The effects of acute and chronic exercise on PGC-1α, irisin and browning of subcutaneous adipose tissue in humans

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Irisin was first identified as a peroxisome proliferator-activated receptor γ co-activator-1α (PGC-1α) dependent myokine with the potential to induce murine brown-fat-like development of white adipose tissue. In humans, the regulatory effect of training on muscle FNDC5 mRNA expression and subsequently irisin levels in plasma is more controversial. We recruited 26 inactive men (13 normoglycaemic and normal weight, controls; and 13 slightly hyperglycaemic and overweight, pre-diabetes group) aged 40–65 years for a 12-week intervention of combined endurance and strength training with four sessions of training per week. Before and after the 12-week intervention period, participants were exposed to an acute endurance workload of 45 min at 70% of VO2max, and muscle biopsies were taken prior to and after exercise. Skeletal muscle mRNA for PGC1A and FNDC5 correlated and both PGC1A and FNDC5 mRNA levels increased after 12 weeks of training in both control and pre-diabetes subjects. Circulating irisin was reduced in response to 12 weeks of training, and was increased acutely (~1.2-fold) just after acute exercise. Plasma concentration of irisin was higher in pre-diabetes subjects compared with controls. There was little effect of 12 weeks of training on selected browning genes in subcutaneous adipose tissue. UCP1 mRNA did not correlate with FNDC5 expression in subcutaneous adipose tissue or skeletal muscle or with irisin levels in plasma. We observed no enhancing effect of long-term training on circulating irisin levels, and little or no effect of training on browning of subcutaneous white adipose tissue in humans.

Introduction

The prevalence of obesity and type 2 diabetes is escalating at an alarming rate in many developed as well as developing countries and is basically a consequence of imbalance between energy intake and energy expenditure. This imbalance is probably due to the combined effect of reduced physical activity and enhanced supply of tasty and energy-dense food at an affordable price. When energy intake is carefully controlled, the magnitude of any weight loss is what would be expected given the energy expenditure due to physical activity [1]. Moreover, physical activity protects against several types of disease including cardiovascular disease [2], type 2 diabetes [3], certain types of cancer [4], depression [5], osteoporosis [6] and sarcopenia.

Abbreviations
PGC-1α, peroxisome proliferator-activated receptor γ co-activator-1 α; UCP1, uncoupling protein 1.
This is clearly demonstrated in murine models [14]. The increased expression of PGC-1α is probably a mechanism for modulating metabolic fluxes in skeletal muscle in response to reduced ATP levels and altered fuel demands [10]. PGC-1α can be induced by acute [11] as well as chronic strenuous training [12,13], and PGC-1α promotes many beneficial effects in murine models [14]. This is clearly demonstrated by overexpression of PGC-1α in murine skeletal muscle causing fibre-type switching, enhanced fatty acid oxidation, mitochondrial biogenesis and angiogenesis [15–17].

Many adult humans have metabolically active brown adipose tissue with the ability to directly transfer energy from nutrients to heat [18–20] via uncoupling protein 1 (UCP1) allowing free flux of protons through mitochondrial membranes in brown adipocytes [21]. Appearance of brown adipose tissue is especially sensitive to low ambient temperatures, promoting high uptake and combustion of glucose and fatty acids [18–20], and human brown adipose tissue activity is known to correlate negatively with body mass index [19,22]. Recent data indicate that ‘brite’ fat cells exist within white adipose depots [23–25]. Murine brite fat cells can enhance expression of UCP1, dissipate energy, and turn on heat production equivalent to classical brown fat cells [25]. Thus, activation of brown or brite adipose tissue may be a way to counteract obesity.

