

Time-Dependent Changes in Increased Levels of Plasma Irisin and Muscle PGC-1 α and FNDC5 after Exercise in Mice

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Exercise induces the expression of peroxisome proliferator-activated receptor gamma co-activator 1- α (PGC-1 α) in skeletal muscle, which promotes the cleavage of fibronectin type III domain-containing protein 5 (FNDC5) to irisin. To explore the relationship between irisin and its regulators, we analyzed the plasma irisin levels and the muscle levels of FNDC5 and PGC-1 α after exercise. Male C57BL/6J mice underwent a treadmill exercise (60% of VO_{2max}) for 30 min or one hour (h), and blood and gastrocnemius samples were collected before exercise (pre-exercise), immediately after exercise, and during 24-h recovery after 1-h exercise. We found that plasma irisin levels were significantly increased during exercise ($P < 0.05$), while FNDC5 protein levels were not significantly increased. Moreover, PGC-1 α mRNA and protein levels were significantly increased during 30-min exercise, but were decreased during 1-h exercise. After 1-h exercise, the irisin levels peaked at 6 h (20.71 ± 0.25 ng/ml) and decreased to pre-exercise levels by 24 h (15.45 ± 0.27 ng/ml). Likewise, PGC-1 α mRNA and protein levels were increased at 1 h and maintained at elevated levels for 6 h; thereafter, the expression levels of PGC1- α protein were decreased to pre-exercise levels at 12 h. Thus, the restoration of PGC-1 α expression to the pre-exercise levels was followed by the decrease in plasma irisin levels. By contrast, during 24-h recovery, the expression levels of FNDC5 mRNA and protein were maintained at elevated levels. These results suggest that the coordinated expression of FNDC5 and PGC-1 α may contribute to the increased levels of plasma irisin after exercise.

Keywords: acute exercise intervention, FNDC5, irisin, PGC-1 α , time points

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Introduction

With the improvement of living standards and changes in life-style, the prevalence of metabolic diseases such as obesity, dyslipidemia and diabetes due to fat accumulation shows a rising trend in many countries (Abraham et al. 2015; Seravalle and Grassi 2017). Regular physical activity is not only an active and effective method for improving health outcomes but is also a beneficial way to reduce the risk of metabolic diseases by increased energy expenditure (Carroll and Dudfield 2004; Vetter et al. 2010; Smith and Adams 2011). Exercise intervention has been well-established as reducing the incidence of cardiovascular disease, type II diabetes and obesity, preventing further deterioration of health (Hodgson et al. 2011). Physical activity can promote muscle contraction and stimulate the

expression and secretion of a variety of muscle factors, but the detailed mechanism remains unclear (Pedersen 2011; Pratesi et al. 2013). Recently, Boström et al. (2012) discovered a new muscle factor named irisin. As a molecule messenger between skeletal muscle and peripheral tissues or organs, irisin can increase energy consumption, induce browning of subcutaneous white adipocytes and regulate the metabolism of glucose and lipid via uncoupling protein 1 (UCP-1) (Boström et al. 2012; Pekkala et al. 2013; Wang and Pan 2016). UCP-1 is responsible for electron transport in mitochondrial oxidative respiration and uncoupling of ATP production, thereby reducing the productivity of fatty acid oxidation metabolism, and increasing energy consumption (Lowell and Spiegelman 2000; Gao et al. 2012). Therefore, the discovery of irisin provides a novel perspective for the prevention and

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treatment of metabolic diseases via regular physical activity.

The fibronectin type III domain-containing 5 (FNDC5) is a membrane protein and its secreted portion, irisin, has remarkable conservation among species, with 100% identity between mice and humans (Boström et al. 2012). FNDC5 consists of three parts, an N-terminal signal peptide, two fibronectin domains and a C-terminal hydrophobic domain (Xu et al. 2011; Boström et al. 2012). Irisin is derived from the fibronectin domain of FNDC5 and released into the circulation from skeletal muscle after exercise training (Boström et al. 2012). Irisin secretion can be caused by many factors such as starvation and cold as well as high temperature, especially due to exercise (Boström et al. 2012; Roca-Rivada et al. 2013; Aydın et al. 2013; Lee et al. 2014). A recent study confirmed that exercise induced the expression of peroxisome proliferator-activated receptor gamma co-activator 1- α (PGC-1 α) in skeletal muscle, which could regulate the conversion of FNDC5 to irisin (Boström et al. 2012). At present, there are two possible explanations for how this occurs. One theory is that PGC-1 α directly causes FNDC5 to be hydrolyzed to irisin (Lin et al. 2002, 2005; Fain et al. 2013; Komolka et al. 2014), and another is that PGC-1 α promotes expression of FNDC5 mRNA in skeletal muscle, leading to the synthesis of FNDC5 and indirectly increasing plasma irisin levels (Boström et al. 2012; Sanchez-Delgado et al. 2015).

Acute exercise has been shown to enhance the secretion of irisin in both mice (Brenmoehl et al. 2014; Liu et al. 2015) and humans (Löffler et al. 2015; Nygaard et al. 2015). The secretion of irisin reached a peak and then returned to baseline levels (Norheim et al. 2014; Kraemer et al. 2014). Kraemer et al. (2014) found that after 90 min of treadmill exercise, the plasma irisin concentration in young people increased only at the beginning of 1 h after exercise, but after resting for 20 min, irisin levels were not elevated. A study by Norheim et al. (2014) showed that the plasma irisin concentration was significantly increased in pre-diabetes patients after 30 min of high-intensity exercise performed one time and then decreased to the baseline level after 2 h. Taken together, these findings indicate that plasma irisin levels may be affected by exercise intensity or exercise duration.

