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Exercise-mimicking treatment fails to increase *Fndc5* mRNA & irisin secretion in primary human myotubes

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ABSTRACT

Irisin, myokine secreted by skeletal muscle, was suggested to mediate some of exercise health benefits *via* "browning" of white adipose tissue. However, mounting evidence contradicts the regulatory role of exercise for muscle irisin production/secretion in humans. Thus, we explored the direct effect of exercise-mimicking treatment on irisin in human primary muscle cells *in vitro*. Human primary muscle cell cultures were established from lean, obese prediabetic and type-2-diabetic individuals. Complex metabolic phenotyping included assessment of insulin sensitivity (euglycemic hyperinsulinemic clamp) and adiposity content&distribution (MRI&MRS). *In vitro* exercise-mimicking treatment (forskolin + ionomycin) was delivered in 1-h pulse/day during differentiation. *Fndc5* mRNA (qRT-PCR) and secreted irisin (ELISA) were determined in cells and media. Exercise-mimicking treatment more than doubled *Pgc1α* mRNA in differentiated muscle cells. Nevertheless, *Fndc5* mRNA was reduced by 18% and irisin in media by 20%. Moreover, *Fncd5* mRNA was increased in myotubes derived from individuals with type-2-diabetes, independent on exercise-mimicking treatment. *Fndc5* mRNA in cells was positively related to fasting glycemia ($p = 0.0001$) and negatively to whole-body insulin sensitivity ($p < 0.05$). Collectively, our data do not support the role of exercise-related signaling pathways in irisin regulation in human skeletal muscle and confirm our previous observations on increased *Fndc5* expression in muscle cells from individuals with type-2-diabetes.

1. Introduction

Exercise is an essential component of healthy lifestyle. Contracting skeletal muscle secretes bioactive substances – myokines – potential mediators of many exercise-induced health benefits. The role of myokines is to provide multilateral support to active muscle by modulating processes related to energy metabolism and muscle regenerative capacity as well as to modify plasticity of other organs and tissues as a part of adaptive response to acute and/or regular exercise. A novel myokine irisin is a proteolytic cleavage product of the fibronectin type III domain-containing protein 5 (FNDC5), transmembrane protein localized in skeletal muscle. In animal models, irisin was shown to be induced by exercise, stimulating UCP1 expression and browning of subcutaneous adipose tissue, with profound effects on the whole body metabolism [2]. In line with these observations, irisin emerged as a promising anti-obesity target. However, results of human studies are not as clear,

Abbreviations: AT, adipose tissue; B2m, B-2-microglobulin; BMI, body mass index; EMCL, extramyocellular lipids; FFA, free fatty acids; Fndc5, fibronectin type III domain-containing protein 5; IMCL, intramyocellular lipids; Ins, insulin; LBM, lean body mass; MRI, magnetic resonance imaging; MRS, magnetic resonance spectroscopy; *M*-value, insulin sensitivity index; oGTT, oral glucose tolerance test; PGC1α, peroxisome proliferator-activated receptor-γ coactivator 1α; qRT-PCR, quantitative reverse transcriptase PCR; REE, resting energy expenditure; Rpl13a, ribosomal protein L13a; T2D, type 2 diabetes; UCP1, uncoupling protein 1.

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and there is no persuasive evidence that exercise regulates circulating or muscle *Fndc5*/irisin levels in humans [16]. On the contrary, many clinical studies point at a lack of exercise-induced effects on irisin production/secretion in humans [9,15,23]. On the other hand, circulating irisin has been shown to be downregulated in type 2 diabetes [9,12], which together with positive associations of muscle *Fndc5* with *in vivo* muscle metabolism, strength and volume [9] suggest a positive role this myokine might play for metabolic health.

Human primary skeletal muscle cells are frequently used as a model for studying muscle secretome and its regulation [4,9,14,19]. Furthermore, differentiated myotubes derived from individuals with distinct obesity & metabolic health phenotypes enable to link clinical information with *in vitro* observations [7,22,24]. To shed more light on effects of exercise on *Fndc5*/irisin expression and secretion, we have explored the regulation of *Fndc5*/irisin by exercise-mimicking treatment of human primary myotubes derived from lean, obese and type 2 diabetic donors *in vitro*.

2. Methods

2.1. Ethics statement

All studies were approved by the Ethics Committee of the Slovak Medical University Bratislava and the Ethics Committee of the Bratislava Region Office and are conforming to the ethical guidelines of the 2000 Helsinki declaration. All participants provided witnessed written informed consent prior entering the study.

2.2. Study population

Human primary skeletal muscle cell cultures were derived from age-matched lean ($n=4$), obese ($n=5$), prediabetic ($n=3$) and T2D individuals ($n=6$) (Table 1). The obesity level in the last three groups was comparable. A complex metabolic phenotyping of cells' donors was performed, as described in detail in [9]. Shortly, bioelectric impedance was used to evaluate total adiposity and to estimate lean body mass (Omron BF511, Omron Healthcare Ltd., Matsusaka, Japan). *Abdominal adipose tissue content* and distribution (visceral and subcutaneous) was measured on 1.5 T Magnetom Symphony MRI scanner (Siemens, Germany). Intra- and extramyocellular lipids were determined by ^{1}H-MRS. *Resting energy expenditure* was measured after an overnight fast and following 30 min bed rest with the Ergostik (Geratherm Respiratory, Germany) for a period of 30 min. *Insulin sensitivity* was determined by frequent sampled euglycemic hyperinsulinemic clamp. A primed (80 mU m^{-2} min^{-1}) continuous (40 mU m^{-2} min^{-1}) insulin (Actrapid 100 IU ml^{-1}, Novo Nordisk, Denmark) infusion was used to achieve hyperinsulinemia. Blood glucose was measured in 5-min intervals and maintained at euglycemia (5 mmol l^{-1} ± 0.25 mmol l^{-1}) using variable infusion rate of 20% glucose. The whole body insulin sensitivity index (*M*-value/insulin) was calculated from the steady state plasma glucose infusion rate required to maintain euglycemia, expressed per kg body weight per minute and normalized to the average steady state insulinemia (μU ml^{-1}) of four samples taken during the last 60 min of the clamp.

