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Fagopyritol, a Derivative of D-*chiro*-inositol, Induces GLUT4 Translocation via Actin Filament Remodeling in L6-GLUT4myc Skeletal Muscle Cells

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Insulin induces glucose transporter 4 (GLUT4) translocation to the muscle cell surface. As fagopyritol has insulin-like effects, the effects of fagopyritol on GLUT4 translocation and filamentous (F) actin remodeling in L6-GLUT4myc skeletal muscle cells were investigated. Fagopyritol significantly increased plasma membrane GLUT4 levels compared with the basal control in L6-GLUT4myc myoblast cells. Phosphatidylinositol (PI) 3-kinase inhibitor (LY294002) treatment prevented GLUT4 translocation to the plasma membrane in the myoblasts. Fagopyritol treatment apparently stimulates F-actin remodeling in myoblasts. In addition, fagopyritol treatment induced GLUT4 translocation and F-actin remodeling in myotubes. Taken together, these results suggest that fagopyritol promotes GLUT4 translocation and F-actin remodeling by activating the PI 3-kinase-dependent signaling pathway.

Key words : Fagopyritol, Glucose transporter 4 (GLUT4) translocation, Actin filament, D-*chiro*-inositol, L6-GLUT4myc myoblasts

Introduction

Insulin-stimulated glucose transporters (GLUTs) play an important role in the regulation of blood glucose levels [3]. Insulin quickly increases the rate of glucose uptake into the primary glucose-consuming tissues, such as skeletal muscle and adipose tissue, in which glucose transporter 4 (GLUT4) is specifically expressed at a significant level [9, 10]. GLUT4 is mainly localized in the intracellular storage pool and translocates to the plasma membrane in response to insulin [22]. This phenomenon is defective in insulin resistance and type 2 diabetes (T2DM) [12]. In T2DM, a defect that is associated with impaired GLUT4 translocation, fat cells and muscle fibers appear to be insensitive to the hormone [31]. The process by which GLUT4 triggers an increase in glucose uptake is a complex signaling cascade, which is still not fully understood.

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In addition, Insulin-dependent GLUT4 translocation requires dynamic remodeling of filamentous (F) actin [2]. In several events, such as vesicle trafficking, the F-actin cytoskeleton plays a major role in insulin-regulated GLUT4 vesicle dynamics [23]. It is well known that insulin causes a rapid and marked remodeling of actin filaments below the plasma membrane, promoting membrane ruffling in cells such as myotubes, adipocytes, and fibroblasts [27]. Disruption of the actin cytoskeleton prevents the arrival of GLUT4 at the cell surface of muscle and adipose cells [6, 31].

Endogenous GLUT4 is not expressed until it is differentiated into myotubes [16]. L6 myoblasts respond poorly to insulin. A myc epitope-tagged GLUT4 (GLUT4myc) into L6 myoblasts is reported to markedly improve insulin sensitivity and responsiveness of glucose uptake in L6 myoblasts [29]. The level of expression of GLUT4myc in myoblasts was similar to the endogenous level of GLUT4 in myotubes [29]. Thus, the myc epitope-tagged GLUT4 (GLUT4myc) into L6 myoblasts was used in this study.

Buckwheat (*Fagopyrum esculentum Moench*) has quite a few beneficial bioactive components such as D-*chiro*-inositol (DCI), flavonoids, and sterols [7, 34]. Fagopyritols are derivatives of the D-*chiro*-inositol (DCI) that accumulates in the seeds of common buckwheat. Fagopyritols have been isolated from not only buckwheat seeds but also soybean, lu-

pine, lentil, chickpea, sugar beet, mung bean, and jojoba [4, 13]. DCI is a component of galactosamine DCI, a putative insulin mediator, known to be deficient in T2DM patients [8]. DCI and its galactosides (fagopyritols) have been reported to lower blood glucose levels [13].

We have previously reported that fagopyritol promotes glucose uptake in L6 myoblast cells [24]. But that mechanism by which this is accomplished is not yet clearly understood. In this study, the effect of fagopyritol on GLUT4 tanslocation and actin filament remodeling in L6-GLUT4myc rat myoblast cells was investigated.

