Research Article

Naringenin and falcarinol stimulate glucose uptake and TBC1D1 phosphorylation in porcine myotube cultures

Sumangala Bhattacharya[a], Martin Krøyer Rasmussen[a], Lars Porskjær Christensen[b], Jette Feveile Young[c], Karsten Kristiansen[c], Niels Oksbjerg[a,*]

[a]Department of Food Science, Aarhus University, Blichers Allé 20, Postbox 50, 8830 Tjele, Denmark
[b]Department of Chemical Engineering, Biotechnology and Environmental Technology, University of Southern Denmark, Niels Bohrs Allé 1, 5230 Odense M, Denmark
[c]Department of Biology, University of Copenhagen, Ole Maaløes Vej 5, 2200 Copenhagen N, Denmark
[*]Corresponding author at: Department of Food Science, Aarhus University, Blichers Allé 20, Postbox 50, 8830 Tjele, Denmark. Phone: + 45 8715 7809, Fax: +45 8715 4891. Email: Niels.Oksbjerg@agrsci.dk

Abstract: Insulin resistance in muscles is a major problem associated with Type 2 diabetes. Bioactive compounds of plant origin have long been known for possessing anti-diabetic properties. We have studied the effect of the bioactive compounds naringenin (dihydroflavonol) and falcarinol (polyacetylene) on glucose uptake (GU) in normal and insulin resistant primary porcine myotubes, in the presence and absence of insulin to identify signaling pathways mediating their effects on GU. The dependence on glucose transporter type 4 (Glut4) activity, insulin signaling and AMP- activated protein kinase (AMPK)-signaling was studied by using the Glut4 inhibitor indinavir, the phosphatidyl inositol-3 kinase (PI3K) and p38 mitogen activated protein kinase (MAPK) inhibitor wortmannin, and the AMPK inhibitor dorsomorphin (DM), respectively. Naringenin and falcarinol stimulated GU was attenuated in the presence of indinavir and wortmannin, indicating a dependence on Glut4 activity as well as PI3K and/or p38MAPK activity. By contrast, DM diminished GU induced by naringenin only, indicating that falcarinol-stimulated GU was independent of AMPK activity. Finally, we show that naringenin and falcarinol enhance phosphorylation of TBC1D1 suggesting that these compounds enhance translocation of Glut4 containing vesicles and thereby GU via a TBC1D1-dependent mechanism.

**Keywords:** naringenin; falcarinol; glucose uptake; type 2 diabetes; TBC1D1; TBC1D4

Introduction

The initiation of insulin resistance in muscles, normally occurs asymptomatically, and is compensated with increased insulin secretion by the pancreatic beta-cells. If left uncontrolled, this can lead to beta-cell exhaustion and failure; leading to an increase in blood glucose level and the manifestation of Type 2 diabetes [1].

Skeletal muscle is the primary site for glucose utilisation, where about 75 % of the insulin stimulated glucose uptake (GU) takes place [2]. Insulin causes GU via the translocation of glucose transporter type 4 (Glut4) vesicles to the plasma membrane [3] via activation of the phosphatidyl inositol-3 kinase (PI3K)-Akt/protein kinase B pathway, which mediates most of the metabolic actions of insulin [4].

Another major signaling pathway responsible for GU and fatty acid oxidation in muscles is the AMP- activated protein kinase (AMPK) signaling cascade. In mammals, AMPK acts as a metabolic energy sensor, maintaining the
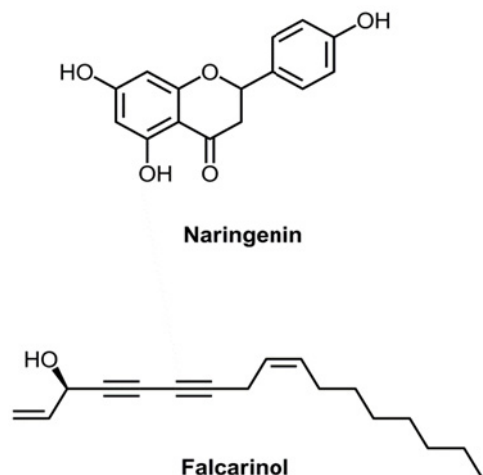
Fig. 1. Chemical structures of naringenin and falcarinol.

cellular energy balance [5]. It has been shown that activation of AMPK by agonists such as AICAR (5-Aminoimidazole-4-carboxamide ribonucleotide) causes Glut4 translocation [6, 7]. Moreover, thiazolidinediones and metformin have been found to enhance GU in skeletal muscle through AMPK phosphorylation [8].

The Rab-GTPase activating protein, TBC1D4 (also known as As160) and its homolog TBC1D1 are downstream targets of Akt and play a major role in insulin and AICAR stimulated Glut4 translocation [9, 10]. It has been suggested that phosphorylation of TBC1D4 and TBC1D1(TBC1D4/1) lead to the activation of small Rab-GTPases, causing cytoskeletal re-organization, which in turn triggers the translocation and docking of Glut4 vesicles to the plasma membrane [11].