Irisin is proposed to be a novel PGC-1α-dependent and exercise-responsive myokine [26]. Irisin is transcribed from the FNDC5 gene and transferred to the cell membrane where it is proteolytically cleaved on the extracellular surface of the muscle cells and released into plasma [26]. Chronic training is shown to enhance irisin production in mice [26], although conflicting results have emerged in humans [26–28]. Whereas Boström et al. [26] showed a twofold increase of circulating irisin after 10 weeks of endurance training, Huh et al. and Pekkala et al. [27,29] found no increase in irisin after 8 weeks of intermittent sprint running or after 21 weeks of combined endurance and strength training, respectively. Timmons et al. [28] demonstrated an induction of muscle FNDC5 in older, but not younger, highly active subjects. However, the study of Timmons et al. has been criticized for including exercise interventions without induction of PGC1A expression in muscle [28]. Thus, an exercise intervention study with chronically increased muscle PGC1A expression would potentially clarify the relation between FNDC5 mRNA in muscle and irisin concentration in plasma. In addition to chronic training, irisin appears to be influenced by a number of phenotypic traits including increased adiposity, lean mass and fasting plasma glucose [28,30–33]. A recent paper by Park et al. [31] showed that circulating irisin level also associates with signs of metabolic syndrome and insulin resistance. Thus, it is of interest if irisin is differentially regulated by training in normal versus overweight subjects with abnormal glucose tolerance.

Irisin is shown to drive browning of subcutaneous white adipocytes in mice [26]. Use of adenoviral vectors to express hepatic FNDC5 promoted enhanced plasma levels of irisin and browning of subcutaneous white adipose tissue [26]. Also culturing of murine primary white adipocytes with recombinant FNDC5 induced a brown-fat-like gene programme [26]. However, Raschke et al. observed no effect of recombinant FNDC5 or irisin on the britening of cultured human primary adipocytes [34]. A potential effect of irisin to induce browning of white adipose tissue in response to chronic training has not been examined in humans.

Our aim was to investigate whether acute and chronic exercise induces transcription of PGC1A and FNDC5 in skeletal muscle and circulating irisin in normal weight and overweight pre-diabetes subjects. Furthermore, we examined if 12 weeks of training may induce browning of abdominal subcutaneous adipose tissue, and if the browning is related to irisin.

**Results**

**Chronic training enhances expression of PGC1A and FNDC5 mRNA**

To investigate the effect of acute as well as chronic exercise, we obtained muscle biopsies and plasma samples before, immediately after, and 2 h post-exercise of 45 min ergometer cycling (70% VO2 max) at baseline and after 12 weeks of combined endurance and strength training. PGC1A transcription was significantly induced after both acute and chronic exercise (Fig. 1A). Chronic training increased PGC1A mRNA expression 1.2-fold and 1.6-fold in the control 

[7]. These beneficial effects of physical activity are probably due to a combination of improved energy balance, reduced adiposity and subclinical inflammation, potentially signalled via proteins released from skeletal muscle (myokines) [8].

Repeated bouts of muscle contraction during physical activity promotes remodelling of muscles and other organs as part of a normal physiological adaptation [9]. One of the exercise-induced adaptations of skeletal muscle might be increased expression of the transcriptional co-activator peroxisome proliferator-activated receptor γ co-activator-1α (PGC-1α) [9].
group and the pre-diabetes group, respectively. At baseline, the PGC1A expression in both groups was about 7.4-fold higher 2 h post-exercise compared with pre-exercise levels (Fig. 1A). After 12 weeks of training, acute exercise increased PGC1A mRNA levels 6.1-fold in the control group and 4.9-fold in the pre-diabetes group (Fig. 1A). The pre-diabetes group exhibited significantly higher PGC1A expression after 12 weeks of training compared with controls (Fig. 1B). Chronic training increased skeletal muscle FNDC5 mRNA expression 1.4-fold in healthy controls (Fig. 1B) and 2-fold in the pre-diabetes group (Fig. 1B). There was no effect of acute exercise on FNDC5 expression during the first 2 h after exercise (Fig. 1B). The pre-diabetes group showed significantly higher FNDC5 expression after 12 weeks of training compared with controls (Fig. 1B). The mRNA expression of PGC1A was strongly correlated with FNDC5 expression both at baseline (Fig. 1C) and after 12 weeks of training (Fig. 1D), and also when calculating fold induction of the gene expression ($r = 0.82$, $P < 0.01$, $n = 26$).

