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Cardiac, skeletal muscle and serum irisin responses to with or without water exercise in young and old male rats: Cardiac muscle produces more irisin than skeletal muscle

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ABSTRACT

Irisin converts white adipose tissue (WAT) into brown adipose tissue (BAT), as regulated by energy expenditure. The relationship between irisin concentrations after exercise in rats compared humans after exercise remains controversial. We therefore: (1) measured irisin expression in cardiac and skeletal muscle, liver, kidney, peripheral nerve sheath and skin tissues, as also serum irisin level in 10 week-old rats without exercise, and (2) measured tissue supernatant irisin levels in cardiac and skeletal muscle, and in response to exercise in young and old rats to establishing which tissues produced most irisin. Young (12 months) and old rats (24 months) with or without 10 min exercise (water floating) and healthy 10 week-old Sprague-Dawley rats without exercise were used. Irisin was absent from sections of skeletal muscle of unexercised rats, the only part being stained being the perimysium. In contrast, cardiac muscle tissue, peripheral myelin sheath, liver, kidneys, and skin dermis and hypodermis were strongly immunoreactivity. No irisin was seen in skeletal muscle of unexercised young and old rats, but a slight amount was detected after exercise. Strong immunoreactivity occurred in cardiac muscle of young and old rats with or without exercise, notably in pericardial connective tissue. Serum irisin increased after exercise, being higher in younger than older rats. Irisin in tissue supernatants (cardiac and skeletal muscle) was high with or without exercise. High supernatant irisin could come from connective tissues around skeletal muscle, especially nerve sheaths located within it. Skeletal muscle is probably not a main irisin source.

1. Introduction

Skeletal muscle produces an exercise-induced hormone, irisin, which is released into the circulation, especially during or immediately after physical activity. It converts white fat into brown fat, enhancing metabolic uncoupling and hence caloric expenditure [5]. Brown adipose tissue dissipates energy stored in triglycerides as heat via an uncoupling protein, UCP1 [4]. Thus irisin could be a new uncoupling agent (like dinitrophenol) that can deplete the body of ATP and increase heat production [5].

Irisin is a product of a fibronectin type III domain-containing 5 (FNDC5). FNDC5 expression is induced by exercise training, and exogenous FNDC5 induces uncoupling protein 1 (UCP1) expression in subcutaneous white adipocytes [5]. Timmons et al. [23] reported that FNDC5 is induced in muscle in only a minority of subjects, whereas all types of training programs led to enhanced cardiovascular function in the vast majority of people; indeed, plasma irisin was elevated only in highly active elderly subjects [23], and doubles Fndc5 mRNA expression in the muscle of older and obese subjects after a 10-week protocol of endurance exercise [5]. Exercise training in pigs does not increase FNDC5 mRNA or protein in the deltoid or triceps brachii of FHM or normal pigs, while increasing

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circulating irisin only in the FHM pigs [10]. Hecksteden et al. [12] also reported that training does not cause an increase in circulating irisin concentration in subjects exercised 3 times per week for 26 weeks. Furthermore, Raschke et al. [20] showed that FNDC5 mRNA expression in muscle biopsies from 2 different human exercise studies was unchanged by endurance or strength training, concluding that it is unlikely that the beneficial effect of irisin seen in mice will also occur in humans.

If it does, some important questions arise. First, is irisin produced in skeletal muscle, or is cardiac muscle its major source? Second, if irisin is produced in skeletal muscle, why is there not a marked increase in young subjects after endurance exercise as seen in older subjects? Third, on the basis of the reported data, is irisin induction after endurance exercise age-dependent? Fourth, are muscle tissues resistant to injury and release of irisin peptide in young subjects, so that irisin is not released into the circulation after exercise? Fifth, is the question of whether irisin is regulated by exercise to shed more light on the functional aspects of this hormone? And whether the increase in plasma irisin has a different source? The last and most important questions are: what are the major irisin-producing tissues, skeletal muscle, cardiac muscle or others (e.g. liver, kidneys)? And is production also induced by endurance exercise?

