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The bone anabolic effects of irisin are through preferential stimulation of aerobic glycolysis

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ABSTRACT

Irisin, a recently identified hormone secreted by skeletal muscle in response to exercise, exhibits anabolic effects on the skeleton primarily through the stimulation of bone formation. However, the mechanism underlying the irisin-stimulated anabolic response remains largely unknown. To uncover the underlying mechanism, we biosynthesized recombinant irisin (r-irisin) using an *Escherichia coli* expression system and used it to treat several osteoblast cell types. Our synthesized r-irisin could promote proliferation and differentiation of osteoblasts as evidenced by enhanced expression of osteoblast-specific transcriptional factors, including Runt-related transcription factor-2 (*Runx2*), Oster (*Osx*), as well as early osteoblastic differentiation markers such as alkaline phosphatase (*Alp*) and collagen type I alpha 1 (*Col1a1*). Furthermore, we showed that the promotion of r-irisin on the proliferation and differentiation of osteoblast lineage cells are preferentially through aerobic glycolysis, as indicated by the enhanced abundance of representative enzymes such as lactate dehydrogenase A (LDHA) and pyruvate dehydrogenase kinase 1 (PDK1), together with increased lactate levels. Suppression of r-irisin-mediated aerobic glycolysis with Dichloroacetate blunted its anabolic effects. The favorite of the aerobic glycolysis after r-irisin treatment was then confirmed in primary calvarial cells by metabolic analysis using gas chromatography–mass spectrometry. Thus, our results suggest that the anabolic actions of r-irisin on the regulation of osteoblast lineage cells are preferentially through aerobic glycolysis, which may help to develop new irisin-based bone anabolic agents.

1. Introduction

Bone is a metabolically dynamic tissue consisting of osteoblasts, osteocytes, and osteoclasts [1]. As the primary bone-forming cells, osteoblasts synthesize a large amount of extracellular matrix proteins, which will then be mineralized to form bone matrix. When the bone matrix is synthetized, the osteoblasts are enveloped by it and become osteocytes, which are the star-shaped bone cells found in the mature bone tissue. On the other hand, the osteoclasts mainly function in bone resorption. Thereby, the homeostatic balance between bone formation (an anabolic process) and bone resorption (a catabolic process) during bone metabolism determines its hardness [2]. It is well known that bone metabolism can be regulated by a range of factors, including physical exercise. Physical exercise acts on the bone directly via mechanical force, or indirectly via an anabolic effect through hormonal factors [3,4]. Among which, parathyroid hormone (PTH) is a well-known hormone involved in both catabolic and anabolic actions on the bone [5]. A recent study has demonstrated that the anabolic actions of PTH on bone were through the stimulations of glucose consumption and lactate production, a hallmark of aerobic glycolysis, and the suppression of mitochondrial oxidative phosphorylation by preventing the entry of glucose into the tricarboxylic acid (TCA) cycle [6].

Abbreviations: Runx2, Runt-related transcription factor-2; *Osx*, Oster; *Alp*, alkaline phosphatase; *Col1a1*, collagen type I alpha 1; *Atf4*, activating transcription factor 4; LDHA, lactate dehydrogenase A; PDK1, pyruvate dehydrogenase kinase 1; TCA, tricarboxylic acid; FNDC5, fibronectin type III domain-containing protein 5; *Ucp1*, uncoupling protein 1; *Ucp2*, uncoupling protein 2; OXPHOS, oxidative phosphorylation; NDUFB8, a subunit of NADH dehydrogenase; SDHB, a subunit of succinate dehydrogenase; UQCRC2, ubiquinol–cytochrome *c* reductase complex; MTCO1, subunit I of cytochrome *c* oxidase; ATP5A, α subunit of F0F1-ATP synthase

