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2,7-Phloroglucinol-6,6-Bieckol Increases Glucose Uptake by Promoting GLUT4 Translocation to Plasma Membrane in 3T3-L1 Adipocytes

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Type 2 diabetes occurs when there is an abnormality in the tissue's ability to absorb glucose. Glucose uptake and metabolism by insulin are the basic mechanisms that maintain blood sugar. Glucose uptake goes through various signaling steps initiated by the binding of insulin to receptors on the cell surface. In line with the foregoing, the purpose of this study was to investigate the effect of 2,7-phloroglucinol-6,6-bieckol (PHB), an active compound isolated from *Ecklonia cava*, on glucose uptake in 3T3-L1 adipocytes. Notably, PHB increased glucose uptake in a dose-dependent manner owing to the enhanced glucose transporter type 4 (GLUT4) expression in the plasma membrane of 3T3-L1 adipocytes. These effects of PHB were attributed to the phosphorylation of insulin receptor substrate-1 and protein kinase B (PKB or AKT), as well as to the phosphoinositide 3-kinase (PI3K) activation in the insulin signaling pathway. PHB also stimulated 5′ AMP-activated protein kinase (AMPK) phosphorylation and activation. The phosphorylation and activation of the PI3K/AKT and AMPK pathways by PHB were identified using wortmannin (a PI3K inhibitor) and compound C (an AMPK inhibitor). In this study, we showed that PHB can increase glucose uptake in 3T3-L1 adipocytes by promoting GLUT4 translocation to the plasma membrane via the PI3K and AMPK pathways. The results indicate that PHB may help improve insulin sensitivity.

Key words : 2,7-phloroglucinol-6,6-bieckol, 3T3-L1 adipocytes, *Ecklonia cava,* glucose uptake, insulin signaling pathway

Introduction

Insulin plays a key role in the stimulation of glucose uptake in muscle and adipose tissues. Type 2 diabetes occurs when insulin is unable to stimulate glucose uptake in the tissues. Insulin-stimulated glucose uptake and metabolism is one of the fundamental mechanisms responsible for the maintenance of glucose homeostasis in the body [28]. Insulin maintains glucose homeostasis by promoting glucose uptake into muscle and adipose tissues. Insulin-mediated glucose uptake involves a series of signaling cascades initiated by insulin binding to its receptor on the cell surface [10]. Glucose uptake in response to insulin in muscle and adipose tissues is mediated by glucose transporter 4 (GLUT4). The mechanism of insulin-stimulated glucose uptake is through the translocation of GLUT4 from intracellular storage sites to the plasma membrane (PM). Insulin initiates the signaling pathway by activating the insulin receptor, leading to the phosphorylation of insulin receptor substrate (IRS) and subsequently, activating phosphatidylinositol-3-kinases (PI3K) that mediates protein kinase B (PKB; AKT) phosphorylation by generating phosphatidylinositol-3,4,5-trisphosphate. The phosphorylated AKT stimulates downstream signaling and finally, stimulates glucose uptake by translocating GLUT4 from intracellular vesicles to the PM [12, 20].

Ecklonia cava (*E. cava*) is a brown alga found mainly in the pacific ocean off Japan and Korea. *E. cava* exerts various biological activities such as antioxidant activity [14], anti-HIV activity [1], anticancer activity [19] and antiallergic effect [29]. In a recent study, dieckol, which is phlorotannin from *Ecklonia cava* increased insulin sensitivity by stimulating phosphorylation of IRS-1 and Akt [13, 17]. 2,7-phloroglucinol-6,6-bieckol (PHB) isolated from *Ecklonia cava*, is newly known substances but only antioxidative effects are known [16]. Thus, in the present study, we investigated its effects on glucose uptake through the regulation of insulin signaling pathway in 3T3-L1 cells.

