Biochemical and Biophysical Research Communications 398 (2010) 178–183

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Naringenin, a citrus flavonoid, increases muscle cell glucose uptake via AMPK

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ARTICLE INFO

Article history:
Received 8 June 2010
Available online 15 June 2010

Keywords:
Naringenin
Myotubes
Glucose uptake
AMPK
Akt
SIRT1

ABSTRACT

Naringenin, a flavonoid found in high concentrations in grapefruit, has been reported to have antioxidant, antiatherogenic, and anticancer effects. Effects on lipid and glucose metabolism have also been reported. Naringenin is structurally similar to the polyphenol resveratrol, that has been reported to activate the SIRT1 protein deacetylase and to have antidiabetic properties. In the present study we examined the direct effects of naringenin on skeletal muscle glucose uptake and investigated the mechanism involved. Naringenin stimulated glucose uptake in L6 myotubes in a dose- and time-dependent manner. Maximum stimulation was seen with 75 μM naringenin for 2 h (192.8 ± 24%, $p < 0.01$), a response comparable to maximum insulin response (190.1 ± 13%, $p < 0.001$). Similar to insulin, naringenin did not increase glucose uptake in myoblasts indicating that GLUT4 glucose transporters may be involved in the naringenin-stimulated glucose uptake. In addition, naringenin did not have a significant effect on basal or insulin-stimulated Akt phosphorylation while significantly increased AMPK phosphorylation/activation. Furthermore, silencing of AMPK, using siRNA approach, abolished the naringenin-stimulated glucose uptake. The SIRT1 inhibitors nicotinamide and EX527 did not have an effect on naringenin-stimulated AMPK phosphorylation and glucose uptake. Our data show that naringenin increases glucose uptake by skeletal muscle cells in an AMPK-dependent manner.

1. Introduction

Skeletal muscle tissue accounts for the majority, ~80%, of insulin-mediated glucose uptake in the post-prandial state and plays a major role in maintaining glucose homeostasis. Insulin increases glucose uptake in skeletal muscle via a signaling cascade that leads to activation of phospatidylinositol-3 kinase (PI3 K), and Akt resulting in increased translocation of intracellular stored GLUT4 glucose transporters to the plasma membrane [1]. Insulin resistance in skeletal muscle coupled with impaired pancreatic β-cell function leads to the development of Type 2 diabetes, the most prevalent form of diabetes [2]. Therefore, skeletal muscle tissue represents an attractive therapeutic target for the treatment of insulin resistance and Type 2 diabetes.

The AMP-activated protein kinase (AMPK) is composed of three subunits, α, β and γ [3]. In mammalian cells AMPK acts as an energy sensor, is activated by an increase in AMP/ATP ratio [3] and has at least two upstream kinases, LKB1 and calmodulin-dependent protein kinase kinase (CaMKKs) [3]. Skeletal muscle AMPK is activated by exercise/contraction [4] and numerous compounds including metformin [5], thiazolidineones [6] and the polyphenol resveratrol [7,8] resulting in stimulation of glucose uptake. The study of novel compounds that activate AMPK and increase skeletal muscle glucose uptake is of major importance as the knowledge gained from such studies could be used towards the development of new treatment of insulin resistance and Type 2 diabetes.

Naringenin is a flavonoid found in citrus fruit and tomatoes that has been reported to have antioxidant [9], anticancer [10] and antiatherogenic [11] properties. Effects on lipid metabolism [12,13] and plasma glucose levels [13–16] have also been reported. *In vitro* studies have shown that naringenin has an insulin-like effect to decrease apoliprotein B (ApoB) secretion in hepatocytes [12]. In *in vivo* studies *Cochlospermum vivifolium* which contains naringenin decreased blood glucose levels in healthy male wistar rats [17]. Furthermore, naringenin administration decreased plasma glucose levels in streptozotocin-induced diabetic rats [14], improved insulin sensitivity in fructose-fed insulin resistant rats [16] and reduced insulin resistance in high fat-fed LDL receptor negative ($Ldlr^{-/-}$) mice [13]. Naringenin has structural similarities [18] with the extensively studied polyphenol resveratrol which has been shown to have antidiabetic properties both *in vitro* and *in vivo* [7,8,19]. Direct effects of naringenin on skeletal muscle glucose transport have not been examined previously and its mechanism of action is not known. In the present study we examined the effects of naringenin on glucose transport in L6 skeletal muscle cells. Naringenin increased glucose uptake in L6 myotubes and increased AMPK phosphorylation. Downregulation of AMPK using small interference RNA (siRNA) abolished the naringenin-stimulated glucose uptake emphasizing the importance of AMPK in this naringenin response.

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0006-291X/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2010.06.048

The SIRT1 enzyme has been suggested to be upstream of AMPK [20,21] and thus we examined its possible involvement in naringenin action using the SIRT1 inhibitors nicotinamide and EX527. The SIRT1 inhibitors did not inhibit naringenin-stimulated glucose transport or AMPK phosphorylation suggesting that SIRT1 is not involved in the mechanism of naringenin.

2. Materials and methods

2.1. Materials

Minimum essential medium (α-MEM), fetal bovine serum (FBS), trypsin, and antibiotic were purchased from GIBCO Life Technologies (Burlington, ON, Canada). Antibodies against phosphorylated and Total Akt and AMPK, HRP-conjugated anti-rabbit and anti-mouse secondary antibodies were purchased from New England Biolabs (Mississauga, ON, Canada). ChemiGlow reagents were purchased from VWR (Mississauga, ON, Canada). Polyvinylidene difluoride (PVDF) membranes, molecular weight protein standards, and electrophoresis reagents were purchased from Bio-Rad (Mississauga, ON, Canada). [^{3}H]2-deoxy-D-glucose, was purchased from PerkinElmer (Boston, MA, US). Cytochalasin B (CB) was from Calbiochem (Gibbstown, NJ, US). All other chemicals, including naringenin, nicotinamide, bovine serum albumin, and cold 2-deoxy-D-glucose, were purchased from Sigma (St. Louis, MO, US). siRNA oligonucleotides were purchased from Dharmacon (Lafayette, CO, US). Calcium phosphate-based transfection reagent (CellPhect transfection kit) was purchased from Amersham Biosciences (Baie d'Urfe, QC, Canada). The SIRT1 inhibitor EX527 was from Tocris Bioscience (Ellisville, MO, US).