Many studies on FNDC5 have indicated that its expression level was not increased immediately after acute exercise in mice (Brenmoehl et al. 2014; Liu et al. 2015) and humans (Nygaard et al. 2015). Instead, the expression of the FNDC5 protein only increased for a short time after exercise (Liu et al. 2015; Nygaard et al. 2015). These results indicated that the effect of acute exercise on the expression of FNDC5 was transient and appeared within a short period of rest after exercise. Based on the results of previous research on irisin and FNDC5 in short-term exercise intervention experiments, the expression levels of FNDC5 do not appear to be linearly correlated with irisin concentrations (Boström et al. 2012; Liu et al. 2015; Nygaard et al. 2015). The expression level of FNDC5 is

up-regulated during rest after exercise but not during exercise, while the plasma level of irisin is elevated during exercise and gradually decrease to normal levels after exercise (Brenmoehl et al. 2014; Norheim et al. 2014; Liu et al. 2015). Thus, the amount of released irisin was decreased gradually after exercise. However, the mechanism by which PGC-1 α regulates the expression of FNDC5 and irisin has not been clearly defined.

In the present study, to investigate the relationship among irisin, FNDC5, and PGC-1 α , we analyzed the effect of treadmill exercise on plasma irisin concentration and the expression levels of FNDC5 and PGC-1 α mRNA and protein in skeletal muscle. Exploring the expression profiles of molecules in this pathway may lead to the better understanding the regulation of the irisin secretion. Such information may provide a basis for exercise therapy in chronic metabolic disease.

Methods

Animal model

Six-week-old male C57BL/6J mice were purchased from the HuaFuKang Experimental Animal Center (Beijing, China) and weighed 20 ± 2 g. The animals were housed six per cage in Jinan University Animal Center under environmentally controlled conditions, with a temperature of 22–25°C and a day and night light cycle. The animals were fed a diet *ad libitum* and had free access to fresh tap water for drinking. After one week of adaptation to exercise, 54 mice were randomly assigned to the exercise group or control group (Pre-exercise, Pre) (Fig. 1). The exercise group was divided into two subgroups: the first group was a one-time exercise group and included a 30-min exercise group (EX0.5) and a 1-h exercise group (EX1). The second group included animals that rested for different duration after 1-h exercise one time, with groups of 6 animals each: animals resting for 1 h after exercise (AF1), 2 h after exercise (AF2), 3 h after exercise (AF3), 6 h after exercise (AF6), 12 h after exercise (AF12), and 24 h after exercise (AF24). All experimental procedures were approved by the Ethical Committee of the Guangzhou Center for Disease Control and Prevention.

Exercise intervention

The treadmill training intensity was set according to a previous relevant literature (Schefer and Talan 1996). After appropriate adjustments, the animals were submitted to a one-time moderate-intensity treadmill running trial (60% of VO_{2max}) at 14 m/min for 30 min or 1 h. The control group did not undergo any exercise training.

Sample collection

After the treadmill exercise intervention, the mice were sacrificed, and their venous blood was collected to obtain plasma. The plasma samples were centrifuged at $3,000 \times g$ at 4°C for 15 min. After centrifugation, the plasma was stored in a freezer at –20°C for future irisin analysis. After mice were anesthetized using 0.1% pentobarbital sodium, the experimenters isolated the gastrocnemius muscle of the left lower limbs in a sterile environment. Then, the gastrocnemius muscle extracts were placed in sterile centrifuge tubes, immediately frozen within 15 min after separation, stored in liquid nitrogen, and then transferred to a –80°C refrigerator for storage for future analyses of protein and mRNA.

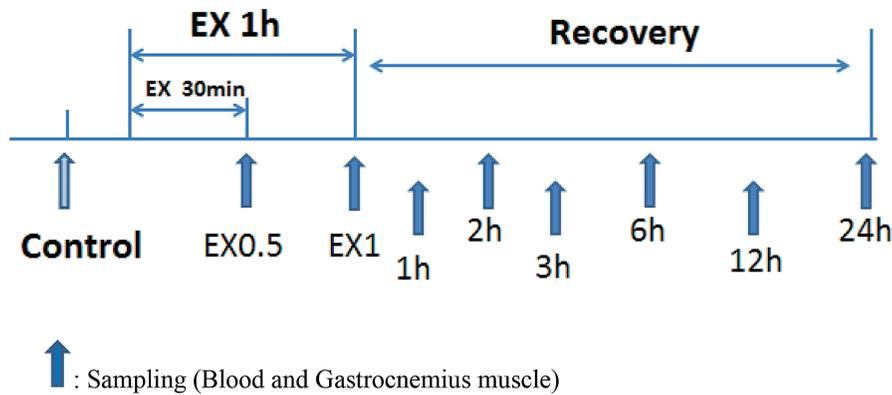


Fig. 1. Experimental protocol.

Mice ($n = 54$) were randomly assigned to the exercise group and control group (Pre-exercise, Pre). The animals in the exercise group were subjected to a one-time moderate-intensity treadmill running trial (60% VO_{2max}); the exercise group included the 30-min exercise group (EX0.5), the 1-h exercise group (EX1), and the groups that rested for the following duration after 1-h exercise: 1 h (AF1), 2 h (AF2), 3 h (AF3), 6 h (AF6), 12 h (AF12) and 24 h (AF24). Each group included 6 animals.

Enzyme-linked immunosorbent assay (ELISA)

Plasma irisin concentrations were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (MM-0623M1, Feiya Biotechnology, China). To produce a standard curve of optical density (OD) versus irisin concentration, we added specimens, standard samples and HRP-labeled antibodies to micropores pre-coated with the irisin antibody, and the OD values of the standard samples and specimens were then detected with a microplate spectrophotometer at a wavelength of 450 nm. The concentration of irisin in the samples was subsequently determined by comparing the OD value of the samples to the standard curve.