Materials and Methods

Materials

Fetal bovine serum (FBS), α-MEM, and other cell culture reagents were purchased from GIBCO (Burlington, Ontario, Canada). Monoclonal anti-myc (9E10) antibody was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Cyanine 3-conjugated goat anti-mouse (Cy3-conjugated goat anti-mouse) and Cyanine 5-conjugated goat anti-mouse (Cy5-conjugated goat anti-mouse) antibodies were purchased from Jackson Immunoresearch Laboratories Inc. (West Grove, PA, USA). Rhodamin-phalloidin (R-415) was purchased from Invitrogen (Carlsbad, CA, USA). Insulin and LY294002 as phosphatidylinositol 3 kinase (PI3K) inhibitors were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of the fagopyritol

Fagopyritol was supplied by S&D Co. (Chuncheon, South Korea) used *in vitro* culture after radio-sterilization. This fagopyritol was extracted from the skin of buckwheat by 50% ethanol at 80℃ and used *in vitro* culture after radio-sterilization. The fagopyritol used in this experiment contained over 9.57% DCI.

Cell culture, differentiation, and treatment

L6 rat myoblasts normally expressing GLUT4 with an exofacial myc epitope (L6-GLUT4myc cells) (kind gift from Amira Klip, the Hospital for Sick Children Toronto, ON, Canada) were cultured according to the following protocols [29]. L6 myoblast cells were grown in α-MEM supplemented with 10% FBS and 1% penicillin/streptomycin (100 U/ml penicillin and 100 microgram/ml streptomycin) in 5% humidified air at 37℃. L6 myoblasts were differentiated into myotubes in α-MEM supplemented with 2% FBS, 1% penicillin/streptomycin, and 1% Fungizone. Before any trials were conducted, the cells were incubated in serum-free α-MEM for 4 hr. Cell viability was not compromised under any of these conditions (data not shown). The treatment dose (1 mM) was chosen according to the results obtained in dose-response and time-course assays [24].

Trafficking of GULT4myc translocation

The cells were placed in a 3 cm confocal dish with cover slip and then incubated in a α-MEM buffer for 30 min at 37℃ and then incubated in insulin for 10 min. After fixation with 2% para-formaldehyde for 10 min at room temperature (RT), the cells were washed with phosphate buffered saline (PBS), and incubated in 0.1 M glycine in PBS for 15 min at RT. The cells were incubated with PBS containing 10% goat serum for 30 min at 4℃. Primary antibody (anti-c-myc, 9E10; Santa Cruz Inc., Santa Cruz, CA, USA) was diluted to 1:50 ratio and incubated for 1 hr at 4℃. The cells were extensively washed with cold PBS. The secondary antibody (Cy3-anti-mouse, Jackson Immunoresearch., West Grove, PA, USA) was diluted to 1:500 ratio and incubated for 1 hr at 4℃. After washing with PBS, the cells were mounted with a mounting medium with DAPI (H-1200, Vector Laboratories. Inc., Burlingame, CA, USA). Immediately, fluorescent images were captured immediately using a confocal imaging system (LSM-510 META NLO, Carl Zeiss, Randburg, Germany). All digital images were captured at the same settings in order to allow direct quantitative comparison of staining patterns. The fluorescent intensity of red GLUT4 translocation was measured by Image J software [5]. Fluorescence intensities of GLUT4myc were normalized by subtracting background intensities.

Filamentous actin staining

To stain actin filaments, the cells were incubated in 2 ml KRH buffer for 30 min at 37℃ and then incubated in 300 nM insulin. After fixation with 3% para-formaldehyde (20 min, RT), the cells were incubated for neutralization in 0.1 M glycine in PBS for 10 min at 4℃. Then, Rhodamin phalloidin (R415, Invitrogen, Carlsbad, CA, USA) was added at 1 unit per dish for 1 hr at RT. After washing with PBS, the cells were mounted with a mounting medium with DAPI (H-1200, Vector Laboratories. Inc.). Fluorescent images were captured Immediately using a confocal imaging system (LSM-510 META NLO, Carl Zeiss).