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Materials and Methods

Isolation of PHB from E. cava extract

The isolation and purification of PHB from the 70% EtOH extract of *E. cava* were performed by using a previously described method [21]. The product isolated was confirmed as PHB by LC–DAD–ESI-MS and 1D NMR spectrum. After purification, its 1H-NMR and 13C-NMR spectra were obtained on a JEOL JNM-LA 300 spectrometer (JEOL Ltd., Tokyo, Japan). FAB-MS and ESI-MS profiles were recorded on a JEOL JMS 700 spectrometer. PHB: amorphous powder, 1H NMR (400 MHz, MeOH-d6) d 5.57 (1H, s), 5.89 (1H, s), 5.74 (1H, m), 5.84 (1H, m), 5.74 (1H, m), 6.25 (1H, s), 6.14 (1H, s), 5.84 (1H, m), 5.89 (1H, m), 5.84 (1H, m), 6.52 (1H, s), 6.14 (1H, m), 6.44 (1H, m), 6.77 (1H, s), 6.72 (1H, s), 8.93 (1H, s), 8.93 (1H, s), 9.19 (1H, s), 9.19 (1H, s), 9.19 (1H, s), 9.04 (1H, s), 8.26 (1H, s), 9.94 (1H, s), 8.59 (1H, s), 9.88 (1H, s), 9.86 (1H, s), 9.25 (1H, s), 9.75 (1H, s), 9.21 (1H, s) ; 13C NMR (400 MHz, MeOH-d6) d 127.6, 143.0, 93.0, 137.1, 125.6, 147.2, 106.5, 152.2, 95.5, 152.4, 127.6, 137.1, 162.0, 98.7, 160.3, 95.5, 160.3, 98.8, 124.3, 147.2, 94.5, 144.1, 124.3, 147.2, 110.0, 144.1, 101.5, 151.8, 137.2, 144.1, 159.7, 96.7, 157.1, 95.5, 157.1, 96.7, 159.8, 97.8, 159.3, 95.2, 159.2, 97.9, 122.5, 153.9, 99.8, 156.8, 99.9, 152.8 (d) ; ESI-MS: [M-H]- at m/z 973.37 [22].

Cell culture and adipocyte differentiation

Mouse 3T3-L1 preadipocytes were purchased from Korean Cell Line Bank (KCLB, Seoul, Korea) and were grown in high glucose (4.5 mM) DMEM with 10% FBS at 37℃ in 5% CO_2 atmosphere. These cells were induced to differentiate into adipocytes as described. Briefly, 2 days after confluence, the medium was changed to 10% FCS DMEM supplemented with 0.5 mM isobutylmethylxanthine, 1 μM dexamethasone, and 10 μg/ml insulin. Subsequently, cells were treated with 10 μg/ml insulin in 10% FCS DMEM for an additional 24 hr and then maintained with 10% FCS with the media being replaced every other day for 8 days. With this protocol, more than 80% of the preadipocytes differentiated into adipocytes [9].

2-Deoxy glucose uptake assay

Glucose uptake assay using a 2-(*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-2 deoxyglucose (2-NBDG) screening system was measured as previously described with some modifications [30]. Briefly, differentiated 3T3-L1 adipocytes were seeded with indicated concentrations of PHB for 24 hr at 1×10^4 cells per well into 96-well plates. After incubation, cells were left untreated or stimulated with 100 nM insulin for 20 min at 37℃ in Kreb's ringer phosphate buffer (128 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 1.25 mM $MgSO_4$, and 10 mM $NaPO_4$; pH 7.4). Glucose uptake was initiated by the addition of 80 μM 2-NBDG to each well. After 1 hr, the supernatant was removed. 2-NBDG uptake was measured by a Multilabel Counter (Perkin Elmer, MA, USA) set at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