Twelve weeks of training does not enhance plasma levels of irisin

In healthy controls circulating irisin levels showed a transient increase peaking immediately after acute exercise (~1.2-fold) before returning to pre-exercise levels after 2 h rest (Fig. 2). This transient increase in plasma irisin was observed before as well as 12 weeks after training (Fig. 2). A similar effect of acute exercise was observed in pre-diabetes subjects (Fig. 2) but it was only statistically significant after 12 weeks of training (~1.2-fold) in the pre-diabetes group (Fig. 2). Chronic training for 12 weeks tended to reduce plasma levels of irisin in both groups (Fig. 2). When we merged the irisin data from both groups, chronic training significantly reduced the plasma levels of irisin (baseline 160 ng·mL$^{-1}$ versus 12 weeks 143 ng·mL$^{-1}$, $P < 0.01$, $n = 26$). To further evaluate the effect of chronic training on plasma irisin levels, we repeated the measurements on nine control subjects and eight pre-diabetes subjects using a different antibody. Again we found that 12 weeks of training had no effect or
tended to reduce plasma levels of irisin (Fig. S1). Circulating levels of irisin were higher in the pre-diabetes group than the control group at baseline and after 12 weeks of training (Fig. 2). The mRNA expression of *FNDC5* tended to correlate with circulating irisin, both before \((r = 0.32, \ P = 0.11, \ n = 26)\) and 12 weeks after training \((r = 0.35, \ P = 0.08, \ n = 26)\). The mRNA expression of *PGC1A* did not show a significant correlation with plasma levels of irisin \((r = 0.100, \ P = 0.627, \ n = 26)\) at baseline or after 12 weeks of training \((r = 0.187, \ P = 0.361, \ n = 26)\).

**No correlation between expression of UCP1 in subcutaneous fat and circulating irisin**

Because training may induce browning of subcutaneous fat in rodents [26], we investigated the effect of training on human abdominal subcutaneous fat. The mRNA levels of *PGC1A* and *FNDC5* were higher in the control group compared with the pre-diabetes group, both before and after 12 weeks of training (Fig. 3A,B). *PGC1A* and *FNDC5* mRNAs were not significantly enhanced in response to chronic training (Fig. 3A,B). The mRNA expression of *UCP1* was not detectable in six and two of 24 subjects before and after 12 weeks of training, respectively. Although we only detected minimum expression of *UCP1* in subcutaneous fat \((\text{mean } C_t = 36.6 \pm 1.8)\), the expression of *UCP1* tended to increase in both groups after training (Fig. 3C), and significantly increased when data from both groups were combined \((1.82\text{-fold}, \ P < 0.05, \ n = 24)\). Neither the brown-fat-selective gene *PRDM16* nor other known browning genes *TBX1*, *TMEM26* and *CD137* [25] were significantly changed in response to chronic training in the control group or the pre-diabetes group (Fig. 3D,E). When we merged the data from both groups, the mRNA expression of *TBX1* was significantly reduced \((0.7\text{-fold}, \ P < 0.05, \ n = 24)\) and the expression of *TMEM26* was significantly enhanced \((1.24\text{-fold}, \ P < 0.05, \ n = 24)\) in response to 12 weeks of training.

Although we did not see much increase in newly discovered browning genes after 12 weeks of training, we observed an enhanced expression of *UCP1* mRNA. Thus, we asked whether *FNDC5* mRNA expression or irisin levels in plasma were correlated with *UCP1* mRNA levels. Neither *FNDC5* mRNA expression in subcutaneous fat (Fig. 3F) nor skeletal muscle (Fig. 3G) nor irisin levels in plasma (Fig. 3H) were correlated with *UCP1* gene expression before (Fig. S2A–C) or after 12 weeks of training (Fig. 3F–H).

