The Effects of Resistance and Endurance Training on the Liver Tissue FNDC5 mRNA Gene Expression in Male Rats

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ABSTRACT

Background. The accumulation of excess triglyceride in the liver and a decrease in brown adipose tissue is related to the fatty liver disease. Regular physical activity can take part in regulating fat oxidation and inhibiting fat accumulation by creating and releasing some myokines. Nevertheless, the effectiveness of each training method in this regard is still not certain. Objective(s). This study aims at comparing the effects of resistance and endurance training on FNDC5 (the precursor of irisin myokine) gene expression in male rat liver tissues. Methods. Fifteen Wistar male rats (aged 10-12 weeks with an average weight of 331.8 ±63.09 gr) were used. After being taken to the animals' laboratory, the subjects were randomly assigned to three equal groups viz: control (N=5), endurance (N=5) and resistance (N=5). For the purpose of tissue sampling, all these subjects were anesthetized 72 hours after the experiment had been completed. The liver tissue was immediately removed and then quickly frozen in liquid nitrogen and kept at a temperature of -80° C until RNA extraction. FNDC5 relative gene expression was identified by Real-time PCR method. Results. Data analysis revealed a significant difference in FNDC5 gene expression among the groups (P=0.008, Chi Square=31.791). No significant difference was observed between the resistance training and endurance training groups in FNDC5 gene expression, though (P=0.959). Conclusion. It can be established that both resistance and endurance training can similarly affect the production and secretion of exercise-induced myokines, including FNDC5 irisin, which can in turn protect against obesity and its impacts on the liver tissue by stimulating the process of browning of white fat.

KEY WORDS: FNDC5, Liver, Resistance Training, Endurance Training.

INTRODUCTION

Mammals provide the energy needed for metabolic functions by storing several macromolecules, such as carbohydrates, proteins and fats as fuel. Organs involved in fuel storage, such as the liver, the skeletal muscle and adipose tissues all communicate with each other and determine fuel selection and delivery under the strict control of neuroendocrine system (1). The liver stores energy as glycogen and stores digested triglycerides in the form of fatty acids (2). On the contrary, the accumulation of excessive triglyceride in the liver can result in hepatic steatosis (3). Adiposity can be seen either in the form of white adipose tissue or
brown adipose tissue. White adipose tissue, the physiological area of triglyceride storage, accounts for a major part of fat in humans. Conversely, brown adipose tissue is the only source of energy expenditure and thermogenesis without trembling in small mammals and human infants (4). In fact, the main function of the brown adipose tissue is energy expenditure, which mostly takes place in an increased expression of the uncoupling protein 1. This protein triggers uncoupling in mitochondrial respiration, promoting energy loss in the form of heat. On the contrary, brown fat appears to be protective for metabolic diseases and its mass increases according to cold exposure or physical activity (5).

Physical exercise stimulates the release of myokines which are soluble factors released by skeletal muscle in response to muscle fiber contraction showing auto-, para-, and endocrine functions. These myokines have been shown to act as messengers among skeletal muscle, liver, adipose tissue, heart, brain, and blood vessels (5). A large list of molecules have been recently considered as myokines, such as irisin, fibroblast growth factor-21 (FGF21), interleukin-6 (IL-6), IL-7, IL-8, IL-15, leukemia inhibitory factor (LIF), myonectin, myostatin, vascular endothelial growth factor (VEGF), brain-derived neurotrophic factor (BDNF) and follistatin-like 1 (FSTL1) (5-7).

