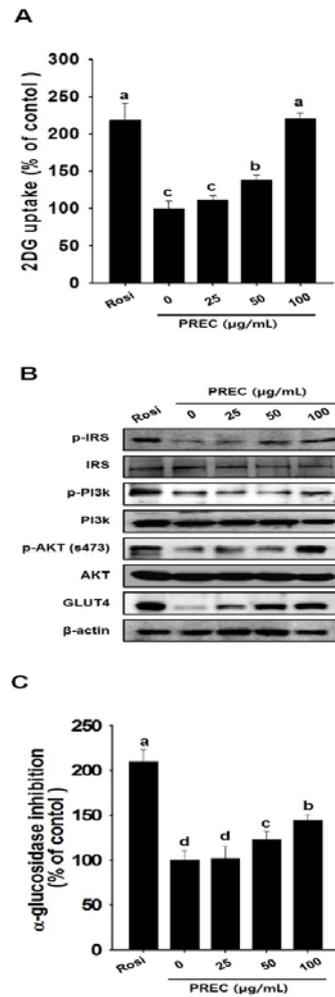

Figure 1. PREC regulate glucose uptake and α-glucosidase enzyme activity in 3T3-L1 adipocytes

3.3. Effect of PREC on Hepatic GLUT4-Associated Gene Expression in db/db Mice
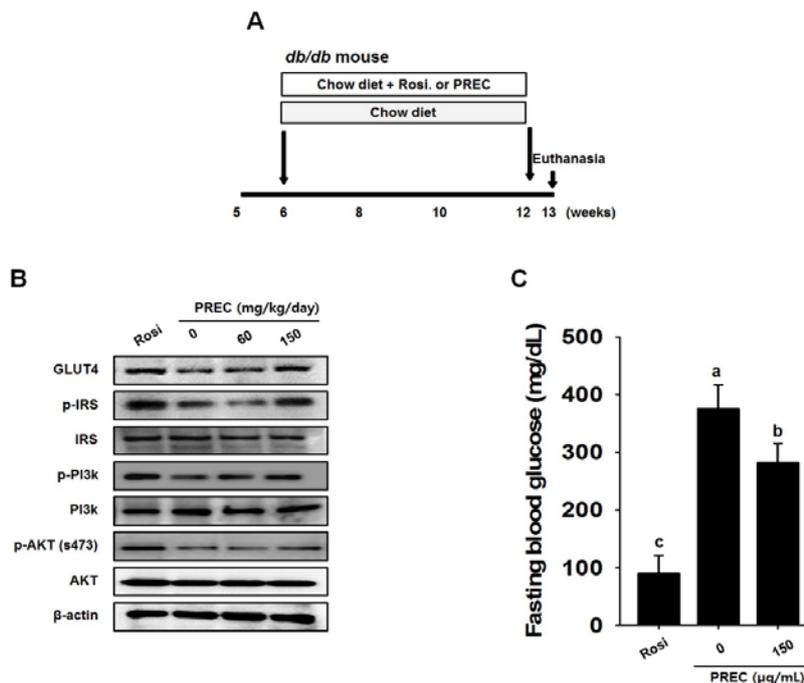

Figure 2. PREC modulate hepatic GLUT4-associated gene expression in db/db mice

The protein expression were determined by western blot to examine whether PREC affect GLUT4-associated gene expression in db/db mice (Figure 2). Western blot analysis revealed small increases in IRS, PI3K, AKT, and GLUT4 protein levels in db/db mice administered with 60mg/kg/day PREC (Figure 2B). However, 150mg/kg/day PREC significantly increased the phosphorylation of IRS, PI3K, and AKT and subsequently enhanced the expression of GLUT4 in the livers of diabetic mice compared to db/db mice in the absence of PREC. Our data suggested that PREC may decrease blood glucose levels through the elevation of glucose uptake in diabetic tissues.

3.4. Effect of PREC on Fasting Blood Glucose Levels in db/db Mice

Next, blood glucose were measured to examine whether PREC regulate the level of fasting blood glucose in db/db mice. As shown in Figure 2C, PREC significantly reduced fasting blood glucose levels compared to the control group.

4. Discussion

The PI3K/AKT pathway, insulin signaling, has been established as an upstream of GLUT4 protein. The phosphorylation of PI3K is promoted by insulin receptor substrate-1 (IRS-1), which is a proximal substrate of the insulin receptor. PI3K subsequently phosphorylates AKT and promotes the translocation of GLUT4 to the cellular membrane. Then, glucose uptake is initiated by GLUT4 trafficking machinery [23,24]. In the present study, GLUT4-associated gene expression and 2DG uptake were increased by PREC. Additionally, α-glucosidase inhibitory activity was elevated by PREC in adipocytes. α-glucosidase, a membrane-bound enzyme, hydrolyses oligosaccharides and disaccharides to glucose and other monosaccharides in the brush border of small intestine, and this enzymatic activity releases glucose into the bloodstream. Therefore, the inhibition of α-glucosidase activity could help to retard the digestion of carbohydrates and suppress the elevation of postprandial blood glucose levels [25,26].

One of the characteristics of type 2 diabetes is chronic hyperglycaemia, or excessively elevated blood glucose levels. Glucose homeostasis is co-ordinated by hepatic glucose mobilization. Thus, glucose uptake-associated signaling, in the liver is responsible for regulating the amount of glucose in the bloodstream and establishing fasting blood glucose levels. Accordingly, the results of this study show that PREC improve glucose uptake through the insulin signaling pathway and therefore result in lower fasting blood glucose levels in db/db mice. Our results indicate that PREC treatment has a beneficial effect on hyperglycaemia in db/db mice. Therefore, further studies should be performed to investigate improving insulin sensitivity by other mechanisms and to identify the components of these additional mechanistic pathways.

5. Conclusions

In conclusion, PREC increase glucose uptake via suppression of α-glucosidase activity *in vitro*. In addition,

PREC suppress fasting glucose through activation of glucose uptake-associated gene expression in db/db mice. These findings indicate that PREC are a potential agent for improving blood glucose homeostasis in diabetes patients.

Acknowledgement

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