2.3. Primary human skeletal muscle cell cultures

Samples of skeletal muscle (*m. vastus lateralis*) were taken by Bergström needle biopsy under local anesthesia in the fasted state. A small piece of muscle (approx. 80 mg) was used to establish primary human skeletal muscle cell cultures, as previously described [24]. Briefly, satellite cells (quiescent mononuclear muscle cells) were isolated by trypsin digestion of the freshly obtained muscle, pre-plated on an uncoated petri dish for 1 h and subsequently transferred to T-25 collagen-coated flasks with growth medium composed of DMEM with 15% fetal bovine serum (FBS), human epidermal growth factor, BSA, dexamethasone, gentamycin, fungizone & fetuin. Cells were passaged once, harvested, and stored frozen in liquid nitrogen.

2.4. Exercise-mimicking treatment of CD56$^+$ human primary muscle cells

Muscle cells derived from 18 individuals (lean, $n=4$; overweight/obese, $n=5$; prediabetics, $n=3$ and type 2 diabetics, $n=6$) were thawed and grown to 80% confluence, trypsinized and mononuclear myoblasts were immunopurified using the mouse monoclonal anti-CD56 (Neural Cell Adhesion Molecule 1, the marker of myoblasts) antibody (Developmental Studies Hybridoma Bank, Iowa City, USA) and magnetic beads attached secondary Ab (Miltenyi Biotech, Germany) as previously described [20]. Sorted cells were grown to 80–90% confluence when differentiation was induced by switching to αMEM supplemented with 0.5% fetuin, 2% FBS, 30 μM palmitate and atb. Exercise-mimicking treatment was initiated from the third day of differentiation. "Exercise-mimicking cocktail" containing 4 μM forskolin (activation of protein kinase A pathway) and 0.5 μM ionomycin (activation of calcium signaling pathway) in 0.01% DMSO (both Sigma–Aldrich, St Louis, MO) was delivered in a single 1-h pulse per day for the last three days of differentiation, as optimized and described in [20]. Control cells were pulse-treated with 0.01% DMSO.

Myotubes and conditioned media were harvested after 5 days of differentiation, when about 80% of mononuclear myoblasts had fused to form multinuclear elongated myotubes. Irisin concentration was determined after 6 h of incubation in serum and fetuin-free conditioned media.

2.5. RNA isolation and real-time PCR

Total RNA from differentiated muscle cells was isolated using TriReagent (Molecular Research Center, Inc., USA). Purified (RNeasy Mini Kit, Qiagen, USA), DNAse treated (Qiagen, USA) RNA was used for gene expression studies. RNA was quantified spectrophotometrically with aid of NanoDrop2000 (Implen, Germany). cDNA was produced with the aid of High Capacity RNA to cDNA kit (Applied Biosystems, USA). Gene expression was measured by qRT-PCR at ABI7900HT (Applied Biosystems, USA) using either pre-designed TaqMan gene expression assays (*Ppargc1A*, Hs01016719_m1, Applied Biosystems, USA) or set of primers designed with the PrimerExpress software (Applied Biosystems, USA); *Fndc5* (fwd: TGAGGTTGTCATCGGATTTGC; rev: GCGGGTGGTGGTGTTCAC); *Myog* (fwd: GCTCAGCTCCCTCAACCA; rev: GCTGTGAGAGCTGCATTCG); *MyoD* (fwd: CACTACAGCGGCGACTCC; rev: TAGGCGCCTTCGTAGCAG); *Myh2* (fwd: TGTCTCACTCCCAGGCTACA; rev: CCAAAAACAGCCAATTCTGAG); *Rpl13α* (fwd: GGACCGTGCGAGGTATGCT; rev: ATGCCGTCAAACACCTTGAGA); and *B2m* (fwd: CGCTCCGTGGCCTTAGC; rev: AATCTTTGGAGTACGCTGGATAGC). Ribosomal protein L13α and B-2-microglobulin were used as internal reference genes to calculate ddCt expression values. qRT-PCR reaction for each primer set was optimized individually.

2.6. Biochemical assays

Irisin concentrations in conditioned media were determined using Irisin, Recombinant Enzyme Immunoassay kit (cat # EK-067-29, Phoenix Pharmaceuticals, USA) with the range 0.1–1000 ng ml^{-1} and linear range between 1.29 and 27.5 ng ml^{-1}. Antibody used in this kit recognizes recombinant full length irisin, irisin (42–112) (100%) and recombinant FNDC5, isoform 4 (9%), but not the irisin precursor C-terminal 48-mer FNDC5 (165–212) and irisin (42–95). All samples were measured in one batch and