Secondary metabolites from different botanicals have the potential to influence different cellular mechanisms, including key signaling pathways balancing energy utilization and storage. More than a thousand plant species have been tested for their efficacy against diabetes [12]. Naringenin, a flavonol, found in citrus fruits (e.g. grapefruits and oranges) has been found to enhance insulin sensitivity and reduce plasma glucose levels in diabetic animal models [13], and cause AMPK activation in L6 myotubes [14]. However, the different steps involved in its mode of action are yet to be elucidated. Falcarinol, a polyacetylene present (among other plants) in carrots, and mostly known for its anti-cancer and anti-inflammatory properties [15, 16], also exhibit cyto protective [17] and growth-stimulatory effects [18] in a biphasic manner. Falcarinol has not been studied yet for its efficacy against diabetes.

In the present study, primary porcine myotube cultures were used as a model for skeletal muscles to test the GU enhancing potential of naringenin and falcarinol in normal and insulin resistant myotubes. GU was measured in the presence of indinavir (a Glut4 inhibitor), wortmannin (a PI3K-inhibitor), and dorsomorphin (DM; an AMPK inhibitor) separately. Simultaneously, the effect of these inhibitors on naringenin and falcarinol induced phosphorylation of TBC1D4/1 was studied.

Mercury (Hg) is a heavy metal that is widespread and persistent in the environment, and infants in the US are exposed to significant levels of environmental Hg through air, water, and breast milk [1]. In addition to environmental Hg exposure and maternal exposures from the mother's Hg body burden, dietary intakes, and Hg-containing pharmaceuticals administered to the mother while the child is developing in utero, and injected organic-Hg from Thimerosal-preserved childhood vaccines have been and, in many countries, remain a significant source of Hg exposure for many infants during the first year of life [1, 2].

Materials and methods

Materials

Falcarinol (> purity 98%) was isolated from carrots as described elsewhere [19] and identified by UV, gas chromatography-mass spectrometry (EI, 70 eV)], NMR (^{1}H and ^{13}C NMR, and [1]H-^{1}H and [1]H-^{13}C correlation spectroscopy recorded in $CDCl_3$ with tetramethylsilane as internal standard), and optical rotation. The spectral data set corresponded fully with literature values for falcarinol [20-22]. Chemical structures of falcarinol and naringenin [23, 24] are shown in Fig. 1. Dulbecco's modified eagles medium (DMEM), fetal calf serum (FCS), horse serum (HS) and Trypsin-EDTA were from GIBCO Life technologies. The antibiotics (amphotericin, penicillin/streptomycin and gentamycin), naringenin, DM and phosphatase inhibitor cocktail (PIC) 2 and 3 were from Sigma-Aldrich. [^{3}H] 2-deoxy-D-glucose (2-DOG) was purchased from Perkin Elmer Inc. Indinavir, wortmannin, and AICAR were from Santa Cruz Biotechnology (Texas, USA), and the primary antibodies against phosphorylated TBC1D4/1 were purchased from Cell Signaling Technology (Danvers, MA, US) and that against α-Tubulin, from Merck Millipore (Darmstadt, Germany). Goat anti-rabbit and anti-mouse HRP-conjugated secondary antibodies were from Dako Denmark A/S (Glostrup, Denmark). Enhanced chemiluminescence reagent (ECL) and chemiluminescence films were from GE healthcare (Buckinghamshire, UK). The polyvinylidene difluoride (PVDF) membranes were from BioRad (CA, USA), protein molecular weight markers from Thermo scientific Inc. (MA, USA) and 4-12 % Bis-Tris gels from Life technologies (Paisley, UK).

Preparation of myotube cultures

Satellite cells were isolated from fast-twitch semimembranosus muscles of female pigs (approximately12

kg) and stored in liquid nitrogen until used. For preparation of myotube cultures, the cells were thawed and evenly seeded on Matrigel matrix (BD Biosciences, cat no. 354230) coated (1:50 v/v) as described elsewhere [25] in 6 or 48 well plates for protein analysis and GU assay, respectively.

