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Original Article

PGC-1 mediates the regulation of metformin in muscle irisin expression and function

Zaigang Yang, Xu Chen, Yujuan Chen, Qian Zhao

Geriatric Department of Endocrinology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou, Henan 450052, China

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Abstract: Background: Overweight and obesity are rapidly becoming major global health, social, and economic problems. Irisin is a newly termed hormone that is related to metabolic diseases. In the present study, the mechanism underlying the effect of Metformin on promoting irisin release from skeletal muscle was investigated. Methods: C57BL/6J-ob/ob was orally administrated with Metformin for 4 weeks. The plasma irisin, insulin, and glucose were detected. Mouse skeletal muscle myoblasts C2C12 cells were treated with Metformin for 24 h. The molecules PGC-1α, FNDC5, AMPK, and ERK mRNA/proteins were quantified by real-time PCR and western blotting *in vivo* and *in vitro*. Results: Metformin elevated FNDC5 mRNA/protein expression of skeletal muscle and plasma irisin concentration in ob/ob mice. PGC-1α, p-AMPK and p-ERK protein expression was up-regulated by Metformin in skeletal muscle and C2C12 cells. In addition, the decrease in irisin concentration and protein expression of FNDC5, p-AMPK, and p-ERK induced by siRNA-PGC-1α could not be reversed by Metformin. Conclusion: Our study demonstrates that Metformin stimulates irisin secretion from skeletal muscle into the circulation system of obese mice, and that PGC-1α is a critical regulator in this process.

Keywords: Metformin, obesity mice, diabetes, FNDC5

Introduction

Overweight and obesity are rapidly becoming major global health, social, and economic problems, and are closely associated with increasing morbidity and mortality in diabetes mellitus, metabolic syndrome, and subsequent cardiovascular diseases [1]. The urgent need for new therapeutic strategies has been highlighted.

Irisin is a newly termed exercise-triggered hormone, released by skeletal muscle and cleaved by fibronectin type III domain containing 5 (FNDC5) into the bloodstream [2]. A recent study showed that white adipose tissue can also secrete irisin. Muscle-adipose tissue crosstalk through a regulatory feedback mechanism plays a role in FNDC5/irisin secretion [3]. Clinical research has demonstrated that circulating levels of irisin are associated with body weight [4], insulin sensitivity [5], hepatic triglyceride [6], and urea nitrogen and creatinine [7], which suggests a potential role in reducing body weight and improving metabolic diseases. It is interesting that irisin has been proven to drive the transformation of white fat to brown fat by increasing energy expenditure, with no changes in movement or food intake [2]. It is also reported that irisin is involved in promoting pancreatic β-cell proliferation and improving glucose tolerance [8]. The circulating irisin levels and FNDC5 gene expression are lower in type 2 diabetes and obesity subjects [9, 10]. Therefore, irisin is considered to be a potential therapeutic target for obesity and its complications.

Metformin is a type of biguanide and is the first-line drug of choice for the treatment of type 2 diabetes, particularly in overweight and obese people [11]. A recent study reported that Metformin increased intramuscular FNDC5 expression and promoted irisin release from murine skeletal muscle into the blood [12]. However, the mechanism underlying the effect of Metformin on promoting irisin release has not been illustrated. It is known that peroxisome proliferator-activated receptor γ co-acti-

