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

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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 polycyclene 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 (1H and 13C NMR, and 1H-1H and 1H-13C correlation spectroscopy recorded in CDC13 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. [3H] 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 Heps, 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 incubation 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 1 μM 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, falcarniol 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 falcarniol 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. Falcarniol 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 falcarniol, 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 falcarniol. Based on these results, the concentrations 10 and 30 μM for both naringenin and falcarniol were chosen for further experiments.

The effects of naringenin and falcarniol 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. Falcarniol, 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 falcarniol 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 IC50 of indinavir in cells expressing Glut4 is 50 to 100 μM [29]. To examine whether the increased GU elicited by naringenin and falcarniol 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 falcarniol 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: ENSSSCCT00000010375 and ENSSSCCT0000009599). 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 IC50 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 TBC1D1 phosphorylation 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.

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Conflict of interests

The authors declare no conflict of interest.

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