Western blot analysis

This assay was performed using a modified version of a protocol previously described [8]. For extracting total proteins from adipocytes, the cells were washed twice with ice-cold PBS and harvested in a lysis buffer (RIPA, 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.1 mM sodium orthovanadate; pH 7.4). After sonication and centrifugation at 13,000 ×*g* for 30 min at 4℃, the protein content of the resulting supernatant was determined using a BCA protein assay kit. The lysate containing 20 μg of protein was subjected to 10% SDS-PAGE. Separated proteins were electroblotted on a pure nitrocellulose membrane, blocked with 5% skim milk for 1 hr, and then incubated with primary antibodies (Abcam, Cambridge, UK; 1:1,000) overnight at 4℃. After washing, the blots were incubated with goat anti-rabbit or goat anti-mouse IgG HRP-conjugated secondary antibody for 1 hr at room temperature. Each antigen-antibody complex was visualized using ECL Western Blotting Detection Reagents and detected by chemiluminescence with LAS-1000 plus (FUJIFILM, Tokyo, Japan). Band densities were determined by an image analyzer (Multi Gauge V3.1, FUJIFILM Corporation, Valhalla, NY, USA) and normalized to β-actin for total protein and nuclear protein.

Isolation of PMs from 3T3-L1 adipocytes

Isolation of plasm membrane was performed as described with some modification [18]. 3T3-L1 adipocytes were homogenized by sonication for 5 min at 3 kHz/130 W (UCD-130TM, Cosmo Bio Co., Tokyo, Japan) in ice-cold HES buffer (0.02 M HEPES, 0.25 M sucrose, and 2 mM EGTA; pH 7.4) and centrifuged at 700×*g* for 7 min to remove unhomogenized cellular debris and nuclei from the homogenate. The harvested supernatant was further centrifuged at 760 ×*g* for

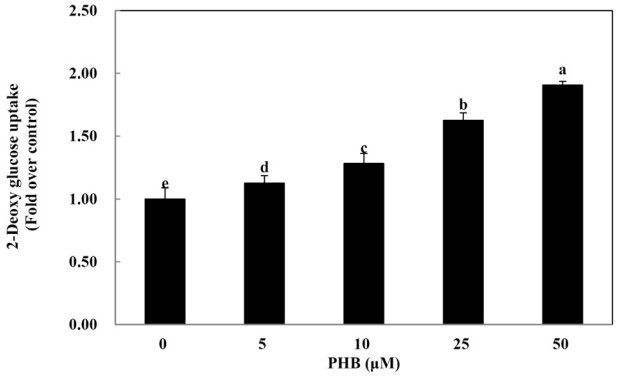

Fig. 1. Effect of 2,7-phloroglucinol-6,6-bieckol on glucose uptake in 3T3-L1 adipocytes. Differentiated 3T3-L1 adipocytes were treated with various concentrations of 2,7-phloroglucinol-6,6-bieckol (PHB) for 2 hr before 2-deoxy glucose uptake measurement. Each value is expressed as mean ± s.d. in triplicate experiments. [a-d]Values with different alphabets are significantly different at $p<0.05$ as analyzed by Duncan's multiple range test.

10 min to remove mitochondria. After recentrifuging at 35,000 ×*g* for 60 min, the resulting pellet was used as the PM fraction of the adipocytes. The supernatant was used as the cytosol fraction. These fractions were subjected to western blotting to detect GLUT4 levels. The amounts of protein in these fractions were quantified by using a BCA protein assay kit.

Statistical analysis

Each experiment was performed in triplicate. The results were expressed as the mean ± SD. Statistical analyses were performed using SPSS software (SPSS version 13.0, USA). Statistical significance was determined by the Student's *t*-test.

Results

Effect of PHB on 2-deoxy glucose uptake

To establish the effect of PHB on glucose uptake, we evaluated its effects on 2-deoxy glucose uptake in differentiated 3T3-L1 adipocytes. Incubation of cells with PHB stimulated glucose uptake in dose-dependent manner. PHB exerted an initial significant effect on glucose uptake at 5 μM and a maximal effect at 50 μM ($p<0.05$; Fig. 1). PHB significantly increased glucose uptake by 1.13, 1.28, 1.63, and 1.91 fold at 5, 10, 25, and 50 μM, (($p<0.05$; Fig. 1). These results indicate that PHB enhances glucose uptake in 3T3-L1 adipocytes.