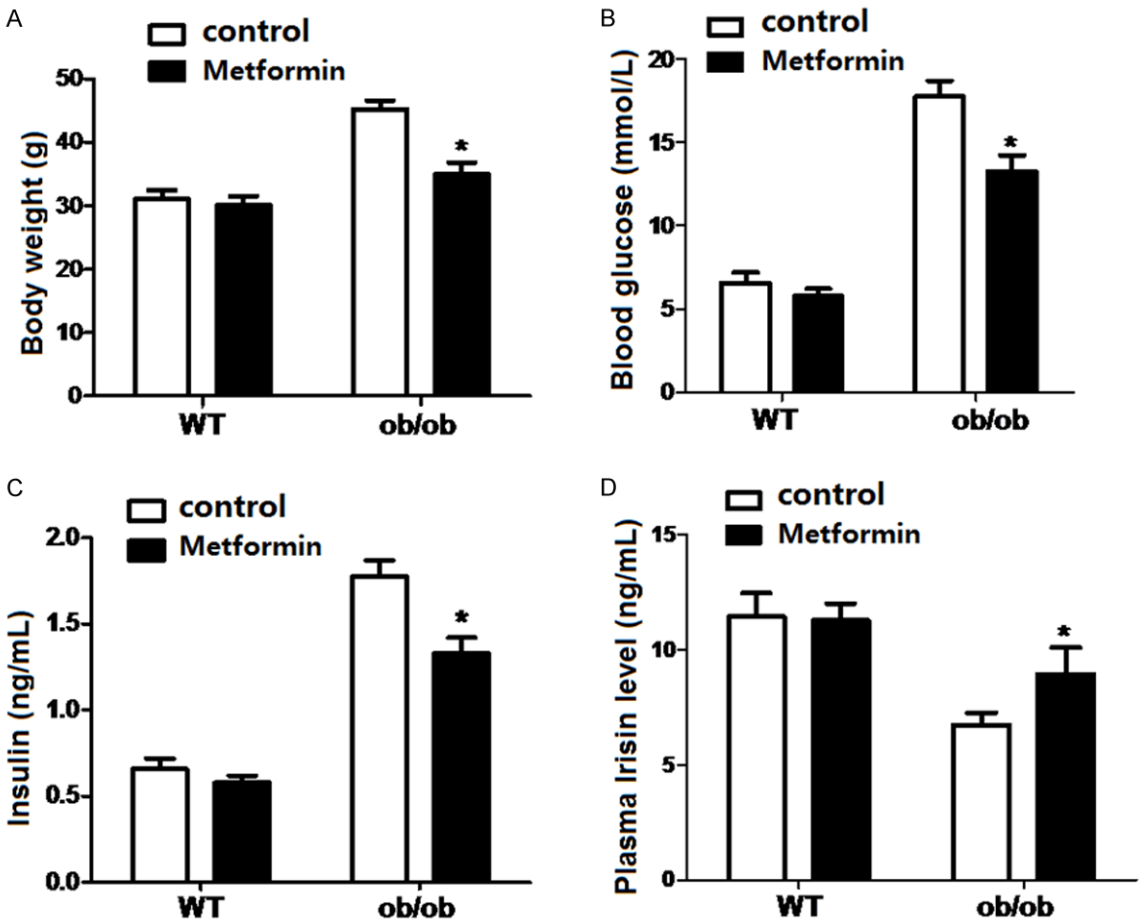

Figure 1. Metformin regulates body weight, blood glucose, and insulin in ob/ob mice. Mice were fed a diet containing Metformin (400 mg/kg/day) or a normal diet for 4 weeks. Weighted mice body weight before sacrificing (A), detected concentrations of blood glucose (B), plasma insulin (C) and plasma irisin (D) after sacrificing; n=6; the data are presented as mean ± SD; *P<0.05 vs control.

vator 1α (PGC1-α)-a transcriptional co-activator that mediates many biological programs related to energy metabolism [13], stimulates an increase in FNDC5 expression and releases irisin from skeletal muscle [2]. It is hypothesized that PGC1-α may be a regulator in the process of releasing irisin stimulated by Metformin. In the present study, *in vivo* and *in vitro* experiments were performed to investigate the hypothesis.

Materials and methods

Animal experiment design

Animal studies were performed according to the Guide for the Care and Use of Laboratory Animals and approved by the Ethics Committee of the first hospital affiliated to Zhengzhou University. Five-week-old male obese C57BL/6J-ob/ob mice and their lean controls C57BL/6J-+/+ (WT) were purchased from Nanjing Pengsheng Biological Technology Development Co., Ltd. (Nanjing, China). After acclimatization for 1 week, the ob/ob were randomly divided into two groups (n=6). Mice were fed with a normal chow diet, with one group supplemented with 400 mg/kg/day of Metformin. The WT group was also divided into two (n=6), with 6 mice fed with a normal chow diet and the remaining 6 mice fed with a normal diet supplemented with 400 mg/kg/day of Metformin. The experiments lasted for 4 weeks. After fasting overnight and weighing, all mice were anaesthetized using 4% phenobarbital sodium and were then sacrificed. Peripheral blood was collected from the mice and skeletal muscle was removed.

