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ORIGINAL PAPER

Prenylflavonoids from fruit of *Macaranga tanarius* promote glucose uptake via AMPK activation in L6 myotubes

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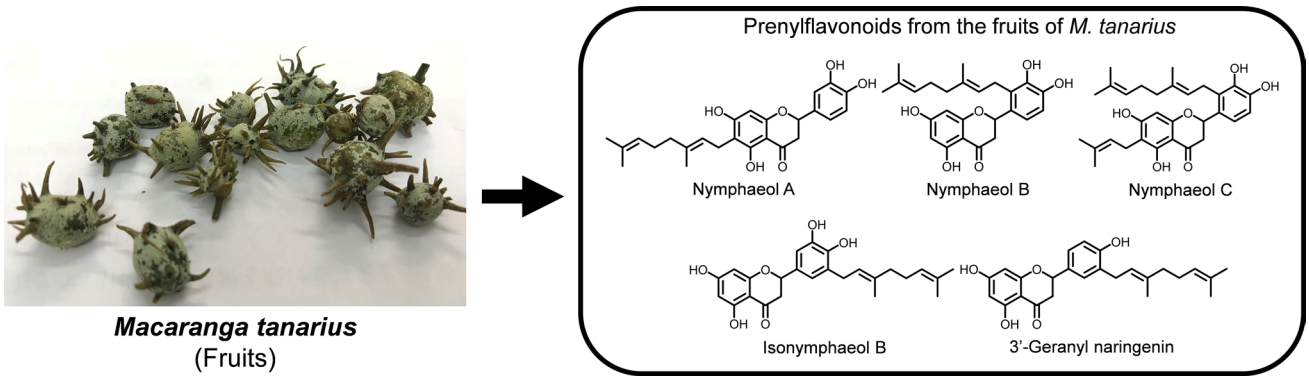
Abstract

Skeletal muscle is a major tissue of glucose consumption and plays an important role in glucose homeostasis. Prenylflavonoids, a component of *Macaranga tanarius* fruits, have been reported to have antioxidant, antibacterial, and anticancer effects. However, the effects of these compounds on skeletal muscle glucose metabolism are unclear. Here, we isolated five prenylflavonoids from *M. tanarius* fruits, and investigated the mechanism of action of these compounds on skeletal muscle cells using L6 myotubes. We found that isonymphaeol B and 3′-geranyl naringenin increased glucose uptake in a dose-dependent manner. Furthermore, both isonymphaeol B and 3′-geranyl naringenin increased AMPK phosphorylation but did not affect PI3K-Akt phosphorylation. Isonymphaeol B and 3′-geranyl naringenin also increased *Glut1* mRNA expression and plasma membrane GLUT1 protein levels. These results suggest that isonymphaeol B and 3′-geranyl naringenin have beneficial effects on glucose metabolism through AMPK and GLUT1 pathway. Isonymphaeol B and 3′-geranyl naringenin may be potential lead candidates for antidiabetic drug development.

Graphical abstract



Keywords Prenylflavonoids · Myotubes · AMPK · GLUT1 · Diabetes

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Introduction

Diabetes is among the top 10 causes of death worldwide. In 2019, the prevalence of diabetes was estimated to be 473 million worldwide and is expected to increase to 578 million by 2030 [1]. Type 2 diabetes is the most common type of diabetes, accounting for around 90% of all diabetes worldwide. Type 2 diabetes mellitus (T2DM) is a metabolic disease characterized by the impairment of insulin-secretion from pancreatic β cells and insulin resistance in peripheral tissues such as liver, adipose tissue, and skeletal muscle [2] causing hyperglycemia which in turn leads to multiple diabetic complications, including nephropathy, neuropathy, retinopathy, ketoacidosis, cardiovascular disease, and hypertension [3], significantly reducing the quality of life.

Skeletal muscle is a major peripheral tissue, accounting for about 40% of the total body mass and more than 30% of total energy expenditure. In addition, over 80% of insulin-stimulated glucose transport is incorporated to skeletal muscle [4]. Any impairment in glucose uptake in skeletal muscle causes hyperglycemia [5]. Therefore, skeletal muscle and insulin plays an important role in maintaining blood-glucose homeostasis.

The uptake of glucose into tissues is mediated to by a carrier protein called glucose transporter (GLUT) 1–12 [6]. In skeletal muscle, GLUT4 is predominantly expressed and regulate glucose uptake in insulin-dependent and -independent pathways [7]. Translocation of intracellular GLUT4 to the cell membrane is important in glucose uptake [8]. On the other hand, GLUT1 is ubiquitously expressed and thought to be responsible for basal glucose uptake [9].

Translocation of GLUT4 into the cell membrane is regulated by two distinct signaling pathways. The first is the insulin-dependent pathway in which phosphatidylinositol-3 kinase (PI3K) is activated by insulin and then downstream protein kinase B (Akt) is activated and increases GLUT4 translocation. The other is the insulin-independent adenosine 5′-monophosphate activated protein kinase (AMPK) pathway, which is a major cellular and whole-body energy sensor and a master regulator of metabolic homeostasis, and this kinase is activated by increases in the AMP/ATP ratio that occurs during energy shortage [10]. AMPK has also been known as an attractive therapeutic target for metabolic disease including obesity and diabetes [11, 12]. Metformin, one of the therapeutic agents for hyperglycemia, increases glucose uptake by activating AMPK in skeletal muscle [13]. It is also reported that AMPK is activated by natural compounds such as resveratrol contained in red wine [14] and curcumin contained in *Curcuma longa* [15]. Therefore, natural products and food ingredients are excellent sources of lead compounds in the search for new drugs for the prevention and treatment of various metabolic diseases including type 2 diabetes.