As stated above, the exact relationship between irisin concentration after exercise in animals when compared with irisin concentration humans is still under discussion and controversial [5,10,12,18,20,23]. Serum irisin concentration increased with also ages increases with endurance exercise [23]. In this research, we wished to see how irisin concentration changed in young and old rats after exercise. This was hopefully to compare with previously reported irisin increase after endurance exercise in humans. Rats rapidly become sexually mature at ~6 weeks of age [1]. In adulthood, every month is approximately equivalent to 2.5 human years [1].

Based on the above information, we examined first the distribution of irisin in striated muscle tissues (cardiac and skeletal muscles), liver, kidneys, and peripheral nerve sheath tissues by immunohistochemistry, and serum irisin by an enzyme-linked immunosorbent assay (ELISA) in 10 week-old rats (2.5 months old rats) without exercise, and also immunohistochemically screened the distribution of irisin in the striated muscle tissues (cardiac and skeletal muscles) of the young (12 months old rats) and old healthy male rats (24 months old rats) in response to 10 min water floating (exercise). Liver, kidney, cardiac and skeletal muscle tissue supernatant and serum irisin concentration were measured by ELISA in rats with or without 10 min water floating. The goal was to see which of these striated muscle tissues (cardiac and skeletal muscles) are the best irisin producer in 2.5 months old rats without exercise, compared with 12 month and 24 month rats responses with or without 10 min water floating exercise.

2. Materials and methods

Experiments involving the animals were conducted according to the policy of the European convention for the protection of vertebrate animals used for experimental and other scientific purposes in accordance with "Recommendations on the Establishment of Animal Experimental Guidelines," and ethical procedures were conducted under Reduction, Replacement and Refinement (the 3 Rs rule). The study was carried out at the Experimental Research Unit of Firat University (FUDAM). Rats rapidly develop and become sexually mature at ~6 weeks of age. In adulthood, every month of the animal is equivalent to ~2.5 human years. The average laboratory rat lives ~3 years [1].

Sprague-Dawley type male rats (260–380 g) were housed under a controlled temperature of 21 °C (±10%) and a humidity of 65% (±5%) with a 12 h day/night cycle. They were fed standard laboratory chow (commercial standard pellets) ad libitum. Each group composed of six Sprague-Dawley type male rats. Rat at 10 weeks of age were randomly assigned, young rats at 12 months of age (n:6); young rats (n:6) received 10 min water floating (stressful exercise); old rats at 24 months (n:6) received the same treatment. After overnight fasting, rats (except for control rats) were exercised in a cylindrical polystyrene container with water at 24–26 °C as previously reported [24,25]. The control rats were also kept at 24–26 °C for 10 min in the cages.

After exercise, rats were decapitated under ketamine-HCl (75 mg/kg) and 10 mg/kg xylazine-HCl anesthesia. To reduce the effect of circadian rhythm, experiments began at 08.00 AM, and were completed by 11.00 AM. The heart, skin, liver, kidneys and gastrocnemius tissue from each rats were excised, cleaned, divided into 2 pieces [one for immunohistochemistry (IHC)] and the other for homogenization for supernatant), washed in ice-cold saline, and tissue samples for IHC staining were immediately placed in 10% formaldehyde. For tissue homogenate, 100 mg were taken from each tissues and placed a tube that with 500 KIU aprotinin before being carefully homogenized in Phosphate-Buffered Saline (PBS, pH 7.4) solution according to published methods [3]. Tissue homogenizations have done twice. Blood was collected in test tubes, and processed for serum preparation by 5 min centrifugation at 4000 rpm; tissue homogenates were centrifuged at 4000 rpm for 10 min. Tissue and serum were measured using a commercially available Rat irisin ELISA Kit (EK-067-52, Phoenix, USA). Intra-assay and inter-assay were <4–6%, and <8–10%, respectively. The quantitation range was between 0.066 and 1024 ng/mL assay. Irisin concentration in the supernatant was assayed as previously published [2,3].

