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Original article

The interaction of auraptene and other oxyprenylated phenylpropanoids with glucose transporter type 4

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ABSTRACT

Background: Glucose transporter 4 (GLUT4) is firmly established to play a pivotal role in glucose metabolism and in particular in modulating the insulin-stimulated glucose transport in several tissues, such as skeletal muscle and adipose tissue. Stimulation of GLUT4 by insulin results in its translocation to the plasma membrane, activation of several kinases, and finally in a large glucose influx into cells.
Purpose: In this study we investigated the modulating properties of four biologically active oxyprenylated ferulic acid and umbelliferone derivatives and of their unprenylated parent compounds on GLUT-4 mediated glucose uptake and translocation.
Methods: Oxyprenylated phenylpropanoids have been synthesized in high yields and purity by already reported methodologies. All the synthesized chemicals were tested for their capacity to modulate GLUT4 mediated glucose uptake and GLUT4 translocation in L6 rat skeletal myoblasts in the concentration range 0.1 – 10 μM. Insulin (0.1 μM) was used as positive control. Western blot analysis was employed to assess if GLUT4 translocation occurred prior to increase of glucose uptake. Statistical analyses were carried out by the Dunnett multiple comparison test.
Results: 4′-Geranyloxyferulic acid (GOFA), 7-isopentenyloxycoumarin, and auraptene (7-geranyloxycoumarin) increased glucose uptake in a concentration-dependent manner, and significant increases were observed at 0.1 μM for GOFA, and 10 μM for 7-isopentenyloxycoumarin, and auraptene. These products also were able to significantly promote the translocation of GLUT4 to the plasma membrane of L6 myotubes. After treatment with compounds for 15 min, the incorporated amounts of GOFA, 7-isopentenyloxucoumarin, and auraptene were 0.15, 0.32, and 1.77 nmols/60-mm culture dish, respectively. A sample of raw Italian propolis, found to be rich in GOFA and auraptene, was also seen to mimic insulin-effect in the concentration range 0.01 – 1.0 mg/ml.
Conclusions: Among the compounds assayed, auraptene showed to possess potentialities to be a potent activator of both translocation of GLUT4 and glucose influx into skeletal muscle cells with the highest bioavailability among effective compounds. Its capacity to modulate sugar metabolism, coupled to its presence in edible *Citrus* fruits, can be regarded as an additional reason to account for the already known stimulating properties of some vegetable (e.g. bitter orange).

Introduction

Glucose transporter 4 (GLUT4) is nowadays well recognized to play a key role in glucose metabolism and in particular in modulating the insulin-stimulated glucose transport in several tissues, such as skeletal muscle and adipose tissue (De Fronzo et al., 1992). In the absence of insulin stimulation, the majority of GLUT4 (> 95%) is sequestered into the cytoplasm. GLUT4 translocation is stimulated by the binding of insulin to its receptor, followed by the activation of several protein kinases involved in insulin signaling (De Fronzo et al., 1992; Yang and Holman, 1993). Activation of the insulin receptor leads to the phosphorylation of phosphatidylinositol 3-kinase (PI3K) followed by the activation of Akt and protein kinase C (PKC). 5′-Adenosine monophosphate-activated protein kinase (AMPK), known to be involved in

Abbreviations: BSA, bovine serum albumin; CREB, cAMP response element-binding protein; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; GLUT4, glucose transporter 4; GOFA, 4′-geranyloxyferulic acid; HPLC, high performance liquid chromatography; MCP, monocyte chemoattractant protein; MEM, modified Eagle's medium; PPAR, peroxisome proliferator-activated receptor

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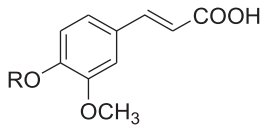
contraction-induced glucose transport in skeletal muscle tissues, is also able to stimulate GLUT4 translocation (Jessen and Goodyear, 2005). The final result of this cascade of events is a large glucose influx into cells (Zhou et al., 1999). Thus, GLUT4 transporter is an important factor in the maintenance of postprandial blood glucose levels through both PI3K and AMPK-dependent pathways in adipose tissue and skeletal muscle. Furthermore recent evidences suggest the presence of GLUT4 in some areas of the central nervous system (e.g. hippocampus). Dysregulation of insulin mediated GLUT4 activity in these regions can cause a decrease in neuronal metabolism and plasticity. Such an effect may be related to the development and progress of depressive like behaviour and cognitive dysfunctions (Patel and Udayabanu, 2014). In recent years oxyprenylated secondary metabolites from plants, fungi, and bacteria and their semisynthetic derivatives were subject of growing interest. These natural products in fact have been characterized as potentially novel lead compounds for a series of pharmacological effects. In particular prenyloxy ferulic acid and umbelliferone derivatives (e.g. boropinic acid **2**, isolated from the Australian shrub *Boronia pinna* Sm., 4′-geranyloxyferulic acid **3**, herein designated with the acronym GOFA, 7-isopentenyloxycoumarin 5, and auraptene 6, all these latters widespread in *Citrus* spp.) (Fig. 1) were found to exert cancer chemopreventive, anti-inflammatory, anti-bacterial, and neuroprotective effects (Genovese et al., 2015; Epifano et al., 2015; Genovese and Epifano, 2011; Epifano et al., 2009).

In this work we wish to report the effects of compounds 2, 3, 5, 6, and the respective unprenylated parent products, ferulic acid 1 and umbelliferone 4, on GLUT-4 mediated glucose uptake and GLUT-4 translocation in L6 rat skeletal myoblasts using insulin (0.1 μM) as a positive control. We also investigated the effectiveness of raw Italian propolis, in which some of the above listed oxyprenylated phenylpropanoids are contained in relatively high concentration, to mimic insulin-effect in the same cell line.

