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Original Paper

Interaction of CREB and PGC-1α Induces Fibronectin Type III Domain-Containing Protein 5 Expression in C2C12 Myotubes

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Key Words
FNDC5 • Irisin • PGC-1α • Myokine • C2C12 • Skeletal muscle • Glucose

Abstract
Background/Aims: Fibronectin type III domain-containing protein 5 (*FNDC5*), also known as irisin, is a myokine secreted from muscle in response to exercise. However, the molecular mechanisms that regulate *FNDC5* expression and the functional significance of irisn in skeletal muscle remain unknown. In this study, we explored the potential pathways that induce *FNDC5* expression and delineated the metabolic effects of irisin on skeletal muscle. ***Methods:*** C2C12 myotubes were treated with drugs at various concentrations and durations. The expression and activation of genes were measured by real-time polymerase chain reaction (qRT-PCR) and Western blotting. Oxidative phosphorylation was quantified by measuring the oxygen consumption rate (OCR). ***Results:*** We found that the exercise-mimicking treatment (cAMP, forskolin and isoproterenol) increased *Fndc5* expression in C2C12 myotubes. *CREB* over-expressed C2C12 myotubes displayed higher *Fndc5* expression. *CREB* over-expression also promoted peroxisome proliferator-activated receptor gamma coactivator 1α (PGC-1α) expression. PGC-1α-induced *Fndc5* expression was blocked when the dominant negative form of CREB (S133A) was present. PGC-1α mutation (S570A) also decreased *Fndc5* expression. Immunoprecipitation showed that overexpressed PGC-1α complexed with CREB in HEK293 cells. C2C12 myotubes treated with forskolin also increased endogenous CREB and PGC-1α binding. Functionally, irisin treatment increased mitochondrial respiration, enhanced ATP production, promoted fatty acid oxidation but decreased glycolysis in myotubes. ***Conclusion:*** Our observation indicates that cAMP-mediated PGC-1α/CREB interaction triggers *Fndc5*

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expression, which acts as an autocrine/paracrine to shape the metabolic phenotype of myotubes.

Introduction

Irisin, the secreted isoform of fibronectin type III domain-containing protein 5 (*FNDC5*), is a myokine that highly expresses in skeletal muscle, with relatively low expression in adipose tissue, pancreas, liver, and brain [1]. In humans, irisin is described as a metabolic myokine that improves obesity and glucose homeostasis by stimulating the browning of white adipose tissue (WAT) [2, 3]. It also improves the endothelial functions in obese subjects [4]. In human skeletal muscle, treatment with irisin (50 nM) for 1 h increased glucose and fatty acid uptake, which was similar to insulin [5]. At lower concentration (5 nM), irisin also stimulated mitochondrial biogenesis and increased UCP3 and GLUT4 levels in C2C12 cells [6]. Moreover, irisin is an AMPK stimulator to promote β-oxidation in muscle [3]. Intraperitoneal injection of irisin (0.5 μg per g of body weight) into obese and diabetic mice increased glucose uptake via stimulating GLUT4 translocation to the skeletal muscle cell membranes [7], suggesting irisin works as an autocrine/paracrine to modulate the energy homeostasis of skeletal muscle.

It was reported that peroxisome proliferator-activated receptor-γ co-activator 1α (PGC-1α) was a major regulator of *FNDC5* expression because irisin was first isolated from the muscle of PGC-1α transgenic mice [2, 8]. PGC-1α is an important regulator of mitochondrial biogenesis, which mediates various metabolic functions in response to different metabolic stresses [9]. Nevertheless, contradictory results were obtained from different laboratories that activation of PGC-1α and *FNDC5* expressions were uncoupled in skeletal muscle during exercise [10]. In 2017, a meta-analysis including 51 studies reported that a solid conclusion could not be made about the link between PGC-1α activity and *FNDC5* expression in response to physical activity [11]. Thus, the molecular mechanisms of exercise-regulated *FNDC5* expression in skeletal muscle are still far from clear.

The present study aims to delineate the regulatory mechanism that controls *FNDC5* expression as well as its metabolic functions in glucose and fatty acid metabolism in skeletal muscle.

