Molecular and Cellular Endocrinology 393 (2014) 99–108

Contents lists available at ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Glabridin induces glucose uptake via the AMP-activated protein kinase pathway in muscle cells

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ARTICLE INFO

Article history:
Received 20 December 2013
Received in revised form 9 June 2014
Accepted 10 June 2014
Available online 19 June 2014

Keywords:
Glabridin
Glucose uptake
Muscle cells
Adenosine monophosphate-activated protein kinase
Glucose transporter type 4

ABSTRACT

The present study demonstrates that glabridin, a prenylated isoflavone in licorice, stimulates glucose uptake through the adenosine monophosphate-activated protein kinase (AMPK) pathway in L6 myotubes. Treatment with glabridin for 4 h induced glucose uptake in a dose-dependent manner accompanied by the translocation of glucose transporter type 4 (GLUT4) to the plasma membrane. Glabridin needed at least 4 h to increase glucose uptake, while it significantly decreased glycogen and increased lactic acid within 15 min. Pharmacological inhibition of AMPK by Compound C suppressed the glabridin-induced glucose uptake, whereas phosphoinositide 3-kinase and Akt inhibition by LY294002 and Akt1/2 inhibitor, respectively, did not. Furthermore, glabridin induced AMPK phosphorylation, and siRNA for AMPK completely abolished glabridin-induced glucose uptake. We confirmed that glabridin-rich licorice extract prevent glucose intolerance accompanied by the AMPK-dependent GLUT4 translocation in the plasma membrane of mice skeletal muscle. These results indicate that glabridin may possess a therapeutic effect on metabolic disorders, such as diabetes and hyperglycemia, by modulating glucose metabolism through AMPK in skeletal muscle cells.

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1. Introduction

Skeletal muscle is one of the important tissues for whole-body insulin-mediated glucose disposal in the postprandial state (Saltiel and Kahn, 2001; Klip and Pâquet, 1990; Kelley et al., 1988), and is, therefore, the most important site for whole-body glucose homeostasis. It is known that in subjects with type 2 diabetes mellitus, essentially all the impairment in insulin-mediated glucose disposal is because of inadequate glucose uptake by muscle (DeFronzo, 1988). Thus, many researchers have investigated the effect of compounds that stimulate muscle glucose uptake in skeletal muscle as a therapeutic target for metabolic disorders (Zhang et al., 2009; Eid et al., 2010; Kawabata et al., 2011).

Glucose transporter type 4 (GLUT4) is a key determinant of whole-body glucose homeostasis, which is highly expressed in skeletal muscle and adipose tissue (Huang and Czech, 2007). Insulin promotes glucose uptake by activating signal transducing proteins, including insulin receptor, phosphoinositide 3-kinase (PI3K), Akt and Akt substrate 160 kDa (AS160), leading to translocation of GLUT4 from intracellular storage vesicles to the plasma membrane (Thong et al., 2005). Muscle contraction and energy depletion also stimulate GLUT4 translocation and glucose uptake through the activation of adenosine monophosphate-activated protein kinase (AMPK) or Ca^{2+}/calmodulin-dependent kinase II (CaMKII) (Rose and Richter, 2005). Insulin and muscle contraction modulate glucose use in muscle cells; the former stimulates anabolic reactions such as glycogen and triglyceride synthesis, and the latter induces their degradation and inhibition of anabolism. Several food factors have been reported to facilitate GLUT4 translocation and anabolic or catabolic reactions via activation of these signaling proteins in skeletal muscle: caffeine activates CaMKII and AMPK, leading to GLUT4-mediated glucose uptake and fatty acid oxidation in rat skeletal muscle (Raney and Turcotte, 2008; Jensen et al., 2007a); and epigallocatechin gallate induces GLUT4

Abbreviations: ACC, acetyl CoA carboxylase; ACO, acyl CoA oxidase; AICAR, 5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide; AMPK, adenosine monophosphate-activated protein kinase; AS160, Akt substrate 160 kDa; BSA, bovine serum albumin; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; 2DG, 2-deoxyglucose; FAS, fatty acid synthase; FBS, fetal bovine serum; G6PDH, glucose-6-phosphate dehydrogenase; GLUT4, glucose transporter type 4; KRH, Krebs–Ringer HEPES buffer; LKB1, liver kinase B1; PI3K, phosphoinositide 3-kinase; PPARγ, peroxisome proliferator-activated receptor γ; TAK1, TGF-β-activated kinase 1; UCP, uncoupling protein.