Irisin expression in the tissues was detected immunohistochemically with Avidin-Biotin-peroxidase Complex (ABC) as per Hsu et al. [13] with minor modification [16], except for the primary antibody step, which was a primary antibody is from Phoenix Pharmaceuticals, Inc., CA, USA (cat no: H-067-17). This was highly specific against the protein being examination and did not cross-react with related proteins. Slides were with hematoxylin and covered with lamella to make them into permanent preparations. They were examined under a light microscope (Olympus BX 50 Olympus Corporation, Tokyo, Japan) and photographed.

2.1. Statistical analysis

Statistical analysis used SPSS (Version 10; SPSS, Chicago, IL). The Kolmogorov–Smirnov test was used for normality of distribution, and the variables were normally distributed. Correlation within groups was calculated using Pearson correlation analysis. Tissue supernatant and serum data are expressed as means ± SD. $p < 0.05$ was considered significant.

3. Results

Close to the ending of 10 min exercise only the head of the rats were above water. Old rats floated less well than young rats floating by the end of each experiment. The minimal irisin detection level in the supernatant of tissues was 0.072 ng/mL (cf. 0.066 ng/mL according to Phoenix's own assay). Dilution of supernatant samples has not been also affected. Intra assay and inter assay were lower than <7 and <10%, respectively. [Note: Phoenix produces 2 different kits to measure irisin, each having a different catalog number and different (intra-assay and inter-assay) detection limits. Thus comparisons of the results with different irisin kits can be invalid.

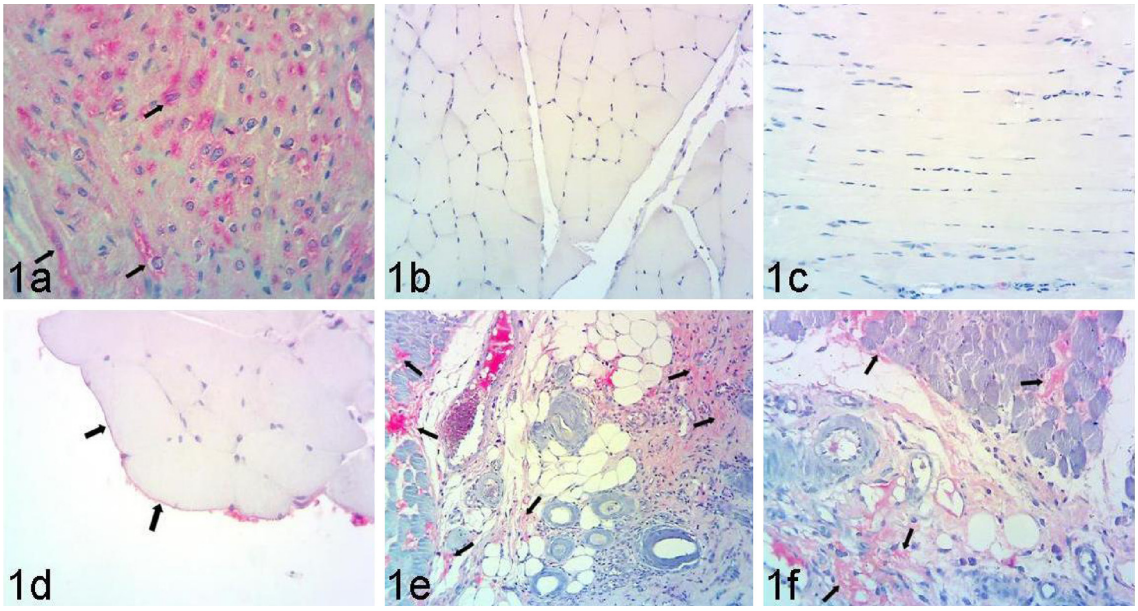

Fig. 1. Irisin immunoreactivity in 2.5 months old Sprague-Dawley male rats without exercise. Strong irisin immunoreactivity in the myocardium (a) but no irisin immunoreactivity in cross (b) or longitudinal (c) sections of skeletal muscle; only trace levels of irisin were observed in the perimysium of the skeletal muscle (d). Significant immunoreactivity is present in the hypodermis and dermis layer of the skin (e and f). Mag. 400×. Arrow shows positive immunoreactivity of irisin.