Materials and Methods

Chemicals and reagents

C2C12 cells were purchased from ATCC (USA). Antibodies against pCREB S133 (cat. no. #9198, diluted 1: 1500), CREB (cat. no. #4820, diluted 1:1000), were purchased from Cell Signaling (USA). Anti-PGC-1α (cat. no. #8934, diluted 1:1000) was obtained from Abcam (USA). Anti-tubulin antibody (cat. no. #T6074, diluted 1:1000) and anti-Flag antibody (cat. no. F3165, diluted 1:2000) were obtained from Sigma-Aldrich (USA). Anti-GFP antibody (cat. no. sc9996, diluted 1: 1000) was obtained from Santa Cruz Biotechnology (USA). Plasmids expressing wild-type CREB, the dominant-negative mutant of CREB (CREB S133A), wild-type PGC-1α, and the dominant-negative mutant of PGC-1α (PGC-1α 570A) were obtained from Addgene (USA). Other chemicals were purchased from Sigma-Aldrich (USA).

C2C12 cell culture and differentiation

C2C12 myoblasts were maintained in high-glucose DMEM with 5% FBS, 15% calf serum, 100 IU/mL of penicillin and 100 μg/mL of streptomycin (Invitrogen, USA). The culture protocol was strictly enforced to avoid cell confluence. Differentiation of myoblasts into myotubes was performed by incubating the confluent myoblasts with differentiating medium (2% horse serum, 100 I.U./mL penicillin, and 100 μg/mL streptomycin) for 4 days. Successful differentiation of the C2C12 was confirmed by morphological changes as previously reported [12].

Cell transfection

C2C12 myotubes transfection was performed using Viromer RED as unstructured (Lipocalyx GmbH, Germany). Briefly, transfection was done in cells seeded in a 6-well plate (80% confluence) with complete antibiotic-free growth media. DNA (18 ng/μl) in 340 μl solution was added to 60 μl of viromer working solution. After mixing and incubation for approximately 15 min at room temperature, the solution was added to the cells. After 24-48 h, the medium was changed to 2% HS media to induce differentiation. Four days after transfection, the myotubes were collected for experiments.

Table 1. Primers used in real-time PCR

Genes	Primer sequence
Mouse Fndc5 5'	ATGAAGGAGATGGGGAGGAA
Mouse Fndc5 3'	GCGGCAGAAGAGAGCTATAACA
Mouse Pgc-1α 5'	ATACCGCAAAGAGCACGAGAA
Mouse Pgc-1α 3'	CTCAAGAGCAGCGAAAGCGTCACA
Mouse Tfam 5'	CAGGAGGCAAAGGATGATTC
Mouse Tfam 3'	ATGTCTCCGGATCGTTTCAC
Mouse Nrf1 5'	TCTCACCCTCCAAACCCAAC
Mouse Nrf1 3'	ATGCTCTTGTACTTTCGCACCA
Mouse β-actin 5'	AACCGTGAAAAGATGACCCAGAT
Mouse β-actin 3'	CACAGCCTGGATGGCTACGT

Quantitative Real-time PCR

Total RNA was isolated using TRIzol Isolation Reagent (Invitrogen, USA). First-strand cDNA was synthesized using 1 μg of total RNA and a reverse transcription reaction mix containing Superscript III reverse transcriptase (Invitrogen, USA) and Oligo-dT17 primer. The expression of genes was detected using RealMasterMix SYBR ROX (5 Prime Inc, USA) and the ABI7500 Real-time PCR System (Applied Biosystems, USA) with gene-specific primers pairs (Table 1). The results were quantified after normalization with β-actin [13].

Mitochondrial respiration

Mitochondrial respiration of C2C12 myotubes was determined by the Seahorse XFe 96 Extracellular Flux Analyzer using the XF Mito Stress Test Kit as previously described (Agilent, USA). The concentrations of oligomycin, carbonyl cyanide-p-trifluoromethoxyphenyl-hydrazone (FCCP), antimycin A and rotenone used were 100 μM, 100 μM, 100 μM and 50 μM, respectively. The oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were recorded, and cellular respiration and ATP production were calculated as described by the manufacturer.

Western blotting

Tissue extracts were prepared by homogenizing the tissues in lysis buffer (50 mM Tris at pH 7.4, 40 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 1.5 mM Na_3VO_4, 50 mM NaF, 10 mM $Na_4P_2O_7$, 10 mM sodium β-glycerol phosphate and protease inhibitor cocktail). Cell debris was removed by centrifugation, and the supernatants were collected for further analysis. Immunoblotting signals within the linear detection range were detected using the G:Box Chemi XRG imager (Syngene, USA) and were analysed by ImageJ (NIH, USA).