Western blot analysis

We extracted 30 mg of the left gastrocnemius muscle from each mouse and added 350 μ l of RIPA lysis buffer (Beyotime Biotechnology, China) with the protease inhibitor phenylmethanesulfonyl fluoride (PMSF, Beyotime Biotechnology, China) for homogenization at 30 rounds per second for 4 min. After homogenization, the samples were placed on ice for 5 min and then centrifuged at 4°C and 12,000 \times g for 4 min. The tissue supernatant was retained, and the protein concentration was determined using the colorimetric bicinchoninic acid (BCA) assay. The proteins were separated through electrophoresis in a 10% SDS-polyacrylamide gel, boiled in 100°C water for 10 min and then transferred to polyvinylidene fluoride (PVDF) membranes. The membranes with the proteins were blocked with 5% fat-free dry milk in 1 \times Tris-buffered saline Tween (TBST) for 1.5 h at room temperature. Then, either rabbit monoclonal anti-PGC-1 α (1:1,000) (Abcam, Cambridge Science Park in England), anti-FNDC5 (1:1,000) (Abcam, Cambridge Science Park in England), or mouse monoclonal anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase, GAPDH) (1:1,000) (ABclonal, Boston in America) was added to the membrane, followed by storage at 4°C overnight. Sequentially, the membranes were washed with TBST buffer and incubated with the corresponding horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After washing the membrane, immunoreactive bands were visualized using an enhanced chemiluminescence light (ECL) detection system. GAPDH was used for normalization of target protein levels. Relative protein expression levels were analyzed using ImageJ2x software.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from 30 mg of the left gastrocnemius muscle from each mouse using 1 ml of the TRIzol reagent (Takara, Japan), according to the manufacturer's instructions. The mRNA expression of PGC-1 α and FNDC5 was assessed using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The extracted RNA was reverse transcribed into cDNA using a reverse transcription kit (Takara, Japan), with incubation for 15 min at 37°C and then for 5 s at 85°C before storage at 4°C. Quantitative real-time PCR was performed using 2 μ l of cDNA template, 10 μ l of SYBR Premix Ex Taq™ II (Takara, Japan), 0.8 μ l of each primer (forward and reverse) and nuclease-free water to obtain a final volume of 20 μ l. The primers were designed with Premier 5.0 and are shown in Table 1. The real-time PCR cycling protocol was conducted with initial denaturation at 95°C for 30 s, which entailed 40 cycles of denaturation at 95°C for 5 s and annealing and elongation at 60°C for 30 s. Relative target mRNA expression levels were normalized to the endogenous housekeeping gene β -actin using the $2^{-\Delta\Delta Ct}$ method (also known as the comparative C_T method).

Statistical Analysis

The statistical analysis was performed using SPSS 22.0 software. All experimental data are presented as the mean \pm S.D. Variable comparisons between the control and exercise groups were performed using Student's *t*-test. At different time points of exercise, the correlation coefficients between the variables were analyzed with Spearman's test. The significance level was set as $P < 0.05$ for all statistical tests.

Results

Plasma irisin levels are elevated during and after exercise

Compared with the pre-exercise (control) levels, the plasma levels of irisin were significantly increased during 30-min and 1-h treadmill exercise (Fig. 2). We also analyzed the time-dependent changes in the plasma irisin levels during 24-h recovery after 1-h exercise. The plasma irisin levels were decreased at 1 h after 1-h exercise, but still higher than the pre-exercise (control) levels (Fig. 2).

Table 1. Primer sequences used for RT-qPCR.

Gene	Primer	Sequence
PGC-1 α	Forward	5'-taggccaggtacgacagcta-3'
	Reverse	3'-ttctgtccgcgttgtgtcag-5'
FNDC5	Forward	5'-gatgtcctggaggatgaagtgg-3'
	Reverse	3'-aagtcctccactgtggtggtg-5'
β -actin	Forward	5'-cattgctgacaggatgcagaagg-3'
	Reverse	3'-ggagtgcaggtggaagtcgt-5'

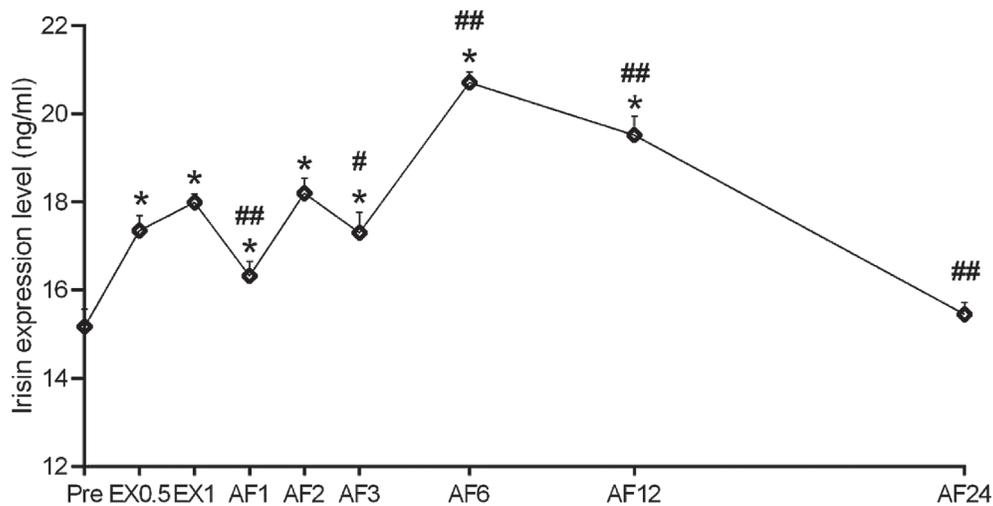


Fig. 2. Changes in plasma irisin levels after acute moderate-intensity exercise.

The data on irisin concentrations in mouse plasma were collected pre-exercise (Pre), during exercise for 30 min (EX0.5) and 1 h (EX1), and post-exercise after resting for 1 h (AF1), 2 h (AF2), 3 h (AF3), 6 h (AF6), 12 h (AF12) and 24 h (AF24). Each group included 6 animals. The data are presented as the mean \pm S.D. *P < 0.05 vs. Pre, #P < 0.05 vs. EX1, ##P < 0.01 vs. EX1.

Table 2. Time-course of changes in blood and muscle parameters after exercise.