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http://dx.doi.org/10.1016/j.mce.2014.06.009
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translocation and glucose uptake in a PI3K-dependent manner in rat skeletal muscle cells (Ueda et al., 2008).

Glabridin is one of the major flavonoids in the hydrophobic fraction of licorice (*Glycyrrhiza glabra* L.), which has been one of the most frequently employed botanicals as a traditional herbal medicine for over 4000 years. Recent reports have shown that an ethanol extract of licorice root, which contains glabridin, suppressed hyperglycemia in genetically obese and diabetic KK-A^y mice (Nakagawa et al., 2004), and abdominal fat accumulation in high-fat diet-induced obese C57BL/6J mice (Aoki et al., 2007). Glabridin has also been demonstrated to have various beneficial effects such as antioxidant activity (Haraguchi et al., 2000; Rosenblat et al., 1999), estrogen-like activity (Tamir et al., 2000), antinephritic activity (Fukai et al., 2003), anti-fatigue properties (Shang et al., 2009), and agonistic activity on peroxisome proliferator-activated receptor (PPAR) γ (Kuroda et al., 2010). Furthermore, glabridin has high bioavailability as it has been reported to escape conjugation during the intestinal absorption process and exists in the free aglycone form in the blood of rats (Aoki et al., 2005; Ito et al., 2007). These facts suggest the direct effect of glabridin on modulation of cellular energy metabolism in peripheral tissues including skeletal muscle. However, the underlying mechanism of the anti-diabetic effect of glabridin remains unclear. In this study, we investigated the effect of glabridin on glucose uptake activity and its underlying mechanism in skeletal muscle cells.

2. Materials and methods

2.1. Materials

Minimum essential medium (MEM), 2-deoxyglucose (2DG), glucose-6-phosphate dehydrogenase (G6PDH), 7-hydroxy-3H-phenoxazin-3-one 10-oxide (resazurin), 5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide (AICAR), Compound C, LY294002 and Akt1/2 inhibitor (Sigma, St. Louis, MO, USA); diaphorase and β-nicotinamide adenine dinucleotide phosphate (NADP)$^+$ (Oriental Yeast, Tokyo, Japan); fetal bovine serum (FBS; Biological Industries, Kibbutz Beit Haemek, Israel) and ImmunoStar® LD (WAKO Pure Chemicals, Osaka, Japan) were purchased from commercial sources. KN-93, anti-GLUT4, anti-GLUT1, anti-IR, anti-goat IgG and anti-rabbit IgG antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-p-AMPK (Thr172), anti-AMPK, anti-phospho-AS160, anti-AS160, anti-p-CaMKII (Thr286), anti-CaMKII and anti-β-actin were purchased from Cell Signaling Technology (Danvers, MA, USA). Glabridin and all other reagents were purchased from WAKO Pure Chemicals, unless otherwise specified. For AMPKα and CaMKII knockdown, following siRNA and transfection reagents were introduced: The siRNA sequences consisting of GCA UAU GCU GCA GGU AGA for AMPKα1and AUU CUA UCA CUAGCG UGA CUU for the unrelated control and Lipofectamine 2000 were purchased from Invitrogen Life Technologies (Burlington, Ontario, Canada); the siRNA sequences consisting of GAA GAA UGA UGG CGU GAA G for CaMKII and CAA GGA UCU GAU CAA UAA G for the unrelated control and siRNA transfection medium were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Cell culture

Rat L6 skeletal muscle cells were maintained in MEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere with 5% CO_2. For each experiment, L6 cells were seeded in 96-well plates (4×10^3 cells/0.2 mL), 12-well plates (1×10^4 cells/mL) or 60-mm dishes (12×10^4 cells/4 mL) in culture medium. To induce differentiation into myotubes, 2 days after plating the medium was replaced with MEM containing 2% FBS and the antibiotics, and was changed every other day. L6 cells were used for experiments after 7 days of differentiation and the subsequent 18 h of serum starvation in MEM containing 0.2% BSA.