Immunoprecipitation

Lysis buffer washed Protein A/G-Agarose beads (50% bead slurry; sc-2003; Santa Cruz, USA) were added to 500 μg of cell lysate in a final volume of 500 μL. After incubation at 4°C for 60 min, the lysates were centrifuged at 12, 000 g for 1 min at 4°C. The supernatant was transferred to a fresh tube with 2 μL of primary antibody and 20 μL protein A/G, followed by an overnight incubation at 4°C with gentle rocking. The Protein A/G beads were then collected by centrifugation and washed three times with cell lysis buffer. After suspended in 20 μl of 2× SDS loading buffer and heated at 95°C for 5 min, the supernatants were used for SDS-PAGE.

Statistical analysis

The results were expressed as means ± S.E.M. and were considered significant when $P \leqslant 0.05$. Statistical analysis was performed using either Student's t-test or one-way ANOVA followed by Tukey's multiple comparison by the computer program Prism (GraphPad Software, USA).

Results

Exercise-mimic increased Fndc5 and Pgc-1α expression in myotubes

To reveal the mechanism of exercise-induced *FNDC5* expression in skeletal muscle, we used Sp-cAMP [14, 15], forskolin [15], and isoproterenol [16, 17] stimulation to mimic the pathways that are induced by exercise. Sp-cAMP, a cell-permeable analogue of adenosine 3',5'-cylcic monophosphorothioate (cAMP), increased *Fndc5* and *Pgc-1α* expressions in a dose-dependent manner (Fig. 1a and 1b). Similarly, increasing the cellular cAMP content by forskolin (a stimulator of adenylyl cyclase) [18] increased *Fndc5* (Fig. 1c and 1d) and *Pgc-1α*