**No effect of caffeine, ionomycin or forskolin on increasing FNDC5 expression in human myotubes**

To further investigate the effect of PGC1-\(\alpha\) on *FNDC5* expression in human skeletal muscles, *in vitro* studies were performed on primary myotubes. Muscle contraction results from a sustained increase in intracellular calcium concentration, which also activates PGC1-\(\alpha\) via the protein phosphatase calcineurin [35]. By increasing cytoplasmic Ca\(^{2+}\) levels, caffeine mimics exercise-induced expression of genes related to mitochondrial function in human myotubes. We performed a dose–response study in primary human myotubes to determine whether changes in *PGC1A* mRNA expression are associated with *FNDC5* expression. Incubation of human myotubes with caffeine for 3 h enhanced *PGC1A* expression dose-dependently up to 5 \(\mu\)M (Fig. 4A). However, we found no effect of caffeine on *FNDC5* expression (Fig. 4A). Thereafter, we incubated myotubes with 5 \(\mu\)M of caffeine for up to 24 h. Once again, *PGC1A* mRNA level increased in response to caffeine (Fig. 4B) with no positive effects on *FNDC5* expression (Fig. 4B). Interestingly, *FNDC5* expression was actually reduced after 24 h incubation with caffeine (Fig. 4B). The PGC1-\(\alpha\) dependent genes [12], *PDK4*, *GLUT4* and *ESRRA*, were increased after incubating human myotubes with caffeine for up to 24 h (Fig. 4C). Whereas PDK4 expression was increased already after 3 h incubation with caffeine, *GLUT4* and *ESRRA* mRNA were increased after 24 h incubation. We next incubated myotubes with the
known PGC1-α inducers ionomycin (1 µM) and forskolin (1 µM) or with a cocktail of the two for 3 and 24 h. Forskolin as well as the forskolin and ionomycin cocktail increased PGC1α expression after 3 and 24 h incubation (Fig. S3A). Ionomycin increased PGC1α expression after 24 h incubation. Neither incubating myotubes with forskolin, ionomycin nor the forskolin and ionomycin cocktail increased the FNDC5 expression (Fig. S3B). Ionomycin and the forskolin and ionomycin cocktail decreased FNDC5 mRNA after 24 h incubation.

**Discussion**

Irisin is a PGC-1α-dependent and exercise-responsive myokine reported to drive browning of subcutaneous white adipocytes in mice [26]. In humans FNDC5 mRNA expression in skeletal muscle and irisin levels in plasma may increase in response to 10 weeks of endurance training [26]. However, other studies have failed to detect a consistent increase in FNDC5 mRNA expression [28] or irisin levels in plasma [27,29] after chronic training. It has been suggested that lack of PGC-1α induction in some of the exercise studies may explain the conflicting results [28].

We present the first human study examining FNDC5 expression in skeletal muscle, irisin levels in plasma, as well as browning of abdominal subcutaneous adipose tissue after long-term training, all samples collected from the same subjects. We observed no effect on plasma concentration of irisin as a chronic exercise-responsive myokine on browning of subcutaneous adipocytes.
white adipocytes in humans. Although we detected increased expression of \textit{PGC1A} and \textit{FNDC5} in muscle after 12 weeks of exercise, we did not detect chronically increased levels of irisin in plasma or a correlation between circulating irisin and \textit{UCP1} mRNA expression in subcutaneous fat.

The effect of chronic training on increasing \textit{FNDC5} mRNA did not translate into increased plasma levels of irisin. We actually detected a reduced level of irisin using two different commercial ELISA kits. Huh et al. also demonstrated a somewhat reduced plasma level of irisin after 8 weeks of intermittent sprint running using an ELISA kit from another vendor (Aviscera Biosciences) [27], whereas Boström et al. showed increased levels of irisin after 10 weeks of endurance training. However, because the antibody used by Boström et al. was made against a peptide corresponding to C-terminal amino acids 149–178 of the human \textit{FNDC5} and does not include any sequence from the irisin peptide [29], their data showing that endurance training increases plasma levels of irisin are highly controversial [34,36]. The reason for different effects of chronic training on \textit{FNDC5} expression in muscle and circulating irisin may be explained by the study of Raschke et al. [34]. They showed that a difference between the human and murine start codon (ATA in humans and ATG in mice) caused most of the translated human \textit{FNDC5} proteins to lose their signal peptides and almost 50% of the irisin sequence so that it was translated with low efficiency in transfected HEK293 cells; this resulted in production of about only 1% full-length protein. Although we used two different ELISA kits with different antibodies, it is possible that we did not detect all the peptide forms translated from the \textit{FNDC5} gene. Future in-depth protein analyses of human plasma are needed to identify all the forms of the \textit{FNDC5} protein which may exist in human plasma.