	Pre	EX0.5	EX1	AF1	AF2	AF3	AF6	AF12	AF24
Irisin (ng/ml)	15.17 \pm 0.39	17.35 \pm 0.35*	17.99 \pm 0.15*	16.33 \pm 0.32*	18.20 \pm 0.34*	17.30 \pm 0.47*	20.71 \pm 0.25*	19.52 \pm 0.42*	<u>15.45 \pm 0.27</u>
FNDC5	0.32 \pm 0.04	<u>0.37 \pm 0.04</u>	<u>0.26 \pm 0.03</u>	0.47 \pm 0.03*	<u>0.18 \pm 0.01</u>	0.42 \pm 0.03*	0.41 \pm 0.02*	0.59 \pm 0.01*	0.81 \pm 0.07*
PGC-1 α	0.14 \pm 0.04	0.22 \pm 0.01*	<u>0.19 \pm 0.01</u>	0.74 \pm 0.05*	0.69 \pm 0.04*	0.36 \pm 0.04*	0.59 \pm 0.02*	<u>0.20 \pm 0.01</u>	<u>0.15 \pm 0.02</u>

Pre, pre-exercise levels; EX0.5, 30-min exercise; EX1, group immediately processed after 1 h of exercise; AF1, resting for 1 h after 1-h exercise; AF2, resting for 2 h after 1-h exercise; AF3, resting for 3 h after 1-h exercise; AF6, resting for 6 h after 1-h exercise; AF12, resting for 12 h after 1-h exercise; AF24, resting for 24 h after 1-h exercise. Each group included 6 animals. The data are presented as the mean \pm S.D. *P < 0.05 vs. Pre. The values, underlined, indicate the levels similar to pre-exercise levels. The data are also shown in Figs. 2, 3C and 4C.

The plasma irisin levels were then increased to reach the highest values by 6 h and then decreased to the control levels by 24 h after 1-h exercise (Fig. 2). Thus, the plasma irisin levels are maintained at the elevated levels for at least 12 h after 1-h exercise. These results suggest that a short-term exercise may increase the secretion of irisin from skeletal muscle. The dynamic changes in the levels of plasma

irisin together with FNDC5 and PGC-1 α proteins are summarized in Table 2.

FNDC5 mRNA levels are elevated during and after exercise

We also analyzed the expression levels of FNDC5 mRNA in the gastrocnemius muscle (Fig. 3A). The relative expression level of FNDC5 mRNA was significantly

increased from the control levels during 30-min and 1-h exercise ($P < 0.05$). At 2 h after 1-h exercise, the expression levels of FNDC5 mRNA reached the highest levels, and then decreased to the control levels at 6 h. Thereafter, the expression levels of FNDC5 mRNA were elevated at 12 h and remained at the elevated levels by 24 h. Thus, the relative expression levels of FNDC5 mRNA were significantly higher than the control levels during 24-h recovery after 1-h exercise (Fig. 3A), except for the time point, 6 h.

FNDC5 protein levels are increased with resting time after exercise

The relative expression levels of FNDC5 protein were not significantly increased during 30-min and 1-h exercise (Fig. 3B, C and Table 2), despite the increased FNDC5 mRNA levels (see Fig. 3A). However, the FNDC5 protein levels after 30-min exercise were significantly higher than the levels after 1-h exercise (Fig. 3C).

At 1 h after 1-h exercise, the relative expression levels of FNDC5 protein were significantly increased, but at 2 h, they were decreased to the levels that were significantly lower than the control levels (Fig. 3C). After 3-h recovery, the relative expression levels of FNDC5 protein were significantly increased, and the elevated levels were maintained for 24 h after 1-h exercise (Fig. 3B, C). Thus, the relative expression levels of FNDC5 protein were significantly higher than the control levels at 1 h, 3 h, 6 h, 12 h and 24 h after 1-h exercise, except for the time point, 2 h ($P < 0.05$, Fig. 3C).

Exercise increases the PGC-1 α mRNA and protein levels with different time courses

The relative expression levels of PGC-1 α mRNA were significantly increased during 30-min exercise, but were decreased during 1-h exercise to the levels that were significantly lower than the control levels (Fig. 4A). Thereafter, during 24-h recovery after 1-h exercise, the expression levels of PGC-1 α mRNA were remained at the elevated levels (Fig. 4A). In parallel with the dynamic changes in PGC-1 α mRNA levels during exercise, the relative expression levels of PGC-1 α protein were significantly increased during 30-min exercise, but were decreased during 1-h exercise to the control levels (Fig. 4B, C). At 1-h recovery after 1-h exercise, the PGC-1 α protein levels were significantly elevated, and the elevated levels were maintained for 6 h. Thereafter, the PGC-1 α protein levels were decreased to the control levels at 12 h (Fig. 4B, C and Table 2), despite the elevated levels of PGC-1 α mRNA (see Fig. 4A).

Correlation analysis of FNDC5, PGC-1 α and irisin levels

Correlation analysis results showed that PGC-1 α protein levels were negatively correlated with FNDC5 protein levels before exercise ($r = -0.888$, $P = 0.02$, Fig. 5A). FNDC5 protein levels were positively correlated with irisin

levels before exercise ($r = 0.266$, $P = 0.61$, Fig. 5B). After 30-min exercise, PGC-1 α mRNA levels were positively correlated with FNDC5 protein levels ($r = 0.806$, $P = 0.05$, Fig. 5C), and FNDC5 protein levels were negatively correlated with irisin levels ($r = -0.577$, $P = 0.23$, Fig. 5D). Additionally, at 1-h exercise, FNDC5 protein levels were positively correlated with irisin levels ($r = 0.310$, $P = 0.55$, Fig. 5E).

After 2-h recovery, the expression levels of PGC-1 α protein were correlated with irisin levels ($r = 0.822$, $P = 0.04$, Fig. 6A), FNDC5 mRNA levels were correlated with irisin levels ($r = 0.910$, $P = 0.01$, Fig. 6B), and FNDC5 protein levels were correlated with irisin levels ($r = 0.623$, $P = 0.19$, Fig. 6C). In addition, FNDC5 mRNA levels were positively correlated with FNDC5 protein levels at 2-h rest ($r = 0.760$, $P = 0.08$, Fig. 6D). PGC-1 α mRNA levels were positively correlated with FNDC5 mRNA levels at 6-h recovery ($r = 0.832$, $P = 0.04$, Fig. 6E), but they were negatively correlated at 24 h ($r = -0.917$, $P = 0.01$, Fig. 6F). At 24 h after exercise, FNDC5 protein levels were negatively correlated with irisin levels ($r = -0.315$, $P = 0.54$, Fig. 6G).