We have investigated the pharmacological activity of various plant resources in Okinawa, Japan. *Macaranga tanarius* is native in Okinawa and known as source of Okinawan propolis [16]. *M. tanarius* characteristically contains prenylflavonoids such as nymphaeol A (6-geranyl eriodictyol), nymphaeol B (2′-geranyl eriodictyol), nymphaeol C (6-dimethylallyl-2′-geranyl eriodictyol), isonymphaeol B (5′-geranyl eriodictyol) and 3′-geranyl naringenin [17]. These prenylflavonoids have been reported to have various pharmacological activities such as antioxidative [18], antibacterial [19], anti-inflammatory and α-glucosidase inhibitive activity [20]. However, the effect of these prenylflavonoids in glucose metabolism is unknown. In this study, we examined the effects of prenylflavonoids from *M. tanarius* fruits on glucose uptake in skeletal muscle cells and the mechanism of action of these compounds.

Materials and methods

Materials

Insulin and Na^+/K^+ ATPase α-1 antibody were purchased from Sigma-Aldrich (St. Louis, MO, USA). Polyclonal antibodies against GLUT1, GLUT4, phospho-Akt (Ser473), Akt, phospho-AMPKα (Thr172), AMPKα, β-actin, horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and HRP-conjugated anti-mouse IgG were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Other reagents were obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan).

Plant material

Macaranga tanarius fruits used in this study was collected at the University of the Ryukyus in Okinawa, Japan., in May 2016. The plant was identified by one of the authors (Y. Saito). A voucher specimen (Saito and Amano 3520, 10 Apr. 2020) has been deposited in the Herbarium of the Faculty of Education (URO), University of the Ryukyus, Okinawa.

Isolation and identification of prenylflavonoids

The dried fruits of *M. tanarius* (2.2 g) were ground with a mixer and then extracted with ethanol (100.0 mL) for 5 days. The ethanol extract solution was filtered and concentrated to obtain *M. tanarius* fruits extract (MFE). MFE (215.4 mg) was separated using reverse-phase HPLC with COSMOSIL 5C18-AR-II (20 × 250 mm) at a flow rate of 5.0 ml/min, UV of 215 nm, gradient condition of 75% methanol containing 0.1% trifluoroacetic acid (TFA) at 0–50 min

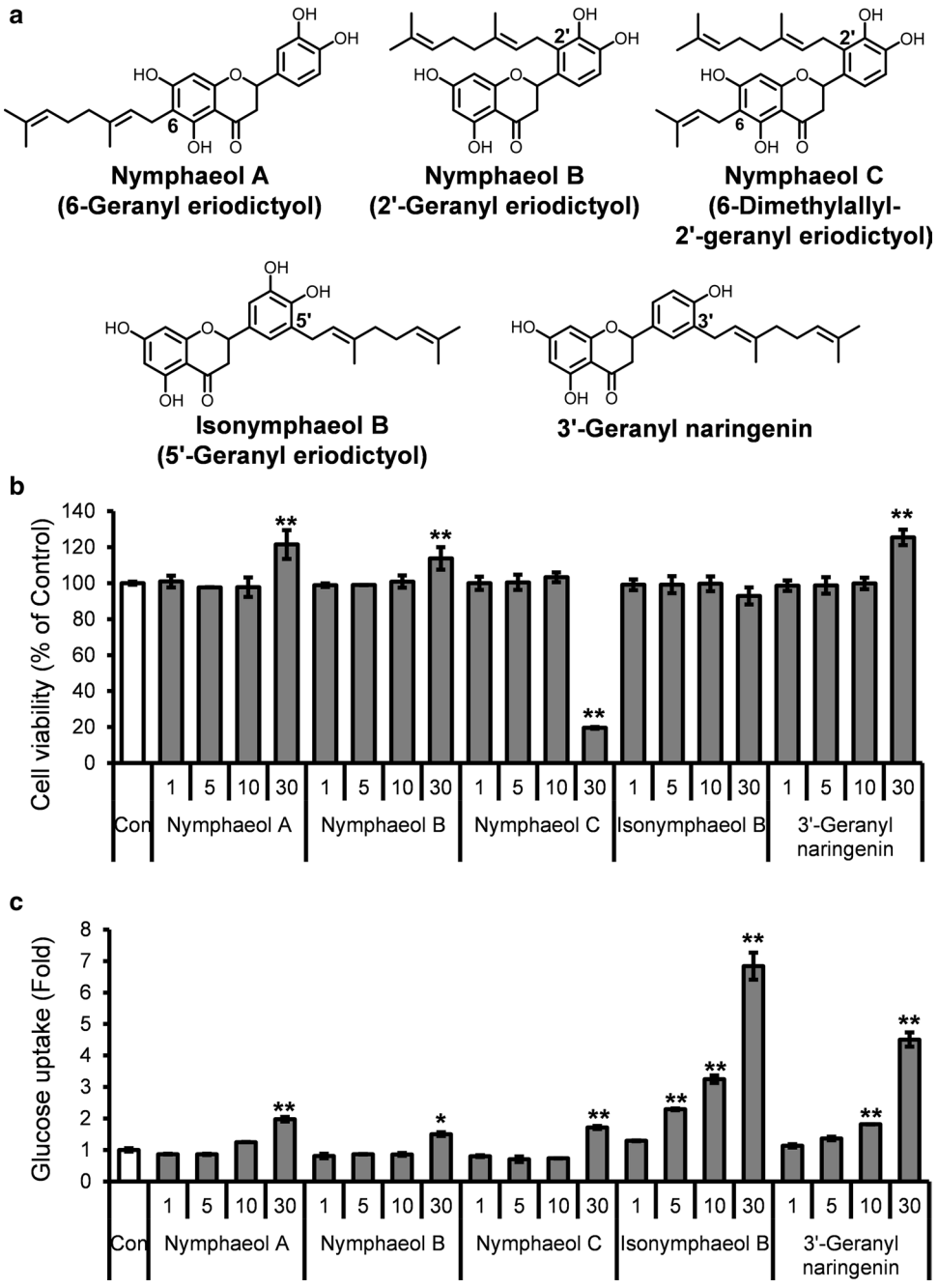

Fig. 1 Effects of prenylflavonoids on glucose uptake in L6 myotubes. **a** Chemical structures of the prenylflavonoids isolated from *M. tanarius* fruits. **b** Effects of prenylflavonoids on the viability of L6 myotubes. Cell viability was measured by the MTT assay. **c** Effects of prenylflavonoids on glucose uptake in L6 myotubes. L6 myotubes were preincubated in glucose-free KHH buffer for 2 h. The cells were then incubated in glucose-containing KHH buffer without or with prenylflavonoids for 24 h, and the glucose uptake was measured as described in Materials and methods. Each value represents the mean ± SD of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ vs Control cells (Con)

