

Irisin Signaling Pathway is up Regulated by Resistance Training in Ovariectomized Rats

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Abstract

Exercise is known to increase the concentrations of irisin, a remarkable myokine that may play an important protective role against metabolic disorders.

Propose: This study investigated the effects of irisin signaling pathway induced by Resistance Training (RT) in ovariectomized (Ovx) rats.

Methods: Thirty-two Holtzman rats were randomly distributed to four experimental groups: Sham-Sedentary (Sed); Ovx-Sed; Sham-RT and Ovx-RT. The RT protocol demanded from the animals a vertical climb. Each session consisted of 4 to 12 climbs with 2 min. of rest during 12 weeks. To quantify mRNA expression the $\Delta\Delta C_t$ method was applied, protein expression was verified by Western Blotting and the analysis of irisin was determined by ELISA. When group averages were different ($p \leq 0.05$), a Tukey post-hoc test was applied.

Results: The Ovx-RT group had higher expression of PGC1 α FNDC5, irisin levels, and UCP1 compared to Ovx-Sed.

Conclusion: RT was led to higher expression of the irisin signaling pathway in the Ovx group showing that the RT seems to be an excellent strategy to counteract the ovariectomy-induced metabolic disorders.

Keywords: PGC1-A, FNDC5; Adipose Tissue; UCP1; Resistance Training

Introduction

Reduction of estrogen levels, a physiological status of female aging, causes problematic deleterious effects, such as loss of muscle mass, changes in fat mass, with the possibility of developing obesity [1-3]. This is a very important issue for public health, once obesity is associated with increased risk of cardiovascular disease and type 2 diabetes [3-5]. In experimental studies with rats, menopause is simulated by ovariectomy, which leads to effects such as loss of muscle mass and increased body and fat mass [6-8].

Physical exercise can be used as an intervention to improve the health of postmenopausal women given that that Resistance Training (RT) has been associated to decreases in fat mass and increases in lean body mass to prevent sarcopenia, cardiovascular diseases, type 2 diabetes and obesity [9-14]. These benefits occur in part because skeletal muscle is an endocrine organ and exercise stimulates skeletal muscle to produce and release myokines, which have endocrine functions [15]. Irisin is a remarkable myokine and its concentration seems to increase in response to both endurance training and RT [16-20]. During physical exercise, the release of this myokine is induced by the activation of PPAR γ co-activator 1 alpha (PGC1- α), which stimulates the fibronectin type III domain containing 5 (FNDC5) to cleave and release irisin into the blood [16].

Irisin is bound to unidentified receptors in the surface of white fat adipocytes and positively regulates the release of UCP1 (Uncoupling Protein 1), which provokes uncoupling in mitochondrial respiration and loss of energy in the form of heat and browning of the White Adipose Tissue (WAT) [16,21,22]. In addition, irisin could stimulate energy expenditure through modulation of hypothalamic neuropeptides and neurotransmitters involved in feeding control [23]. Thus, the thermogenic changes in white adipose tissue may play an important protective role against metabolic disorders, such as cardiovascular diseases, type-2 diabetes and obesity [24,25]. In view of these benefits, the activation of the irisin pathway could play an important protective role against the deleterious effects caused by reduced levels of estrogen. However, to date there are no studies that demonstrate the effect of RT on the irisin signaling pathway in Ovariectomized (Ovx) rats. Our hypotheses in the present study were: 1) the irisin signaling pathway would be lower in Ovx rats, and 2) the Sham and Ovx rats would have greater irisin signaling pathway after RT.

Methods

Animals and Experimental Conditions

This study used 32 female Holtzman rats. During the experimental period, animals were kept in collective cages. Their environment was controlled by reversed light cycle (12 h light cycle starting at 08:00 PM), at constant temperature ($22 \pm 2^\circ\text{C}$) and water and chow *ad libitum*. The study was approved by Ethics Commission of Animals Use-UFSCar (protocol n. 005/2013). All procedures were conducted according to the Guide for the Care and Use of Laboratory Animals [26].