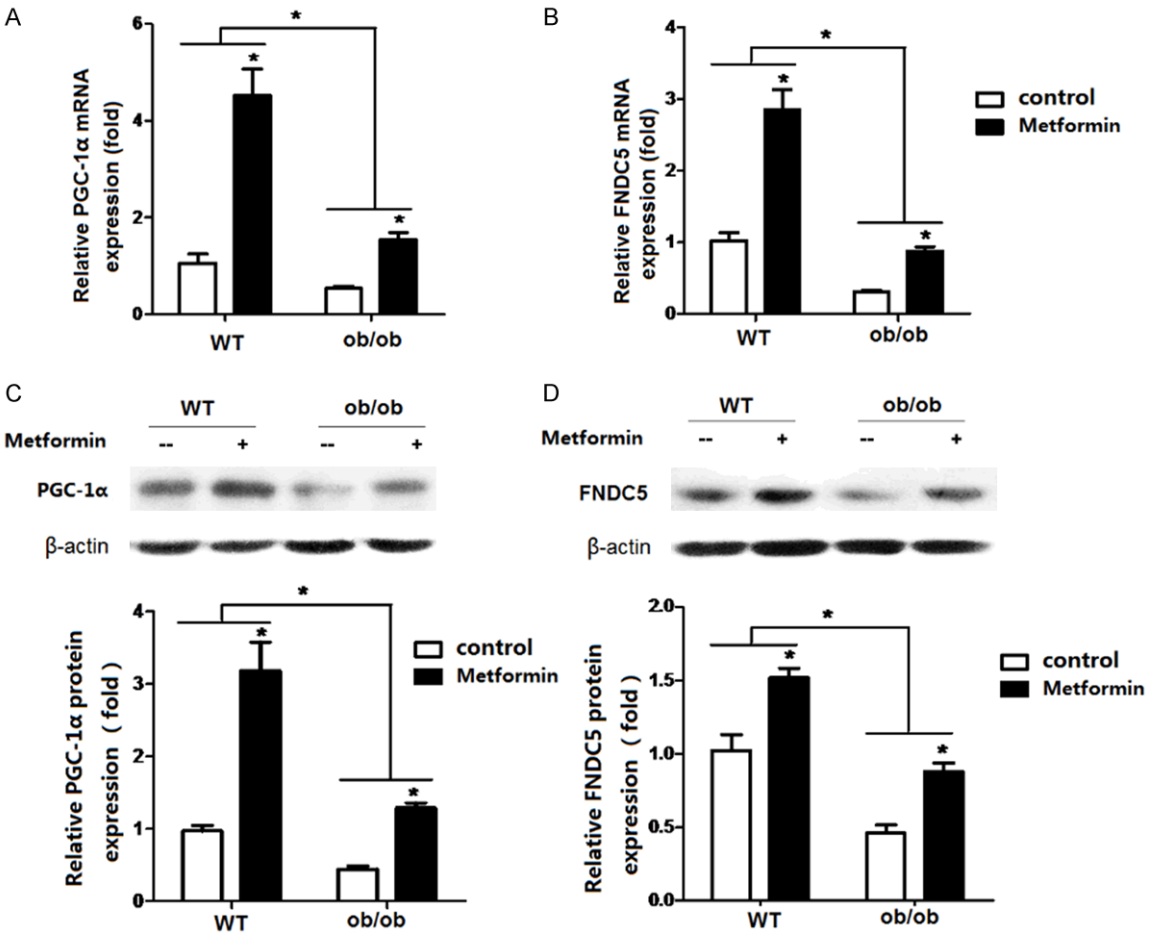

Figure 2. Metformin increases muscular PGC-1α and FNDC5 expression in mice. Mice were fed a diet containing Metformin (400 mg/kg/day) or a normal diet for 4 weeks, and skeletal muscle was isolated after sacrifice; the relative mRNA expression of PGC-1α and FNDC5 was quantified by real-time PCR (A, B); the protein expression of PGC-1α and FNDC5 was quantified by western blotting (C, D); n=6; the data are presented as mean ± SD; *P<0.05 vs control in same types of mice (or the same treatment in different types of mice).

Detection of blood glucose, plasma insulin, and irisin

The blood was collected in tubes pretreated with heparin sodium and then centrifuged at 3500 rpm/min for 10 min at 4°C to obtain plasma. Plasma glucose was detected by a Hitachi 7020 automatic analyzer (HITACHI, Japan). Plasma insulin concentration was detected using commercial ELISA kits (GenStar, China) according to the manufacturer's protocol. Irisin concentrations in the plasma and cell mediums were determined using commercial ELISA kits (GenStar, China) following the manufacturer's instruction.

Cell culture

C2C12 mouse skeletal muscle myoblasts cells were obtained from American Type Culture Collection. The C2C12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Gibco, USA) and 1% penicillin/streptomycin at 37°C with 5% CO_2. After growing to 80%-90% confluence, DMEM containing 10% horse serum was used for cell differentiation into myotubes for 3 days. Medium renewal was conducted every three days. When grown with 60%-70% confluence, the cells were transfected with siRNAs for 24 h. Then 2 mM Metformin was supplemented into medium for another 24 h. The C2C12 cells were harvested and following experiments were performed.

siRNA transfection

When the C2C12 cells had grown to 60%-70% confluence, the cells were transfected with