and 100% methanol at 50–70 min. The fraction eluted with 100% methanol was further separated using reverse-phase HPLC with COSMOSIL 5C18-AR-II (20 × 250 mm) at a flow rate of 5.0 ml/min, UV of 215 nm, gradient condition of 90% methanol containing 0.1% TFA at 0–10 min and 100% methanol at 10–20 min. The fraction eluted with 75% methanol containing 0.1% TFA resulted in the isolation of Compound 1 (33.7 mg), 2 (13.6 mg), 3 (43.8 mg) and 4 (19.7 mg), and the fraction eluted with 90% methanol containing 0.1% TFA resulted in the isolation of compound 5 (24.8 mg). The chemical structures of Compound 1 (nymphaeol B), 2 (isonymphaeol B), 3 (nymphaeol A), 4 (3′-geranyl naringenin) and 5 (nymphaeol C) were determined from their ^{1}H NMR and ^{13}C NMR spectra measured in $CDCl_3$, CD_3OD and acetone-d_6 by comparison with reported data [21–24] (Fig. 1a). The ^{1}H and ^{13}C NMR spectra were obtained on AVANCE III 500 NMR spectrometer (Bruker BioSpin Corporation, Billerica, MA, USA).

Cell culture

L6 rat myoblasts (JCRB Cell Bank, Osaka, Japan) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS) (Gibco, BRL, NY) and antibiotics (100 units/mL penicillin and 10 µg/mL streptomycin (Sigma-Aldrich) at 37 ℃ under a humidified 5% CO_2 atmosphere. To differentiate myotubes, L6 myoblasts were seeded in 96-well plates (5×10^3 cells/well) or 6-well plates (1×10^5 cells/well) and cultured to confluence for 2 days. Then, the cells were cultured with DMEM supplemented with 0.5% FBS for 9 days. The medium was renewed every 3 days.

Glucose uptake assay

Differentiated L6 myotubes in 96-well plates were washed with PBS and incubated in filter-sterilized glucose-free Krebs–Henseleit-HEPES (KHH) buffer (119 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$•$2H_2O$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 0.1% bovine serum albumin, 10 mM HEPES, and 2 mM sodium pyruvate, pH 7.4) for 2 h. The cells were then cultured for 24 h in KHH buffer containing 11 mM glucose (glucose-containing KHH buffer) with or without prenylflavonoids. To measure the amount of glucose uptake into the cells, the glucose concentrations in the KHH buffer before and after culture were measured with a microplate reader (Bio Tek, absorbance at 490 nm) using the glucose CII test kit (FUJIFILM Wako). The amounts of glucose uptake were calculated from the differences in glucose concentrations between before and after incubation.

Cell viability

Cell viability was determined using a MTT reagent (Sigma-Aldrich) after the Glucose uptake assay. The absorbance at 570 nm was measured using a microplate reader.

Subcellular fractionation and GLUTs translocation analysis

To assesses the translocation of GLUT4, plasma membrane and cytoplasmic fractions were prepared as described previously [25] with slight modification. L6 myotubes were harvested with buffer A (50 mM Tris–HCl, pH 8.0, 0.5 mM DTT) containing 0.1% (v/v) Nonidet P-40 (NP-40), protease inhibitors (1 mM PMSF, 5 µg/mL leupeptin and 5 µg/mL aprotinin), and a phosphatase inhibitor (10 mM NaF and 1 mM Na_3VO_4) and homogenized by 10 passages through a 27-gage needle. Each homogenate was centrifuged at 1000 g for 10 min at 4 ℃, and the pellet was resuspended in NP-40-free buffer A. After incubation on ice for 10 min with occasional mixing, the samples were centrifuged at 1000×*g* for 10 min at 4 ℃. The supernatants from the first and second centrifugations at 1000×*g* were pooled and centrifuged at 16,000×*g* for 20 min at 4 ℃. This supernatant was collected as the cytoplasm fraction. The pellet was resuspended in buffer A containing 1% (v/v) NP-40, incubated for 24 h at 4 ℃, and centrifuged at 16,000×*g* for 20 min at 4 ℃. The supernatant was collected as the plasma membrane fraction.