Irisin, a 112-amino acid protein (12587 kDa), is a thermogenic protein that triggers energy consumption by converting white adipose tissue to brown adipose tissue. Irisin is a cleavage product that is produced when fibronectin domain containing 5 (FNDC5) is cleaved (8). It was initially discovered and characterized by two independent groups in 2002. In 2011 FNDC5 was introduced by Boström and colleagues as the precursor of irisin (9). The expression of FNDC5 has been traced in the heart, brain, ovary, testicles, kidneys, rectum, epiglottis, ovixiduct, adrenal, tonsil, esophagus, vena cava, hypophys, retina, thyroid, small intestine, spinal cord, skeletal muscle and the liver, which has also attracted lots of attention (8, 10, 11). One of the target proteins of PPARγ coactivator-1α (PGC1α) is FNDC5 (6). PPARγ are nucleonc receptors that function to modify the expression of the genes involved in lipid and glucose metabolism and have also been referred to as atherosclerosis inhibitors (12). Conversely, recent groundbreaking experiments in mice indicate that the transcriptional coactivator, PGC-1α, which promotes biogenesis of mitochondria, drives the expression of FNDC5, which in turn leads to increased brown adipose tissue, increased VO2, insulin sensitivity, and glucose tolerance (13).

Physical exercise is one of the major non-pharmacological technique for controlling frequently encountered metabolic disorders; yet the mechanisms which enact exercise-induced effects remain elusive. It was suggested that during muscular exercise, peroxisome proliferator-activated receptor-gamma (PPAR-γ) coactivator-1α (PGC1α) expression is increased in the muscle [3]. This induces the expression of fibronectin type-III domain-containing 5 (FNDC5), which is then cleaved and secreted as irisin (14). This can trigger energy hemostasis and metabolism, improve obesity and glucose intolerance, which is why it is known as a beneficial factor in recovering from disease (2). Physical activity is the basis of change in lifestyle which is of great importance for patients with non-alcoholic fatty liver disease (15). A recent research in this regard made it evident that physical activity can result in the lowering of liver osteotasis. The co-activator of this lowering is unknown, but it was recently shown that the levels of plasma irisin is inversely related to the triglyceride content of the liver and it was suggested that irisin was involved (16).

In fact, irisin is known as an exercise-induced myokine (17) and several studies have documented the relationship between FNDC5/irisin expression and intensity, duration and type of exercise (11, 18, 19). The expression that irisin is an exercise-induced myokine dependent on PGC-1α is based on the research carried out by Boström et al. (2012), who managed to demonstrate that the levels of FNDC5 mRNA in mice’s skeletal muscle had an increase of about 2.8 times after 3 weeks of optional training on the treadmill. Furthermore, they reported that FNDC5 expression co-occurred with an increase in PGC-1α mRNA (20).

In spite of extensive examinations of the metabolic functions of this novel protein, few

Rashidlamir, A., Hoseinzadeh, M., Zeiaddini Dashtkhaki, L. (2017). Ann Appl Sport Sci, 5(2): 51-60.
studies have examined the mechanisms by which acute exercise/muscle contractions increase FNDC5 expression (21). Therefore, it remains unclear which kind of exercise protocol, intensity or duration, if any, would be optimal to increase the circulation of serum irisin levels in the general population (22). Specifically, the comparison of different exercises and their correspondent influence on FNDC5 expression has, thus far, led to no reliable results, particularly in terms of the liver as a major glucose-regulating organ and adipocyte metabolism. All the same, the current study seeks to compare the effects of two protocols of endurance training and resistance training on the liver FNDC5 gene expression in male rats.

**MATERIALS AND METHODS**

**Animals.** This is an experimental study in which 15 male rats (Wistar race, 10-12 weeks old with an average weight of 331.8± 63.09 gr) were equally allocated into three groups of control, aerobic exercise and resistance (5 in each group). These rats were kept in controlled light conditions (12 hours of dark after 12 hours of light), temperature (22±1 degrees centigrade) and humidity (50%) in transparent polycarbonate cages meant for rodents and they had free access to food and water. All animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985).

**Exercise Training Protocol.** Before the commencement of the training protocol, the subjects spent a week getting acquainted with the procedure. The familiarization process for the aerobic group included 5 sessions of walking on the treadmill at a speed of 15-20 meters per minute and a zero slope which lasted for 20-40 minutes. For the resistance group, it comprised 5 sessions of climbing a one-meter-high ladder with a slope of 60-80 degrees. This is followed by the attachment of the clipper and the connected weight to the rat’s tail.