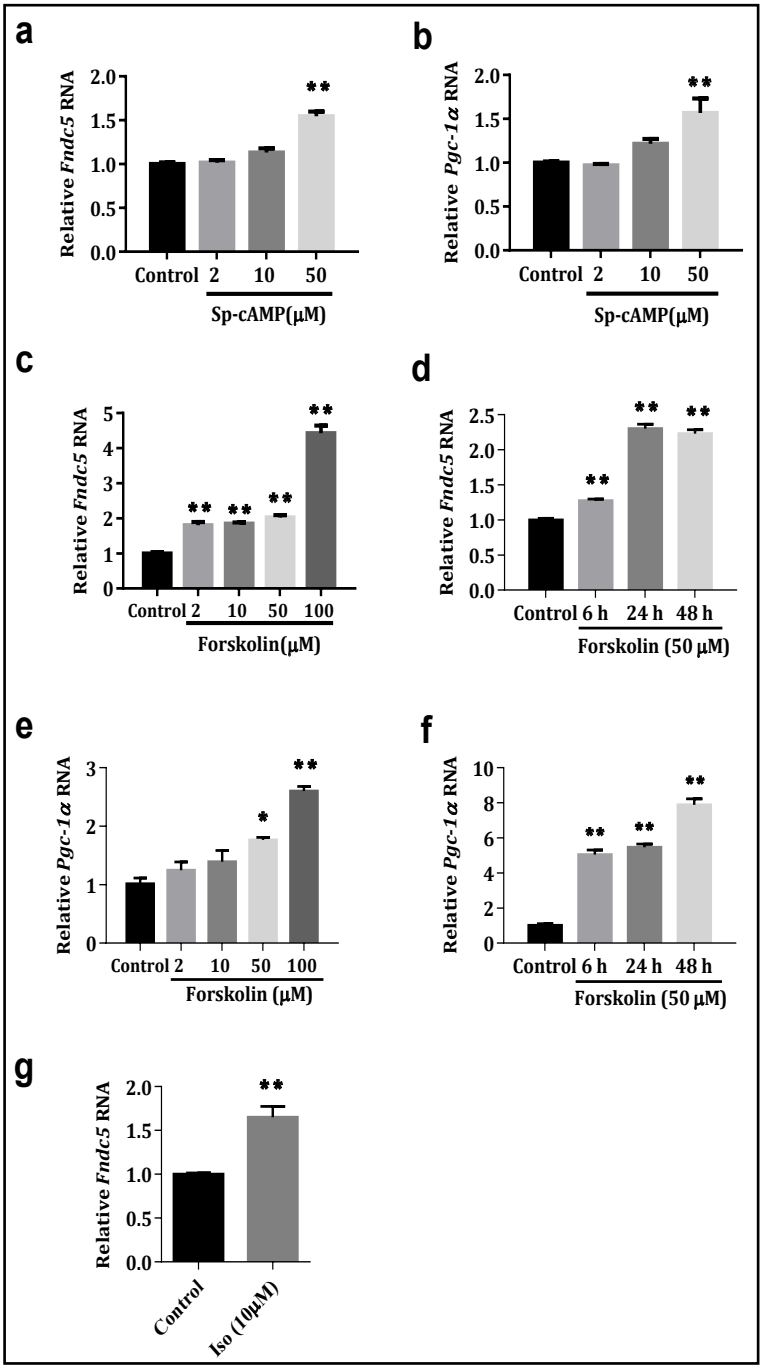

Fig. 1. cAMP increased *Fndc5/Pgc-1α* expression in myotubes. (a-b) Myotubes were treated with various concentrations of Sp-cAMP for 24 h. The transcription levels of Fndc5 (a) and Pgc-1α (b) were measured by qRT-PCR (n=3, **: P<0.01 vs control, one-way ANOVA). (c) *Fndc5* expression in C2C12 myotubes after stimulated with forskolin at various concentrations for 24 h was measured by qRT-PCT (n=3, **: P<0.01 vs control, one-way ANOVA). (d) *Fndc5* expression in C2C12 myotubes after stimulated with 50 μM forskolin for various time intervals was measured by qRT-PCT (n=3, **: P<0.01 vs control, one-way ANOVA). (e) *Pgc-1α* expression in C2C12 myotubes after stimulated with forskolin at various concentrations for 24 h was measured by qRT-PCT (n=3, *: P<0.05, **: P<0.01 vs control, one-way ANOVA). (f) *Pgc-1α* expression in C2C12 myotubes after stimulated with 50 μM forskolin for various time intervals was measured by qRT-PCT (n=3, **: P<0.01 vs control, one-way ANOVA). (g) *Fndc5* expression in C2C12 myotubes after isoproterenol (10 μM) stimulation for 24 h was measured by qRT-PCT (n=3, **: P<0.01 vs control, one-way ANOVA).

expressions (Fig. 1e and 1f). Isoproterenol is a non-selective β adrenoreceptor agonist that also elevates the intracellular cAMP level [19, 20]. Because β adrenoreceptor activation is associated with exercise [21], isoproterenol is commonly used as a pharmacological agent to study the exercise-induced metabolic changes in various tissues [22, 23]. Interestingly, isoproterenol stimulation provoked a comparable induction of *Fndc5* to that induced by 50 μM forskolin challenge (Fig. 1g). These data suggest that exercise-induced *Fndc5* expression is possibly mediated via the cAMP signalling.

CREB overexpression increased Fndc5 expression in myotubes

cAMP response element-binding protein (CREB) is a cAMP-regulated transcription factor that controls energy homeostasis. In C2C12 myotubes, forskolin or isoproterenol stimulation increased CREB phosphorylation (Fig. 2a). Suppressing, forskolin-induced *Fndc5* expression was diminished when the CREB was inhibited by cyclosporine (Fig. 2b), suggesting CREB plays a role in regulating *Fndc5* expression. When wild-type CREB or CREB S133A mutant was expressed in C2C12 myotubes (Fig. 2c), augmented *Fndc5* expression was only observed in wild-type CREB-transfected cells (Fig. 2d). We also found that overexpression of wild-type CREB, but not the CREB S133A mutant, in C2C12 myotubes upregulated *Pgc-1α* expression (Fig. 2e), which aligned with the previous report that CREB was a transcriptional regulator of *PGC-1α* expression [24].

PGC-1α increased Fndc5 expression through interacting with CREB in myotubes

While over-expression of wild-type PGC-1α increased *Fndc5* expression, the presence of PGC-1α dominant-negative (S570A) mutant reduced the levels of *Fndc5* in C2C12 myotubes (Fig. 3a), confirming that PGC-1α activity is critical for *FNDC5* expression [2, 8]. Interesting, co-expression of inactive CREB also abolished PGC-1α-induced *Fndc5* expression (Fig. 3a),