Discussion

In skeletal muscle, exercise activates a signaling pathway that promotes the expression of PGC-1 α and the cleavage of FNDC5 to irisin (Castillo-Quan 2012; Choi et al. 2014). Exercise can elevate the level of PGC-1 α , which can accelerate the cleavage of FNDC5 protein to irisin; however, there is a difference in the regulation of the expression of FNDC5 mRNA and protein after different lengths of exercise (Liu et al. 2015). In the present study, we detected the coordinated changes in the plasma irisin levels and in the muscle levels of FNDC5 and PGC-1 α during acute exercise intervention and/or during the 24-h recovery after 1-h exercise in mice. Importantly, the expression levels of PGC-1 α protein were returned to control levels by 12 h after 1-h exercise, and the plasma irisin levels were returned to control levels by 24 h. The apparent time-lag of the decreases in the elevated levels of muscle PGC-1 α and plasma irisin is consistent in part with the hypothesis that PGC-1 α may regulate plasma irisin via the cleavage of FNDC5.

After an exercise intervention of 30 min or 1 h, we detected the increases in the plasma irisin levels and the expression levels of FNDC5 mRNA, compared with the control levels. By contrast, FNDC5 protein levels were not significantly increased during 30-min and 1-h exercise, compared with the control levels. Furthermore, the expression levels of PGC-1 α mRNA and protein were increased after 30-min exercise and then decreased after 1-h exercise. The levels of PGC-1 α mRNA after 1-h exercise were lower than the control levels, while the PGC-1 α protein levels were still higher than the control levels. These results suggest the important role of exercise duration for the expression of FNDC5 and PGC-1 α in skeletal muscle.

To explore the possible relationship between FNDC5

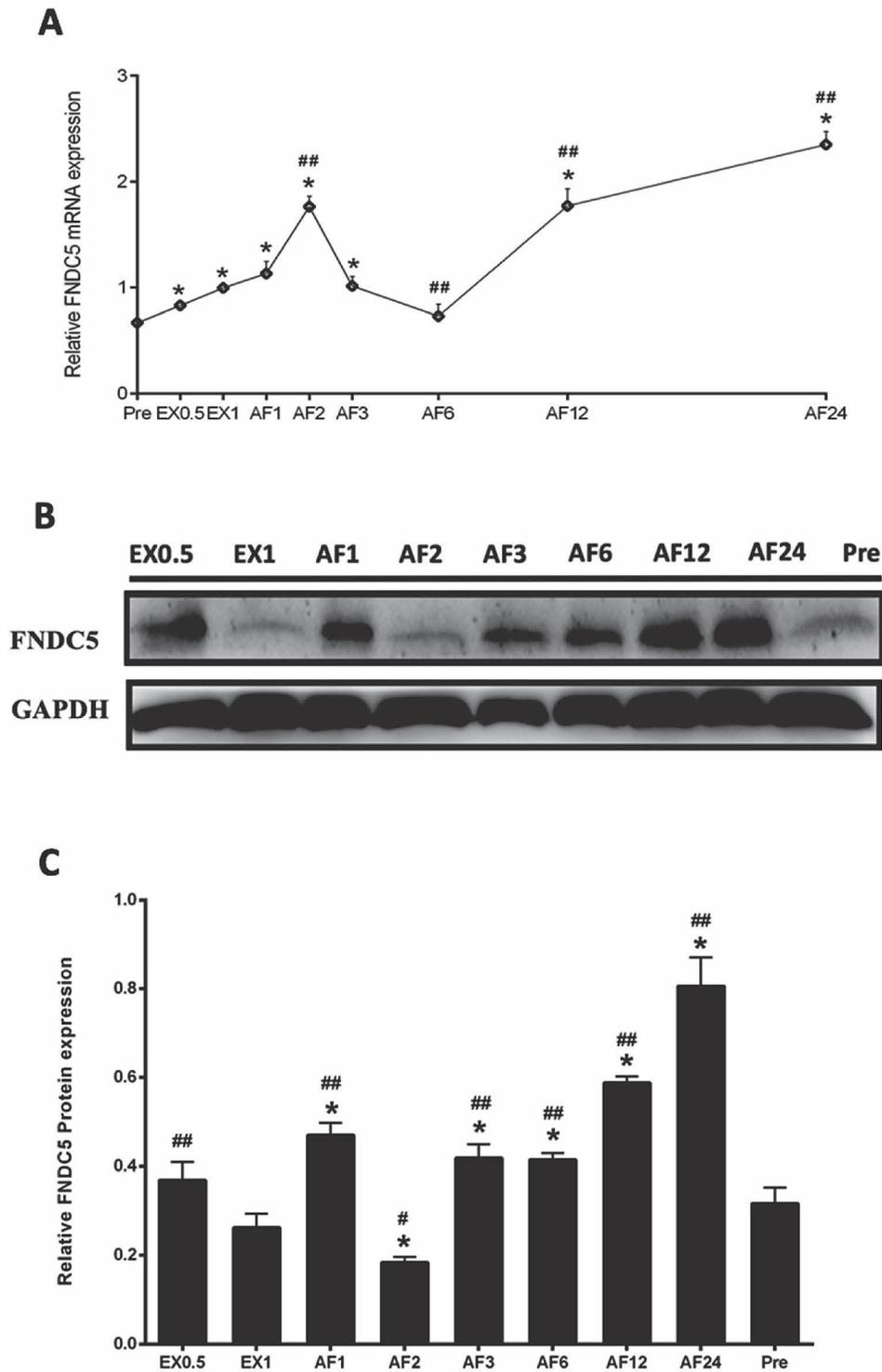


Fig. 3. Expression levels of FNDC5 mRNA and protein in the gastrocnemius muscle after acute moderate-intensity exercise. All the data were collected pre-exercise (Pre), during exercise for 30 min (EX0.5) and 1 h (EX1), and post-exercise after resting for 1 h (AF1), 2 h (AF2), 3 h (AF3), 6 h (AF6), 12 h (AF12) and 24 h (AF24). (A) Relative mRNA expression of FNDC5 in the gastrocnemius muscle. (B) Corresponding western blot of FNDC5 protein (25 kDa) in the gastrocnemius muscle. (C) Relative expression levels of the FNDC5 protein. A comparison between the control and exercise groups was performed using Student's t-test. Each group included 6 animals. The data are presented as the mean \pm S.D. * $P < 0.05$ vs. Pre, # $P < 0.05$ vs. EX1, ## $P < 0.01$ vs. EX1.