Subsequently, the endurance group performed the aerobic exercise, which was made up of 8 weeks’ (5 days a week) running at a speed of 20 m/min for 60 minutes in each session. The first 10 minutes was allocated for warmup and the final 10 minutes to cool down at a speed of 15 m/min (23).

The resistance training was carried out in 3 sessions per week for 8 weeks. During the first week, the weight connected to each subject’s tail equaled 50% of the rat’s bodyweight, and it had a 10% increase per week. The training was done on a one-meter-high ladder so that a weight was attached to the clipper on the rat’s tail, and the animal had to climb up the ladder. This was done in three rounds and repeated five times. The rounds’ intervals were 3 minutes and the interval for repetitions was 1 minute. This training method was adapted from reliable sources (24, 25). The control group performed no exercise during this period.

**Tissue Biopsies.** 72 hours after the final exercise session, the animals were anaesthetized in the specialized sampling site (sterile space) with a combination of ketamine (30-50 mg for each kg of weight) and xylazine (3-5 mg for each kg of weight) (25). When anesthesia was ensured via not backing of the leg on touch, a rupture was made on the skin in the abdominal region and when the liver appeared, tissue sampling was conducted. The sample was then immediately cleansed using cooled physiological serum and inserted in a microtube and then frozen in liquid nitrogen. Liver samples were then transferred to a freezer at a temperature of −80 °C.

**RNA, cDNA Synthesis and Real-time PCR.** In order for mRNA to be taken, the tissue was washed twice with warm PBS and then placed in 1.5 microliter microtubes. 1 ml of Tripure was added to each tube. The tissue was pipetted severally for the cells to be perfectly lubricious. The homogenized samples were kept at room temperature for 5 minutes. Then 200 microliters of chloroform was added to each tube and they were thoroughly shaken. The tubes were incubated for 15 minutes and then centrifuged at a speed of 12000 g at a temperature of 4°C for 15 minutes. The topmost phase was smoothly transferred to another sterile microtube and 500 microliters of isopropanol was added and reversed several times. The samples were incubated at room temperature for 10 minutes. The samples were centrifuged at a speed of 12000 g at a temperature of 4°C for 10

Rashidlamir, A., Hoseinzadeh, M., Zeiaddini Dashtkhaki, L. (2017). Ann Appl Sport Sci, 5(2): 51-60.
54

Effects of Resistance and Endurance Training on Liver FNDC5 mRNA

Rashidlamir, A., Hoseinzadeh, M., Zeiaddini Dashtkhaki, L. (2017). Ann Appl Sport Sci, 5(2): 51-60.

minutes. The topmost solution was discarded and 1 ml of ethanol 75% was added. The RNA plate was washed in ethanol by vortexing and the samples were centrifuged at a speed of 7500 g for 5 minutes. The topmost solution was discarded and was allowed for the RNA deposit to become semi-arid. This deposit was dissolved in 20 microliters of DEPC water. This solution was incubated at a temperature of 55°C for 15 minutes and immediately subjected to –80 °C. Subsequently, by the means of a cDNA synthesis kit (BIONEER, Korea), cDNA was synthesized from the RNA. The nucleotide primer sequences of FNDC5 gene (25) and the reference gene (26) are given in Table 1. Real Time PCR was run to measure the level of gene expression and was programmed with an initial step of 5 mins at 95°C (1 cycle), followed by 40 cycles of 15 seconds at 95°C and 30 seconds at 60°C. The parameter for melting curve program was 72°C for 30 seconds.

| Gene    | Forward and reverse | Forward and reverse primer sequences | Annealing temperature |
|---------|-------------------|-------------------------------------|-----------------------|
| FNDC5   | F                 | 5′-GTCTCCCACCACCACTTCTTT-3          | 63                    |
|         | R                 | 5′-TCTGCTCTGAGTGAGCCCTTAC-3         | 63                    |
| GAP.DH  | F                 | 5′-GTCGACGCTCCTCCTAGTCATAG          | 60                    |
|         | R                 | 5′-GACTGTGCCTGTTAGAATTGC           | 60                    |

After the completion of the reaction, the threshold cycle (Ct) was calculated for each sample. The proportion of the gene Ct to that of the housekeeping gene gave the level of gene expression via the method 2-ΔΔCt by means of the following equation (27): the Ct for each sample’s housekeeping gene is subtracted from the Ct for that very sample’s gene.