Material and methods

Chemistry

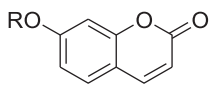
Ferulic acid and umbelliferone were purchased from Sigma-Aldrich (St. Louis, MI, USA) and used after crystallization (water). Boropinic acid, GOFA, 7-isopentenyloxycoumarin, and auraptene have been synthesized in our laboratories as previously reported (Bruyere et al., 2011), their purity (> 97.4%) assessed by HPLC, elemental analysis, and ^{1}H NMR. HPLC analysis were performed using a Waters 600 HPLC system equipped with a Waters 2996 PDA detector, a Rheodyne manual syringe-loading valve injector model 7125 (Cotati, CA., USA) fitted with a 20 μl loop. Data acquisition was monitored by Waters Empower software (ver. 2.0). Chromatographic separation was achieved employing a GraceSmart RP_{18} (5 μm particle size, 250 mm x 4.6 mm, Grace, Deerfield, IL, USA). Column temperature was maintained at 25 ± 1 °C using a cool pocket chiller (ThermoScientific, Waltham, USA). The detection was set at 322 nm for each analyte . Elution mixture consisted of H_2O and acetonitrile both with 0.03% of acetic acid (eluent A and eluent B, respectively). Mobile phase was directly on-line degassed by using Infinity Agilent model 1260 (Agilent Technologies, Santa Clara, CA, USA). The flow rate was 1.20 ml/min. Chromatographic separation was carried out using the following gradient elution: 60% A – 40% B from 0.01 min. to 18.0 min., 60% A – 40% B to 20% A – 80% B from 18.01 min. to 22.0 min., 60% A – 40% B from 22.01 min. to 25.0 min., 20% A – 80% B to 60% A – 40% B from 25.01 min. to 29.0 min., 60% A – 40% B from 29.01 min. to 33.0 min., 60% A – 40% B to 100% B from 33.01 min. to 37.0 min., 100% B from 37.01 min. to 38.0 min., 100% B to 60% A – 40% B from 38.01 min. to 39.0 min., and finally 60% A – 40% B from 39.01 min. to 50.0 min.



1 R = H

2 R = 3,3-dimethylallyl

3 R = geranyl



4 R = H

5 R = 3,3-dimethylallyl

6 R = geranyl

Fig. 1. Chemical structures of oxyprenylated phenylpropanoids and unprenylated parent compound under investigation.

Pharmacology

Anti-GLUT4 mouse polyclonal antibody (#2212) and horseradish peroxidase-conjugated anti-mouse (#7076) and anti-rabbit (#7074) IgG antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). All other reagents used were of the highest grade available from commercial sources.

Cell culture. L6 myoblasts were maintained in MEM supplemented with 10% FBS, 100 μg/ml streptomycin, and 100 U/ml penicillin at 37 °C in a 5% CO_2 atmosphere. When the myoblasts reached confluence, the medium was replaced every 2 days with differentiation medium consisting of MEM supplemented with 2% FBS. Experiments were performed with fully differentiated myotubes 8 days after having reached confluence.

Glucose uptake assay. Glucose uptake assay was performed as described previously (Yamamoto et al., 2015). Briefly, L6 myotubes on a 96-well plate were serum-starved for 18 h in MEM containing 0.2% (w/v) BSA at 37 °C. The cells were incubated with compounds under investigation in MEM containing 0.2% BSA for 4 h The cells were washed twice with Krebs–Ringer phosphate-HEPES buffer (KRH; 50 mM HEPES, pH 7.4, 137 mM NaCl, 4.8 mM KCl, 1.85 mM $CaCl_2$, 1.3 mM $MgSO_4$) containing 0.1% BSA and treated with 100 μM of 1 mM 2-deoxy-D-glucose in KRH buffer containing 0.1% (w/v) BSA for 20 min. Cells were subsequently washed twice with KRH buffer containing 0.1% (w/v) BSA, lysed with 50 μl of 0.1 M NaOH at 60 °C for 10 min, dried at 85 °C for 50 min., solubilized with 50 μl of 0.1 M HCl and 50 μl of 200 mM triethanolamine (TEA) buffer (pH 8.1), and finally shaken by a microplate shaker. An aliquot of 30 μl of the lysate was mixed with 100 μl of assay cocktail [50 mM TEA, pH 8.1, 50 mM KCl, 0.02% (w/v) BSA, 0.1 mM β-$NADP^+$, 2 units diaphorase, 150 unites glucose-6-phosphate dehydrogenase, and 2 μM rezazurin] on another 96-well plate and incubated at 37 °C for 80 min. The fluorescence of resorufin formed from rezazurin was measured (Ex: 530 nm and Em: 570 nm) by a Wallac 1420 ARVOsx (Perkin-Elmer Life Sciences, Boston, MA).

Preparation of the plasma membrane fraction. Preparation of the plasma membrane fraction from L6 myotubes was performed as described by Yamamoto and coworkers with slight modifications (Yamamoto et al., 2016). Briefly, L6 myotubes were serum-starved for 18 h in MEM containing 0.2% (w/v) BSA at 37 °C. The cells were incubated with prenyloxyphenylpropanoids in MEM for 15 min., washed twice with PBS, added 180 μl of buffer A [50 mM Tris, pH 8.0, 0.5 mM dithiothreitol (DTT), 10 mM NaF, 1 mM Na_3VO_4, 5 μg/ml aprotinin, 1 mM PMSF, and 5 μg/ml leupeptin] containing 0.1% (v/v) Nonidet® P-40 (NP-40), and homogenized with a hand-held pestle homogenizer. The homogenate so obtained was transferred into a 1-ml syringe and