Glucose uptake assay

The differentiated myotubes were treated with serum free media (SFM; DMEM with 7 mM, glucose, antibiotics, and 1 µM cytosine arabinoside, 1 % FCS) for 2-5 h, incubated with various treatments for 1 h, washed with (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) HEPES buffered saline (20 mM Hepes, 140 mM NaCl, 5 mM KCl, 2.5 mM $MgSO_4$, 1 mM $CaCl_2$, adjusted to pH 7.4), and incubated with 250 µL/well 2-DOG for 30 min; washed with phosphate buffered saline (PBS, 500 µL/well), lysed by adding 0.05 M NaOH (37°C, 250 µL/well) and placed on a
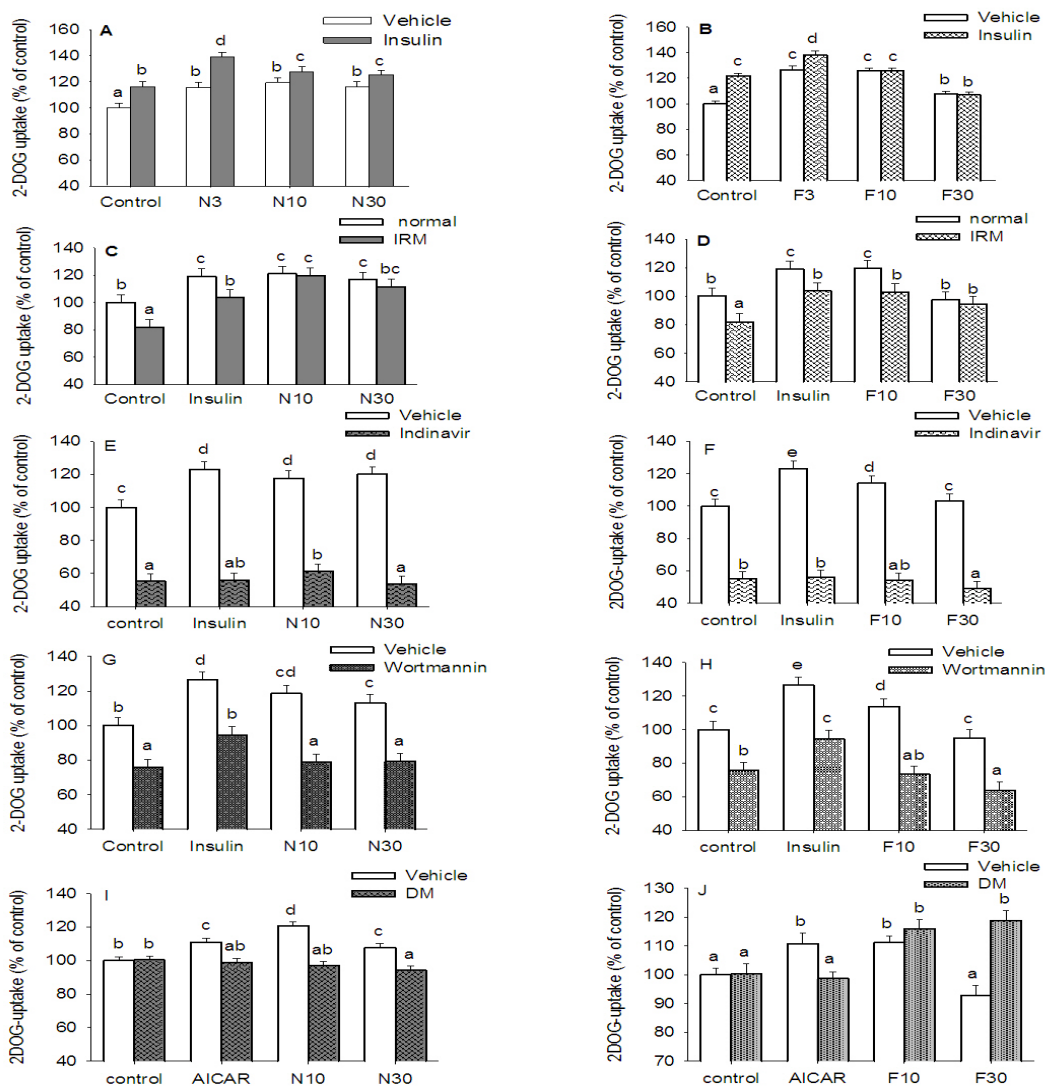
Fig. 2. Effect of naringenin and falcarinol on glucose uptake. The differentiated myotubes were incubated with 3, 10 and 30 µM (A) naringenin and (B) falcarinol, in presence and absence of 10 nM insulin (closed and open bars respectively) for 1 h; or incubated for 24 h with either 7 mM or 12 mM glucose (open and closed bars respectively) and then treated with 10 and 30 µM of (C) naringenin and (D) falcarinol for 1 h, following which glucose uptake was measured. Myotubes were incubated with indinavir (100 µM) for 5 minutes prior to 2DOG addition (E & F); 1µM wortmannin for 1 h (G & H) or 10 µM DM for 5 minutes (I & J), prior to treatment addition. For A – H, insulin (10 nM) and for I & J, AICAR (1 mM) was used as the positive control. N10, N30 = 10 and 30 µM naringenin and F10, F30 = 10 and 30 µM falcarinol respectively. Vehicle = cells treated with DMSO only. In the plot DM = dorsomorphin. Values are LS means ± SEM of experiments conducted with satellite cells from 3 pigs, expressed as percent of control. Number of replicates per pig (n) = 6. Different letters indicate significant differences between and within groups.

shaking board for 30 min. The cell lysate was mixed with scintillation mix (1:10) and counted in a Win spectral 1414 liquid scintillation counter. Following serum deprivation, the differentiated cells were pre-incubated with 1μM wortmannin for 1 h or 10 μM DM for 5 min, prior to treatment addition. Indinavir (100 μM) was added 5 min prior to 2DOG addition. AICAR (1 mM) and insulin (10 nM) were used as positive controls (1 h incubation). To make insulin resistant myotubes (IRM), myotubes were incubated with differentiation media for 24 h and then with differentiation media containing 12 mM as compared to 7 mM glucose for 24 h. These myotubes were not treated with SFM, before treatment addition. Controls received DMSO.