Quantification and statistical analysis

Several representative images of GLUT4myc translocation from three separate trials were quantified with the use of Image J software (≥30 cells per condition). The data are presented as the mean ± SEM. To determine statistical significance, the data were analyzed using student's *t* test. A value of $p<0.05$ was considered to be statistically significant.

Results

Fagopyritol stimulates the translocation of GLUT4 to the plasma membrane in L6-GLUT4*myc* myoblast cells

This study shows the morphological changes and immunoreactivities on GLUT4 translocation and filamentous (F) actin remodeling in L6-GLUT4myc myoblasts and myotubes treated with a 1 mM concentration of fagopyritol. Fagopyritol treatments stimulated GLUT4 translocation and filamentous (F) actin remodeling of L6-GLUT4myc myoblasts and myotubes. In a previous study, fagopyritol promoted glucose uptake in L6 myoblast cells [24]. The present study, thus, investigated whether fagopyritol can induce GLUT4 translocation, causing increased glucose uptake in L6-GLUT4myc myoblasts. Fagopyritol significantly increased plasma membrane GLUT4 levels compared with the basal control condition, $p<0.05$ (Fig. 1A and 1B). L6-GLUT4myc myoblasts were treated with LY294002, insulin, or fagopyritol for 20 min. This treatment prevented the insulin-stimulated association of GLUT4myc with insulin or fagopyritol (Fig. 1A-d, f, and 1B).

Fagopyritol stimulates filamentous actin remodeling in L6-GLUT4*myc* myoblast cells

To examine whether the cell surface was remodeled, rhodamine-phalloidin was used to stain F-actin. In the basal state, stress fiber was evenly distributed across the myoblast surface (Fig. 2a, d, and g; white arrows). Upon insulin or fagopyritol stimulation, a correspondence in the actin aggregates was observed (Fig. 2b, c, e, f, h, and i; white triangles).

Fagopyritol induces GLUT4 translocation and filamentous actin remodeling in L6-GLUT4*myc* myotubes

The myoblasts differentiate spontaneously after confluency into multinucleated myotubes when cultured in low concentrations of serum [15]. The multiple myonuclei were confirmed by DAPI staining (blue) (Fig. 3d, e, and f). The cells were fixed and double-stained for F actin (green, rhodamine-phalloidin) and GLUT4myc (red, an anti-myc antibody followed by a Cy5-conjugated secondary antibody). Upon insulin or fagopyritol stimulation, a marked correspondence was observed in the actin aggregates on the plasma membrane of cells (Fig. 3a, b, c, j, k, and l; white triangles). Also, fagopyritol apparently increased plasma membrane GLUT4 levels compared with the basal control condition in myotubes (Fig. 3g, h, and i). In both myoblasts and myotubes, fagopyritol stimulated GLUT4 translocation and F actin remodeling. In both myoblasts and myotubes, fagopyritol stimulated GLUT4 translocation and F actin remodeling.

Discussion

In this study, we have shown the morphological changes and immunoreactivities on GLUT4 translocation and filamentous (F) actin remodeling in L6-GLUT4myc myoblasts and myotubes treated with a 1 mM concentration of fagopyritol. It was found that this remodeling was positively stimulated by fagopyritol treatment.

Fagopyritols are derivatives of DCI. Body stores, mainly in muscle and urine, of DCI gradually decrease in patients with diabetes (both type 1 and type 2) and obesity [19]. This symptom may be in part due to increased urinary losses through an unknown mechanism of DCI [33]. DCI, an inositol derivative, has been shown to act as an anti-diabetic compound by improving insulin sensitivity in patients exhibiting insulin resistance due to diabetes or polycystic ovary syndrome [1, 25].