and PGC-1 α in the muscle, we conducted a correlation analysis. There was a significant positive correlation ($r = 0.806$) between PGC-1 α mRNA and FNDC5 protein levels

after 30-min exercise (Fig. 5C), indicating that exercise can simultaneously increase the expression levels of PGC-1 α and FNDC5. The FNDC5 protein levels and plasma irisin

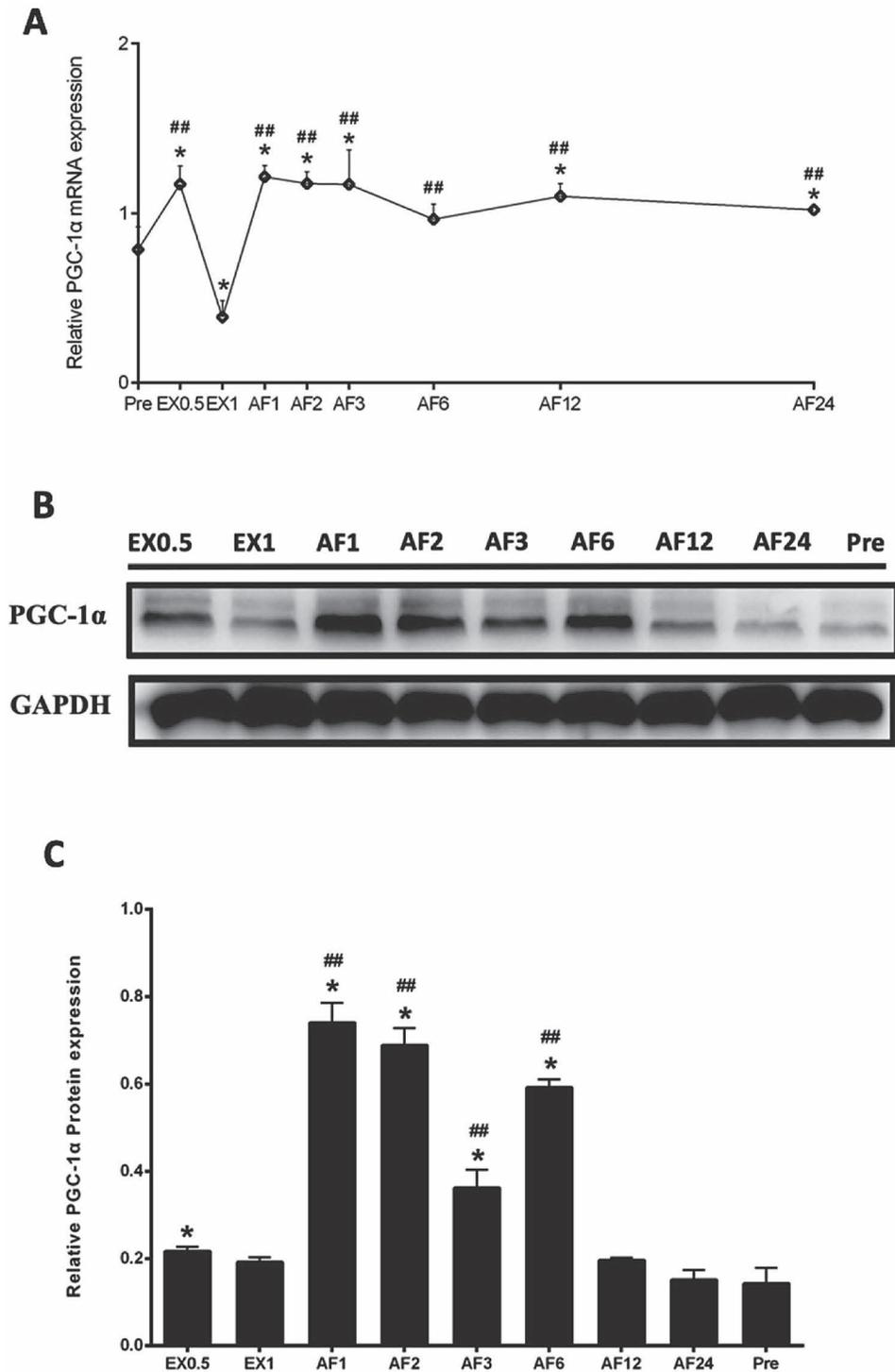


Fig. 4. Expression levels of PGC-1 α mRNA and protein in the gastrocnemius muscle after acute moderate-intensity exercise. All the data were collected pre-exercise (Pre), during exercise for 30 min (EX0.5) and 1 h (EX1), and post-exercise after resting for 1 h (AF1), 2 h (AF2), 3 h (AF3), 6 h (AF6), 12 h (AF12) and 24 h (AF24). (A) Relative expression levels of PGC-1 α mRNA in the gastrocnemius muscle. (B) Corresponding western blot of PGC-1 α protein (92 kDa) in the gastrocnemius muscle. (C) Relative expression levels of the PGC-1 α protein. A comparison between the control and exercise groups was performed using Student's t-test. Each group included 6 animals. The data are presented as the mean \pm S.D. * P < 0.05 vs. Pre, * P < 0.05 vs. EX1, $^{##}$ P < 0.01 vs. EX1.

levels showed negative correlation ($r = -0.577$) after 30-min exercise (Fig. 5D). However, there was a positive correlation ($r = 0.310$) between FNDC5 protein and irisin

levels during 1-h exercise (Fig. 5E). It is, therefore, conceivable that the production of irisin via the cleavage of FNDC5 protein may be increased during 30-min exercise,