Western blotting

Differentiated cells were treated with various treatments for 2.5 h, washed with PBS, harvested using 0.25 % Trypsin-EDTA, and frozen at -80°C. Lysis buffer (4 % SDS, 10 mM Tris-HCl, and 1 mM EDTA) containing PIC 2 and 3 was used to lyse the cells. Cell lysates containing equal amounts of protein were separated by SDS-PAGE using 4-12 % Bis-Tris gels. Proteins were transferred to a PVDF membrane, stained with Ponceau S and visually inspected for equal loading and blotting efficiency [26]. The membranes were blocked using 2 % (w/v) BSA in 0.1 % TBS-T buffer (0.05 M Tris-base, 0.5 M NaCl, 0.1 % (v/v) Tween-20, pH adjusted to 7.4) for 1 h, at room temperature, and washed in 0.1 % TBS-T. Thereafter, the membrane was incubated with primary antibody (1:1000) at 4 °C overnight or 1 h at room temperature (RT), washed, incubated with HRP-conjugated anti-mouse or anti-rabbit secondary antibody for 1 h at RT, and washed again. All washing steps were done 6 times, 10 min each. ECL reagent was used to detect the primary antibody and was visualized by exposure to chemiluminescence films. Bands were analysed using the ImageJ software. The relative protein expression was normalized against the α-Tubulin as a housekeeping protein. Statistical analysis

Statistical analysis of data was conducted, using the 'Mixed' procedure of SAS statistical programming software (Ver. 9.2; SAS Institute Inc., Cary, NC, USA). The models used included fixed effects of treatments and their interactions. Data representing Fig. 2A-J were tested separately, where the model included fixed effects of insulin, glucose concentration, indinavir, wortmannin, DM, AICAR, naringenin, falcarinol and insulin as well as their interactions. Myotube cultures (triplicates) and replicates (n=4) nested within were used as random effects. When overall effects were significant, Least Square Means (LSMeans) was separated by pairwise comparison (pdiff option in SAS). For the western blot, differences between treatments were determined by Student's unpaired t-test. P value < 0.05 was considered statistically significant.

Results

GU was determined in differentiated myotubes incubated with 3, 10 or 30 μM of naringenin and falcarinol separately, in presence or absence of 10 nM insulin (Fig. 2A and 2B) for 1 h. Naringenin significantly increased GU in the absence of insulin at 3, 10 and 30 μM concentrations by 15.6 (p = 0.001), 19.5 (p < 0.001) and 16.4 % (p < 0.001), respectively compared to control. An increase of 23.0 (p < 0.001), 11.5 (p < 0.01), and 9.2 % (p = 0.02) in GU at 3, 10, and 30 μM naringenin concentrations was observed compared to 10 nM insulin only. Falcarinol significantly increased GU at 3, 10 and 30 μM concentrations in the absence of insulin by 26.5 (p = 0.001), 26.0 (p < 0.001), and 7.6 (p < 0.01) %, respectively, compared to control; whereas in presence of 10 nM insulin, an increase of 15.8 (p < 0.01), 4.0 (p = 0.1) and a decrease of 15.1 (p < 0.001) % in GU, was observed at 3, 10 and 30 μM falcarinol, respectively, compared to 10 nM insulin only. Here, the GU stimulating effect in the presence of insulin was only observed at 3 μM, while GU was inhibited at concentration of 30 μM falcarinol. Based on these results, the concentrations 10 and 30 μM for both naringenin and falcarinol were chosen for further experiments.

The effects of naringenin and falcarinol on IRM are illustrated in Fig. 2C and 2D. Insulin sensitivity of the myotubes was reduced by pre-incubation with 12 mM glucose for 24 h, as has been demonstrated elsewhere [27]. Basal GU was significantly reduced in IRM (18.1 %, p = 0.02) compared to control. A significant increase in GU was observed in presence of 10 nM insulin (21.8 %, p =0.005), as well as 10 and 30 μM naringenin (37.7, and 29.0 %; p < 0.001) in IRM. The increase in GU in presence of 10 but not 30 μM naringenin was significantly higher (p = 0.03) than that caused by 10 nM Insulin. Falcarinol, at the concentration s of 10 (21.0 %, p = 0.007) and 30 μM (13.0 %, p = 0.09), significantly increased GU in IRM. At 10 μM concentration, the increase in GU caused by falcarinol was not significantly different from that caused by 10 nM Insulin.

Indinavir, a specific inhibitor of Glut4 mediated glucose transport, directly binds and blocks the Glut4 transporter [28]. The IC_{50} of indinavir in cells expressing Glut4 is 50 to 100 μM [29]. To examine whether the increased GU elicited by naringenin and falcarinol was dependent on Glut4 transporters, myotubes were incubated in presence or absence of 100 μM indinavir for 35 min (Fig. 2E and 2F). Incubation with indinavir significantly reduced basal GU by 44.7 % (p < 0.001). There was no significant increase in GU in presence of 10 nM insulin or 30 μM naringenin in the indinavir treated cells; whereas 10 μM naringenin caused a minute, but significant increase in GU (6 %, p = 0.03). GU was unaffected at 10 μM and further reduced at 30 μM falcarinol concentrations in indinavir treated cells.

Activation of PI3K is necessary for both basal and insulin stimulated Glut4 translocation to the plasma membrane, and is inhibited by wortmannin [30]. In order to test whether the GU induced by naringenin and falcarinol is affected by the inhibition of PI3K, myotubes were incubated with 1 µM wortmannin for 1 h (Fig. 2G and 2H), which significantly decreased the basal and insulin stimulated GU by 24.2 and 31.8 % ($p < 0.001$) respectively. Naringenin induced GU at 10 and 30 µM concentrations was reduced by 39.9 and 33.9 % ($p < 0.001$) respectively. A similar reduction in GU for 10 and 30 µM falcarinol in wortmannin treated myotubes (40.0 and 31.2 %, $p < 0.001$) was

observed.