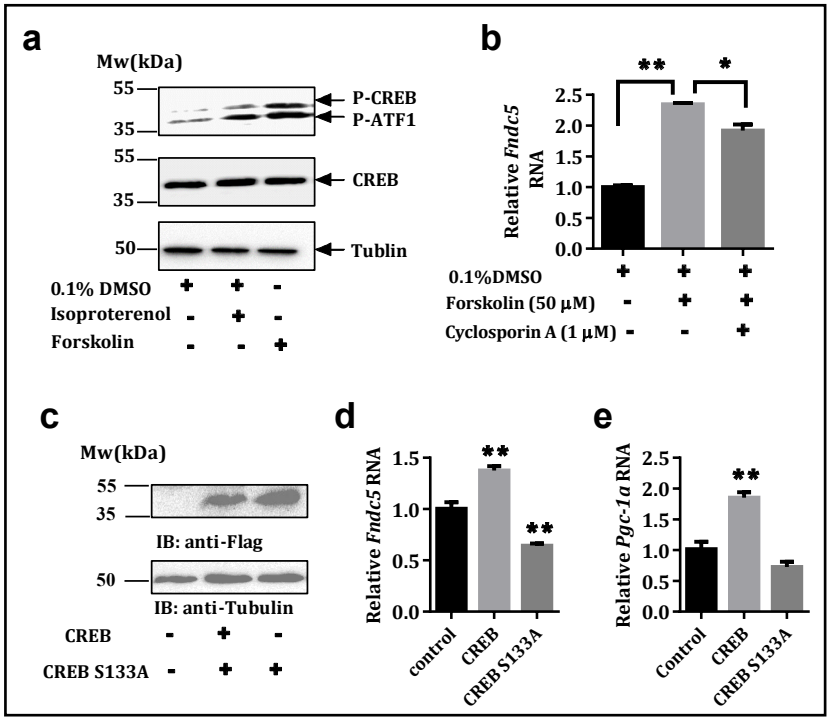

Fig. 2. CREB overexpression increased *Fndc5* and *Pgc-1α* expression in myotubes. (a) C2C12 myotubes were stimulated with isoproterenol (10 μM) or forskolin (50 μM) for 30 min and the phosphorylation of CREB was examined by Western blotting. (b) *Fndc5* expression in forskolin-stimulated C2C12 myotubes with or without cyclosporine A pre-treatment was detected by qRT-PCR (n=3, *: P<0.05, **: P<0.01, Student's t-test). (c) Expressions of various CREB constructs and tubulin in C2C12 myotubes were verified by immunoblotting. (d) *Fndc5* expression in wild-type CREB or CREB S133A mutant-transfected C2C12 myotubes were examined by qRT-PCT (n=3, **: P<0.01 vs control, one-was ANOVA). (e) *Pgc-1α* expression in wild-type CREB or CREB S133A mutant-transfected C2C12 myotubes were examined by qRT-PCT (n=3, **: P<0.01 vs control, one-was ANOVA).