Western blotting analysis

L6 myotubes cultured in a 6-well plate were washed with PBS and then incubated with glucose-free KHH buffer for 2 h. Next, glucose-free KHH buffer was removed and incubated with glucose-containing KHH buffer for 2 h. The cells were then cultured for prescribed time in glucose-containing KHH buffer without or with prenylflavonoids. The cells were washed with ice-cold PBS and solubilized in 1 × SDS Loading Buffer (Cell signaling) to obtain total cell lysate. The lysates were sonicated for 10 s, boiled at 100 ℃ for 10 min and centrifuged at 15,000 rpm for 10 min. The protein concentrations of the supernatants were measured using RC DC ™ Protein Assay kit (Bio-Rad laboratories Inc., Hercules, CA, USA). Equal amounts of protein (20 µg per lane) were loaded onto 10% Mini-PROTEAN TGX precast gels (Bio-Rad), separated by electrophoresis, and transferred to nitrocellulose membranes. The membranes were washed with Tris buffered saline (TBS) and blocked with 5% BSA in TBS containing 1% Tween 20 (TBS-T) for 1 h. After the blocking, the membranes were washed in TBS-T and incubated with anti-phospho-AMPK, anti-AMPK, anti-phospho-Akt, anti-Akt, anti-β-actin, anti-GLUT1, anti-GLUT4 or anti-Na^+/K^+ ATPase antibodies overnight at 4℃. The membranes were then washed with TBS-T and incubated with HRP-conjugated anti-rabbit IgG or HRP-conjugated anti-mouse IgG at room temperature for 1 h. Immunoreactive bands were detected using Clarity Western ECL Substrate (Bio-Rad). The intensity of each band was analyzed with a ChemiDoc XRS Plus system coupled with Image Lab software (Bio-Rad).

Table 1 Primer sequence used in Real-time RT-PCR

Target gene	Forward primer (5′→3′)	Reverse primer (5′→3′)
Glut1	ACGTCCATTCTCCGTTTCAC	TCCCACGGCCAACATAAG
Glut4	ACAGGGCAAGGATGGTAGA	TGGAGGGGAACAAGAAAGT
β-actin	GGCTGTGTTGTCCCTGTATG	AATGTCACGCACGATTTCC

Real-time reverse transcription-polymerase chain reaction (RT-PCR)

L6 myotubes cultured in a 6-well plate were subjected to total RNA isolation using RNeasy plus mini kit (QIAGEN). The total RNA was reverse transcribed into cDNA using the ReverTra Ace qPCR RT kit (TOYOBO, Osaka, Japan) according to the manufacturer's instructions. Real-time PCR was performed with FastStart SYBR green Master Mix (Roche Diagnostics, Mannheim, Germany) in a StepOnePlus System (Applied Biosystems, USA). The relative expression levels of the target genes against the endogenous reference gene β-actin was calculated using the delta cycle threshold method. The primer sequences are listed in Table 1.

Statistical analysis

All the data are presented as mean ± standard deviation. One-way analysis of variance (ANOVA) followed by Dunnett's test was used to determine the statistical significance of the differences between the experimental groups. Statistical significance was assumed at $p < 0.05$, $p < 0.01$.

Results

Effect of prenylflavonoids from *M. tanarius* fruits on glucose uptake in L6 myotubes

First, cytotoxicity of prenylflavonoids was assessed by MTT assay. Nymphaeol C decreased cell viability only at highest concentration (30 μM). In contrast, nymphaeol A, nymphaeol B, isonymphaeol B and 3′-geranyl naringenin did not show cytotoxicity up to 30 μM (Fig. 1b). To investigate the effect of prenylflavonoids on glucose metabolism, glucose uptake was measured in L6 myotubes. Nymphaeol A, nymphaeol B and nymphaeol C increased glucose uptake at a concentration of 30 μM. Isonymphaeol B and 3′-geranyl naringenin increased glucose uptake of L6 myotubes in a concentration-dependent manner (Fig. 1c). These results suggest that prenylflavonoids from *M. tanarius* have a stimulatory effect on glucose uptake with the highest activity shown by isonymphaeol B.

Effects of isonymphaeol B and 3′-geranyl naringenin on PI3K/Akt and AMPK signaling molecules

To study the mechanism of action of prenylflavonoids in the upregulation of glucose uptake, we investigated whether isonymphaeol B and 3′-geranyl naringenin affect insulin

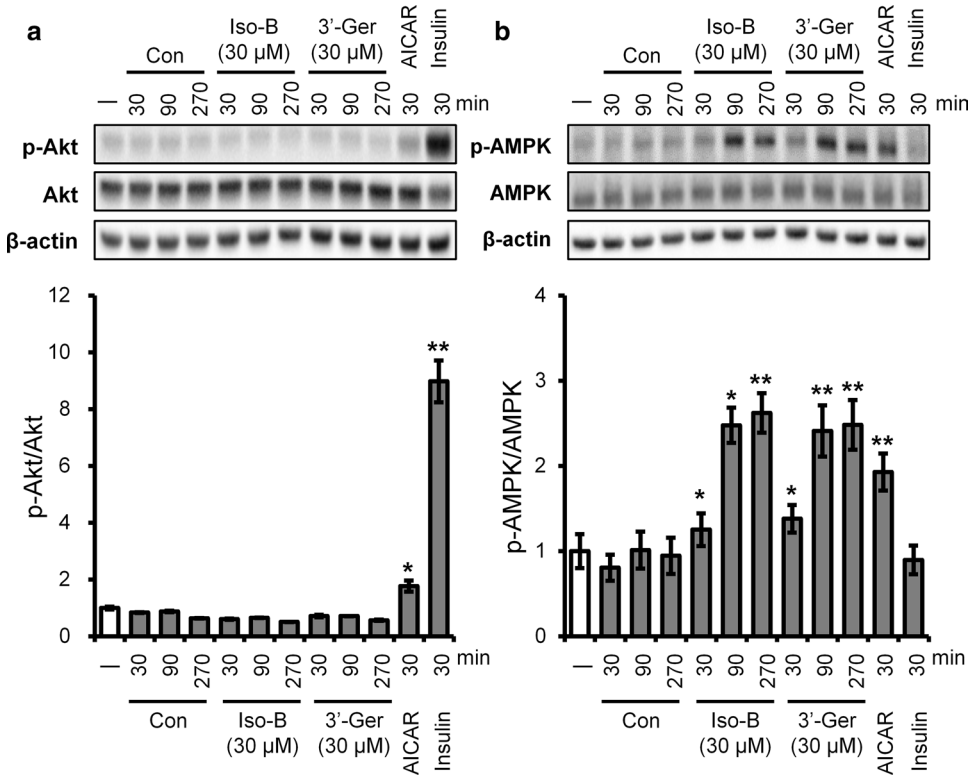

Fig. 2 Effects of isonymphaeol B and 3′-geranyl naringenin on the phosphorylation of Akt and AMPK in L6 myotubes. **a**, **b** Cells were incubated for the indicated times with isonymphaeol B (Iso-B) and 3′-geranyl naringenin (3′-Ger) and the cell lysates were subjected to Western blotting. Each value represents the mean ± SD of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ vs non-treatment cell (−)