2.2. Cell culture

L6 rat myotubes were grown in α-MEM containing 5 mM glucose, 2% (v/v) FBS, and 1% (v/v) antibiotic–antimycotic solution (100 U/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B) in a humidified atmosphere of 5% CO_2–95% air at 37 °C as previously described [8]. Undifferentiated myoblasts grown in 10% FBS containing α-MEM media were also used. Prior to experiments, the cells were serum deprived for 3–5 h.

2.3. Treatment and glucose uptake assay

Naringenin stock solutions were prepared using ethanol. A vehicle-treated control group was used. At the end of the treatment period, the cells were rinsed with HEPES-buffered saline solution (HBS) followed by [^{3}H]2-deoxy-D-glucose uptake measurements as performed previously [8]. All experiments were assayed in triplicate and performed at least three times. The Bio-Rad protein assay was used to measure cellular protein levels.

2.4. Western blotting

Protein samples (15 μg) were separated by SDS–PAGE, transferred to a PVDF membrane which was blocked for 1 h with 5% (w/v) dry milk in Tris-buffered saline, and incubated overnight at 4 °C with the primary antibody. The primary antibody was detected with HRP-conjugated anti-rabbit or anti-mouse secondary antibody and ChemiGLO reagent and visualized by FluroChem software (ThermoFisher).

2.5. siRNA oligomer transfection

L6 cells were transfected with a combination of siRNA targeting the α1 and α2 subunits of AMPK as reported in Konrad et al. [6]. The siRNA sequences used were: α1:GCA UAU GCU GCA GGU AGA UdTdT and α2:CGU CAU UGA UGA UGA GGC UdTdT. Cells were seeded in antibiotic-free media and on day 2 (>60% confluency) were transfected with siRNA AMPK α1/α2 (90 nM of each oligonucleotide sequence), or 45 nM scramble sequence (an unrelated siRNA) as per the manufacturer's instruction. Fourteen hours following transfection, cells were washed and the media was changed to fresh α-MEM supplemented with 2% FBS. Experiments were performed 72 h post-transfection. Myoblast fusion into myotubes and protein recovery was not affected by siRNA treatment.

2.6. Statistical Analysis

The results are the mean ± SE of the indicated number of independent experiments. Analysis of variance (ANOVA) and/or student's paired *t*-test was used. Statistical significance was assumed at $p < 0.05$. SPSS v15.0–16.0 or SAS v9.0 software were used for the calculations.

3. Results

Myotubes were exposed to different naringenin concentrations for 2 h followed by glucose uptake measurements (Fig. 1A). Naringenin at 10 and 20 μM did not affect glucose uptake (93 ± 3.5% and 108 ± 12.5% of control, $p > 0.05$, respectively). However, naringenin at 50 μM significantly increased glucose uptake (141 ± 5.5% of control, $p < 0.05$) and maximum stimulation was observed at 75 μM (165 ± 4.9% of control, $p < 0.01$). Higher concentrations of naringenin (100 μM and 150 μM) did not further increase glucose uptake.

The effect of naringenin was time-dependent (Fig. 1B) with a significant response seen at 60 min (144 ± 8.7%, $p < 0.05$) and a maximum response at 2 h of stimulation (168 ± 20.1%, $p < 0.01$). Importantly, our data show that the effect of naringenin on glucose uptake in myotubes is at the same level as seen with maximum insulin stimulation (192.8 ± 24%, $p < 0.01$ and 190.1 ± 13%, $p < 0.001$, respectively, Fig. 1C). We also examined whether naringenin increases glucose uptake in myoblasts that are known to not respond to insulin due to low GLUT4 expression [22]. Naringenin did not significantly increase glucose uptake in L6 myoblasts (106.3 ± 15.2%), an effect similar to insulin (122.3 ± 11.8%), indicating that the effect of naringenin may be due to modulation of GLUT4 translocation and/or activity (Fig. 1C). Visual microscopic inspection of all experimental groups showed that cell morphology was not affected by any treatment.

Given the similarity of naringenin's effect with that of insulin we next examined the effect of naringenin on Akt phosphorylation, a molecule involved in the insulin-stimulated glucose uptake in myotubes. Insulin strongly stimulated phosphorylation of both residues associated with Akt activation, Ser473 and Thr308, (367 ± 12.5%, $p < 0.05$ and 262.5 ± 7.5%, $p < 0.05$, Fig. 2A) while naringenin (15–120 min) had no effect.

The effect of naringenin on insulin-stimulated glucose uptake was examined next. Insulin dose-dependently increased glucose uptake (Fig. 2B). Pretreatment with naringenin significantly increased glucose uptake compared to treatment with insulin alone at sub-maximal (1, 3 and 10 nM) levels (Fig. 2B). At maximal (100 nM) levels of insulin no significant differences were observed between the response to insulin alone vs. insulin combined with naringenin (Fig. 2B). Furthermore as expected insulin treatment increased Akt phosphorylation in a dose-dependent response that was not affected by naringenin (Fig. 2C and D).

Since we [8] showed previously that the polyphenol resveratrol, that has structural similarities with naringenin [18], activates