We were unable to increase \textit{FNDC5} transcription in primary human myotubes using some compounds known to increase PGC-1\textsubscript{\alpha} transcription. Incubating the cells for up to 24 h with caffeine, ionomycin, forskolin or a cocktail of forskolin and ionomycin did not induce \textit{FNDC5} expression. \textit{FNDC5} mRNA was actually reduced after incubating myotubes with caffeine, ionomycin and the ionomycin and forskolin cocktail for 24 h. The transcription of \textit{PDK4}, \textit{GLUT4} and \textit{ESRRA}, which are known to be PGC1-\alpha dependent in cultured murine muscle cells [12], were all increased after 24 h caffeine incubation. One explanation of the discrepancy between \textit{in vivo} and \textit{in vitro} data is that \textit{FNDC5} is not a direct PGC1-\alpha target gene but rather...
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is upregulated in skeletal muscle in vivo via secondary mechanisms. Another option is that caffeine and ionomycin have inhibitory effects on FNDC5 transcription that are stronger than the potential positive effect of PGC-1α. It is also possible that longer incubations with the PGC1-α inducers could upregulate FNDC5. However, this is rather unlikely because FNDC5 mRNA in human myotubes seems to be reduced rather than enhanced after incubation with caffeine or ionomycin.

We observed that the plasma concentration of irisin was acutely increased after 45 min ergometer cycling (~1.2-fold) and then decreased after 2 h rest. This acute increase in circulating irisin in response to aerobic exercise was independent of an increase in FNDC5 mRNA, suggesting that the observed increase of irisin in plasma is caused by increased translation of FNDC5 mRNA in skeletal muscle. Huh et al. showed an acute increase in irisin concentration in plasma after sprint training [27]. However, the acute effect of sprint training on circulating irisin was not seen after 8 weeks of intermittent sprint running [27]. The ATP concentration in skeletal muscle was decreased when circulating irisin was increased, prompting the authors to hypothesize that irisin has a short-term effect to restore ATP homeostasis [27]. On the other hand, acute strength training for up to 30 min did not increase serum levels of irisin [29]. The discrepancy between the studies might be explained by type of exercise, intensity or blood sampling time point.

Production of irisin in other organs than muscle may be important determinants of plasma irisin levels. It is also possible that 12 weeks of training has an effect on other factors such as insulin sensitivity [37], ATP concentration in muscle [27], high-density lipoprotein [33] or adiposity [27,30,32], which might be regulators of circulating irisin independent of PGC-1α. Lean mass, fat mass and plasma concentration of glucose have been shown to correlate positively with plasma concentration of irisin [27,32,37], and a recent study showed that circulating irisin correlated positively with increased risk of metabolic syndrome and insulin resistance as assessed with homeostasis model assessment for insulin resistance (HOMA-IR) [31]. In agreement with these findings we observed higher plasma levels of irisin in overweight pre-diabetes subjects compared with healthy controls. The fact that circulating levels of irisin are increased in pre-diabetes subjects compared with healthy controls, and decreased after chronic training, might be explained by a role of irisin in glucose metabolism. It should be mentioned, however, that irisin levels in subjects with metabolic syndrome/type 2 diabetes are reported to be enhanced [31] as well as reduced [38].

We examined whether 12 weeks of training may induce mRNA expression of UCP1 and other browning genes within subcutaneous white fat in humans, and if a potential effect of exercise on brite fat recruitment could be conveyed by irisin. Although we only detected minimum expression of UCP1 in subcutaneous fat, we did detect an increase of UCP1 mRNA. However, the other genes related to browning of fat like PRDM16, TBX1, TMEM26 and CD137 showed no consistent change. It should be noted that our training intervention started in the fall and ended in the winter, which may give falsely positive effects on brite gene markers due to low outdoor temperatures perhaps capable of inducing brown adipose tissue [18]. However, we did not observe much browning of subcutaneous white fat in response to chronic training, and the level of irisin in plasma could not predict brite fat recruitment. Although we did not find a correlation between plasma irisin with UCP1 mRNA in subcutaneous fat, it is possible that irisin might have paracrine and autocrine effects. However, we did not detect a correlation between FNDC5 and UCP1 mRNA expression in subcutaneous fat. Again our results are consistent with the study of Raschke et al. observing no effect of recombinant FNDC5 and irisin on browning of cultured human primary adipocytes [34]. Moreover, a previous study reported a weak but significant correlation between FNDC5 and UCP1 mRNA expression in visceral and subcutaneous adipose tissue [30]. It is noteworthy that different populations of adipocytes might be affected by different external stimuli to induce browning [39], and human brite fat cells might only be located in specific anatomical depots like the supraclavicular region [25,40]. Thus, an exercise intervention study monitoring browning of different fat depots is needed to further investigate the effect of physical activity on inducing browning of adipocytes, and there was no convincing browning of subcutaneous abdominal adipocytes in the present study. Our finding that 12 weeks of chronic training decreased circulating irisin indicates that a potential browning in specific adipose depots would not be conveyed by irisin.