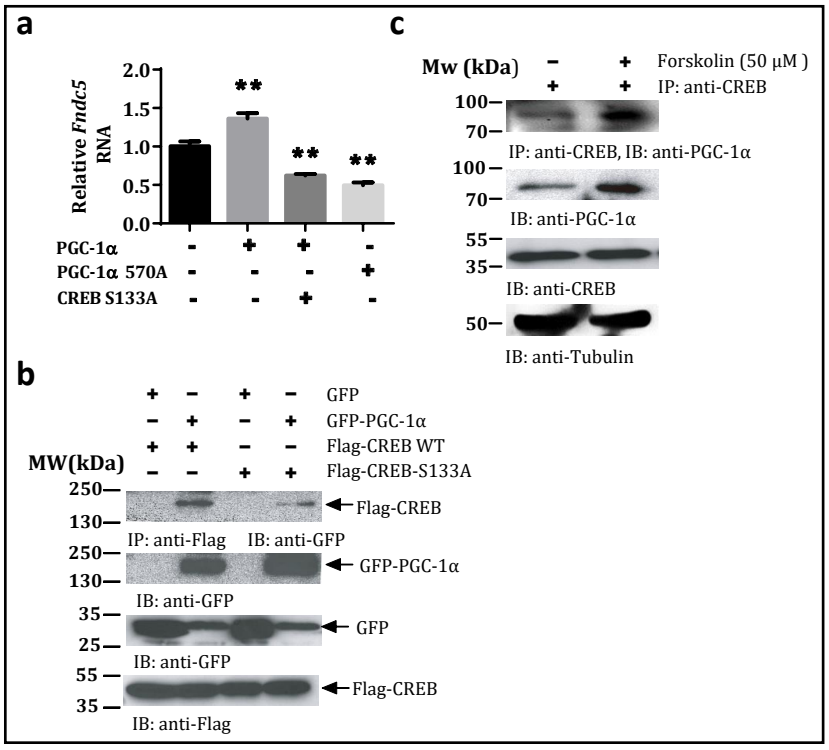

Fig. 3. PGC-1α increased *Fndc5* expression through interacting with CREB in myotubes. (a) Expression of *Fndc5* in various PGC-1α or CREB mutants-expressed C2C12 myotubes were determined by qRT-PCR (n=3, **: P<0.01 vs control, one-way ANOVA). (b) Co-immunoprecipitation of overexpressed PGC-1α and CREB in HEK 293 cells. Lysates from the transfected HEK 293 cells were immunoprecipitated with anti-Flag antibody. The pulled-down proteins were analysed by immunoblotting. Expression of CREB and tubulin were also verified by Western blotting. Expression of GFP-PGC-1α, Flag-CREBs, and tubulin were also verified by immunoblotting. (c) Co-immunoprecipitation of endogenous PGC-1α and CREB in C2C12 myotubes treated with forskolin (50 μM, 24 h). C2C12 lysates were immunoprecipitated with anti-CREB antibody and the pulled-down proteins were analysed by SDS-PAG using anti-PGC-1α antibody. Expression of PGC-1α, CREB, and tubulin were also verified by Western blotting.

suggesting that PGC-1α may interact with CREB to control *Fndc5* expression. Agreeing with this hypothesis, immunoprecipitation showed that PGC-1α complexed with CREB, and the presence of CREB S133 mutant downregulated their interaction (Fig. 3b). The endogenous CREB/PGC-1α interaction could be readily detected in C2C12 myotubes, which was increased after forskolin stimulation (Fig. 3c).

Irisin increased mitochondrial respiration in myotubes

To investigate the metabolic functions of irisin, we monitored the oxidative and glycolytic metabolism in C2C12 myotubes. Treatment with various concentrations of irisin (10, 50, 100 ng/ml) for 24 h increased the oxygen consumption rate (OCR) (Fig. 4a), ATP production (Fig. 4b), basal respiration (Fig. 4c), and maximal respiration (Fig. 4d). The ATP-coupling efficiency was not altered after irisin stimulation (Fig. 4e), suggesting the increased ATP production was not caused by enhanced enzymatic activities in oxidative phosphorylation but an augmented mitochondrial number. Indeed, treatment with irisin significantly provoked the expression of *Pgc-1α, Tfam,* and *Nrf1* expressions in C2C12 myotubes (Fig. 4f), which are the key transcriptional regulators of mitochondrial biogenesis [25]. On the other hand, irisin stimulation lowered basal ECAR (Fig. 4g and 4h). Therefore, the OCR/ECAR ratio in C2C12 myotubes was significantly augmented after irisin stimulation (Fig. 4i), which represented a glycolysis-to-oxidative phosphorylation shift for cellular ATP generation [26].

Irisin increased myotube fatty acid oxidation

Exercise increases free fatty acid (FFA) uptake and oxidation in muscle [27]. To test whether exercise-induced irisin production is responsible for fatty acid metabolism in skeletal muscle, we evaluated the effect of irisin on FFA oxidation. C2C12 myotubes were