Irisin in the 10 week rats was intensely localized in the myocardium of the heart tissue (Fig. 1a) but no irisin immunoreactivity in cross sections (Fig. 1b) or longitudinal sections (Fig. 1c) of skeletal muscle; only trace levels of irisin were present in the perimysium of the skeletal muscle (Fig. 1d). Rat skin expressed irisin in the hypodermis and dermis (Fig. 1e and f). Skeletal muscle expressed less irisin than cardiac muscle, only the perimysium showing as much irisin (Fig. 1). Thus it is questionable whether whole skeletal muscle releases irisin and is its highest source.

We have also investigated whether irisin is increased in skeletal muscle (as previously reported [5]) after exercise, or whether it is increased in striated muscles other than skeletal muscle, e.g. cardiac muscle, and which are the highest producers of irisin. The immunohistochemical data are given in the Fig. 2 there was no immunoreactivity in the skeletal muscle of young rats without exercise (Fig. 2a), but +2 severity irisin (black stars) after water exercise (Fig. 2b). Strong immunoreactivity (+3 severity) was seen in the cardiac muscle of young rats without exercise (Fig. 2c), irisin (black arrows) being especially increased in the connective tissues in the vicinity of heart tissue (Fig. 2d). There was also no irisin in skeletal muscle of old rats whether exercised or not (Fig. 2e and 2f). There was a strong immunoreactivity (+3 severity) was observed in the cardiac muscle of young rats with and without water floating (Fig. 2g), and especially irisin immunoreactivity (Fig. 2f) was elevated in the connective tissues in the vicinity of heart tissue after water floating experiments. Increased serum irisin was seen after exercise in young and old male Rats, the increment being more pronounced in young rats than old rat whether these were exercised or not (Fig. 2).

Irisin was also expressed in peripheral myelin sheath (nerve sheath), liver, and kidneys of 10 week rats without exercise (Fig. 3). This shows that irisin (black arrows) was present in the distal tubules of the kidneys (Fig. 3a), in hepatocytes (black arrow), and sinusoidal cells (red arrows) of the liver (Fig. 3b). Interestingly there was significant immunoreactivity (black arrow) in the peripheral myelin sheath of the skeletal section of Sprague-Dawley type male rats (Fig. 3c). These tissues are important sources of irisin. Serum irisin in 10 week rats without exercise was 197 ± 19.3 ng/mL, which was higher than those of young and old rats; the oldest rat had the lowest serum irisin levels (Fig. 4). Thus serum irisin concentration is negatively correlated with age. Irisin concentration in the supernatants of liver, kidney, cardiac and skeletal muscle tissues of young (Fig. 5) and old rats (Fig. 6) increased after exercise, but not markedly so, except in the kidney tissues of old rats after exercise (Fig. 6).

4. Discussion

Irisin was originally reported in skeletal muscle, increasing heat production and circulation after endurance exercise [5]; however, the production of irisin by different tissues and its physiological role have been the subjects of much debate. The initial findings have been questioned [5], primarily because irisin can induce FNDC5 in skeletal muscle after exercise programs, and its other effects have not gained consensus [10,12,18,20,23]. Some evidence supports the report of Boström et al. [5] that FNDC5 increases in highly active elderly subjects, but there has been no confirmation of such a change in young subjects after exercise [23].

The peptide was identified in various tissues of 2.5 months old rats without exercise; these are usually preferably chosen by researchers because of lower cost. Rats at 2.5 months had irisin densely concentrated in the dermis and hypodermis of the skin (Fig. 1). It is also highly expressed in cardiac muscle, but not skeletal muscle, except for low-level expression in the perimysium connective tissue. Dun et al. [9] found that cardiac and skeletal muscle cells express different amounts of irisin. In skeletal muscle fibers, irisin was more densely distributed around the cell membrane, with lower immunoreactivity close to the middle of the muscle cells [9]. Our observations agree with this regarding cardiac muscle, but not in skeletal muscle. Also, the figures in their article were in black and white, making it difficult to compare the results. Location of irisin in the cardiac and skeletal muscle cells was not visible in their published pictures. Here, our colored pictures give direct evidence of the location of irisin expression in skin cells, cardiac muscle and the perimysium of skeletal muscle. Also, the original reports only demonstrated irisin expression in the muscle by western blotting, making for no comparison with our results [5]. Possibly their irisin-positive results for skeletal muscle originated from connective