Experimental Groups

Rats were randomly distributed into four experimental groups as follows: 1) Sham-operated (Sham) Sedentary (Sed) (Sham-Sed); 2) Ovariectomized (Ovx) Sedentary (Ovx-Sed); 3) Sham Resistance Training (Sham-RT); 4) Ovariectomized Resistance Training (Ovx-RT). Rats first underwent a bilateral ovariectomy or a bilateral sham operation. Thereafter, one group of Ovx and one group of Sham rats remained sedentary while one group of Ovx and one group of Sham rats were submitted to a 12-week RT program.

Surgery

Ovariectomy and sham surgeries were performed when animals reached ~250 g body weight. Surgical procedures were performed as described by Kalu [6]. Animals were anesthetized with an intra peritoneal mixture of ketamine-zylazine (61.5-7.6 mg/kg). The Ovx groups had their ovaries removed and sham groups only had their ovaries exposed. All animals had 1 week of recovery after surgery and 92 days of from surgery to euthanasia, that is, all rats were euthanized at 26 weeks of age.

Resistance Training (RT)

RT was performed in a vertical ladder with a cage at the top where animals rested for 2 min between climbs [27]. Three 50 ml Falcon tubes containing weights were attached to their tails with adhesive tape as overload apparatus. Two familiarization sessions were performed. The rats were considered familiarized when they performed three consecutive climbs. Maximum Load was tested the first day after familiarization. The first climb was realized with an overload corresponding to 75% of animal's body weight and additional climbs with systematic increases of 30 g were performed until the rat was unable to climb the entire ladder even after three successive small stimuli in the tail. The larg-

est successful load was considered the Maximum Carrying Load (MCL). Training sessions were performed every three days and consisted of four climbs with 50%, 75%, 90% and 100% of the MCL determined on the previous session. From the 5th to the 9th climb load was added 30 g until a new MCL was determined [27]. This pattern was made for 12 weeks in a total of 27 sessions of RT.

Euthanasia and tissue collection

At the end of the experimental period, animals were euthanized by decapitation. RT groups were euthanized 48 h after the last training session. Left gastrocnemius muscle, mesenteric fat, uterus and blood were taken, weighted and stored. Both the muscle tissue and fat were initially stored in liquid nitrogen and they were frozen at -80°C . For blood analysis, 8 mL of blood were collected, of which 4 mL were placed in a 13x75 mm vacuum tube with EDTA k3, mixed with gentle inversions and centrifuged at 3000 rpm for 10 min. at 4°C . The obtained plasma was taken and stored at -20°C . The remaining of the blood was put in a 15 mL falcon tube, mixed with gentle inversions and centrifuged at 3000 rpm for 10 min. at 4°C . The obtained serum was withdrawn and stored at -20°C .

Quantitative Real-time polymerase chain reaction (RT-PCR)

Total RNA was extracted with Trizol Invitrogen (Carlsbad, California, USA) according to manufacturer's recommendation. Briefly, 100 mg of tissue were placed in 1 mL of Trizol and homogenized with the tissue homogenizer (Polytron - Fisher Scientific). Quantification of RNA was made by spectrophotometry (NanoDrop 2000 - Thermo Scientific, Waltham, Massachusetts, USA) with 1 μl of each sample. All used samples had the 260nm/280nm ratio [28]. RT-PCR was performed in duplicate for each gene using a CFX 96 real-time PCR detection system (Bio-Rad, San Francisco, Calif., USA). All steps followed the manufacturer's recommendation and stored at -20°C . Reactions were made in 15 μl of solution containing 1 μl of the sample cDNA, 0.5 μl of each primer sequence Forward and Reverse, 5.5 μl of RNase-free diethyl pyrocarbonate treated water and 7.5 μl of the EvaGreen PCR Master mix (dNTP, reaction buffer, TaqDNA polymerase and EvaGreen). The genes analyzed were PGC-1 α , FNDC5 and UCP1. Glyceraldehyde-3-Phosphate dehydrogenase (GAPDH) was used as an endogenous control (Table 1). The relative expression of the quantitative RT-PCR products was determined by the $2^{-\Delta\Delta\text{Ct}}$ method [29].