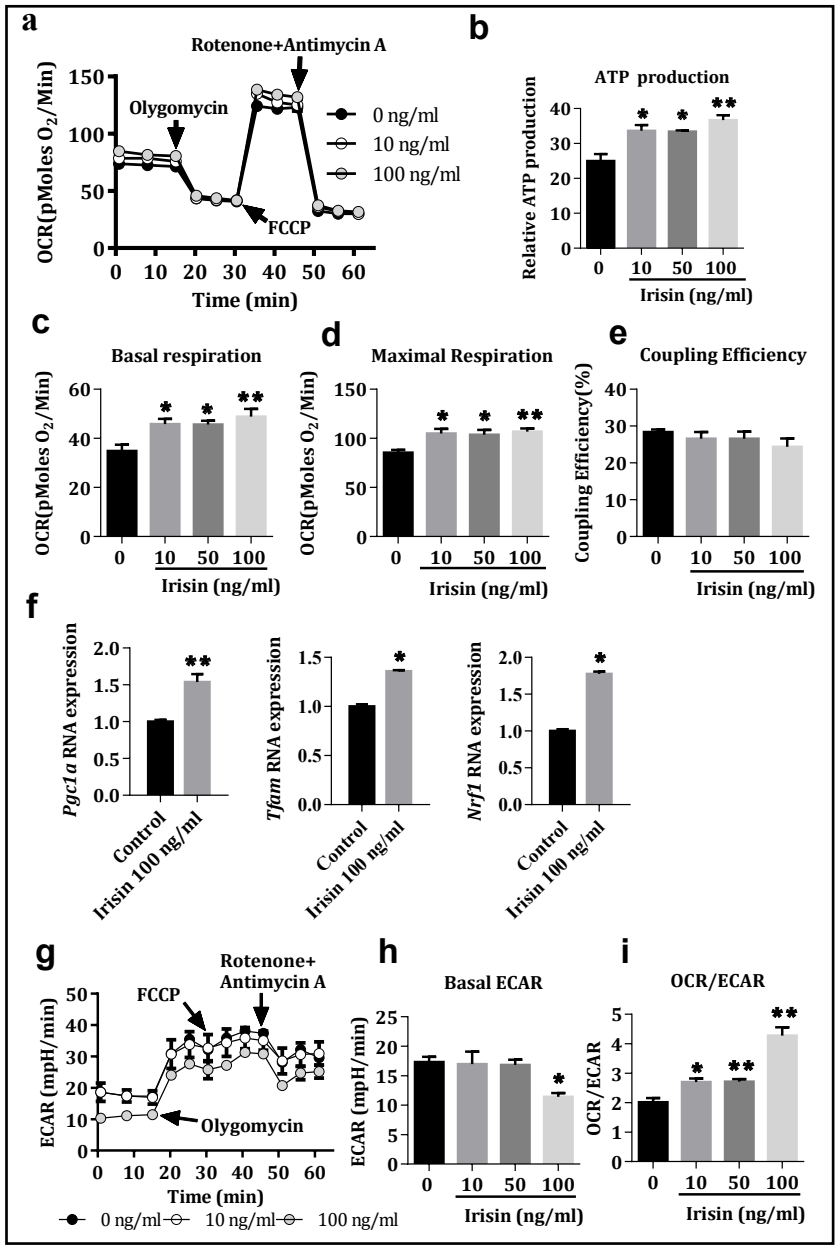

Fig. 4. Irisin increased mitochondrial respiration but decreases extracellular acidification in C2C12 myotubes. (a-e) After treated with irisin for 24 h, the kinetic profile of mitochondrial respiration (a), ATP production (b), basal respiration (c), maximal respiration, (d) maximal respiration, and (e) coupling efficiency of C2C12 myotubes were determined by the Extracellular Flux analyser (n = 4, *: P<0.05, **: P<0.001, one-way ANOVA). (f) Expression of Pgc-1α, Tfam and Nrf1 in irisin-stimulated (24 h) C2C12 myotubes were measured by qRT-PCR (n=3, *: P<0.05, **: P<0.01, Student's t-test). (g-h) After treated with irisin for 24 h, the kinetic profile (g) and basal ECAR (h) of C2C12 myotubes were detected by the Extracellular Flux analyser (n = 4, *: P<0.05, one-way ANOVA). (i) OCR to ECAR ratio of C2C12 myotubes after irisin treatment (24 h) as recorded by the Extracellular Flux Analyser (n = 4, *: P<0.05, **: P<0.001, one-way ANOVA).

stimulated by 100 ng/ml of irisin for 24 h, and FFA oxidation was measured using OCR as an indicator [28]. We found that irisin increased cellular oxygen consumption when palmitic acid was supplied as the sole energy source (Fig. 5a). The ATP production (Fig. 5b), basal respiration (Fig. 5c), and maximal respiration (Fig. 5d) of myotubes were also elevated.

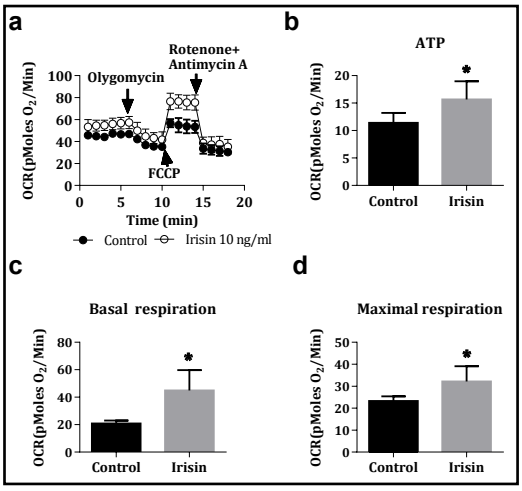

Fig. 5. Irisin increased fatty acid oxidation in C2C12 myotubes. After stimulated with irisin for 24 h, the kinetic profile of mitochondrial respiration (a), ATP production (b), basal respiration (c), and maximal respiration (d) in the presence of palmitic acid (200μM) were recorded by the Extracellular Flux Analyser (n = 4, *: P<0.05, Student's t-test).