Insulin caused a twofold increase in cell surface content in GLUT4*myc* and in glucose transport activity in L6-GLUT4myc myoblasts [24]. Thus, in L6 myoblasts, ectopic expression of GLUT4 generated an insulin-responsive phenotype [32]. GLUT4 translocation to the plasma membrane is mostly regulated by two distinct signaling pathways, the PI3K/Akt- and AMP-activated protein kinase (AMPK)-dependent signaling pathways [17]. As PI3K is required for insulin-mediated recruitment of GLUT4 [26], the interaction of GLUT4myc with fagopyritol was examined in the presence of the PI3K inhibitor LY294002. LY294002 treatment prevented insulin or fagopyritol from stimulating GLUT4myc translocation. This result indicates that fagopyritol, in part, promotes GLUT4 translocation by activating the

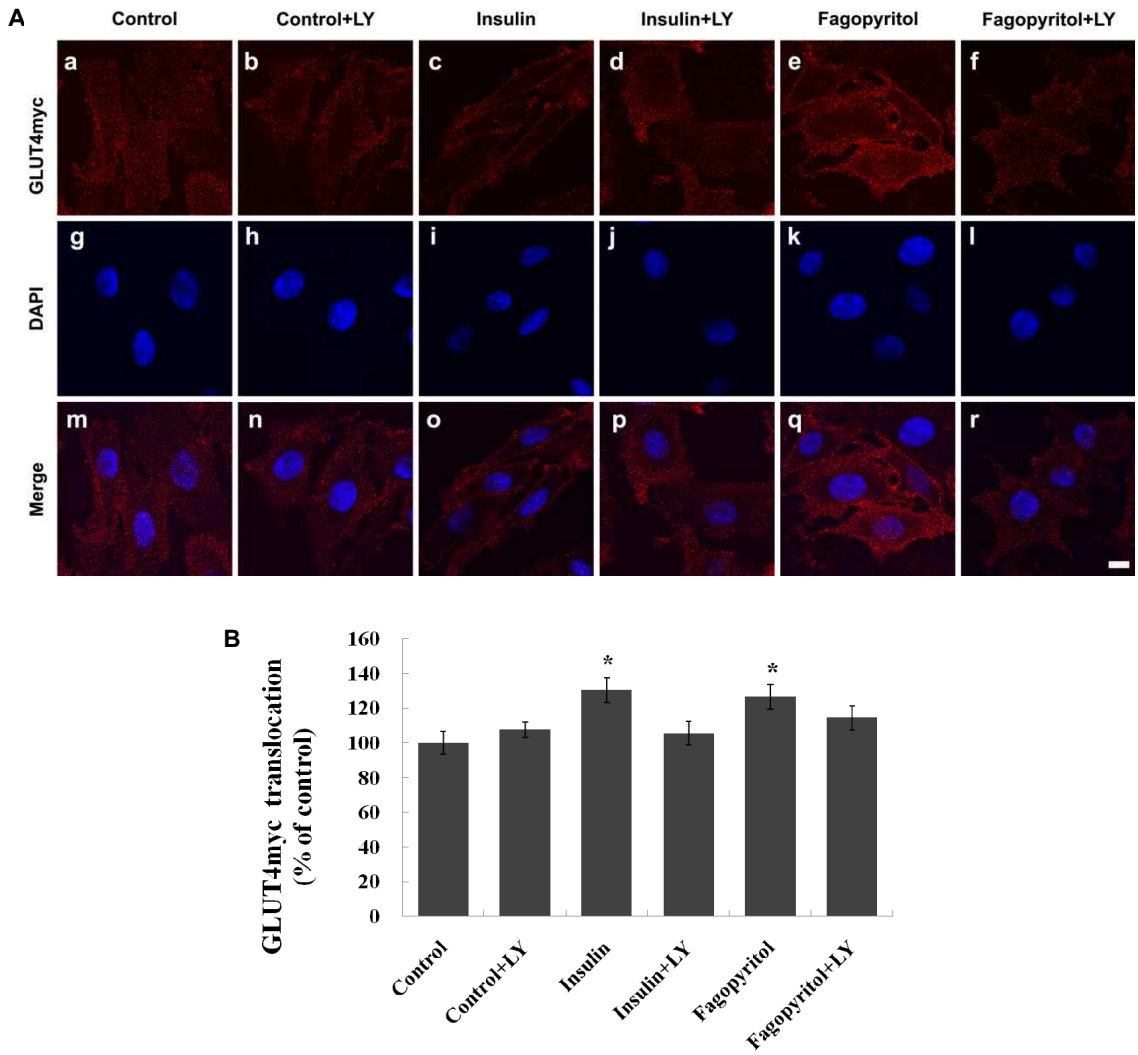

Fig. 1. Fagopyritol treatment induces GLUT4 translocation. LY294002 as PI3 kinase inhibitor treatment prevents GLUT4 translocation. (A) Immunostaining of GLUT4 in non-permeabilized L6-GLUT4myc cells. Intact L6-GLUT4myc cells were stained with anti-myc mouse IgG (9E10), and subsequently with Cy3 goat anti-mouse IgG to detect surface GLUT4myc (red). The myonuclei were identified by DAPI staining (Blue). Insulin (100 nM), fagopyritol (1 mM), and LY294002 (0.01 mM) were treated for 10min. Representative immunofluorescence images of 3 independent experiments. Scale bar, 10 μm. (B) Quantification of cell surface GLUT4myc content. Data are means ± SEM and expressed relative to the values in basal control cells. *p value <0.05 as compared to the basal control.

PI3K-dependent signaling pathway.