Table 1:

Gene	Forward Primer	Reverse Primer	Access number
PGC-1 α	GGCCCGGTACAGTGAGTGTT	ATTGCTCCGGCCCTTCTT	NM_031347.1
FNDC5	CTCTTCATGTGGGCAGGTGTC	GCTGGTCTCTGATGCACTCTTG	NM_001270981.1
UCP1	GGCATCCAGAGGCAAATCAGC	CCAATGAATACGCCACGCC	NM_012682.2
GAPDH	GATGCTGGTGCTGAGTATGTCG	GTGGTGCAGGATGCATTGCTGA	NM_017008.3

PGC-1 α : Peroxisome Proliferator-Activated Receptor-Gamma Coactivator 1 alpha; FNDC5: Fibronectin type III Domain-Containing protein 5; UCP1: Uncoupling Protein 1; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase

Western Blotting assay

Protein analyzes were performed by the Western blotting method [30]. Aliquots of gastrocnemius muscle and mesenteric fat (~100mg) were separated by SDS-PAGE gel and transferred to nitrocellulose membrane. Before either procedure, the membrane was blocked with albumin solution 5%. After this, each membrane was incubated overnight at 4° C with appropriate dilutions of primary antibodies, including FNDC5 (#174833 - Abcam®), UCP1 (#23841- Abcam®) and GAPDH (#8245 - Abcam®) and β-actin (#4970 - Cell Signaling Technology®). The membrane was washed in TBST (3 × 5 min.) and then incubated with the appropriate secondary antibody conjugated with HRP for 120 min. at room temperature. Antibody binding was detected by enhanced Super Signal® West Pico Chemiluminescent Substrate (PIERCE, IL, USA), as described by the manufacturer. Blots were scanned (Epson Expression 1600), and the densitometry of protein bands was determined by pixel intensity using Scion Image (Scion Corporation®, Frederick, Maryland, USA).

Elisa Assay

Quantification and measurement of irisin was per-

formed using Irisin enzyme-linked immunosorbent assay (ELISA) Kit (EK-067-16, Phoenix Pharmaceuticals, CA, USA) and micro-reader [20]. 17β-Estradiol dosage was made with the commercial kit according to manufacturer’s specifications (ADI-900-174, Enzo Life Sciences, Farmingdale, New York, USA). Assay sensibility was 10 pg/ mL. All samples were tested in duplicate. Quantification was given in relation to the standard curve using logistic 4-parameter nonlinear regression.

Statistical Analysis

Results were submitted to the Shapiro-Wilk normality test. As variables presented normal distribution, the one-way analysis of variance (ANOVA) was applied. When group averages were different (p ≤ 0.05), Tukey post-hoc analysis was used. Statistical analyses were made using the software SPSS Statistics 17.0. Results are presented in mean ± Standard Deviation (SD).

Results

The results of body weight, left gastrocnemius, mesenteric fat, 17β-Estradiol and uterus mass are presented in Table 2.

Table 2:

Experimental Groups	Sham-Sed	Ovx-Sed	Sham-RT	Ovx-RT
Body Weight (g)	312.71 ± 16.66	373.98 ± 20.70*	311.29 ± 8.75	355.93 ± 15.44* & #
Left Gastrocnemius (g)	1.56 ± 0.14	1.66 ± 0.12	1.65 ± 0.10	1.85 ± 0.10* & #
Mesenteric Fat (g)	2.99 ± 0.064	3.45 ± 0.33*	1.92 ± 0.33*	3.08 ± 0.55&
17β-Estradiol (pg/ml)	34.05 ± 0.28	14.91 ± 0.56*	34.13 ± 0.28	14.63 ± 0.51* &
Uterus Mass (mg)	0.65 ± 0.14	0.09 ± 0.02*	0.62 ± 0.06	0.09 ± 0.02* &