(ΔCt= Ct Target- Ct Housekeeping)

As for the next step, ΔCt for each sample is subtracted from that of the sample with which it will be compared with, and the negative result is applied as the power of two so that the relevant gene expression is achieved.

(ΔΔCt= ΔCt Target - ΔCt Reference)

E= 2-ΔΔCt

Statistical Analysis. Shapiro Wilk test did not yield a normal distribution of data. Thus, to make a comparison of the mean of the groups, Kruskal Wallis non-parametric test was run. The level of significance was set to P≤0.05. The data was analyzed using SPSS 16.

RESULTS

Results of a Shapiro Wilk test revealed no normal distribution of data and Kruskal Wallis test demonstrated a significant difference in FNDC5 gene expression among the groups (P=0.008 and Chi-Square= 9.27). A subsequent LSD test revealed a significantly higher FNDC5 gene expression of the liver both in the endurance and resistance groups than in the control group (P<0.033 and P<0.030, respectively) (Figure 1). However, no significant difference was found in FNDC5 gene expression between the aerobic exercise group and the resistance training group (P=0.959).

Figure 1. Real-Time PCR of Liver FNDC5 Relative mRNA Expression in Control (C), Endurance -Training (END), Resistance – Training (RES) Groups, in male Rats. Data expressed as mean ± SEM. Each column is for each group including 5 rats.

Table 1. Oligonucleotide Primer Sequences and Real-time PCR Amplification Parameters Temperature

| Gene    | Forward and reverse | Forward and reverse primer sequences | Annealing temperature |
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| FNDC5   | F                 | 5′-GTCTCCCACCACCACTTCTTT-3          | 63                    |
|         | R                 | 5′-TCTGCTCTGAGTGAGCCCTTAC-3         | 63                    |
| GAP.DH  | F                 | 5′-GTCGACGCTCCTCCTAGTCATAG          | 60                    |
|         | R                 | 5′-GACTGTGCCTGTTAGAATTGC           | 60                    |
DISCUSSION

The findings of this study concerning the level of liver NDC5 gene expression revealed a significant increase of that gene in response to endurance and resistance training.

Recent studies showed that FNDC5 was also expressed in other tissues such as heart, adipose tissue and liver, which indicates supplementary functions of this hormone (3). The liver stores energy as glycogen and stores digested triglycerides in the form of fatty acids (2).