FNDC5 has been identified as a PGC-1α target gene in mice [26]. Expression of FNDC5 in skeletal muscle was increased in mice with transgenically increased PGC-1α in skeletal muscle and reduced in mice with muscle-specific deletion of PGC-1α [26]. Our present work as well as previous studies have shown a strong correlation between the mRNA levels of PGC1α and FNDC5 in skeletal muscle [27,41], supporting the role of PGC-1α as a regulator of FNDC5 transcription. A
limitation of our present study is that biopsies and blood samples were only taken the first 2 h after acute exercise and later responses may have occurred. Although our data suggest that increased PGC-1α transcription is not translated into irisin production directly, we have to bear in mind that PGC-1α is a co-activator and requires transcription factor and protein regulating histone acetylation for activation of gene expression. The strong correlation between mRNA levels of PGC1A and FNDC5 in skeletal muscle both before and after the training intervention supports that PGC-1α regulates FNDC5 transcription, although the regulation of irisin synthesis from FNDC5 may be the more important step in humans for regulating irisin concentration in blood. Accordingly, we found no significant correlation between mRNA levels of PGC1A in skeletal muscle and irisin levels in plasma using two different commercial ELISA kits, although mRNA of PGC1A correlated with FNDC5 mRNA.

In conclusion, we present the first human study examining FNDC5 expression in skeletal muscle, irisin levels in plasma, as well as browning of subcutaneous adipose tissue after long-term training, and we have demonstrated that, although both PGC1A and FNDC5 mRNA expression was increased in skeletal muscle in response to 12 weeks of training, the plasma concentration of irisin was reduced. There was little or no effect of long-term training on selected browning genes and we observed no correlation between circulating irisin with UCP1 mRNA in subcutaneous adipose tissue.

Material and methods

Cell culture

Primary human myoblasts taken from musculus obliquus internus abdominis of healthy kidney donors were isolated and dissociated as previously described [42]. Myoblasts (at passage 5) were proliferated on collagen I coated dishes in DMEM/Ham’s F12 1:1 (Gibco, Life Technologies, Grand Island, NY, USA; Sigma-Aldrich, St Louis, MO, USA) containing glutamax (Gibco), 50 U·mL⁻¹ penicillin, 50 g·mL⁻¹ streptomycin, 5 mM glucose, 10% fetal bovine serum, 10 µg·mL⁻¹ insulin, 10 ng·mL⁻¹ epidermal growth factor, 2 ng·mL⁻¹ basic fibroblast growth factor and 0.4 µg·mL⁻¹ dexamethasone (Sigma-Aldrich). When the cultures were near confluency, the myoblasts were differentiated into multinucleated myotubes by changing the medium to DMEM/Ham’s F12 1:1 (5 mM glucose) containing glutamax, 50 U·mL⁻¹ penicillin, 50 µg·mL⁻¹ streptomycin and 2% horse serum (Sigma-Aldrich). After 6 days of differentiation, myotubes were incubated with caffeine or a combination of forskolin and ionomycin (Sigma-Aldrich), known to activate PGC-1α transcription.

Ethical approval

The study adhered to the standards set by the Declaration of Helsinki and was approved by the National Committee for Research Ethics, Oslo, Norway (NCT01803568). Informed consent was obtained from all participants.