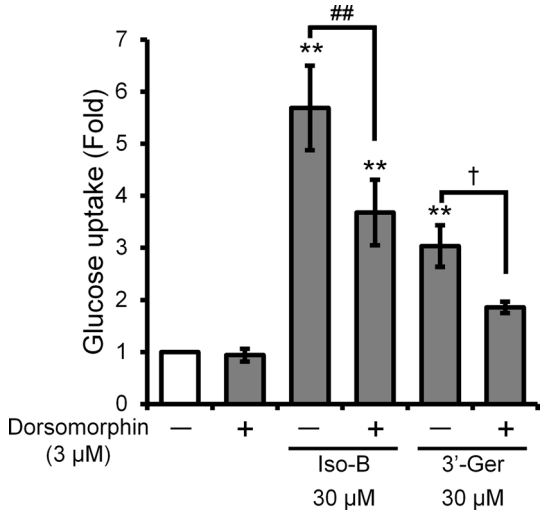

Fig. 3 Effects of AMPK inhibitor on isonymphaeol B and 3′-geranyl naringenin increased glucose uptake. L6 myotubes were preincubated in glucose-free KHH buffer without or with dorsomorphin (AMPK inhibitor) for 2 h. The cells were then incubated in glucose-containing KHH buffer without or with dorsomorphin (AMPK inhibitor) in presence of isonymphaeol B (Iso-B) and 3′-geranyl naringenin (3′-Ger) for 24 h, and the glucose uptake was measured as described in Materials and methods. Each value represents the mean ± SD of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ vs non-treatment cells (−)

PI3K/Akt pathway or noninsulin AMPK pathways by Western blot analysis. Insulin increased the amount of phosphorylated Akt in L6 myotubes, whereas isonymphaeol B and 3′-geranyl naringenin had no effect on phosphorylated Akt (Fig. 2a). In contrast, 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), an AMPK activator used as a positive control, significantly increased the amount of phosphorylated AMPK. Isonymphaeol B and 3′-geranyl naringenin also increased phosphorylated AMPK in a time-dependent manner (Fig. 2b). These results suggest that isonymphaeol B and 3′-geranyl naringenin activate AMPK pathway but not PI3K/Akt pathway.

Furthermore, to reveal whether the activation of the AMPK pathway is involved in isonymphaeol B and 3′-geranyl naringenin-increased glucose uptake, we examined the effects on isonymphaeol B and 3′-geranyl naringenin-increased glucose uptake using AMPK inhibitor (dorsomorphin). Dorsomorphin partly decreased isonymphaeol B and 3′-geranyl naringenin-increased glucose uptake (Fig. 3). These results suggest that the activation of AMPK contributes to isonymphaeol B and 3′-geranyl naringenin-increased glucose uptake.

Effects of isonymphaeol B and 3′-geranyl naringenin on GLUT1 and GLUT4 in L6 myotubes

GLUT1 and GLUT4 is the major glucose transporters in muscle. The amount of glucose transporters in plasma membrane is important in the activity of glucose uptake. We prepared protein sample of the plasma membrane and cytoplasmic fraction from L6 myotubes and the expression levels of GLUT1 and GLUT4 proteins were determined by Western blot analysis. Na^+/K^+ ATPase and β-actin were used as loading control proteins for the plasma membrane fraction and the cytoplasmic fraction, respectively. In the plasma membrane fraction, isonymphaeol B and 3′-geranyl naringenin significantly increased the expression level of GLUT1 protein as compared with control. On the other hand, the expression level of GLUT4 protein did not significantly increased as compared with the control. In addition, isonymphaeol B and 3′-geranyl naringenin significantly increased the expression level of Na^+/K^+ ATPase protein in the cell membrane fraction as compared to control (Fig. 4a and b). In the cytoplasmic fraction, isonymphaeol B and 3′-geranyl naringenin did not significantly change the protein expression levels of GLUT1 and GLUT4 as compared with the control (Fig. 5a and b). Next, we investigated the effects of isonymphaeol B and 3′-geranyl naringenin on *Glut1* and *Glut4* mRNA expression in L6 myotubes. Isonymphaeol B and 3′-geranyl naringenin increased *Glut1* mRNA expression (Fig. 6a). On the other hand, *Glut4* mRNA expression was increased by isonympaheol B but not 3′-geranyl naringenin (Fig. 6b). From these results, it is considered that isonymphaeol B and 3′-geranyl naringenin increased GLUT1 protein by increasing *Glut1* mRNA expression, and as a result, the amount of GLUT1 protein on the cell membrane also increased.

Discussion

Many flavonoids have been reported to have antidiabetic effects [26]. In this study, we focused on prenylflavonoids from *M. tanarius*, and investigated the antidiabetic effects of these prenylflavonoids. We first found that prenylflavonoids isolated from *M. tanarius* fruits increased glucose uptake in L6 myotubes. In particular, isonymphaeol B and 3′-geranyl naringenin promoted glucose uptake at lower concentrations in a concentration-dependent manner compared to the others. These results indicated that isonymphaeol B and 3′-geranyl naringenin might exert some metabolic roles as glucose uptake enhancers within skeletal muscles.