Effect of PHB on PI3K/AKT pathway signaling

To evaluate the mechanism of PHB-induced glucose uptake, we measured the expression of IRS-1, PI3K, and AKT. Results shown in Fig. 2 demonstrate that PHB significantly increases the phosphorylation of IRS-1 and activation of IRS-1. At 50 μM, PHB increased the phosphorylation of IRS-1 to 173.3% of control, respectively. We also determined the activation of phosphorylation of Akt. As expected, insulin stimulation resulted in augmentation of phosphorylation of Akt. PHB also increased the phosphorylation. In regards to enhancement of Akt phosphorylation, PHB was almost potent as insulin. The activation of PI3K is necessary for in-

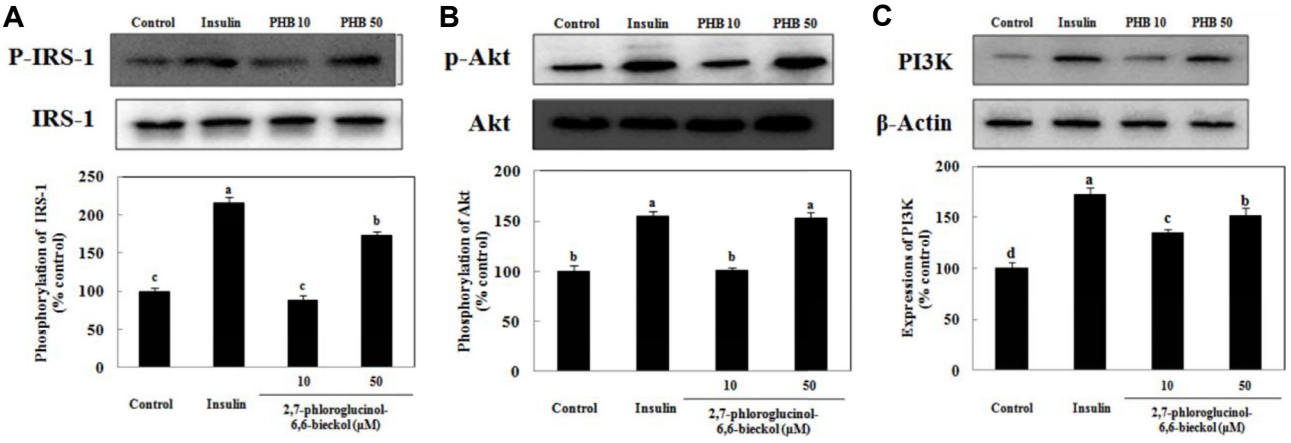

Fig. 2. Effects of 2,7-phloroglucinol-6,6-bieckol on insulin signaling in 3T3-L1 adipocytes. Differentiated adipocytes were incubated with 10 or 50 μM 2,7-phloroglucinol-6,6-bieckol or 100 nM insulin for 2 hr, and then, the cells were extracted and subjected to immunoblot analysis. (A) phosphorylation level of insulin receptor substrate-1 (IRS1). (B) Phosphorylation level of AKT. (C) Expression levels of PI3K. Each value is expressed as mean ± s.d. in triplicate experiments. [a-d]Values with different alphabets are significantly different at $p<0.05$ as analyzed by Duncan's multiple range test.

sulin-stimulated glucose uptake. We examined the activation of PI3K on PHB-stimulated glucose uptake. PI3K activation was remarkably enhanced by PHB. Treatment with 10 and 50 μM of PHB increased PI3K activation to 134.5% and 151.2% of control, respectively. These results suggested that glucose uptake by PHB is possibly mediated via a PI3K-Akt pathway.