Data are presented as mean ± SD. Sham: Sham operated; Ovx: Ovariectomized; RT: Resistance Training. p ≤ 0.05 compared to the group * Sham-Sed; & Sham-RT; # Ovx-Sed,

Regarding the irisin pathway signaling, the ovariectomy induced lower (p ≤ 0.05) PGC1-α mRNA expression (Figure 1) when compared with Sham-Sed group. The results of gene and protein expression of FNDC5 (Figure 2a and 2b) and irisin concentration (Figure 3) were not significantly different between Ovx-Sed and Sham-Sed groups. The final stepwise performed was UCP1 expression in mesenteric fat (Figure 4a and 4b) that was lower (p ≤ 0.05) in the Ovx-Sed group when compared to the Sham-Sed group in both mRNA and protein expression, respectively.

The groups that performed RT presented higher (p ≤ 0.05) PGC1-α mRNA expression when compared to their sedentary control groups. The Ovx-RT group presented higher (p ≤ 0.05) PGC1-α mRNA expression compared to Sham-Sed groups (Figure 1). FNDC5 mRNA expression (Figure 2a) was higher (p ≤ 0.05) on trained animals compared to their sedentary control groups. The date of FNDC5 protein expression (Figure 2b) was also higher (p ≤ 0.05) on trained animals compared to their sedentary control groups.

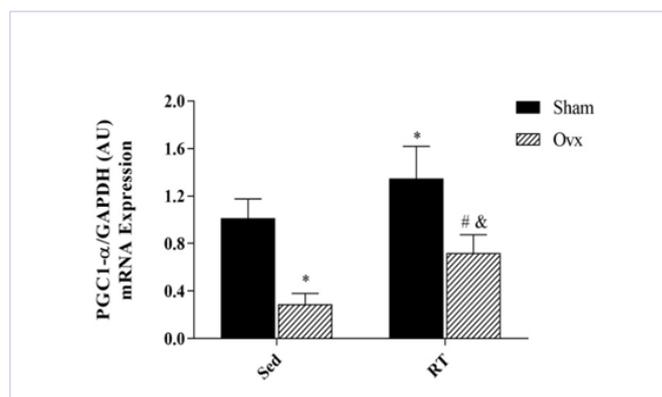


Figure 1: 1PGC1-α mRNA expression in the gastrocnemius muscle relative to GAPDH mRNA expression (n = 6). Values are mean ± SD (p ≤ 0.05). Sham: sham-operated; Ovx: ovariectomized; Sed: sedentary; RT: resistance training. PGC-1α: Peroxisome Proliferator-activated Receptor-γ Coactivator-1α.
*(p < 0.05) compared to Sham-Sed;
& (p < 0.05) compared to Sham-RT;
(p < 0.05) compared to Ovx-Sed.