In order to test the AMPK dependence, myotubes were incubated with DM (10 µM), for 65 min (Fig. 2I, J), and AICAR (1 mM) was used as a positive control. There was no reduction in basal GU, in DM treated myotubes, but AICAR stimulated GU was significantly reduced (12.1 %, $p < 0.001$) in presence of DM. A significant reduction of 23.7 and 13.4 % ($p < 0.001$) for 10 and 30 µM naringenin, and an increase of 5.0 and 24.3 ($p < 0.001$) % at 10 and 30 µM falcarinol was observed in DM treated myotubes compared to vehicle.

Activation of TBC1D4/1 by naringenin and falcarinol
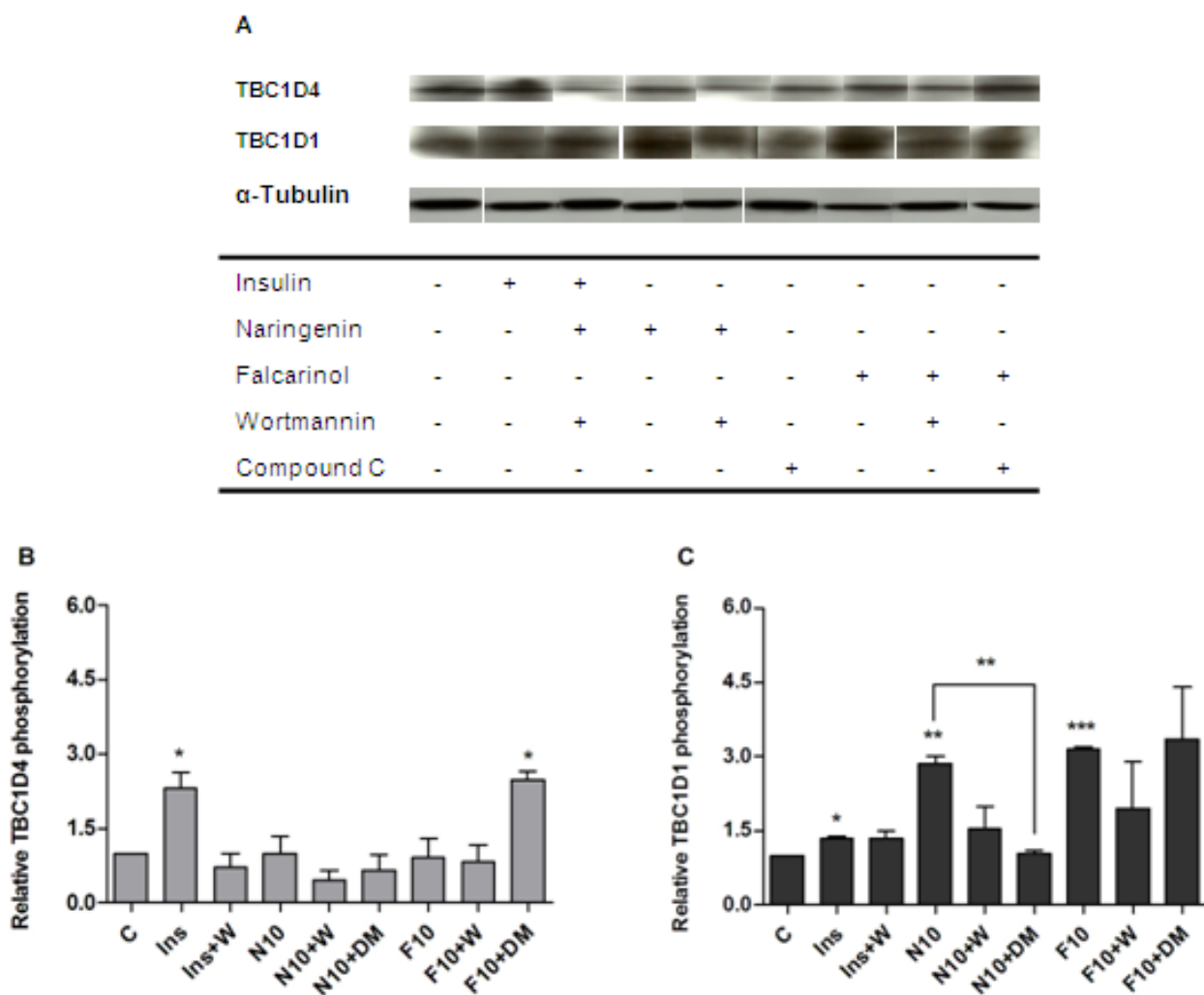
Fig. 3. TBC1D4 and TBC1D1 phosphorylation by naringenin and falcarinol. Differentiated myotubes were incubated for 2.5 h with 100 nM insulin (Ins), 10 µM naringenin (N10), and 10 µM falcarinol (F10); in presence or absence of 1 µM wortmannin (W) and 10 µM dorsomorphin (DM). Cells were harvested, lysed, and equal amounts of protein were used for SDS-PAGE, followed by immunoblotting (representative blots shown in A) with specific antibodies of recognizing phosphorylated (Thr642) TBC1D4 and (Thr590) TBC1D1 (plots B and C, respectively). α-Tubulin was used as housekeeping protein. Bands were analysed using the ImageJ software. Values are given as Mean ± SEM of experiments conducted with satellite cells from 3 pigs. Levels of significance, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