Prenyl groups can affect the cellular uptake and biological function of flavonoids by increasing the hydrophobicity of flavonoids and facilitating interaction with the phospholipid bilayers of biological membranes [27, 28]. In fact, 8-prenyl

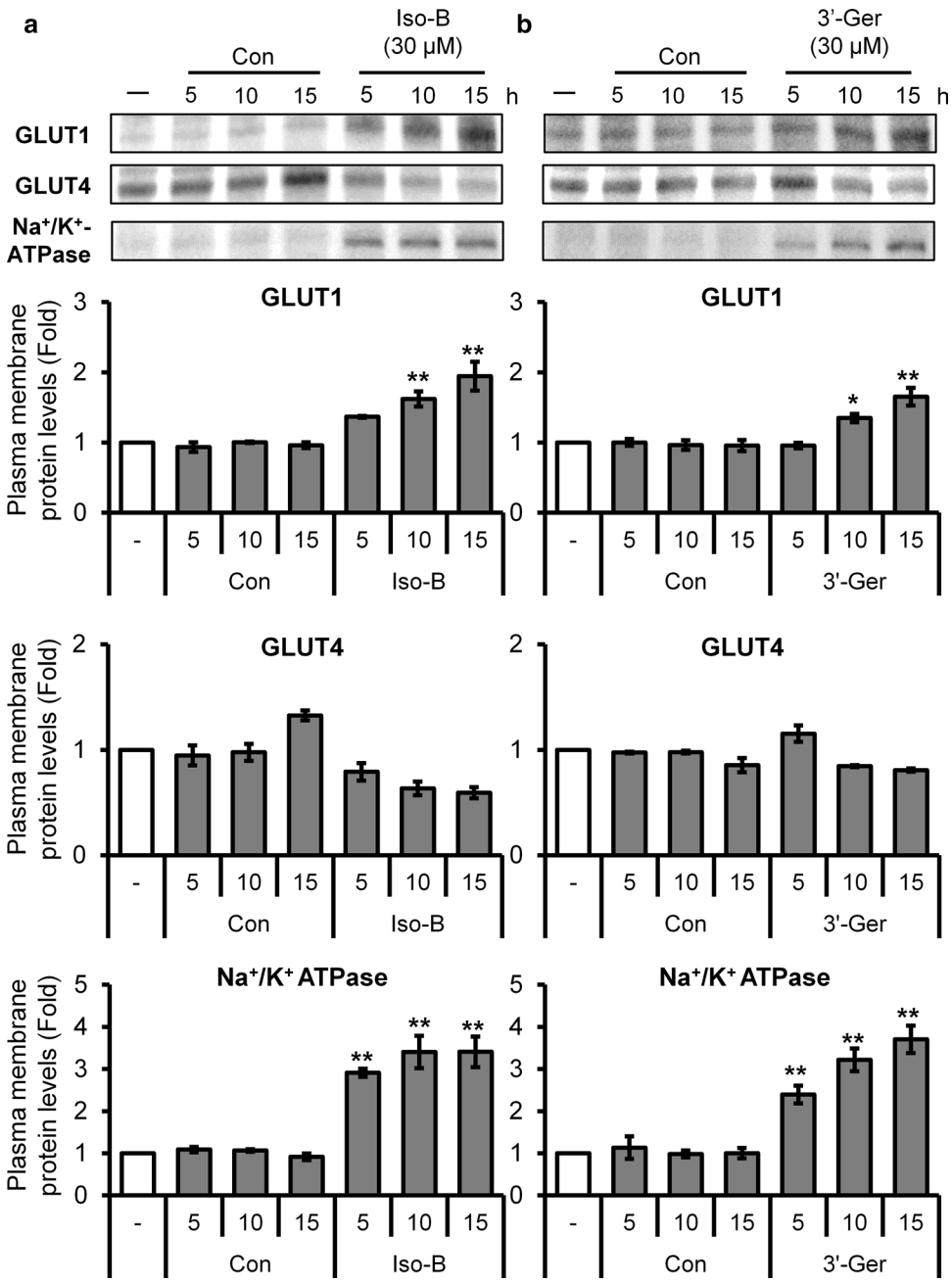

Fig. 4 Effects of isonymphaeol B and 3′-geranyl naringenin on the protein expression of GLUT1 and GLUT4 in the plasma membrane of L6 myotubes. **a**, **b** Cells were cultured for the indicated times with isonymphaeol B (Iso-B) and 3′-geranyl naringenin (3′-Ger). The plasma membrane fraction was subjected to Western blotting and GLUT1, GLUT4 and Na^+/K^+ ATPase were detected. Each value represents the mean ± SD of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ vs non-treatment cells (−)

quercetin and 8-prenyl naringenin have been reported to accumulate more in cells and tissues than quercetin and naringenin [29, 30]. In addition, prenylation has been reported to enhance the estrogenic activity in naringenin and genistein [31]. The five prenylflavonoids isolated from *M. tanarius* are prenylated derivatives of eriodictyol and naringenin. The theoretical value of the Octanol/Water partition coefficient (log Pow), which are an indicator of the hydrophobicity of a compound, is higher in nymphaeol A (logP = 5.8), nymphaeol B (logP = 5.8), nymphaeol C (logP = 7.7), isonymphaeol B (logP = 5.8) and 3′-geranyl naringenin (logP = 6.4) than eriodictyol (logP = 2.0) and naringenin (logP = 2.4). Eriodictyol and naringenin have been reported to increase glucose uptake in adipocytes [32] or skeletal muscle [33]. We have confirmed that isonymphaeol B and 3′-geranyl naringenin increase glucose uptake in L6 myotubes at lower concentrations compared to eriodictyol and naringenin (data not shown). In addition, isonymphaeol B showed a higher glucose uptake effect on L6 myotubes than nymphaeol A, nymphaeol B and nymphaeol C. These results provide that the presence and location of prenyl groups in flavonoids may affect glucose metabolism in skeletal muscle.