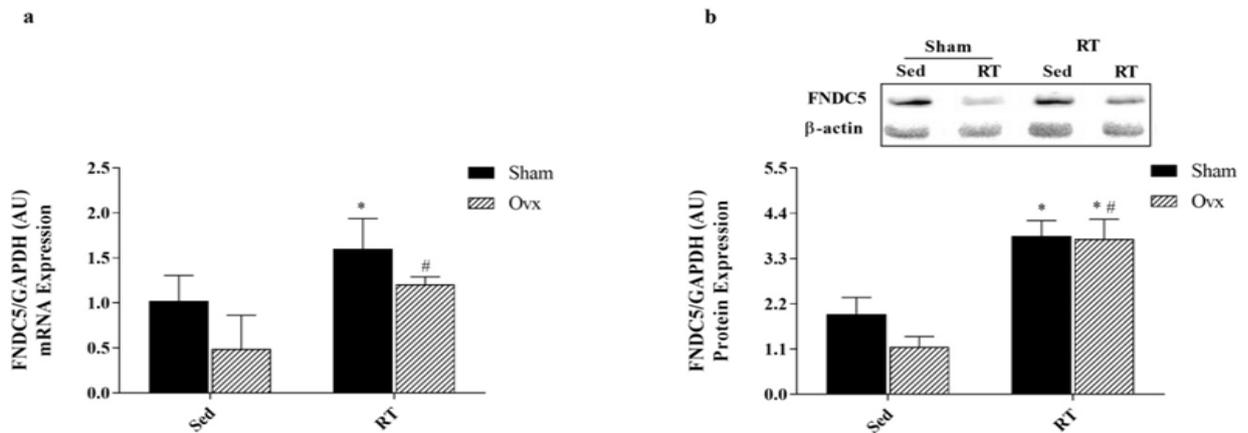


Figure 2: (a) FNDC5 mRNA expression relative to GAPDH mRNA expression (n = 6). (b) FNDC5 protein expression relative to mRNA expression (n = 4) in the gastrocnemius muscle. Values are mean ± SD (p ≤ 0.05). Sham: sham-operated; Ovx: ovariectomized; Sed: sedentary; RT: resistance training. FNDC5: Fibronectin type III domain-containing protein. * (p < 0.05) compared to Sham-Sed; & (p < 0.05) compared to Sham-RT; # (p < 0.05) compared to Ovx-Sed.

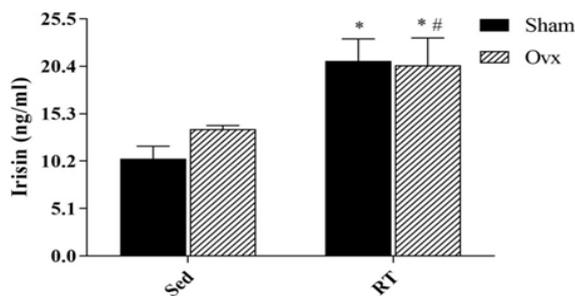


Figure 3: (a) Irisin Levels (ng/ml) in plasma (n = 4). Values are mean ± SD (p ≤ 0.05). (b) Protein concentration bands of FNDC5/GAPDH and UCP1/β-actin. Sham: sham-operated; Ovx: ovariectomized; Sed: sedentary; RT: resistance training. * (p < 0.05) compared to Sham-Sed; # (p < 0.05) compared to Ovx-Sed.

Moreover, the Ovx-RT group had higher (p ≤ 0.05) FNDC5 protein expression compared to the Sham-Sed. Although there was no significant difference between the Sham and Ovx groups for irisin plasma concentration (Figure 3), after 12 weeks of RT both trained groups presented higher (p ≤ 0.05) irisin concentration when compared to their sedentary controls.

When Ovx-RT was compared to Sham-Sed group the irisin concentration was significantly higher (p ≤ 0.05). UCP1 protein expression in mesenteric fat (Figure 4b) presented higher values (p ≤ 0.05) in Sham-RT group compared to Sham-Sed. The

Ovx-RT group presented higher (p ≤ 0.05) mRNA and protein expression of UCP1 compared to the Ovx-Sed group. Finally, Ovx-RT presented higher (p ≤ 0.05) protein expression of UCP1 compared to Sham-Sed (Figure 4).

Discussion

The main finding of the study was that the Ovx group submitted to RT had higher expression of the irisin signaling pathway, indicating the potential benefits of RT as an alternative strategy to control the deleterious effects of menopause. This is the first study to analyze the chronic effects of RT on irisin signaling pathway of Ovx rats.

Ovariectomy

In the present study, the highest body weight was found in the Ovx-Sed group (Table 1). Ovariectomized animals present body weight gain, and these effects indicate that estrogens play a pivotal role in preventing excess body weight gain in females [31-35].