Effect of PHB on 5′ AMP-activated protein kinase (AMPK) activation

We investigated the effects of PHB on AMPK activation by western blotting. As shown in Fig. 3, PHB stimulated AMPK phosphorylation to 126.3% and 157.6% of control at PHB concentrations of 10 and 50 μM, respectively. Insulin did not significantly increase AMPK phosphorylation (p< 0.05, Fig. 3). To examine whether the PHB stimulation of glucose uptake was mediated by AMPK activation, we pretreated the cells with compound C (10 μM). Compound C markedly decreased AMPK phosporylation to 20.05% and 28.34% in the presence of 10 and 50 μM PHB, respectively (Fig. 3). These results indicated phosphorylation of AMPK might play an important role in the PHB mechanism of stimulation of glucose uptake.

Effect of PHB on PM-GLUT4 expression

To investigate the effect of PHB on PM-GLUT4 expression, the differentiated adipocytes were fractionated and subjected to immunoblotting. Fig. 4 shows that PHB significantly increased the expression of PM-GLUT4 to 348% of control. To elucidate the signaling pathways involved in PHB-mediated activity, the effect of PHB on GLUT 4 activation was analyzed in the presence of specific inhibitors for PI3K (wortmannin) and AMPK (compound C). Pretreatment of adipocytes with wortmannin, or compound C markedly decreased the PM-GLUT4 expression induced by PHB (Fig. 4). As shown in FIG. 4, when compared with the level of PM-GLUT4 treated with PHB alone, wortmannin was inhibited to 156% and compound C was inhibited to 173%. These results imply that PHB stimulated glucose uptake and enhances PM-GLUT4 expression by activating the PI3K/AKT and AMPK pathways in 3T3-L1 adipocytes.

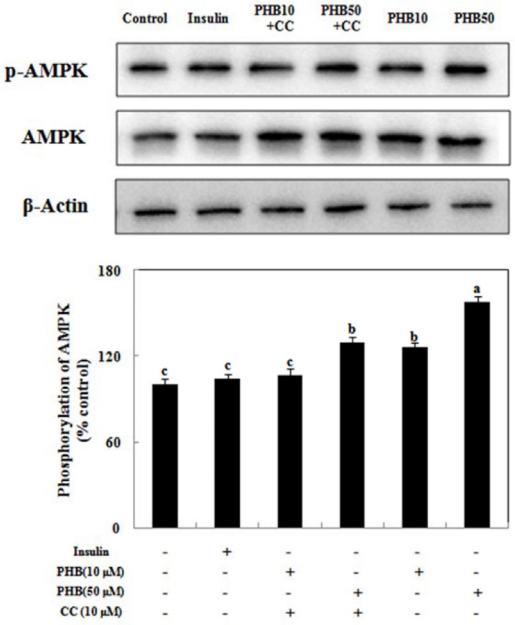

Fig. 3. Effects of 2,7-phloroglucinol-6,6-bieckol on AMPK expression in 3T3-L1 adipocytes. Differentiated adipocytes were incubated with 10 or 50 μM 2,7-phloroglucinol-6,6-bieckol (PHB) or 100 nM insulin for 2 hr or in combination with 10 μM compound C (CC) and then, the cells were extracted and subjected to immunoblot analysis. Each value is expressed as mean ± s.d. in triplicate experiments. [a-c]Values with different alphabets are significantly different at p<0.05 as analyzed by Duncan's multiple range test.