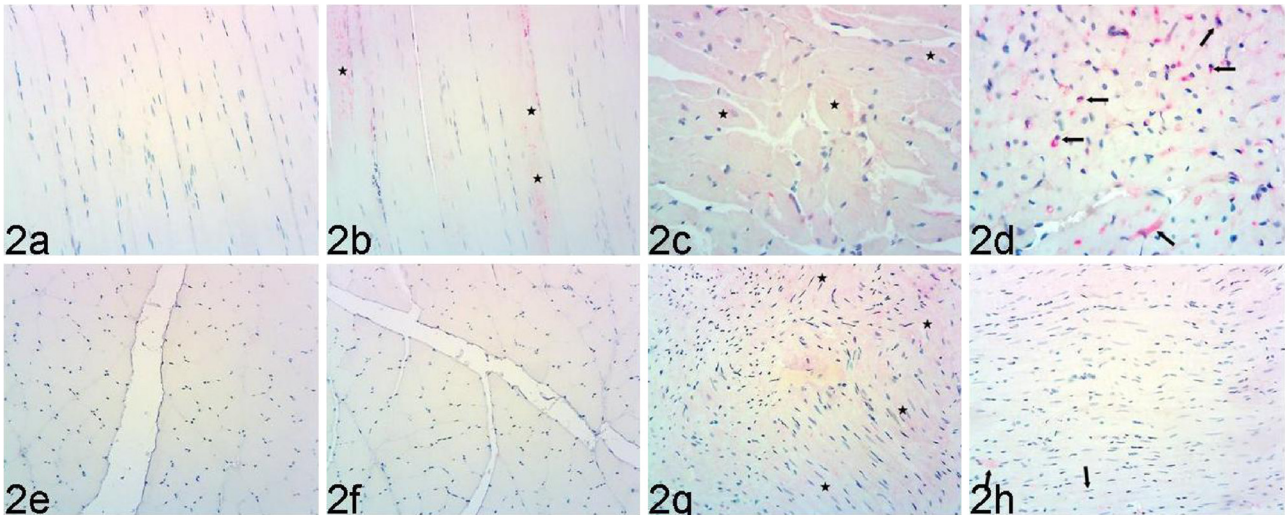

Fig. 2. Irisin immunoreactivity in cardiac and skeletal muscle in young and old Sprague-Dawley rats with and without exercise. No immunoreactivity is seen in the skeletal muscle of young rats without exercise (a), but +2 severity (black stars) was seen after exercise (b). Strong immunoreactivity (+3 severity) is seen in cardiac muscle of young rats without exercise (c) and immunoreactivity (black arrows) is especially increased in the connective tissues in the vicinity of heart tissue after exercise (d). No immunoreactivity is seen in skeletal muscle of old rats whether exercised or not (e and f). Strong immunoreactivity (+3 severity) is seen in the cardiac muscle of young rats with and without exercise (g), and especially so in the connective tissues in the vicinity of heart tissue after exercise (f). Mag. 400×. Arrow shows positive immunoreactivity of irisin.

tissue, most likely the skeletal muscle perimysium and nerve sheaths distributed within skeletal muscle (Fig. 3) [5].