was examined, with 100 nM insulin as the positive control. Naringenin and falcarinol solely increased TBC1D1 phosphorylation (Fig. 3), which had a tendency to decrease in presence of wortmannin. Insulin significantly increased the phosphorylation of TBC1D4/1, where the former was induced to a higher degree. Insulin stimulated TBC1D4 (not TBC1D1) phosphorylation was significantly reduced by wortmannin. In DM treated myotubes, naringenin stimulated TBC1D1 phosphorylation was significantly reduced, and a similar tendency was observed for TBC1D4 phosphorylation; whereas falcarinol showed a significant increase in TBC1D4 phosphorylation in the presence of DM. TBC1D4/1 were found to have an approximate molecular weight (m. wt.) of 65-70 kDa. Human and murine TBC1D4/1 has a m. wt. of 160 kDa [31, 32]. The m. wts of these proteins in pigs have not yet been established. However, according to Ensembl sources, based on their mRNA transcripts, porcine TBC1D4/1 are predicted to have an approximate m. wt. of 56-70 kDa (*Ensembl: ENSSSCT00000010375* and ENSSSCT00000009599). Two other studies [33, 34] have attempted to detect TBC1D4 in porcine muscles, but used a different approach where phosphorylated Akt substrate antibody was used to detect the protein.

Discussion

In the current study, naringenin and falcarinol were found to enhance GU in primary porcine myotube cultures autonomously. In presence of insulin, both naringenin and falcarinol showed a higher increase in GU at the lowest concentration tested (3 μM); which was reduced at 10 and 30 μM concentrations. A possible explanation could be a shift in the sensitivity range of the compounds in the presence or absence of insulin, due to competition for common pathway proteins at higher concentrations.

In IRM, basal GU was significantly reduced, while the naringenin induced effect was maintained. This indicates an insulin-independent mechanism of GU which correlates with the fact that naringenin activates AMPK in L6 muscle cells [14]. However, a reduced GU was observed in naringenin exposed MCF-7 breast cancer cells and myelocytic U937 cells [35, 36], indicating a cell-type specific effect of this flavonol. Falcarinol induced GU was not maintained, in IRM after 10 μM exposure. This could be due to down-regulation of signaling proteins required for falcarinol stimulated GU at this concentration.

A significantly reduced GU was observed in indinavir treated cells, where naringenin (10 μM) caused a minute but significant increase in GU, although insulin treatment did not. A similar observation was obtained for naringenin treated IRM, suggesting that although naringenin mostly depends on Glut4 for GU, it might be capable of partially inducing other glucose transporters (like Glut1) and/or the activity of the small number of Glut4 still available for transport. However, falcarinol did not increase GU in the indinavir treated cells; which might be indicative of its complete dependence on Glut4, as is also the case for insulin.

Neither naringenin nor falcarinol increased GU in wortmannin treated cells, suggesting PI3K dependence. However, it is important to note that in earlier studies, wortmannin has also been found to inhibit MAPK [37] with an IC_{50} of 300 nM. This could link GU by naringenin and falcarinol to MAPK-inhibition as well; since, other than being a downstream target of AMPK [38], p38-MAPK is involved in full activation of Glut4 [39].

Treatment with DM did not cause any significant change in basal GU; but AICAR and naringenin mediated GU was diminished in its presence. This also corroborates well with naringenin induced AMPK activation [14] and unchanged naringenin induced GU in IRM compared to normal myotubes. The inability of DM to reduce falcarinol induced GU and TBC1D1 phosphorylation indicates AMPK independence. The significant DM induced increase in GU observed at 30 μM falcarinol is surprising. However a cross talk between different signaling pathways could provide a rationale. Moreover, DM has been shown to participate in other signaling cascades, independent of the AMPK pathway [40]. Furthermore, intracellular reactive oxygen species (ROS) has been implicated in GU during exercise/muscle contraction [41] and the activation of p38 MAPK [42], stimulating GU [39]. The fact that falcarinol induces ROS formation at low concentrations (1.6 to 25 μM) [17] could explain the falcarinol stimulated AMPK-independent increase in GU.

The activation of TBC1D1 by naringenin and falcarinol was reported for the first time in this study. TBC1D1 is relatively more abundant in fast-twitch and TBC1D4 in slow-twitch muscles [10]. However, TBC1D4 was more responsive to insulin induced phosphorylation than TBC1D1. Mass spectrometry analysis on TBC1D1 from mouse skeletal muscle has revealed phosphorylation sites, that are consensus or near consensus sites for AMPK; and AICAR was found to be a stronger regulator of TBC1D1 phosphorylation than insulin [10]. This explains the inhibition of naringenin induced TBC1D1phosphorylation by DM.

Overall, it can be concluded that both naringenin and falcarinol depend predominantly on Glut4 and PI3K and/or p38MAPK activity for the induction of GU. Naringenin (not falcarinol) induced GU, is dependent on AMPK activation. Treatment with wortmannin and DM indicate that naringenin and falcarinol differ in their mechanism of action, but both increase GU via TBC1D1 phosphorylation.