Generally, a small amount of the fat and carbohydrates coming from the meals is stored in the liver. Lipolysis (found in the adipose) and lypogenesis in the liver also take part in the liver fat mass storing, but in return, the process of fat disposal maintains this mass at a low level. Nevertheless, if the adipose tissue is unable to store additional free fatty acids, the liver will be transformed into a place where fat can be stored and a lot of fat will be accumulated in the liver (28). Furthermore, changes in the amount of fat produced and fat removal through the oxidation of fatty acids or secretion of lipoproteins rich in triglyceride from the liver take part in this imbalance (29, 30). In cases such as obesity, excessive nutrition intake and resistance to insulin, the balance between adipose collection and fat removal is disturbed, which can result in NAFLD (29). The collection of excess fat in the adipose tissue and the liver causes inflation and metabolic stress, which brings about NAFLD (31). NAFLD can be enhanced through weight reduction and exercise, without medical therapy (2). FNDC5 mRNA/irisin is produced not only in skeletal muscles of humans and animals, but also in other tissues including the liver, though to a limited extent (32-35), which is involved in energy homeostasis and the metabolism of the body; hence, a connector of the skeletal muscle and the other tissues (32, 36). Particularly with respect to the liver, it appears that irisin is produced by the liver cells, Kupfer cells and endothelial sinusoids (34, 35). The function of the liver-derived irisin is not yet known. It may play a local role through the autocrine and paracrine routes and an endocrine role through the circulation system. The irisin in the liver may be related to fat and carbohydrate metabolism and also resistance to liver insulin (37). People with NAFLD often have an iatrogenic lipid profile identifying them with high LDL and low HDL (38). In other words, an important aspect of the disease in such people is an increase in intra-liver triglyceride resources with/without inflation or fibrosis. Intra-liver triglyceride is strongly connected with BMI, waist circumference, high blood pressure, level of insulin and trans aminazes (ALT, AST) (40). HDL cholesterol acts against artherosclerosis through its anti-inflation and control function on the process of cholesterol transfer (40). In accordance with previous research, the indirect correlation between the level of intra-liver triglyceride and irisin and the direct correlation between irisin and HDL can reinforce irisin’s protective potential particularly in people with such chronic diseases as NAFLD, which generate cardiovascular risks (41). Irisin can directly or indirectly prevent the accumulation of triglyceride in the liver. With this regard, irisin may manipulate PPAR-α, which is an instrumental regulator in fat metabolism that modifies fat oxidation through a thermogenesis mechanism (42). Additionally, PPAR-α regulates fibroblast 21 growth factor (FGF21), which can ameliorate liver osteatosis and insulin sensitivity (43). Therefore, irisin can regulate the amount of intra-liver triglyceride through FGF21. Furthermore, a decline in the plasma irisin causes a rise in ALT and AST, signifying that irisin can function as a protective against liver steatosis (16).

The beneficial effects of a balanced diet along with physical activity are well-documented in that they comprise the basis of non-pharmacological treatment of cardiovascular and metabolic diseases. Apart from enhancing resistance and strength, physical activity promotes energy expenditure, which reduces the adipose tissue and has protective power against chronic diseases such as obesity and type 2 diabetes. However, the mechanisms leading to these effects are not definitely known (5).

Eight weeks of endurance training and resistance training demonstrated that both protocols can similarly manipulate the expression of the liver FNDC5 mRNA, which was consistent with the previous research (13, 17, 22, 25). But it is inconsistent with Fain et al. (2012), who reported that 16-20 weeks physical training caused no significant effects on FNDC5,
Effects of Resistance and Endurance Training on Liver FNDC5 mRNA

Rashidlamir, A., Hoseinzadeh, M., Zeiaddini Dashtkhaki, L. (2017). Ann Appl Sport Sci, 5(2): 51-60.
the most probable mechanism that increased the expression of PPARα. PPARα is a member of the family of PPAR ligand-activated receptors, which play roles in the control of lipid and glucose metabolism. Over-expression of FNDC5 increases the expression of PPAR mRNAs in white adipocytes up to 3-fold. Furthermore, the pharmacological inhibition of PPARα stops fat browning, signifying a possible role of PPAR in mediating the effects of FNDC5 (5). This relationship between irisin and PPARα signaling is very interesting, given that this signaling pathway is known to play a vital role in hepatic β-oxidation. Irisin may modulate the PPARα signaling pathway, a key regulator of lipid metabolism that coordinates fat oxidation through a thermogenesis mechanism (5). Previous studies have reported an increase in PPARα following physical training (26, 49, 50). It seems practicable that endurance training and resistance training are beneficial in adipose metabolism by elevating the expression of FNDC5 and the subsequent increase in PPARα expression stimulation. However, PPAR gene expression was not measured in the present study.