In the second part of our study, we measured the effect of exercise on young and old Sprague-Dawley male rats. There was no immunoreactivity in the skeletal muscle of young (12 months) and old (24 months) rats whether exercised or not, as in skeletal muscle tissues of 2.5 month rats without exercise. The only exception of weak irisin immunoreactivity was in the skeletal muscle of young rats after exercise. In contrast, our ELISA results (skeletal muscle tissues supernatants) indicate that irisin is present in the skeletal muscle of young and old rats whether exercised or not. Possibly this irisin in the supertanant of skeletal muscle tissues originated from connective tissue, e.g. the skeletal muscle perimysium, the connective tissues in the vicinity of skeletal muscle tissue, and especially from peripheral myelin sheaths, which express irisin abundantly (Fig. 3). With the current technology, it was difficult to obtain pure skeletal muscle, since myelin sheath is distributed within it, and the amounts of connective tissues around skeletal muscle might also be an obstacle to getting pure skeletal preparations. Keeping this in mind, therefore, our present results suggested that all previously recorded irisin expression in the muscle biopsy might be also come from the myelin sheath, not from pure skeletal muscle; previous irisin data in the muscle biopsy might be contaminated with myelin sheath expression. Levels of irisin in muscle biopsy after exercise might be due to an indeterminate amount of myelin sheath originating inside skeletal muscle.

There is a strong irisin immunoreactivity in the cardiac muscle of young rats whether exercised or not, and especially in the connective tissues in the vicinity of heart tissue after exercise. Irisin was also presenting supernatant of the cardiac muscle of young rats whether exercised or not and it was increased in both serum and the supernatants of cardiac muscle of young rats after exercise. A slight increase in cardiac and skeletal muscle tissue irisin in young and old rats was in agreement with Boström et al. [5] who reported that FNDC5/irisin mRNA expression in the skeletal muscle increased after exercise. It also agrees with Lecker et al. [17] who found that FNDC5/irisin in the skeletal muscle increased with high aerobic exercise; however this result was contrary to Raschke et al. [20] who reported that FNDC5 mRNA expression in muscle biopsies from 2 different human exercise studies was not changed by endurance or strength training, as also with Fain et al. [10] who reported that exercise in pigs does not increase FNDC5 mRNA or protein in the deltoid or triceps brachii of familial hypercholesterolemic (FHM) or normal pigs. Exercise in young and old human

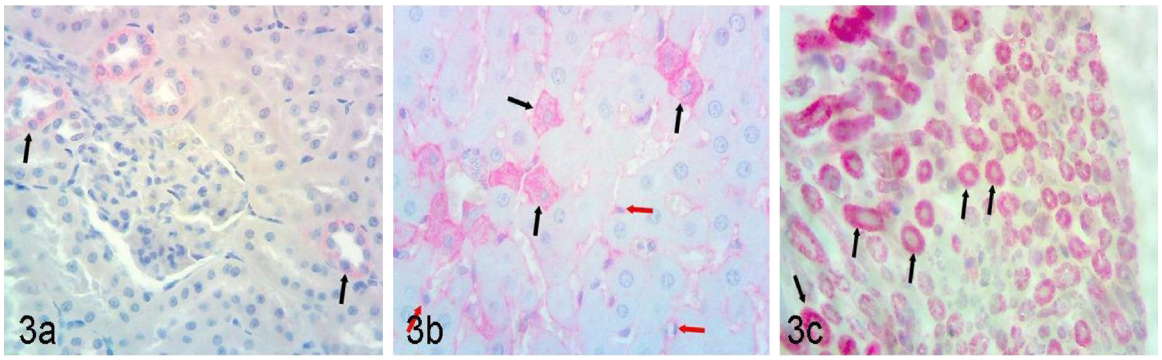

Fig. 3. Irisin immunoreactivity in kidneys liver, peripheral myelin sheath (nerve sheath) in unexercised 2.5 months Sprague-Dawley rats. There was significant irisin (black arrows) in the distal tubules of the kidneys (a); irisin occurred in the hepatocytes (black arrow) and sinusoidal cells (red arrows) of the liver (b). There was significant immunoreactivity (black arrow) in the peripheral myelin sheath of the skeletal section (c). Mag. 400×. Arrow shows positive immunoreactivity of irisin. (For interpretation of the references to color in this legend, the reader is referred to the web version of the article.)