The results on gene expression of PGC1-α in the Ovx-Sed group represent that low estrogen levels could have a negative impact on skeletal muscle energy metabolism as mitochondria are estrogen-sensitive organelles [36]. This result corroborates with the findings of Barbosa, Shiguemoto, et al. which seems to be the first study to present some changes in mitochondrial function in an experimental model of menopause [37]. Our results strengthen the evidence that reduction of estrogen levels is a problem of female physiology that requires further investigation. Thus, our results suggest a link between ovariectomy and reduction of PGC1-α expression in the muscle.

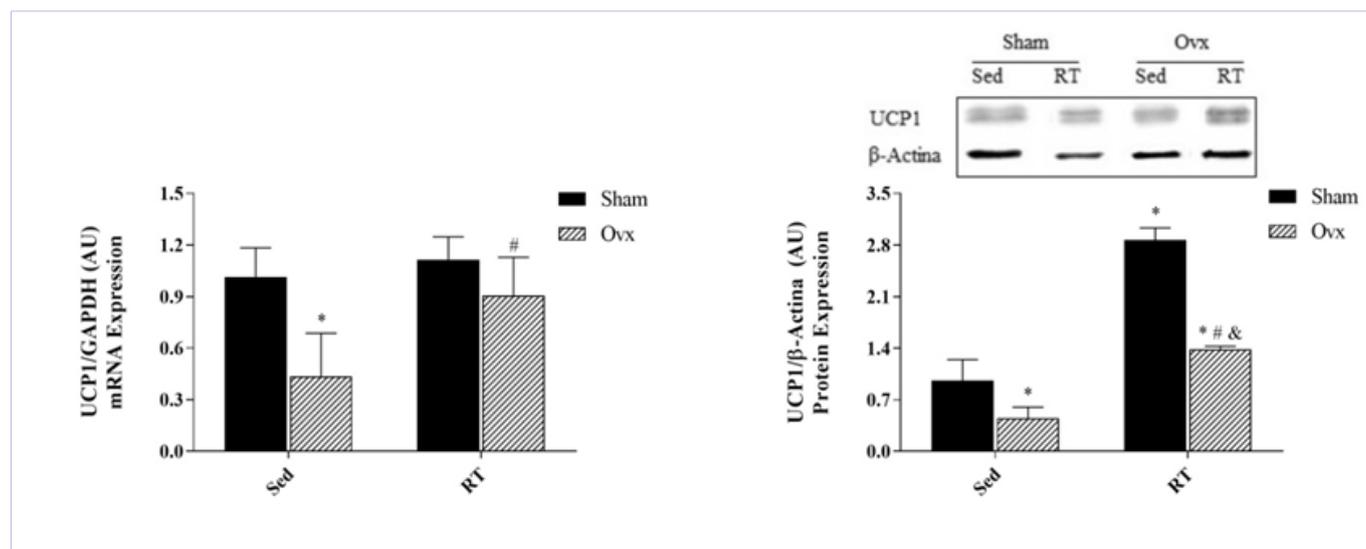


Figure 4: (a) UCP1 mRNA expression relative to GAPDH mRNA expression (n = 6). (b) UCP1 protein expression relative to β -actin mRNA expression (n = 4) in the mesenteric fat. Values are mean \pm SD ($p \leq 0.05$). Sham: sham-operated; Ovx: ovariectomized; Sed: sedentary; RT: resistance training. UCP1: Uncoupling Protein.

* ($p < 0.05$) compared to Sham-Sed;

& ($p < 0.05$) compared to Sham-RT;

($p < 0.05$) compared to Ovx-Sed.