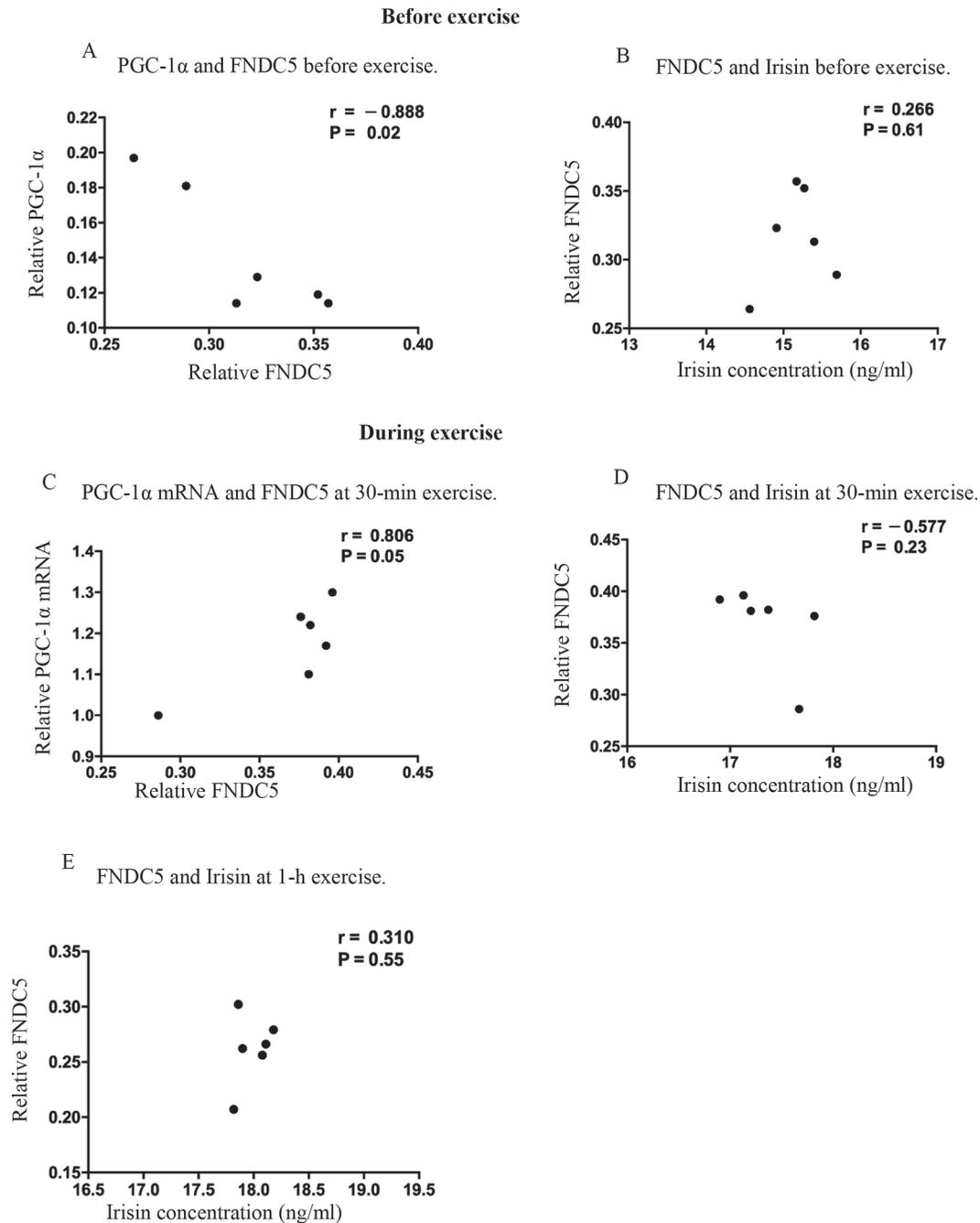


Fig. 5. Correlation analysis.

Scatterplots generated for irisin, FNDC5, FNDC5 mRNA, PGC-1 α , and PGC-1 α mRNA before exercise, during exercise for 30 min (EX0.5) and 1 h (EX1), with Spearman's correlation coefficients. (A) Scatterplot between PGC-1 α and FNDC5 before exercise; (B) Scatterplot between FNDC5 and irisin before exercise; (C) Scatterplot between PGC-1 α mRNA and FNDC5 at 30-min exercise; (D) Scatterplot between FNDC5 and irisin at 30-min exercise; (E) Scatterplot between FNDC5 and irisin at 1-h exercise. Each group included 6 animals.

but decreased with the prolonged exercise.

The plasma irisin levels were transiently decreased at 1 h after 1-h exercise (Fig. 2), the levels of which were higher than the pre-exercise levels. Additionally, compared with pre-exercise levels, the expression levels of FNDC5 mRNA and protein were higher at 1-h recovery after exercise (Fig. 3). These findings are consistent in part with the previous reports that exercise could increase the production

of irisin from FNDC5 (Ellefsen et al. 2014; Liu et al. 2015; Nygaard et al. 2015). However, the FNDC5 protein levels were significantly decreased after 2-h recovery, despite the increase in its mRNA levels. Thereafter, the levels of FNDC5 mRNA and protein were maintained at higher levels during 24-h recovery. Thus, the increased expression levels of FNDC5 mRNA may be responsible for the elevated levels of FNDC5 protein during 24-h recovery. On

the other hand, the expression levels of PGC-1 α protein were increased at 1 h after exercise and then decreased to the levels of pre-exercise levels by 12 h. By contrast, the expression levels of PGC-1 α mRNA were increased at 1 h after exercise and remained at higher levels than the pre-

exercise levels during 24 h after exercise. Furthermore, the levels of the PGC-1 α protein and irisin were positively correlated at 2 h ($r = 0.822$, Fig. 6A), when FNDC5 protein levels were positively correlated with irisin levels ($r = 0.625$, Fig. 6C). These results are consistent in part with