Acknowledgements

Conflict of interests

The authors declare no conflict of interest.

References

[1] Polonsky KS. Dynamics of insulin secretion in obesity and diabetes. Int J Obes 2000; 24: 29-31.

[2] Saltiel AR, Kahn CR. Insulin signalling and the regulation of glucose and lipid metabolism. Nature 2001; 414: 799-806.

[3] Pessin JE, Saltiel AR. Signaling pathways in insulin action: molecular targets of insulin resistance. J Clin Invest 2000; 106: 165-169.

[4] Taniguchi CM, Emanuelli B, Kahn CR. Critical nodes in signalling pathways: insights into insulin action. Nat Rev Mol Cell Bio 2006; 7: 85-96.

[5] Viollet B, Lantier L, Devin-Leclerc J, Hebrard S, Amouyal C, Mounier R, et al. Targeting the AMPK pathway for the treatment of Type 2 diabetes. Front Biosci 2009; 14: 3380-3864.

[6] Koistinen HA, Galuska D, Chibalin AV, Yang J, Zierath JR, Holman GD, et al. 5-amino-imidazole carboxamide riboside increases glucose transport and cell-surface GLUT4 content in skeletal muscle from subjects with type 2 diabetes. Diabetes 2003; 52: 1066-1072.

[7] Kristensen M, Rasmussen MK, Juel C. Na(+)-K (+) pump location and translocation during muscle contraction in rat skeletal muscle. Pflugers Arch 2008; 456: 979-989.

[8] Musi N, Goodyear LJ. AMP-activated protein kinase and muscle glucose uptake. Acta Physiol Scand 2003; 178: 337-345.

[9] Sakamoto K, Holman GD. Emerging role for AS160/TBC1D4 and TBC1D1 in the regulation of GLUT4 traffic. Am J Physiol-Endoc M 2008; 295: E29-E37.

[10] Taylor EB, An D, Kramer HF, Yu H, Fujii NL, Roeckl KSC, et al. Discovery of TBC1D1 as an insulin-, AICAR-, and contraction-stimulated signaling nexus in mouse skeletal muscle. J Biol Chem 2008; 283: 9787-9796.

[11] Roach WG, Chavez JA, Miinea CP, Lienhard GE. Substrate specificity and effect on GLUT4 translocation of the Rab GTPase-activating protein Tbc1d1. Biochem J 2007; 403: 353-358.

[12] Marles RJ, Farnsworth NR. Antidiabetic plants and their active constituents. Phytomedicine1995; 2: 137-189.

[13] Kannappan S, Anuradha CV. Naringenin enhances insulin-stimulated tyrosine phosphorylation and improves the cellular actions of insulin in a dietary model of metabolic syndrome. Eur J Nutr 2010; 49: 101-109.

[14] Zygmunt K, Faubert B, MacNeil J, Tsiani E. Naringenin, a citrus flavonoid, increases muscle cell glucose uptake via AMPK. Biochem Biophys Res Commun 2010; 398: 178-183.

[15] Metzger BT, Barnes DM, Reed JD. Purple carrot (Daucus carota L.) polyacetylenes decrease lipopolysaccharide-induced expression of inflammatory proteins in macrophage and endothelial cells. J Agric Food Chem 2008; 56: 3554-3560.

[16] Zaini RG, Brandt K, Clench MR. Effects of Bioactive Compounds from Carrots (*Daucus carota* L.), Polyacetylenes, Beta-Carotene and Lutein on Human Lymphoid Leukaemia Cells. Anticancer Agents Med Chem 2012; 12: 640-652.

[17] Young JF, Christensen LP, Theil PK, Oksbjerg N. The polyacetylenes falcarinol and falcarindiol affect stress responses in myotube cultures in a biphasic manner. Dose-Response 2008; 6: 239-251.

[18] Hansen SL, Purup S, Christensen LP. Bioactivity of falcarinol and the influence of processing and storage on its content in carrots (*Daucus carota* L). J Sci Food Agric 2003; 83: 1010-1017.

[19] Kidmose U, Hansen SL, Christensen LP, Edelenbos M, Larsen E, Norbaek R. Effects of genotype, root size, storage, and processing on bioactive compounds in organically grown carrots (*Daucus carota* L.). J Food Sci 2004; 69: 388-394.

[20] Bernart MW, Cardellina JH, Balaschak MS, Alexander MR, Shoemaker RH, Boyd MR. Cytotoxic falcarinol oxylipins from Dendropanax arboreus. J Nat Prod 1996; 59: 748-753.

[21] Czepa A, Hofmann T. Structural and sensory characterization of compounds contributing to the bitter off-taste of carrots (Daucus carota l.) and carrot puree. J Agric Food Chem 2010; 51: 3865-3873.

[22] Hirakura K, Morita M, Nakajima K, Ikeya Y, Mitsuhashi H. The Constituents of Panax Ginseng .3. 3 Acetylenic-Compounds from Roots of Panax-Ginseng. Phytochemistry 1992; 31: 899-903.

[23] Roman M, Dobrowlski JC, Baranska M, Baranski R. Spectroscopic studies on bioactive polyacetylenes and other plant components in wild carrot root. J Nat Prod 2011; 74: 1757-1763.