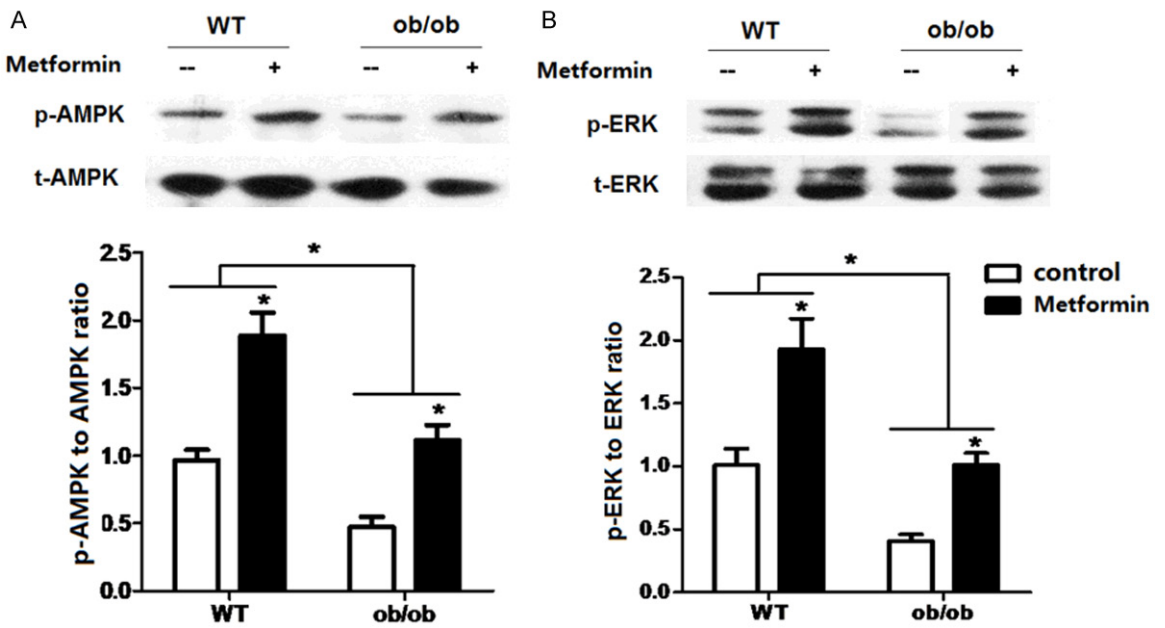

Figure 3. Metformin increases muscular p-AMPK and p-ERK expression in mice. Mice were fed a diet containing Metformin (400 mg/kg/day) or a normal diet for 4 weeks, and skeletal muscle was isolated after sacrifice; The p-AMKP/AMPK (A) and p-ERK/ERK (B) proteins were quantified by western blotting; n=6; the data are presented as mean ± SD; *P<0.05 vs control in same types of mice (or the same treatment in different types of mice).

siRNA-PGC-1α or siRNA-scramble (negative control) using Lipofectamine LTX Reagent (Invitrogen, USA). SiRNA-PGC-1α and siRNA-scramble were synthesized by Inovogen Tech. Co., Ltd. (Beijing, China) following the manufacturer's instruction.

RNA isolation and quantitative PCR

The total RNA in skeletal muscle tissue and in C2C12 cells was isolated using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. The concentrations of RNA were quantified by the ultraviolet spectroscopy method and checked by agarose gel electrophoresis. Precisely 2 μg of RNA was used to perform the reverse transcription to synthesize cDNA. The relative mRNA of PGC-1α and of FNDC5 was quantified by an ABI PRISM® 7000 Sequence Detection System with SYBR® Green PCR Master Mix (Applied Biosystems, USA). The relative mRNA was calculated using the ΔΔCt method. β-actin acted as a reference gene.

Western blotting

The skeletal muscle tissues were washed with ice-cold normal saline and homogenized in phosphate buffered saline (PBS). After discarding the supernatant, the homogenate was incubated in a lysis buffer containing a protease inhibitor. C2C12 cells were washed with ice-cold PBS and lysed in a whole lysis buffer containing a protease inhibitor. The protein contents of the samples were quantified using a Bradford Protein Assay Kit (Beyotime, China). Equal amounts of protein samples were boiled for 15 min and run on a 12% SDS-PAGE. The proteins were transferred onto a nitrocellulose membrane and were blocked with 5% milk in PBS-0.05% Tween. The membrane was co-incubated overnight with primary antibodies (Cell Signaling Technology, USA) at 4°C. All antibodies were diluted to a suitable concentration according to the manufacturer's protocol. After washing three times in TBST, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for 1 h. The protein detection was visualized and quantified using Quantity One software system (Bio-Rad Laboratories, USA). All experiments were repeated three times.

Statistical analysis

Data are expressed as mean ± standard deviation (SD). The differences among three or more groups were determined by one-way ANOVA followed by the Tukey post-hoc test, and the difference between two groups was evaluated with