It was found that insulin-stimulated F-actin membrane ruffles in L6 myoblasts accumulated at localized plasma membrane sites and directed the localization of both PI3 kinase and GLUT4 protein [30]. Insulin also stimulation induced a rapid reorganization of actin filaments into a cortical mesh in muscle cells. Some studies have shown that actin remodeling is required for GLUT4 translocation and glucose uptake into cells either by connecting signaling or by making the tracks on which GLUT4 vesicles can move in myocytes [14, 20, 28] and adipocytes [11, 18]. It has been reported that disruption of the actin cytoskeleton prevents the arrival of GLUT4 at the cell surface of muscle and adipose cells [21, 27].

Yap et al. have also reported that DCI, L-*chiro*-inositol (LCI), *epi*-inositol, *muco*-inositol, and D-pinitol induced GLUT4 translocation in the L6 myotubes and skeletal muscles of rats ex vivo [35]. This result suggests that fagopyritol stimulates GLUT4 translocation via actin filament remodeling.

In conclusion, our current findings provide strong evidence to support the hypothesis that fagopyritol improves

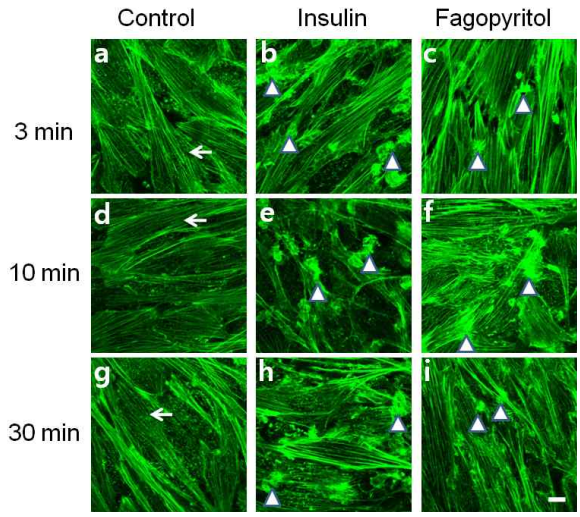

Fig. 2. Fagopyritol treatment induces F actin remodeling in L6-GLUT4myc myoblast cells. Serum-deprived L6-GLUT4myc myoblast cells were treated with 100 nM insulin or 1 mM fagopyritol for 3, 10, and 30 min at 37℃. Afterward, the cells were fixed, permeabilized, and stained for F-actin (green, rhodamine-phalloidin). White arrows indicate stress fiber (a, d, and g). White triangles indicate the F actin aggregates caused by remodeled actin filaments (b, c, e, f, h, and i). Scale bar, 10 μm. The images are representative of three experiments.