Moreover, since only nymphaeol C showed cytotoxicity at 30 μM, it is possible that the dimethylallyl group at the

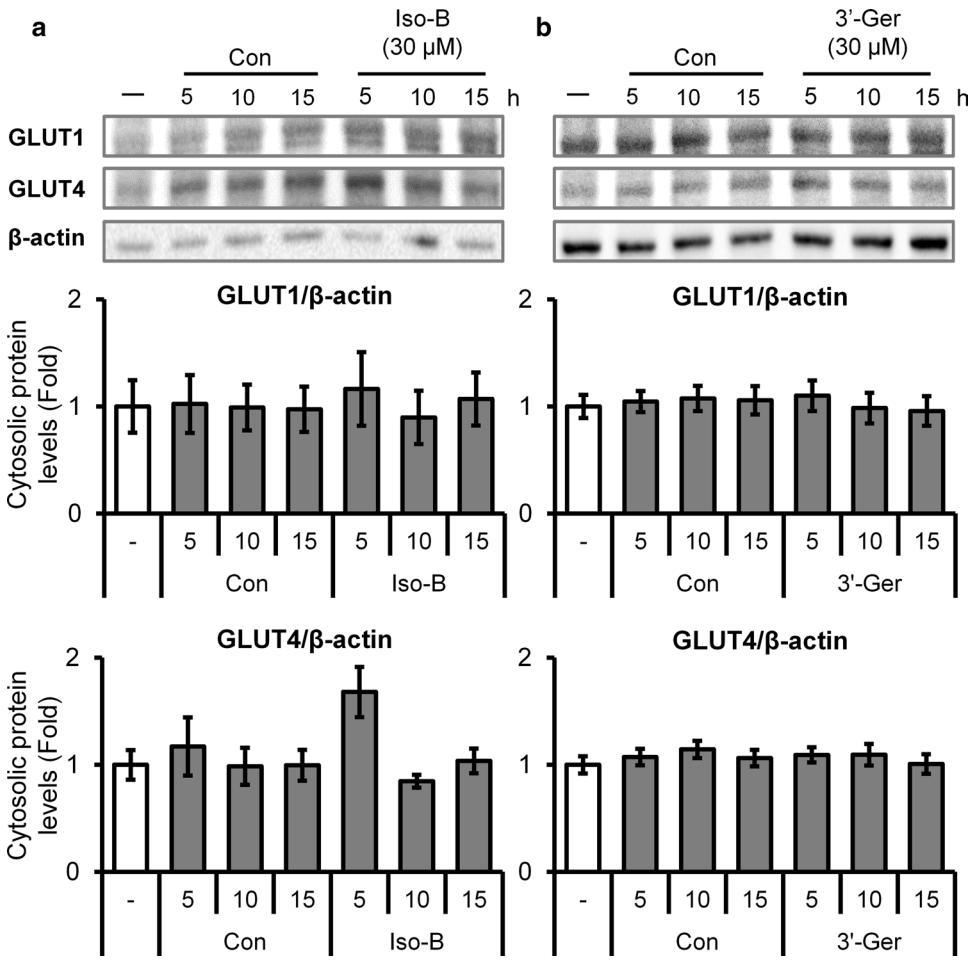

Fig. 5 Effects of isonymphaeol B and 3′-geranyl naringenin on the protein expression of GLUT1 and GLUT4 in the cytoplasm of L6 myotubes. **a**, **b** Cells were cultured for the indicated times with isonymphaeol B (Iso-B) and 3′-geranylnaringenin (3′-Ger). The cytoplasm fraction was subjected to Western blotting and GLUT1, GLUT4 and β-actin were detected. Each value represents the mean ± SD of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ vs non-treatment cells (−)

6-position is involved in the expression of cytotoxic activity. Nymphaeol C has been reported to activate caspase 3 involved in the induction of cell apoptosis more strongly than nymphaeol A, nymphaeol B and isonymphaeol B [34]. Therefore, nymphaeol C may have shown cytotoxicity by inducing apoptosis in L6 myotubes.

Glucose transport in skeletal muscle is mediated by GLUT1 and GLUT4. GLUT1 is localized primarily at the plasma membrane and, is thought to participate mainly in basal glucose transport in skeletal muscle [9]. The activation of GLUT1 is mainly occurred by the cell stressors such as azide [35, 36], osmotic stress [37, 38], methylene blue [39] and glucose deprivation [40, 41]. Among them, the activation of GLUT1 by hypoxia or azide has been attributed to activation of AMPK [38, 42, 43]. Studies of cultured human skeletal muscle cells have reported a decrease in basal glucose uptake and GLUT1 protein levels in cells from patients with type II diabetes [44]. In contradistinction to GLUT1, under basal conditions, GLUT4 is localized mainly in intracellular vesicles and is translocated to the plasma membrane in response to insulin. In particular, GLUT4 is responsible for most of the glucose transport from blood following a meal. GLUT4 translocation to the plasma membrane is mainly regulated by insulin-dependent and -independent signal pathways. The insulin-dependent signaling pathway induces GLUT4 translocation by activating PI3K and downstream Akt phosphorylation when insulin binds to the insulin receptor [45]. On the other hand, the insulin-independent signaling pathway induces translocation of GLUT4 to the plasma membrane through activation of AMPK [46]. AMPK is activated by an increase in the AMP/ATP ratio due to exercise and muscle contraction, adiponectin secreted by adipocytes [47], and liver kinase B1 (LKB1) and Ca^{2+}/calmodulin-dependent kinase β (CaMKKβ) upstream of AMPK [48]. It was suggested that isonymphaeol B and 3′-geranyl naringenin did not affect PI3K phosphorylation, but increased phosphorylation of AMPK, leading to insulin-independent activation of AMPK. It has been reported that naringenin also induced AMPK activation in L6 myotubes without affecting PI3K, and it is possible that isonymphaeol B and 3′-geranyl naringenin promotes glucose uptake by a similar mechanism [33]. To further investigate the mechanism of AMPK activation, it is necessary to investigate the