Regarding the results of gene and protein expression of FNDC5 and the levels of irisin in the Ovx-Sed group, we expected lower expression of the entire irisin pathway, however our hypothesis was not confirmed, since the Ovx-Sed group did not present lower expression of FNDC5 and levels of irisin when compared to Sham-Sed group. Tsuchiya, Ando, et al. assume that it is possible that another pathway is involved in this process however, more studies need to be conducted to discover such pathways [38]. FNDC5 has been discovered and characterized by Bostrom, Wu, et al. nevertheless, other modulating factors of FNDC5 are not well known, although studies that analyze this membrane receptor are directly associated to PGC1- α and exercise [16,21]. Irisin is a recently discovered hormone and there is little evidence on factors that may modulate its signaling. Is well established in the literature that RT promotes irisin release, and also increases cellular thermogenesis and reduces fat storage by suppressing formation of new adipocytes [19,20,39-41]. Therefore, maintaining optimal irisin concentrations seems to be important, especially in our ovariectomy model, which has strong evidences of being a precursor of obesity and other metabolic disorders [42-45]. We found that the Ovx-Sed group presented similar values of irisin levels when compared to Sham-Sed group (Figure 3). However, it is already known that irisin increases cellular thermogenesis by up regulating UCP1, thus it is important to analyze and consider the UCP1 concentrations in WAT [41,46].

The results of UCP1 gene and protein expression in mesenteric fat of the Ovx-Sed group (Figure 4a,b) were lower when compared to the Sham group. This result shows part of the deleterious effects caused by reduced estrogen, once UCP1 expression is related to changes in the thermogenesis of WAT [24]. Nowadays, there are no studies in the literature that show these results, however, according to Pedersen, Bruun, et al. ovariectomy

induces lower expression of the UCP1 gene in BAT [47]. Furthermore, our study confirms these findings on the effects of ovariectomy leading to lower UCP1 expression in the WAT of Ovx-Sed rats. Our results show that in the Ovx-Sed group, even in normal irisin concentrations it does not produce the same stimulatory effect of UCP1 expression. As demonstrated in a previous study, the lower expression of UCP1 in brown adipose tissue seems to be associated with reduced levels of estrogen in rats [47]. Our study is the first to demonstrate that despite the fact that irisin levels are not impaired by the reduction of estradiol, UCP1 expression is reduced in Ovx-Sed rats. Thus, we can speculate that the Ovx-Sed group did not have a thermogenic effect of WAT, once body and mesenteric fat weights were significantly greater compared to the Sham-Sed group (Table 2).

Resistance Training

12 weeks of RT were effective in leading to higher expression of the irisin signaling pathway in Sham and Ovx groups. Our study showed that the ovariectomy-induced reduction of estrogen levels leads to lower expression of PGC1- α and UCP1. However, RT yielded significantly higher the expression of PGC1- α as well as UCP1 in Ovx-RT. Our results corroborate with the findings of Barbosa, Shiguemoto, et al. that also reported lower PGC1- α in Ovx rats and their study showed that the reduction of PGC1- α expression are accompanied by significant reductions in genes and proteins as TFAM and NRF1, which favor mitochondrial biogenesis and also improve the thermogenic machinery [37,48-50]. These data represent an important contribution to understand the relation of exercise, higher mitochondrial biogenesis and thermogenic process as important strategy to prevent the effects of ovariectomy and development of some obesity-related dis-

eases. In addition, studies showed that RT increases circulating irisin human and mice. Kim, Lee, et al. demonstrated an increase in circulating plasma irisin in overweight/ obese adults after 8 weeks of RT [19]. In a study with aging mice, Kim, et al. So demonstrated that circulating irisin was significantly increased after 12 weeks of RT [20]. The results of the present study evidence the benefits of RT for metabolic responses when estrogen levels are reduced. We also suggest that although ovariectomy does not interfere with irisin levels, RT is able to enhance the expression of factors related to the irisin signaling pathway in Ovx rats, once UCP1 expression in WAT was significantly higher in Ovx-RT compared to Ovx-Sed.

Conclusion

In summary, our hypothesis that RT would lead to higher expression of the irisin signaling pathway in both Sham and Ovx groups was confirmed. Thus, we conclude that RT seems to be an excellent strategy to counteract ovariectomy-induced metabolic disorders, since irisin signaling was not affected by ovariectomy. In addition, RT promotes alterations at UCP1 of WAT in ovariectomized rats, a crucial final stepwise of the whole irisin signaling pathway.

Acknowledgement

Conflict of interest

The authors do not have any conflict of interest related to this manuscript.

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