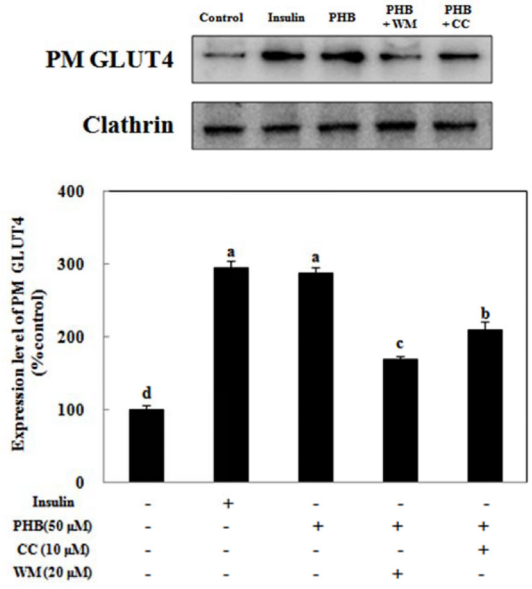

Fig. 4. Effects of 2,7-phloroglucinol-6,6-bieckol on GLUT4 protein expression in 3T3-L1 adipocytes. Differentiated adipocytes were incubated with 50 μM 2,7-phloroglucinol-6,6-bieckol (PHB) or 100 nM insulin for 2 hr or with PHB in combination with 10 μM compound C (CC) or 20 μM wortmannin (WM) and then, the cells were extracted and subjected to immunoblot analysis. Each value is expressed as mean ± s.d. in triplicate experiments. [a-d]Values with different alphabets are significantly different at p<0.05 as analyzed by Duncan's multiple range test.

Discussion

PHB isolated from *E. cava* has been known to have antioxidant properties, but its effect on glucose uptake has not been confirmed. Thus, this study investigated the effect of PHB on glucose uptake in 3T3-L1 adipocytes and the underlying cellular mechanism.

Glucose homeostasis is determined by glucose production and utilization in insulin-sensitive organs and tissues, including the muscle, liver, and adipose tissue [11]. Glucose uptake in the adipose tissue plays a critical role in glucose homeostasis. Insulin stimulates glucose uptake by binding to the insulin receptor at the cell surface, leading to the activation of GLUT4 [24]. In this study, PHB significantly enhanced glucose uptake in 3T3-L1 adipocytes. The mechanism of glucose uptake in adipocytes is generally associated with PI3K/AKT and AMPK pathways [23, 25]. Among the two mechanisms, the PI3K/AKT pathway is crucial in mediating the hypoglycemic effect of insulin through IRS activation. The AMPK pathway also activates GLUT4 translocation to the PM, which increases intracellular glucose uptake [5].

To identify the mechanism of the PHB-stimulated increase in glucose uptake, we investigated the activation of PI3K/AKT and AMPK pathways. In the present study, treatment with PHB in 3T3-L1 adipocytes markedly increased the phosphorylation of IRS-1 and AKT. Additionally, PHB increased PI3K activation, which is important for GLUT4 activation. In the PI3K/AKT signaling cascade for glucose uptake, the activation of insulin receptor β subunit results in the subsequent phosphorylation of the downstream signaling molecule, IRS-1. Phosphorylated IRS-1 activates PI3K formation, which stimulates the phosphorylation of its downstream effector, AKT. AKT phosphorylation then induces the translocation of GLUT4 from the cytoplasm to the PM, which promotes the uptake of glucose into the cell [2, 7, 26]. In addition to the PI3K pathway, AMPK signaling influences GLUT4 translocation and glucose uptake in muscles and adipocytes [4]. In this study, PHB increased p-AMPK level, and compound C (AMPK inhibitor) suppressed this PHB-mediated increase in p-AMPK level. Therefore, PHB seemed to increase glucose uptake by activating PI3K/AKT and AMPK pathways. Its effect on PI3K/AKT signaling was greater than that on AMPK signaling. Therefore, it could be considered that glucose uptake by PHB occurs mainly through the PI3K/AKT pathway.