[24] Abad-García B, Garmón-Lobato S, Berrueta LA, Gallo B, Vicente F. On line characterisation of 58 phenolic compounds in *Citrus* fruit juices from Spanish cultivars by high-performance liquid chromatography with photodiode-array detection coupled to electrospray ionisation triple quadrupole mass spectrometry. Talanta 2012; 99: 213-224.

[25] Theil PK, Sorensen IL, Nissen PM, Oksbjerg N. Temporal expression of growth factor genes of primary porcine satellite cells during myogenesis. Anim Sci J 2006; 77: 330-337.

[26] Rasmussen MK, Juel C, Nordsborg NB. Exercise-induced regulation of muscular N+-K+ pump, FXYD1, and NHE1 mRNA and protein expression: importance of training status, intensity, and muscle type. Am J Physiol Regul Integr Comp Physiol 2011; 300: 1209-1220.

[27] Che BN, Oksbjerg N, Hellgren LI, Nielsen JH, Young JF. Phytanic acid stimulates glucose uptake in a model of skeletal muscles, the primary porcine myotubes. Lipids Health Dis 2013; 12.

[28] Hertel J, Struthers H, Horj CJ, Hruz PW. A structural basis for the acute effects of HIV protease inhibitors on GLUT4 intrinsic activity. J Biol Chem 2004; 279: 55147-55152.

[29] Murata H, Hurz PW, Mueckler M. Indinavir inhibits the glucose transporter isoform Glut4 at physiologic concentrations. Aids 2002; 16: 859-863.

[30] Tsakiridis T, Mcdowell HE, Walker T, Downes CP, Hundal HS, Vranic L, et al. Multiple Roles of Phosphatidylinositol 3-Kinase in Regulation of Glucose-Transport, Amino-Acid-Transport, and Glucose Transporters in L6 Skeletal-Muscle Cells. Endocrinology 1995; 136: 4315-4322.

[31] Gerhard DS, Wagner L, Feingold EA, Shenmen C M, Grouse LH, Schuler G, et al. The status, quality, and

expansion of the NIH full-length cDNA project: the Mammalian Gene Collection (MGC). Genome Res2004; 14: 2121-2127.

[32] Strausberg RL, Feingold EA, Grouse LH, Derge JG, Klausner RD, Collins FS, et al. Generation and initial analysis of more than 15,000 full-length human and mouse cDNA sequences. Proc Natl Acad Sci U S A 2002; 99: 16899-16903.

[33] Essen-Gustavsson B, Granlund A, Benziane B, Jensen-Waern M, Chibalin AV. Muscle glycogen resynthesis, signalling and metabolic responses following acute exercise in exercise-trained pigs carrying the PRKAG3 mutation. Exp Physiol 2011; 96: 927-937.

[34] Granlund A, Kotova O, Benziane B, Galuska D, Jensen-Waern M, Chibalin AV. Effects of exercise on muscle glycogen synthesis signalling and enzyme activities in pigs carrying the PRKAG3 mutation. Exp Physiol 2010; 95: 541-549.

[35] Harmon AW, Patel YM. Naringenin inhibits glucose uptake in MCF-7 breast cancer cells: a mechanism for impaired cellular proliferation. Breast Cancer ResTreat 2004; 85: 103-110.

[36] Park JB. Flavonoids are potential inhibitors of glucose uptake in U937 cells. Biochem Biophys ResCommun 1999; 260: 568-574.

[37] Ferby IM, Waga I, Hoshino M, Kume K, Shimizu T. Wortmannin inhibits mitogen-activated protein kinase activation by platelet-activating factor through a mechanism independent of p85/p110-type phosphatidylinositol 3-kinase. J Biol Chem 1996; 271: 11684-11688.

[38] Cheng Z, Pang T, Gu M, Gao AH, Xie CM, Li JY, et al. Berberine-stimulated glucose uptake in L6 myotubes involves both AMPK and p38 MAPK. BBA.Gen. Subjects 2006; 1760: 1682-1689.

[39] Konrad D, Somwar R, Sweeney G, Yaworsky K, Hayashi M, Ramlal T, et al. The antihyperglycemic drug alpha-lipoic acid stimulates glucose uptake via both GLUT4 translocation and GLUT4 activation - Potential role of p38 mitogen-activated protein kinase in GLUT4 activation. Diabetes 2001; 50: 1464-1471.

[40] Jin JF, Mullen TD, Hou Q, Bielawski J, Bielawska A, Zhang XM, et al. AMPK inhibitor Compound C stimulates ceramide production and promotes Bax redistribution and apoptosis in MCF7 breast carcinoma cells. J Lipid Res 2009; 50: 2389-2397.

[41] Sandstrom ME, Zhang SJ, Bruton J, Silva JP, Reid MB, Westerbald H, et al. Role of reactive oxygen species in contraction-mediated glucose transport in mouse skeletal muscle. J Physiol-London 2006; 575: 251-262.

[42] Kim JY, An JM, Chung WY, Park KK, Hwang JK, Kim DS, et al. Xanthorrhizol Induces Apoptosis Through ROS-Mediated MAPK Activation in Human Oral Squamous Cell Carcinoma Cells and Inhibits DMBA-Induced Oral Carcinogenesis in Hamsters. PhytotherRes 2013; 27: 493-498.