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Irisin, a newly identified hormone-like molecule, is secreted by skeletal muscle in response to exercise. It is the extracellular domain of fibronectin type III domain-containing protein 5 (FNDC5). Initially, the existence of human irisin was questioned because of the unconventional ATA start codon of FNDC5 and the poor specificity of the antibodies [7,8]. However, it was finally identified in plasma via mass spectrometry [9], and is mainly translated from a non-canonical start codon. Previous studies have shown that irisin plays important roles in converting white adipose tissue to brown adipose tissue, and in regulating energy expenditure, inflammation, hippocampal neurogenesis, aging, and other metabolic conditions [10–14]. Recent studies have indicated that irisin displays anabolic actions on the skeleton through the stimulation of bone formation [15,16]. However, the mechanism underlying the irisin-stimulated anabolic response has not been fully understood.

To explore the underlying mechanism, here we biosynthesized irisin in *Escherichia coli* using recombinant DNA technology and characterized the synthesized protein after purification. We showed that our biosynthesized r-irisin stimulated osteoblastic bone formation by preferentially enhancing aerobic glycolysis while the TCA cycle was suppressed. Thus, our results suggest that the anabolic actions of r-irisin on the regulation of osteoblast lineage cells were preferentially through the stimulating of aerobic glycolysis.

2. Materials and methods

2.1. Antibodies

Antibodies for p-ERK (Thr202/Tyr204) (Cat#9101), ERK (Cat#9102), p-P38 (Thr180/Tyr182) (Cat#4511), P38 (Cat#9212), LDHA (Cat#2012), and PDK1 (Cat#5662) were purchased from Cell Signaling Technologies (Danvers, MA, USA). FNDC5 antibody (ab131390) and total OXPHOS antibody cocktail (ab110413) were purchased from Abcam (Cambridge, UK). GAPDH (Cat#OAEA00006) antibody was from Aviva Systems Biology (San Diego, CA) and α-tubulin (Cat#05829) antibody was from Merck Millipore (Danvers, MA). Horseradish peroxidase (HRP)-conjugated anti-rabbit (Cat#7074) and HRP-conjugated anti-mouse (sc-2005) secondary antibodies were from Cell Signaling Technologies and Santa Cruz Biotechnology (CA, USA), respectively.

2.2. Plasmid construction

The pcDNA3.1 + C-6His + FNDC5 plasmid was purchased from GenScript (George Town, Cayman Islands). The irisin-coding region (amino acid residues 32–143) was amplified via PCR from the pcDNA3.1 + C-6His + FNDC5 plasmid, which contained a 555-bp sequence of human *FNDC5*. First, a TEV protease target sequence was inserted upstream of the irisin-encoding sequence, and then these two sequences were amplified together using the following primers: 5′-CGAAAACCTGTATTTTCA GGGCGACAGTCCCTCAGCCCCAGTG AAC-3′ (forward) and 5′-CCGCTCGAGTCACTC TTTCATGGTTACCTC-3′ (reverse, containing a *Xho*I site). The PCR product was purified and amplified using the following primers: 5′-GGGGGATCCGAAAACCTGT ATTTTCAG-3′ (forward, containing a *Bam*HI site) and 5′-CCGCTC GAGTCACTCTTTCATGGTTACCTC-3′ (reverse, containing a *Xho*I site). To create pET32a-TEV-irisin, the digested PCR product was subcloned into the pET-32a vector (Novagen) such that an N-terminal hexahistidine (His)-tag was included in the protein product for purification.