To support these findings, we further investigated the effect of specific inhibitors on PM-GLUT4 expression. GLUT4 plays a major role in regulating glucose transport in muscles and adipocytes. GLUT4 translocation to the PM is triggered by the insulin receptor, followed by the activation of various protein kinases involved in the PI3K/AKT pathway [6]. Moreover, GLUT4 translocation is triggered by AMPK, which increases glucose uptake by cells [27]. In experiments using inhibitors, PHB-mediated increased in PM-GLUT4 expression was suppressed by both wortmannin (PI3K inhibitor) and compound C (AMPK inhibitor). The effect was significant when the PI3K inhibitor was used, indicating that the increase in glucose uptake by PHB mainly occurs by PI3K/AKT pathway activation.

PHB is a type of marine algal polyphenols (phlorotannins) isolated from *E. cava*, a brown alga. Polyphenols have been reported to exert insulin-like effects *in vivo* and *in vitro* [12, 14, 31]. Insulin generally acts via two major signaling pathways. One is the mitogen-activated protein kinase (MAPK) pathway and the other is the PI3K/AKT pathway. Among the two mechanisms, the PI3K/AKT pathway is important in mediating the hypoglycemic effect of insulin [3]. The polyphenol-rich extract of *E. cava* is reported to activate insulin signaling pathways i.e., increase AKT and AMPK phosphorylation and activation; particularly, the ratio of p-AKT/AKT after treatment with the extract was comparable to that after insulin treatment in this report [15]. Taken together, PHB appears to increase glucose uptake by activating PI3K/AKT and AMPK pathways and translocating GLUT4 from the cytoplasm to the PM.

In conclusion, PHB enhances glucose uptake by activating the PI3K/AKT pathway, IRS-1 phosphorylation, and the AMPK pathway in 3T3-L1 adipocytes. The phosphorylation and activation of PI3K/AKT and AMPK pathways by PHB were confirmed using wortmannin (PI3K inhibitor) and compound C (AMPK inhibitor); these inhibitors inhibited PHB-induced PM-GLUT4 expression. These results suggest that PHB may help to improve insulin sensitivity.

Acknowledgment

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The Conflict of Interest Statement

The authors declare that they have no conflicts of interest

with the contents of this article.

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초록 : 2,7-Phloroglucinol-6,6-Bieckol의 3T3-L1 지방세포에서 GLUT4 활성화를 통한 포도당 흡수 증진 효과

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제 2 형 당뇨병은 조직의 포도당 흡수 능력에 이상이 있을 때 발생하며, 인슐린에 의한 포도당 섭취와 신진대사는 혈당을 유지하는 기본 활동이며 포도당 섭취는 인슐린이 세포 표면의 수용체에 결합하여 시작되는 다양한 신호 단계를 거친다. 본 연구는 *Ecklonia cava*에서 분리된 활성 화합물 인 2,7-phloroglucinol-6,6-bieckol이 3T3-L1 지방 세포에서 인슐린 신호전달체계에 따른 포도당 흡수 증가에 미치는 영향에 대한 것이다. 2,7-phloroglucinol-6,6-bieckol 은 3T3-L1 지방 세포에서 농도의존적으로 GLUT4의 발현을 증가시켜 원형질막에서의 glucose uptake를 증가시켰다. 이는 인슐린 신호 전달 경로에서 2,7-phloroglucinol-6,6-bieckol 에 의한 IRS-1, AKT의 인산화 및 PI3K 활성화에 의한 것이다. PHB는 또한 AMPK 인산화와 활성화를 자극했다. 2,7-phloroglucinol-6,6-bieckol에 의한 PI3K/AKT 및 AMPK 경로의 인산화 및 활성화는 wortmannin (PI3K 억제제) 및 화합물 C (AMPK 억제제)를 사용하여 확인하였다. 본 연구에서 2,7-phloroglucinol-6,6-bieckol 이 3T3-L1 지방 세포에서 PI3K 및 AMPK 경로를 통해 원형질막으로의 GLUT4 전위를 촉진함으로써 포도당 흡수를 증가시킬 수 있음을 나타내었다. 이러한 결과는 2,7-phloroglucinol-6,6-bieckol 가 인슐린 감수성을 개선하는 데 도움이 될 수 있음을 시사한다.