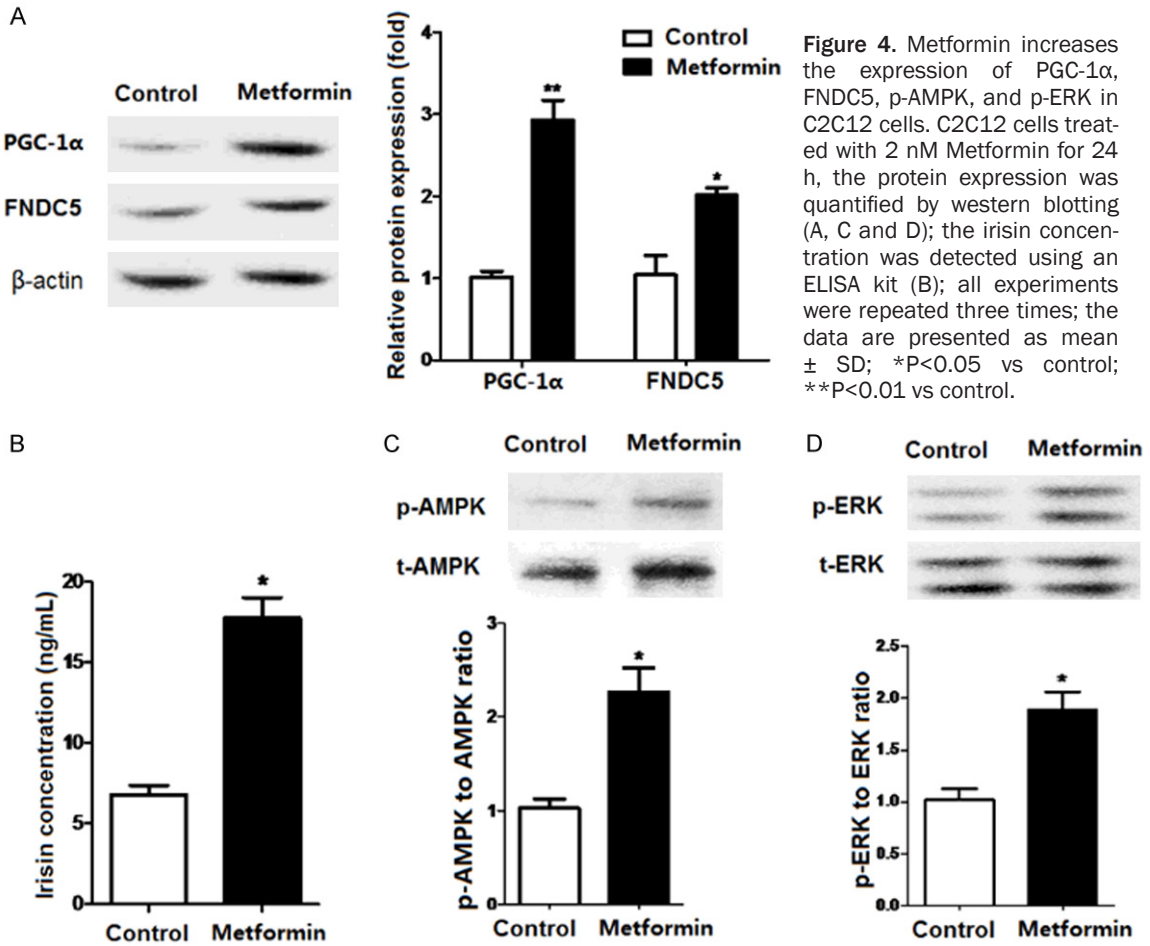

Figure 4. Metformin increases the expression of PGC-1α, FNDC5, p-AMPK, and p-ERK in C2C12 cells. C2C12 cells treated with 2 nM Metformin for 24 h, the protein expression was quantified by western blotting (A, C and D); the irisin concentration was detected using an ELISA kit (B); all experiments were repeated three times; the data are presented as mean ± SD; *P<0.05 vs control; **P<0.01 vs control.

an independent-samples T test. All statistical analysis was conducted by SPSS 18.0. A value of $p<0.05$ was considered statistically significant.

Results

Metformin regulates body weight, blood glucose, and insulin in ob/ob mice

This study administrated Metformin to WT and ob/ob mice for 4 weeks. No differences in body weight, blood glucose, plasma insulin, or plasma irisin were observed between the Metformin group and controls in WT mice. However, in ob/ob mice, Metformin significantly reduced body weight, blood glucose, and insulin levels, while markedly elevating plasma irisin levels (**Figure 1**).

Metformin increases muscular PGC-1α and FNDC5 expression in mice

The mRNA and protein expression of PGC-1α and FNDC5 were determined in mice. Compared with WT mice, ob/ob mice had significantly lower PGC-1α and FNDC5 expression. Metformin significantly increased the PGC-1α mRNA level in WT (4.3 times the control level) and in ob/ob mice (3.0 times the control level) (**Figure 2A**). The PGC-1α protein expression was also elevated in WT (3.4 times the control level) and in ob/ob mice (3.3 times the control level) (**Figure 2C**). The up-regulation of Metformin on FNDC5 mRNA and protein was observed in WT and ob/ob mice (**Figure 2B**, **2D**).

Metformin increases muscular p-AMPK and p-ERK expression in mice

The level of phosphorylated AMPK (p-AMPK) and phosphorylated ERK (p-ERK) of skeletal muscle was quantified with western blotting. The ob/ob mice had markedly lower p-AMPK and p-ERK expression. As shown in **Figure 3**, p-AMPK and p-ERK levels were significantly increased by Metformin in WT and ob/ob mice. The p-AMPK/AMPK ratios in Metformin group