2.3. Preparation of plasma membrane fraction and western blot analysis

Serum-starved L6 myotubes plated on 60-mm dishes were treated with glabridin (DMSO as vehicle control), insulin or 5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide (AICAR), and harvested with lysis buffer [50 mmol/L Tris, pH 8.0, 0.1% (v/v) Nonidet P-40, 0.5 mmol/L DTT, protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride, 5 μg/mL leupeptin, and 5 μg/mL aprotinin) and phosphatase inhibitors (10 mmol/L NaF and 1 mmol/L Na_3VO_4)]. The obtained cell lysate was fractionated into a plasma membrane fraction as described previously (Kawabata et al., 2011). Aliquots of 3–10 μg of cell lysate or 5 μg of plasma membrane fraction were separated on 10% SDS–PAGE gels and transferred to polyvinylidene difluoride membranes (Biotrace, Pall Corporation, Port Washington, NY, USA). After blocking with Blocking One® (Nakarai Tesque, Kyoto, Japan), the membrane was treated with the appropriate specific primary antibodies (1:10,000) overnight at 4 °C, followed by the corresponding horseradish peroxidase-conjugated secondary antibody (1:20,000) for 1 h at room temperature. Specific immune complexes were detected using ImmunoStar® LD.

2.4. Glucose uptake assay

Serum-starved L6 myotubes in a 96-well plate were treated with glabridin and/or other compounds in 0.2% (w/v) BSA/MEM. The final concentration and the time of incubation of each compound are indicated in the figures. For inhibition of signal pathways, L6 myotubes were pre-incubated with 40 μM Compound C (an AMPK inhibitor), 10 μM LY294002 (a PI3K inhibitor), 1 μM Akt1/2 inhibitor, 10 μM KN-93 (a CaMKII inhibitor), or 10 μM BAPTA/AM (a cell-permeable Ca^{2+} chelator) for 30 min. The cells were treated with 100 nM insulin, 1 mM AICAR, or 10 or 30 μM glabridin for 4 h. DMSO was used as a vehicle control (final concentration was 0.1%). The treated cells were further incubated with 1 mM 2DG for 20 min in Krebs–Ringer HEPES buffer (KRH; 50 mM HEPES, pH 7.4, 137 mM sodium chloride, 4.8 mM potassium chloride, 1.85 mM calcium chloride and 1.3 mM magnesium sulfate) containing 0.1% (w/v) BSA. The amount of 2DG incorporated into the cells was enzymatically determined as described previously (Yamamoto et al., 2006, 2010).

2.5. Measurement of L-lactic acid, glycogen and ATP

L6 myotubes in a 96-well plate (for measurement of L-lactic acid and ATP) or a 12-well plate (for glycogen measurement) were serum-starved for 18 h. The cells were treated with 3, 10 or 30 μM glabridin or 100 nM insulin as a positive control in 200 μl of 0.2% (w/v) BSA/MEM for up to 4 h. For the L-lactic acid assay, 1 μl of culture medium was collected and mixed with 100 μl of an assay cocktail (500 mM glycine, 400 mM hydrazine, pH 9.0, 1 unit/ml lactate dehydrogenase from pig heart, 270 μM β-nicotinamide adenine dinucleotide, 2 units/ml diaphorase and 2 mM resazurin) in another 96-well plate and incubated at 37 °C for 30 min. The fluorescence of resorufin was measured at 570 nm with excitation at 530 nm using a Wallac 1420 ARVOsx (Perkin–Elmer, Boston, MA, USA). Lactate concentration in each well was calculated from a standard curve generated with an L-lactic acid standard solution. For the glycogen assay, the treated cells were washed three times with KRH and heated at 105 °C for 10 min. The cells were

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