One possible scenario concerning the effect of long-term resistance training on body composition is that these exercises create signals which activate PGC-1α in the skeletal muscle and this, in turn, induces the expression of FNDC5. Irisin operates on white adipose tissue and elevates UCP1, which signals an increased thermogenesis and energy expenditure (2, 25). However, as there is no established relationship between FNDC5 and PGC-1α alterations, some studies have suggested the possibility that there may be other factor(s) involved in the regulation of FNDC5 expression, with PGC-1α being the only major regulator of energy metabolism (21).

These contradictory findings allow for an alternative path for the regulation of FNDC5 expression. Mo et al. (2015) showed that a novel path regulates FNDC5/irisin expression through CAR in the mouse liver. It is revealed that FNDC5/irisin is the direct target of CAR transcription. CAR (constitutive androstane receptor) is a member of nucleionic receptors that induces FNDC5 expression in the mouse liver and increases serum irisin. In other words, CAR is a xenobiotic sensor that controls the expression of genes involved in the metabolism of exogenous molecules and endogenous compounds such as bile acids, bilirubin and thyroid hormones. Activation of CAR inhibits hepatic steatosis by repression of lipogenesis and increases fatty acid β-oxidation. Activation of CAR also inhibits diet- and genetic-induced obesity and insulin resistance by reducing the expression of glucose-6-phosphatase (G6p) and phosphoenol pyruvate carboxykinase (Pepck), two key enzymes in gluconeogenesis (3).

Mo et al. (2015) elucidated the involvement of this pathway in the regulation of FNDC5/irisin expression and also determined the physiological role that this hormone plays in glucose and adipose hemostasis. As a result, they explained the role of irisin in adipose and hepatic glucose metabolism, providing a potential treatment target for metabolic diseases. It is important that FNDC5/irisin regulation was reported in mouse liver, but its expression was not changed in skeletal muscle or adipose tissue. According to prominent studies, CAR is mainly expressed in the liver and intestine (3).

Generally, the metabolic function of irisin is known to be increasing energy expenditure and glucose hemostasis. Thus, it is possible that FNDC5 and irisin are increased in response to endurance training and aerobic training, which can be noticed by the increase in oxidation and metabolism (22). Another beneficial exercise-induced effect of FNDC5/irisin is that irisin could inhibit the synthesis of hepatic cholesterol via AMPK-dependent inhibition of SREBP2 and its downstream target genes. Irisin increases the phosphorylation of AMPKα in hepatocytes. Blockage of irisin-induced AMPK activation by compound C or knockdown of AMPKα1 reverses the suppressive effects of irisin on: 1) hepatic cholesterol synthesis; 2) mRNA expression of SREBP2 and its downstream target genes crucial for cholesterol synthesis; and 3) nuclear translocalization of SREBP2. Irisin inhibits the synthesis of hepatic cholesterol via the mechanism dependent of AMPK-SREBP2 signaling. These findings may give insight in the treatment of diseases related to hypercholesterolemia, such as atherosclerosis (51).

In summary, previous research support that training is an influential factor in the expression of...
of genes involved in energy metabolism (20). With respect to different physical trainings, metabolic adaptations originating from aerobic training have attracted the most attention; however, the potential value of resistance training in health and fitness have also been recognized. The significance of such training is that resistance training can, through the medium of a similar cell path, lead to adaptations similar to those that occur in endurance training while maintaining or increasing total muscle mass (25).

**CONCLUSION**

As it was shown here, resistance and endurance training can influence the liver FNDC5 gene expression similarly, considerably increasing liver FNDC5 mRNA in comparison to the control group. Thus, it is apparent that performing either aerobic or resistance training can stimulate the process of browning of white fat and improve the effects of fat amassment on the liver tissue. However, further research needs to be conducted so that the mechanisms involved in the effectiveness of different trainings on gene expression and irisin co-activator are fully comprehended and more helpful and efficient training protocols are put forward.

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Rashidlamir, A., Hoseinzadeh, M., Zeiaddini Dashtkhaki, L. (2017). Ann Appl Sport Sci, 5(2): 51-60.
Effects of Resistance and Endurance Training on Liver FNDC5 mRNA

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