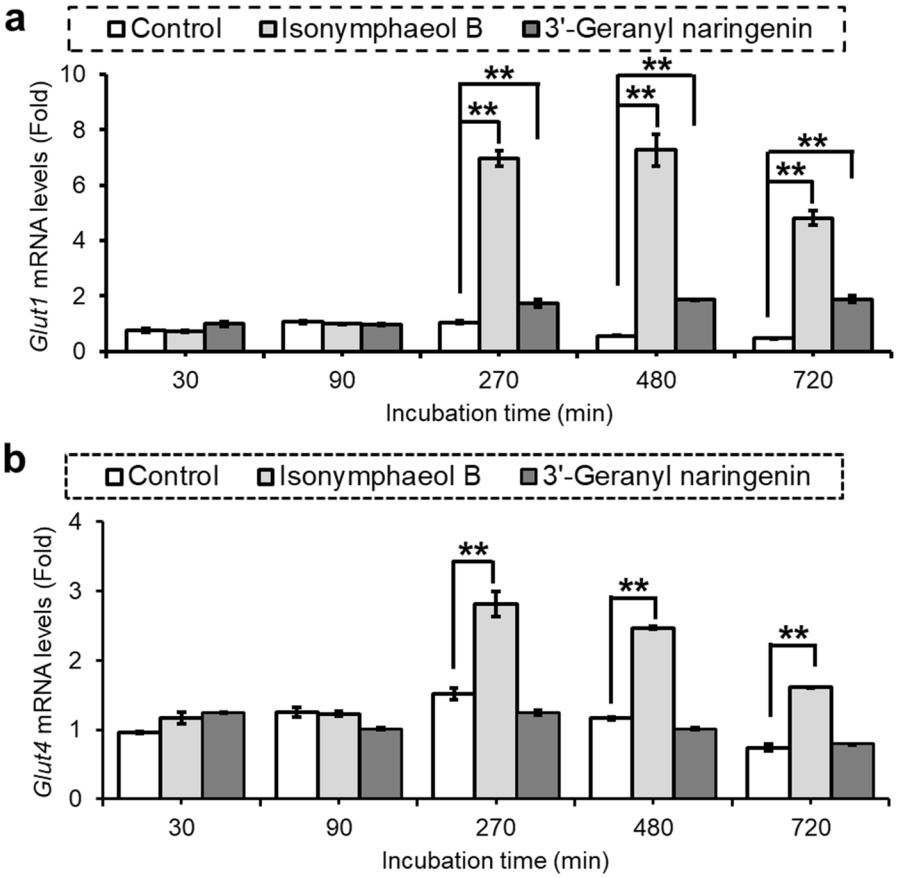

Fig. 6 Effects of isonymphaeol B and 3′-geranyl naringenin on the mRNA expression of *Glut1* and *Glut4* in L6 myotubes. (**a**, **b**) Cells were cultured for the indicated times with isonymphaeol B (30 μM) and 3′-geranyl naringenin (30 μM). Total RNA was isolated and reverse transcribed to cDNA for RT-PCR analysis. The expression levels of each mRNA were normalized to the level of β-actin. Each value represents the mean ± SD of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ vs Control cells (Control)

effects of isonymphaeol B and 3′-geranyl naringenin on LKB1, CaMKKβ, and AMP/ATP ratio.

We measured Na^+/K^+ ATPase as a loading control in plasma membrane fraction analysis. However, Na^+/K^+ ATPase protein has been reported to increase with activation of AMPK in L6 myotube cells [49]. Therefore, it is suggested that isonymphaeol B and 3′-geranyl naringenin also increased Na^+/K^+ ATPase protein through activation of AMPK. Isonymphaeol B and 3′-geranyl naringenin enhanced GLUT1 protein in the plasma membrane of L6 myotubes. Thioredoxin-interacting protein (TXNIP) reduces glucose uptake by promoting GLUT1 endocytosis and suppressing *Glut1* mRNA expression [50]. However, it has been reported that phosphorylation of TXNIP by activated AMPK promotes the degradation of TXNIP, resulting in an increase GLUT1 protein in plasma membrane, *Glut1* mRNA expression and glucose uptake [50]. Therefore, isonymphaeol B and 3′-geranyl naringenin may have increased GLUT1 protein in plasma membrane and *Glut1* mRNA expression by promoting TXNIP degradation via AMPK activation. On the other hand, isonymphaeol B and 3′-geranyl naringenin did not increase GLUT4 protein in plasma membrane. Interestingly, although isonymphaeol B increased *Glut4* mRNA expression, it did not increase GLUT4 protein in plasma membrane. Therefore, it is necessary to further investigate the mechanism by which isonymphaeol B and 3′-geranyl naringenin selectively increase GLUT1 protein in plasma membrane. In addition, Na^+/K^+ ATPase is known to regulate glucose transport via sodium glucose cotransporters (SGLTs) according to the established sodium gradient [51]. Therefore, it is necessary to verify whether SGLT1 expressed in skeletal muscle [52] is involved in the glucose uptake increasing effect of isonymphaeol B and 3′-geranyl naringenin. These results suggest that isonymphaeol B and 3′-geranyl naringenin take up glucose into the cell via GLUT1.

In conclusion, we demonstrated that isonymphaeol B and 3′-geranyl naringenin isolated from *M. tanarius* fruits increase glucose uptake, expression of *Glut1* mRNA and plasma membrane GLUT1 protein in muscle cells by activating the AMPK pathway. We propose that isonymphaeol B and 3′-geranyl naringenin and material (*M. tanarius*

fruits and Okinawan propolis) containing these components are potential lead candidates for the development of antidiabetic drugs.

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