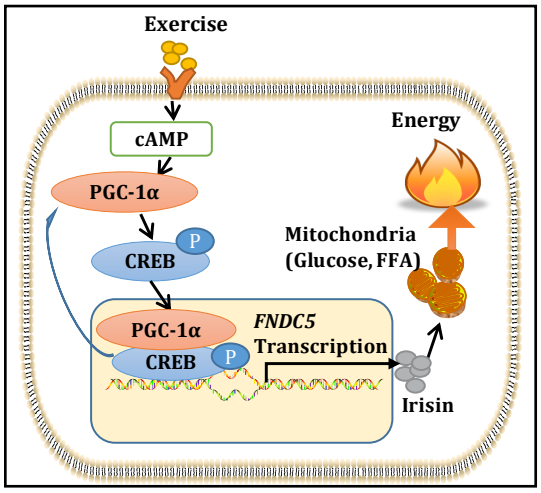

Fig. 6. Proposed model of exercise-induced FNDC5 expression. Exercise increases the intracellular content of cAMP, which triggers the CREB/PGC-1α interaction to induce the transcription FNDC5. The newly synthesized irisin will serve as an autocrine/paracrine to enhance mitochondrial respiration and FFA oxidation in skeletal muscle.

Discussion

Skeletal muscle is not only responsible for locomotion but also acts as an endocrine organ [29]. In response to muscular contractions or different metabolic demands, it secretes myokines to communicate with other tissues [29, 30]. Irisin is one of the most prominent metabolic myokines that is generated from contracting muscle during exercise [31]. Previous studies have demonstrated that irisin stimulation increased the metabolic rate, mitochondrial content [6, 32], and FFA oxidation in myocytes [3, 7]. Moreover, a positive correlation of mortality risk in acute heart failure and serum irisin was also observed, suggesting irisin can be used as a predictive biomarker for cardiovascular diseases [33]. In this study, we demonstrated that exercise may enhance cAMP to stimulate muscular *Pgc-1α* expression, which interacts with the transcription factor CREB to induce *Fndc5* expression. Consequently, irisin may serve as an autocrine/paracrine to increase mitochondrial respiration and shift the metabolic preference from glucose to FFA for ATP production in muscle cells (Fig. 6).

The signalling pathways that control *FNDC5* expression have not been fully elucidated with contradictory results from different *in vitro* studies [10, 11]. For instance, it was reported that exercise-mimicking treatment failed to increase *Fndc5* expression in myotubes [34]. Adrenaline stimulation also showed no significant effects on *Fndc5* expression in cultured myotubes [17]. These conflicting results might be attributed to the difference in experimental protocol or choice of anti-irisin antibodies [10]. Nevertheless, our study demonstrated that cAMP treatment increased both *Fndc5* and *Pgc-1α* expressions in myotubes, probably because of longer stimulation (24 h) than in other *in vitro* models (1 h) [6, 32].

Skeletal muscle-specific *Pgc-1α* knockout animals show reduced endurance capacity as well as other signs of motility defects and metabolic dysfunctions [35, 36]. On the other hand, over expressing *Pgc-1α* in muscle increased *Fndc5* expression and irisin release, eventually causing higher UCP1-dependent thermogenesis and energy expenditure [2]. In our study, we found that *Pgc-1α* over expression induced *Fndc5* expression in myotubes. We also proved that CREB promoted both *Fndc5* and *Pgc-1α* expression. More importantly, CREB inactivation suppressed PGC-1α-induced *Fndc5* transcription. The results suggest that

intact CREB is essential for PGC-1α to control *Fndc5* transcription. Previous studies have reported that CREB controls the transcription of *PGC-1α* through binding to its promoter [37]. Our results further suggest that PGC-1α may physically interact with the transcription factor CREB to stimulate *Fndc5* expression.

Conclusion

Our study demonstrates that the expression of *Fndc5* in skeletal muscle is mediated by the cAMP-induced PGC-1α/CREB interaction. Our functional studies also indicated that irisin shapes the metabolic phenotype of myotubes by increasing fatty acid oxidation but reducing glycolysis.

Acknowledgements

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Disclosure Statement

The authors declare no conflicts of interest.

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