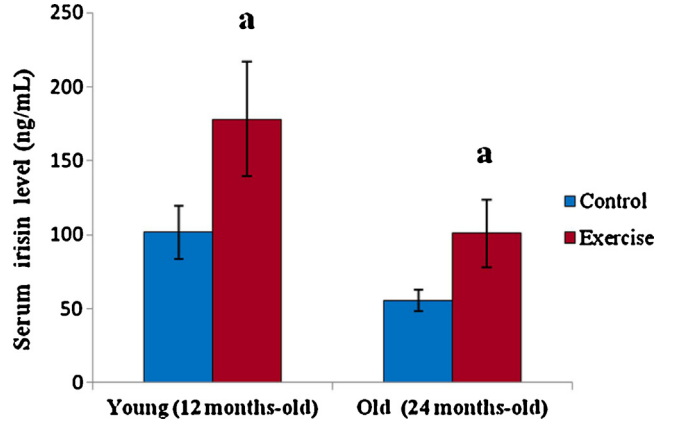

Fig. 4. Serum irisin concentration in young and old young Sprague-Dawley male rats with and without water exercise. Each data point is an average of 3–6 rat sera. [a] $p < 0.05$.

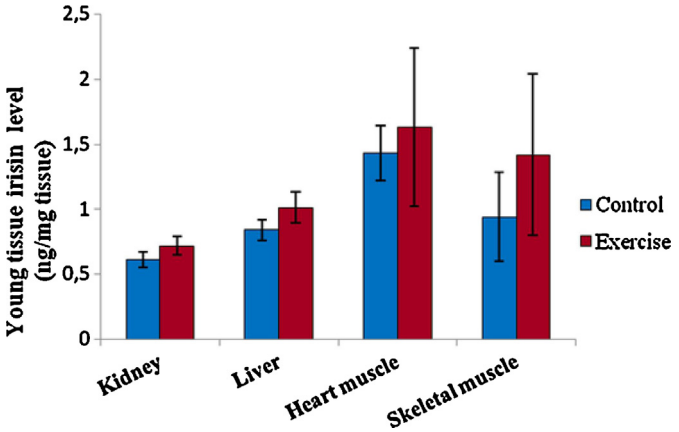

Fig. 5. Liver, kidney, cardiac and skeletal muscle tissue supernatant irisin concentrations in Sprague-Dawley type male rats (12 months old) with and without exercise. Each data point is an average of 3–5 rat tissues.

subjects does not increase FNDC5 mRNA after 6 weeks intense endurance exercise and 20 weeks of supervised resistance exercise [23]. It seems that different species (human, pigs, rats) gives are not comparable with regard to these parameters.

Although both cardiac and skeletal muscles are striated, it is not known why irisin cannot be synthesized in skeletal muscles (except for a small amount in the perimysium). The data are insufficient

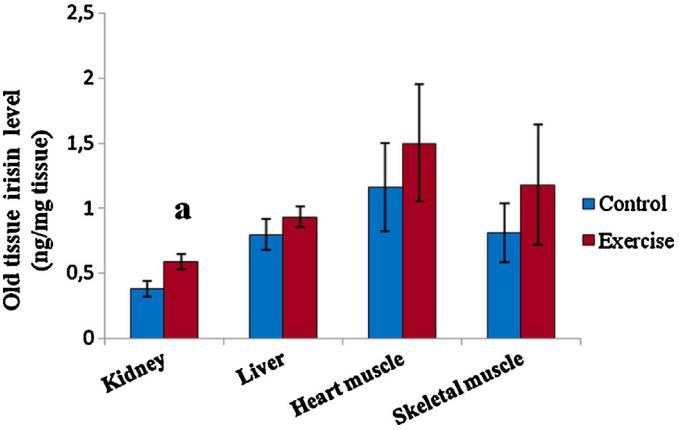

Fig. 6. Liver, kidney, cardiac and skeletal muscle tissue supernatant irisin concentrations in Sprague-Dawley type male rats (24 months old) with and without exercise. Each data point is an average of 3–5 rat tissues. [a] $p < 0.05$.