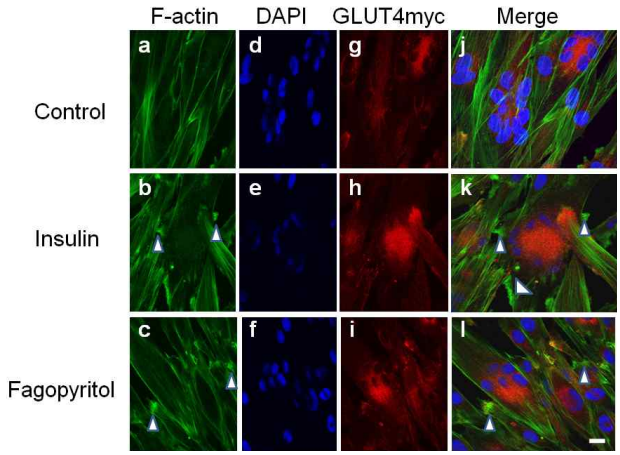

Fig. 3. Fagopyritol treatment induces GLUT4 translocation and F actin remodeling in L6-GLUT4myc myotubes. Serum-deprived L6-GLUT4myc myotubes were treated with 300 nM insulin or 1 mM fagopyritol for 10 min at 37℃. Afterward, the cells were fixed and double-stained for actin (green, rhodamine-phalloidin) and GLUT4myc (red, anti-myc antibody followed by Cy5-conjugated secondary antibody) as described in Methods. The multiple myonuclei were identified by DAPI staining (blue; d, e, and f). White triangles indicate the F actin aggregates (b, c, k, and l). Scale bar, 10 μm. The images are representative of three experiments.

glucose uptake mainly by promoting GLUT4 translocation and F-actin remodeling in skeletal muscle. Therefore, the consumption of fagopyritol might ameliorate the symptoms of diabetes mellitus and polycystic ovary syndrome.

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초록 : 랫드 근육세포에서 fagopyritol이 액틴 필라멘트 구조와 포도당 수송체 4에 미치는 영향

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인슐린은 근육세포 표면으로 포도당 수송체 4(glucose transporter 4, GLUT4)를 유도하여 혈액 속의 포도당을 세포 내로 유입시키도록 작용한다고 알려져 있다. Fagopyritol은 인슐린과 유사한 작용을 하는 것으로 알려져 있으므로, 본 연구에서는 혈당강하 효과가 있다고 알려진 fagopyritol을 랫드의 근육세포주(L6GLUT4myc 세포)에 처리하여, 아직 명확하게 밝혀지지 않은 fagopyritol의 혈당강하 기전을 규명하고자 수행하였다. Fagopyritol의 혈당강하 기전을 규명하기 위하여 근원세포(myoblast)와 근관세포(myotube)에 fagopyritol을 처리하여 액틴 필라멘트의 구조와 GLUT4에 미치는 영향을 분석하였다. Fagopyritol을 myoblast에 처리하였을 때, GLUT4가 처리군에서 대조군과 비교하여 유의 있게 원형질막 쪽으로 유도되는 것을 확인하였고, 액틴 필라멘트의 구조가 재조정되면서 GLUT4의 이동을 돕는 것으로 생각된다. 또한 fagopyritol이 인슐린과 유사한 작용 경로를 가지는지 확인하기 위하여, 인슐린 작용 경로에서 중요한 역할을 하는 것으로 알려진 phosphatidylinositol 3-kinase (PI3K)의 억제제인 LY294002를 fagopyritol과 함께 처리하였을 때 GLUT4가 원형질막 쪽으로 유도되지 않는 것을 확인하였다. Fagopyritol을 myotube에 처리하였을 때, myoblast에 처리하였을 때와 유사한 결과를 나타내었다. 이러한 결과를 종합하면 fagopyritol이 인슐린과 유사한 작용을 하여 액틴 필라멘트의 구조 변경과 GLUT4의 이동을 촉진시키는 것으로 사료된다.