2.3. Expression and purification of r-irisin from E. coli

The plasmid pET32a-TEV-irisin was transformed into *E. coli* BL21 (DE3) cells for expression. Single colonies were separately incubated into Luria–Bertani broth medium. After incubation at 37 °C for 2 h, the optical density (OD) at 600 nm reached 0.6–0.7, following which isopropyl β-D-1-thiogalactopyranoside was added to a final concentration of 0.8 mM. All bacterial cells were harvested after an overnight growth. The cell pellet was resuspended and sonicated in lysis buffer (25 mM NaPi, 300 mM NaCl, and 5 mM β-mercaptoethanol). The supernatant was separated by centrifugation (14,000 rpm for 30 min at 4 °C) and then passed through a His-tag affinity Ni-nitrilotriacetic acid resin. The affinity column resin was washed out twice using washing buffer containing 60 mM imidazole in lysis buffer. The TEV-irisin fusion peptide was eluted in lysis buffer containing 500 mM imidazole and then desalted using a desalting column. Then, the thioredoxin and His-tag were removed from the target protein by treatment with a TEV protease (2:1 molar ratio, overnight at room temperature), and r-irisin was re-eluted in the flow-through fraction during reversed-phase column. To increase the protein purity, size-exclusion chromatography was performed using a Superdex™ 200 column (GE Healthcare, Little Chalfont, UK) in PBS buffer. The purity of the target protein was identified by 15% SDS-PAGE. The target protein, r-irisin, was verified by western blotting analysis using an anti-FNDC5 antibody, and its concentration was determined by a Nanodrop 2000/2000c spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

2.4. Determination of endotoxin

The endotoxin content of purified r-irisin was measured by using the ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit (Genscript, Piscataway, NJ, USA). This examination was performed according to the manufacturer's protocol, and the concentrations of endotoxins were calculated according to its guidelines.

2.5. Semi-quantitative RT-PCR and quantitative reverse transcription PCR

Total RNA was extracted from cells treated with or without r-irisin using TRIsure™ reagent (Bioline Reagents Ltd, London, UK) according to the manufacturer's instructions. The reverse transcription of 2 μg total RNA was performed using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA). The gene-specific primers used in this study are listed in Table 1. PCR amplification was performed using 2 μL first-strand cDNA, 10 pmol primers, and GoTap DNA polymerase (Promega, WI, USA). The following cycles were used for Ucp1: initial denaturation at 94 °C for 5 min; followed by 32 cycles of 95 °C for 30 s, 55 °C for 1 min, and 72 °C for 1 min; and a final extension at 72 °C for 10 min. For β-actin: initial denaturation at 94 °C for 5 min; followed by 25 cycles of 95 °C for 30 s, 59 °C for 1 min, and 72 °C for 1 min; and a final extension at 72 °C for 10 min.

The qRT-PCR was performed in triplicate with SensiFAST™ SYBR® Hi-ROX kit (Bioline Reagents Ltd, London, UK) using a one-step system. The real-time PCR conditions were as follows: 95 °C for 10 min, then 40 cycles at 95 °C for 15 s, 60 °C for 1 min; and a melt curve cycle at 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative gene expression, and β-Actin was used

Table 1
The PCR primers.

Gene	Forward (5′–3′)	Reverse (5′–3′)
Ucp1	AGGCTTCCAGTACCATTAGGT	CTGAGTGAGGCAAAGCTGATTT
Atf4	GAGCTTCCTGAACAGCGAAGTG	TGGCCACCTCCAGATAGTCATC
$Runx_2$	CCGTGGCCTTCAAGGTTGT	TTCATAACAGCGGAGGCATTT
Osx	CCCTTCTCAAGCACCAATGG	AAGGGTGGGTAGTCATTTGCATA
Alp	TGACCTTCTCTCCTCCATCC	CTTCCTGGGAGTCTCATCCT
Col1a1	GCTCCTCTTAGGGGCCACT	CCACGTCTCACCATTGGGG
Sost	ATCCCAGGGCTTGGAGAGTA	ACATCTTTGGCGTCATAGGG
Ucp2	CAGGTCACTGTGCCCTTACCAT	CACTACGTTCCAGGATCCCAAG
β-Actin	GCTACAGCTTCACCACCACAG	GGTCTTTACGGATGTCAACGTC

Ucp1: uncoupling protein 1, Atf4: transcription factor 4, $Runx_2$: Runt-related transcription factor-2, Sox: Oster, Alp: alkaline phosphatase, Col1a1: collagen type I alpha 1, Ucp2: uncoupling protein 2.