to explain this, even when the differences between cardiac muscle (involuntarily controlled, loosely bound, semi-spindle-shaped, short, one or two nuclei, endomysium and mitochondria dense, fewer but wider T-tubules, presence of gap junctions) and skeletal muscle (voluntarily controlled, tightly bound, cylindrical in shape, long, multi-nucleated, endomysium and mitochondria not dense, more and narrower T-tubules, no gap junctions, and contraction affecting the entire syncytium) are considered [11]. The data also show another difference between cardiac muscle (one of best irisin producing tissues) and skeletal muscles. We suggest that irisin peptides synthesized in the cardiac muscle could directly oppose the formation of atheromata by stimulating the conversion of white adipose tissue (WAT) into brown adipose tissue (BAT), thereby enhancing metabolic uncoupling and hence caloric expenditure in the heart. Irisin expression in heart muscle might therefore indicate a more marked effect on its physiology than of the rest of the body.

Serum irisin level in young and old rats increased after exercise. The circulation irisin increase agrees with other studies [5,14]. Irisin also upregulates with exercise as with other peptides hormones [15,19]. Serum and saliva irisin concentration was upregulated with heat [2]. Serum irisin concentration might have risen from a number of tissues, including nerve sheath, liver and kidneys, as newly reported in Fig. 4. Irisin also be might transferred to the serum from other tissue, e.g. adipose, salivary gland and skin [2]. Exercise significantly increases ANP and BNP levels in healthy men [19] and short-term aerobic exercise increases post-prandial pancreatic polypeptide in obese individuals [15]. Numerous peptide hormones can be upregulated with exercise [15,19]; and therefore it is possible that irisin is just of many showing the same trend, thereby leading to controversy over its relevance.

Although liver and kidney of Sprague-Dawley rats synthesis irisin, a number of other peptides hormone are also synthesized by them. The function of locally synthesized irisin is currently unknown, but might be related to the regulation of the metabolic pathways, especially carbohydrate and fat metabolism, thereby altering energy metabolism (possibly through paracrine and autocrine action). The fact that peripheral nerve sheath within skeletal muscle tissue synthesizes irisin is particularly interesting, but its significance is unclear. However, we suggested the following: the transfer of a nerve impulse is affected by 3 factors: (1) axon diameter – the larger the diameter, (2) the faster the speed [6,22] saltatory propagation [22]; and (3) temperature – the higher the temperature, the faster the speed [6,22]. Irisin in peripheral myelin sheath (nerve sheath) might increase local heat release when necessary. Since irisin is a mediator of non-shivering thermogenesis in the tissues, this local heating leads to faster nerve impulses and may govern neuronal excitability. Supporting this notion, warm-blooded animals have faster responses than cold-blooded ones [7,21]. Since irisin expression decreases with age, contrary to Timmons et al. [23] who reported that serum irisin concentration increased with also ages increases with endurance exercise, it might be possible that older people have lower responses than young ones. Age-induced decrease in cardiac mitochondrial function has also been reported in healthy rats [8].

In summary, there is presently no explanation for the discrepancies between the study and those of others, which calls for further investigation into these differences, especially as to whether skeletal muscle is a major producer of irisin. Skin connective tissue and cardiac muscle, liver, kidneys and peripheral myelin sheath (nerve sheath) are other major irisin producers, skeletal muscle being only a minor irisin producer. However, contrary to the immunohistochemical results, skeletal muscle homogenate contains abundant irisin. This might be due to irisin coming from the connective tissue, most likely the skeletal muscle perimysium, and nerve sheaths within skeletal muscle. Higher serum irisin and supernatant irisin

concentrations of cardiac and skeletal muscle, liver, kidney tissues have been found in both young and old rats after exercise. A higher serum irisin concentration was found in young rats compared to old rats, i.e. irisin decreases with age. Changes in skeletal muscle concentrations were smaller than in cardiac muscle after exercise. Thus irisin is not significantly upregulated in the supernatants of cardiac and skeletal muscle with exercise, and in other tissues supernatants (except for liver). The failure to replicate strong irisin immunoreactivity in skeletal muscle should lead to further investigations of this finding by others.

Conflict of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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