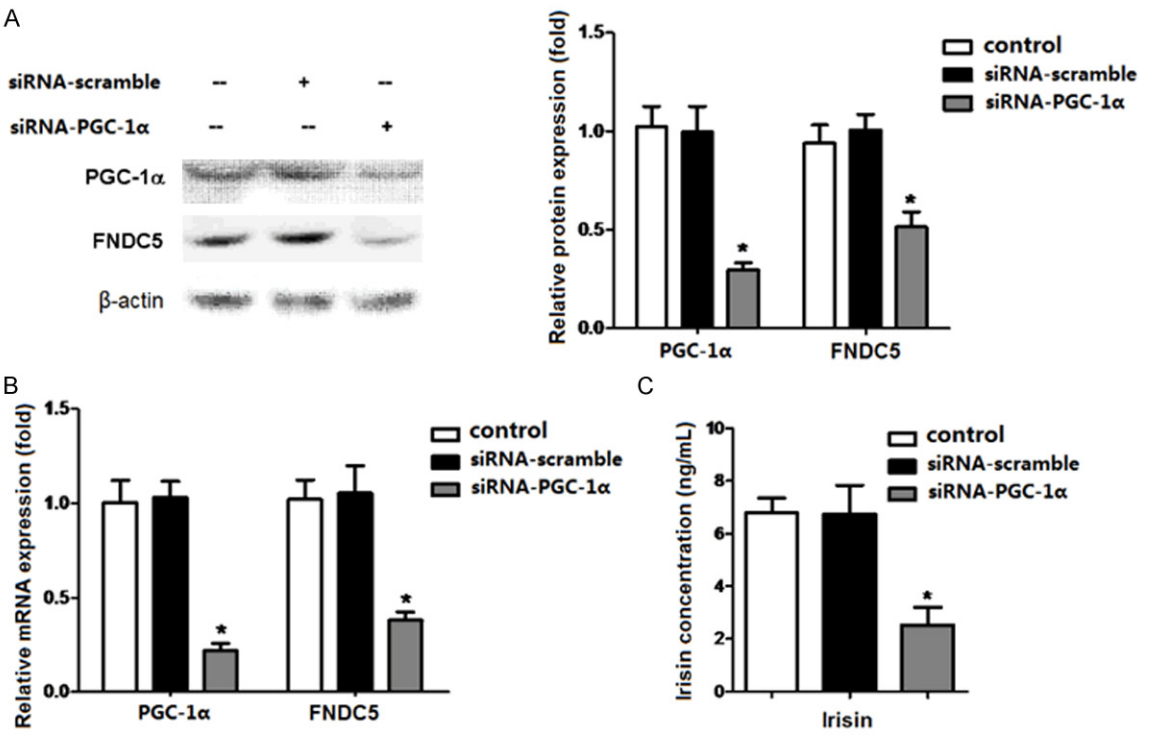

Figure 5. Down-regulation of PGC-1α decreases FNDC5 and irisin levels in C2C12 cells. C2C12 cells treated with siRNA-PGC-1α, and siRNA-scramble acted as a negative control; the protein expression of PGC-1α and FNDC5 was quantified by western blotting (A); relative mRNA of PGC-1α and FNDC5 was quantified using real-time PCR (B); the irisin concentration was detected using an ELISA kit (C); all experiments were repeated three times; the data are presented as mean ± SD; *P<0.05 vs group with siRNA-scramble treatment.

were 1.9 and 2.4 times those in the controls, and the p-ERK/ERK ratios were 2.0 and 2.5 times those in the controls in WT and ob/ob mice, respectively.

Metformin increases the expression of PGC-1α, FNDC5, p-AMPK, and p-ERK in C2C12 cells

Mouse myoblast cell line C2C12 was treated with or without Metformin *in vitro*. The protein expression of PGC-1α and FNDC5 in cells was up-regulated (**Figure 4A**). The irisin concentration of cells was also elevated by 2 nM Metformin compared with the control (17.8 ng/ml vs 6.9 ng/ml, p<0.05). In addition, the western blotting results showed that p-AMPK/t-AMPK and p-ERK/t-ERK levels were also increased by Metformin.

Down-regulation of PGC-1α decreases FNDC5 and irisin levels in C2C12 cells

To further investigate the regulation effect of PGC-1α on irisin expression and its function, PGC-1α was down-regulated by siRNA-PGC-1α. As shown in **Figure 5A** and **5B**, PGC-1α expression was effectively inhibited by siRNA-PGC-1α, which significantly down-regulated FNCC5 expression in protein and mRNA levels. Irisin concentration was decreased in C2C12 cells with the inhibition of PGC-1α.

Down-regulation of PGC-1α decreases FNDC5 and irisin levels in C2C12 cells exposed to Metformin

Metformin significantly elevated PGC-1α and FNDC5 expression of C2C12 cells (**Figure 6A-C**). Cells were transfected with siRNA-PGC-1α, and were then co-incubated with 2 nM Metformin. The decrease in PGC-1α and FNDC5 induced by siRNA-PGC-1α could not be reversed by Metformin. In addition, Metformin did not cancel the decrease in irisin concentration induced by siRNA-PGC-1α (**Figure 6D**).

Down-regulation of PGC-1α decreases p-AMPK and p-ERK levels in C2C12 cells exposed to metformin

The levels of p-AMPK and p-ERK were also determined in C2C12 cells exposed to Metformin. As shown in **Figure 7**, p-AMPK and p-