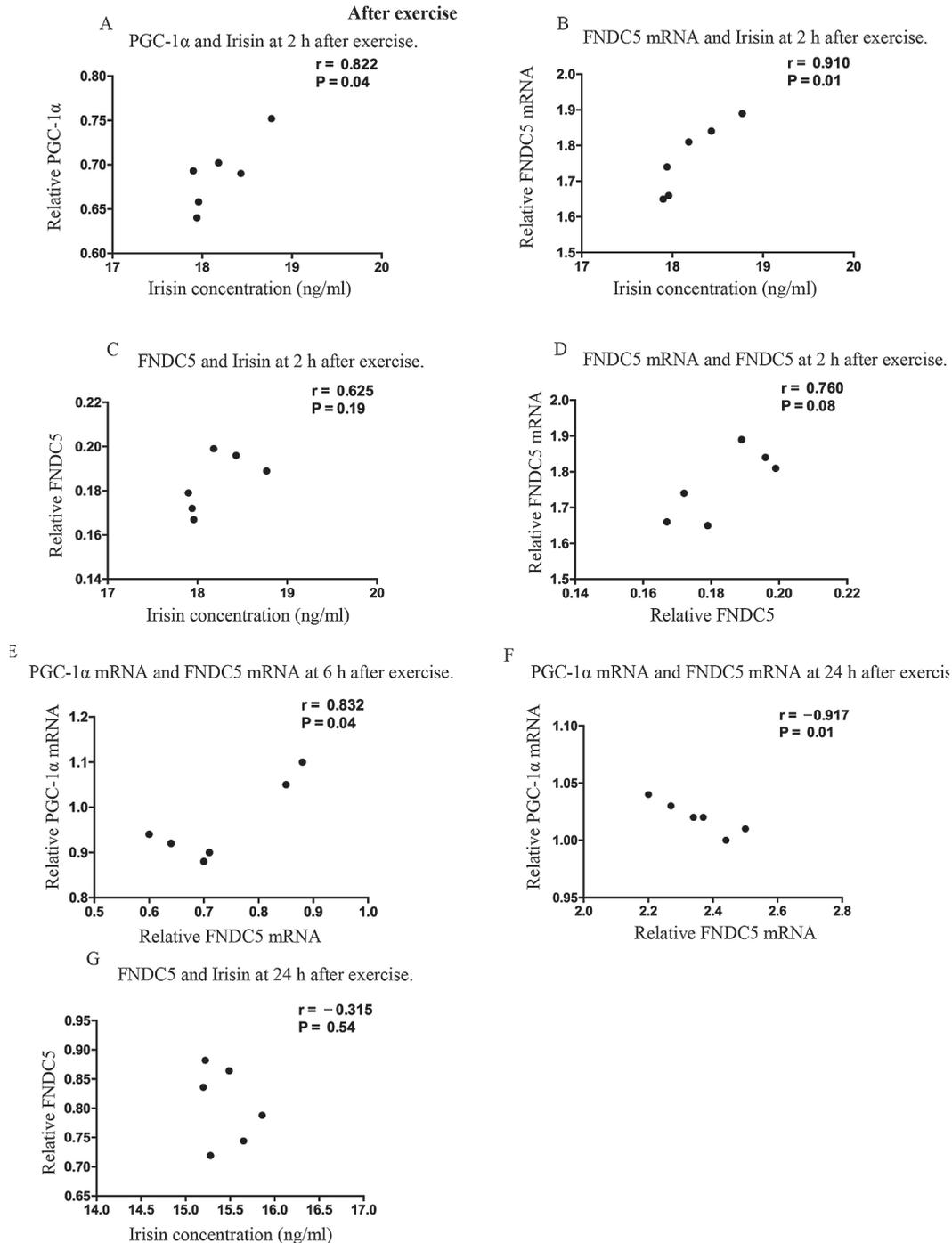


Fig. 6. Correlation analysis.

Scatterplots generated for irisin, FNDC5, FNDC5 mRNA, PGC-1 α , and PGC-1 α mRNA post-exercise after resting for 1 h (AF1), 2 h (AF2), 6 h (AF6) and 24 h (AF24), with Spearman's correlation coefficients. (A) Scatterplot between PGC-1 α and irisin at 2 h after exercise; (B) Scatterplot between FNDC5 mRNA and irisin at 2 h after exercise; (C) Scatterplot between FNDC5 and irisin at 2 h after exercise; (D) Scatterplot between FNDC5 mRNA and FNDC5 at 2 h after exercise; (E) Scatterplot between PGC-1 α mRNA and FNDC5 mRNA at 6 h after exercise; (F) Scatterplot between PGC-1 α mRNA and FNDC5 mRNA at 24 h after exercise; (G) Scatterplot between FNDC5 and irisin at 24 h after exercise. Each group included 6 animals.

the notion that PGC-1 α may promote the cleavage of FNDC5 protein to irisin.

We observed another interesting phenomenon. The plasma irisin level peaked at 6 h after 1-h exercise. Incidentally, a related study has shown that the browning phenomenon of white adipose tissue peaks at 6 h after exercise (Ringholm et al. 2013). Furthermore, there was a significant positive correlation between PGC-1 α mRNA and FNDC5 mRNA levels only at 6 h after exercise ($r = 0.832$, Fig. 6E). Thus, the timing around 6 h after exercise may be important for achieving the irisin's action.

Lastly, the expression levels of PGC-1 α protein were returned to control levels by 12 h after 1-h exercise, and subsequently, the plasma irisin levels were returned to control levels by 24 h. By contrast, expression levels of PGC-1 α mRNA and FNDC5 mRNA and protein were maintained at higher levels for 24 h after 1-h exercise. We speculate that the decreased levels of PGC-1 α protein may lead to the decrease in the cleavage of FNDC5 protein to irisin, which is consistent with a negative correlation between irisin and FNDC5 protein levels at 24 h after exercise ($r = -0.315$, Fig. 6G). In fact, at 24 h after exercise, high levels of the FNDC5 protein were maintained, with pre-exercise levels of irisin (Table 2).

In summary, plasma irisin levels are elevated after 30-min and 1-h exercise of moderate intensity (60% VO_{2max}), while FNDC5 protein levels were not significantly changed during exercise. By contrast, the expression levels of PGC-1 α mRNA and protein are elevated during 30-min exercise, but decreased to the pre-exercise levels during 1-h exercise. During 24-h recovery after 1-h exercise, plasma irisin levels were returned to the control levels by 24 h, with the elevated FNDC5 levels and the decreased PGC-1 α levels. Our study suggests the regulatory link between FNDC5 and PGC-1 α expression that may contribute to achieving the proper levels of plasma irisin after exercise.

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Conflict of Interest

The authors declare no conflict of interest.

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