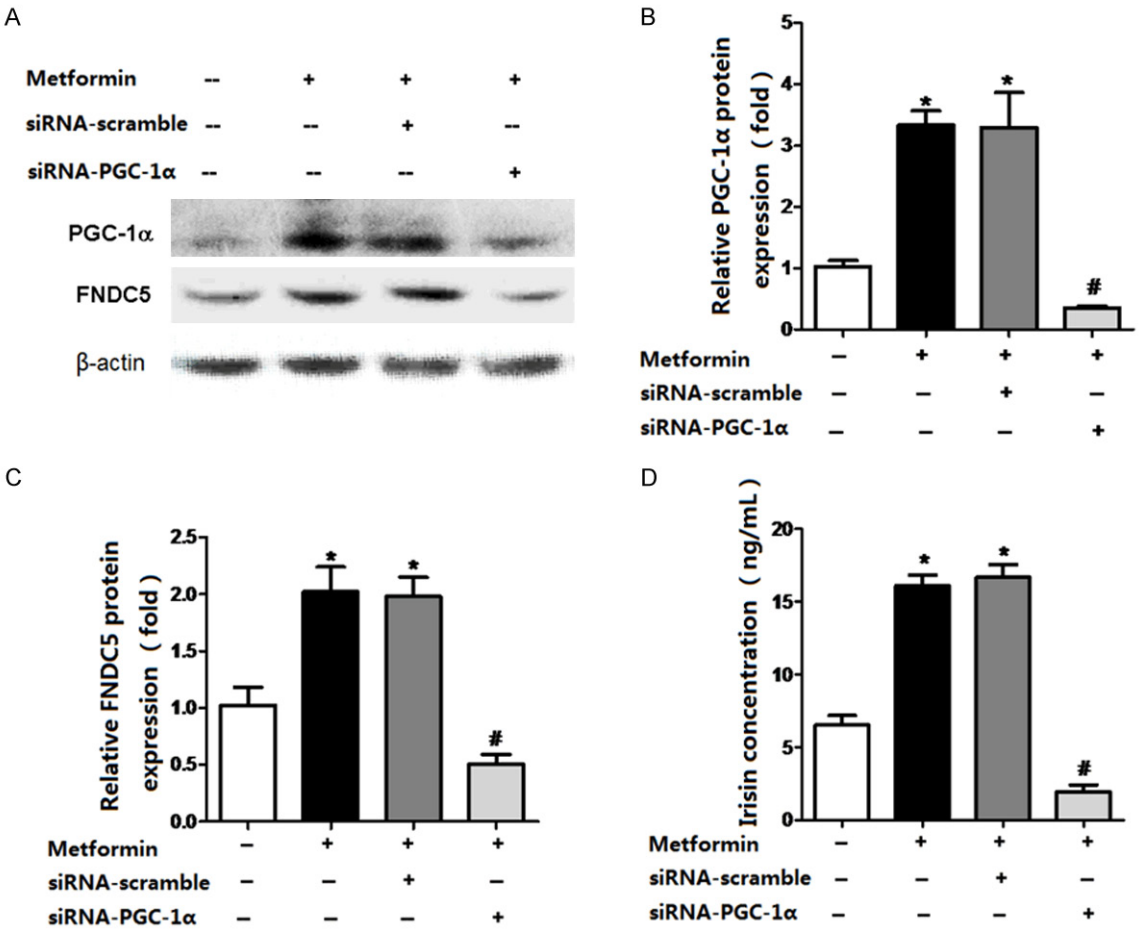

Figure 6. Down-regulation of PGC-1α decreases FNDC5 and irisin levels in C2C12 cells exposed to metformin. C2C12 cells were transfected with siRNA-scramble or siRNA-PGC-1α, and were then co-incubated with 2 nM Metformin for 24 h; the protein expression of PGC-1α and FNDC5 was quantified by western blotting (A-C); the irisin concentration was detected using an ELISA kit (D); all experiments were repeated three times; the data are presented as mean ± SD; *P<0.05 vs group without any treatment; #P<0.05 vs group treated with Metformin and siRNA-scramble.

ERK protein expression was significantly increased by Metformin. However, Metformin could not reverse the effect of siRNA-PGC-1α on decreases in p-AMPK and p-ERK expression.

Discussion

Since the discovery of the exercise-triggered hormone irisin, there has been great interest in its effect on reducing body weight and improving metabolic disease [2, 10]. In the present study, we found that plasma irisin and FNDC5 mRNA/protein expression of skeletal muscle was significantly decreased in ob/ob mice compared to WT mice. Further, Metformin elevated plasma irisin and FNDC5 expression through activation of PGC-1α in ob/ob mice.

Irisin has been identified as an exercise-triggered hormone secreted by muscle and white adipose tissue in mice and humans [2, 3], possibly mediating some exercise health benefits via the 'browning' of white adipose tissue. Bostrom et al., demonstrated that irisin is a cleaved and secreted fragment of FNDC5. The irisin of mice and humans is 100% identical, suggesting a highly conserved function [2]. Clinical studies found that plasma irisin concentration was decreased in obese and type 2 diabetic subjects [10, 14, 15]. The decreased FNDC5 expression of muscle and adipose tissue was also observed in type 2 diabetics [10, 16] and FNDC5 Single Nucleotide Polymorphism was associated with body mass index [17]. In the present study, we found that plasma iri-

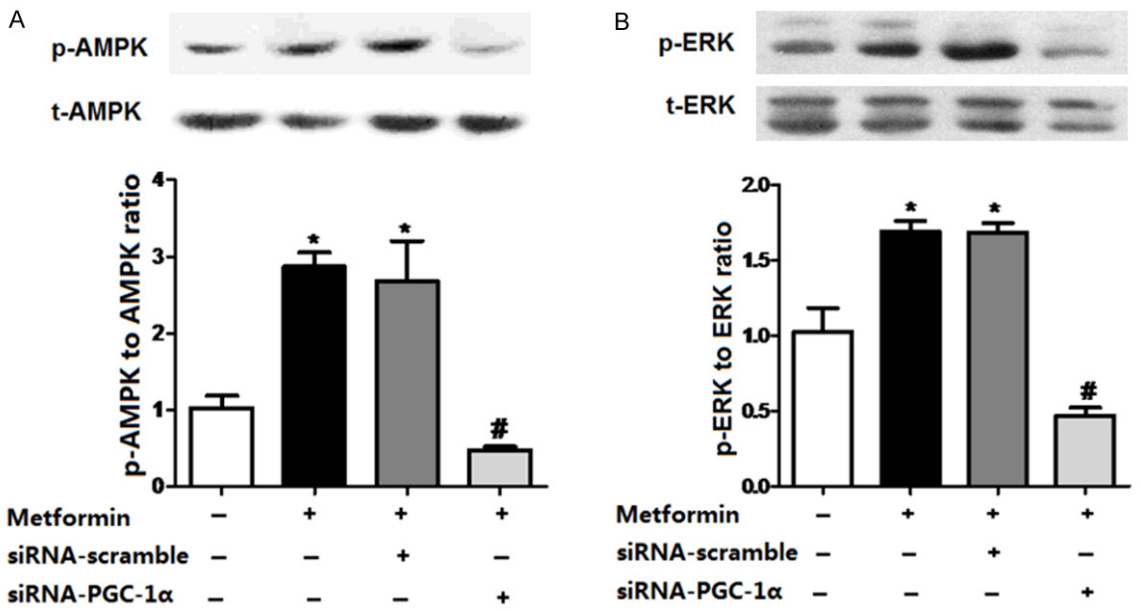

Figure 7. Down-regulation of PGC-1α decreases p-AMPK and p-ERK levels in C2C12 cells exposed to metformin. The C2C12 cells were transfected with an siRNA-scramble or siRNA-PGC-1α, and were then co-incubated with 2 nM Metformin for 24 h; the protein expression of AMPK and ERK was quantified by western blotting (A and B); all experiments were repeated three times; the data are presented as mean ± SD; *P<0.05 vs group without any treatment; #P<0.05 vs group treated with Metformin and siRNA-scramble.

sin concentration and FNDC5 mRNA/protein expression in skeletal muscle were reduced in ob/ob mice compared with WT mice, which was in accordance with the results of previous studies.

Li et al., recently found Metformin promotes irisin release from murine skeletal muscle *in vivo* and *in vitro,* but this was not observed in glibenclamide, another drug commonly used to treat diabetes [12]. The results implied that irisin was one possible mechanism for lowering blood glucose by Metformin. However, the cellular and molecular mechanisms of Metformin in treatment regimens for diabetics are not fully understood. PGC-1α plays a role in the regulation of exercise-related muscle function [18]. Bostrom et al., demonstrated that FNDC5 is induced with forced PGC-1α expression and turns on brown fat gene expression [2]. It was important that we found PGC-1α is a crucial molecule in the process of promoting irisin release by Metformin. *In vivo*, the skeletal muscle PGC-1α mRNA/protein expression was markedly up-regulated by Metformin in both ob/ob and WT mice. *In vitro*, the up-regulation of PGC-1α mRNA/protein by Metformin was also observed in cell line C2C12 of mouse skeletal muscle myoblasts. However, the decrease in FNDC5 expression and irisin concentration induced by siRNA-PGC-1α could not be reversed by Metformin, which suggested that PGC-1α activation by Metformin in skeletal muscle cells is critical for the promotion of irisin release by Metformin.

AMPK is an enzyme that plays a role in cellular energy homeostasis, which is expressed in various tissues including the liver, heart, kidney, and skeletal muscle. It is known that Metformin activates the AMPK pathway to promote mitochondrial biogenesis and protects against apoptosis attenuation via the PGC-1α pathway [19, 20]. Phosphorylated AMPK increases glucose uptake and cellular energy and benefits diabetics and reduces body weight [21]. In addition, the phosphorylation of the extracellular signal-related kinase (ERK) pathway is involved in stimulating the browning of white adipocytes when induced by irisin [22]. In the present study, Metformin elevated p-AMPK and p-ERK levels in WT and ob/ob mice and was accompanied by an increase in irisin concentration. The down-regulated PGC-1α resulted in a decrease in p-AMPK and p-ERK expression in C2C12 cells exposed to Metformin. The data

suggested that AMPK and ERK participated in the release of irisin from skeletal muscle.

In conclusion, our study demonstrates that Metformin stimulates irisin secretion from skeletal muscle into the circulation of obese mice, and PGC-1α is a critical regulator in this effect. This study provides a theoretical basis for better understanding the beneficial effects of Metformin on obesity-related metabolic diseases. The role of PGC-1α and irisin as potential therapeutic targets in obesity and diabetes needs further study in both *in vivo* and *in vitro* settings.

Disclosure of conflict of interest

None.

Address correspondence to: Qian Zhao, Geriatric Department of Endocrinology, The First Affiliated Hospital of Zhengzhou University. The No. 1 Road of Construction, Zhengzhou, Henan 450052, China. Tel: +86 13526770037; E-mail: zhaoqian880914@163.com

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