Strength and endurance training intervention

Healthy and physically inactive (<1 bout of exercise per week the previous year) men (40–65 years) were recruited into two groups: controls with normal weight (23.5 ± 2.0 kg·m⁻²) and normal glucose response or overweight (29.0 ± 2.4 kg·m⁻²) with abnormal glucose metabolism (pre-diabetes group). Abnormal glucose metabolism was defined as impaired fasting glucose ≥5.6 mmol·L⁻¹ and/or impaired glucose tolerance (2 h serum glucose ≥7.8 mmol·L⁻¹). Both groups (n = 26) underwent combined strength and endurance training for 12 weeks, including two endurance bicycle sessions (60 min) and two whole-body strength training sessions (60 min) per week. A 45-min bicycle test at 70% of VO₂max was performed before and after the 12-week training period. In advance a 10-min warm-up at three different workloads was performed, corresponding to 50% (4 min), 55% (3 min) and 60% (3 min) of VO₂max. Measurements of blood lactate and glucose were done at 7.5, 15, 25, 35 and 45 min. VO₂ consumption, respiratory exchange ratio, heart rate and rating of perceived exertion values were measured at 5, 10, 20, 30, 40, 45 min. Encouragement was given during the 70% test. For the post-test a new workload was calculated corresponding to the post-test of VO₂max.

Blood and tissue sampling

Blood and muscle samples were taken before, directly after, and 2 h after the 70% bicycle test, before as well as after 12 weeks of training. A single subcutaneous adipose tissue biopsy was taken 30–60 min after the bicycle session. Blood sampling was performed by standard antecubital venous puncture.

Plasma was immediately separated from blood cells in tubes with added EDTA, by centrifugation at 1400 relative centrifugal force for 10 min at 4 °C, and stored at −80 °C until analysis. In duplicate plasma samples, irisin concentration (catalog EK-067-52, and, when repeated, EK-067-29) was measured using ELISA according to the manufacturer’s protocol (Phoenix Pharmaceuticals Inc., Burlingame, CA, USA). The optical density for the ELISA kits was determined using a microplate reader (Titertec Multiscan Plus; EFLAB, Helsinki, Finland) set to 450 nm. Standard
curves were generated with a four-parameter logistics curve-fitting method (MyAssays.com).

Biopsy samples from *musculus vastus lateralis* were immediately transferred to RNA-later (Qiagen, Hilden, Germany) overnight and stored at −80 °C. Subcutaneous fat biopsies were immediately frozen in liquid nitrogen and stored at −80 °C until further processing.

**Tissue RNA isolation and cDNA synthesis**

Total RNA was isolated from human myotubes with the RNeasy Mini Kit (Qiagen) as previously described [42]. Frozen human muscle biopsy pieces were cooled in liquid nitrogen and then crushed to powder in a liquid nitrogen cooled mortar and pestle. Muscle tissue powder was poured into 1 mL QIAzol Lysis Reagent (Qiagen) and homogenized using TissueRuptor (Qiagen) at full speed for two times 15 s. Total RNA was then isolated using miRNeasy Mini Kit (Qiagen). Frozen adipose tissue biopsy pieces were homogenized using TissueRuptor (Qiagen) at full speed for two times 15 s in 1 mL QIAzol Lysis Reagent (Qiagen), and then total RNA was isolated using RNeasy Lipid Tissue Mini Kit (Qiagen). RNA integrity and concentration were determined using Agilent RNA 6000 Nano Chips on a Bioanalyzer 2100 (Agilent Technologies Inc.).

**Reverse transcription polymerase chain reaction (RT-PCR)**

RNA from fat (200 ng), muscle (500 ng) and primary human muscle cells (550 ng) was reverse transcribed into cDNA on a Gene Amp PCR 9700 thermal cycler with the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). The cDNA reaction mixture was diluted in water and an equivalent of 10 ng RNA from fat and 25 ng from muscle was analysed in each sample. Quantitative real-time PCR was performed with reagents and instruments from Applied Biosystems in the 96-well format using a 7900HT Fast instrument and the SDS 2.3 software (Applied Biosystems). Predeveloped primers and probe sets (TaqMan Assays, Applied Biosystems) were used to analyse mRNA levels of tumour necrosis factor receptor superfamily, member 9 (*CD137* (TNFRSF9), HS00155512_m1), oestrogen-related receptor *α* (*ESRRA*, HS1067166_g1), *FNDC5* (HS00401006_m1), insulin-responsive glucose transporter 4 (*GLUT4*, HS00168696_m1), pyruvate dehydrogenase kinase, isozyme 4 (*PDK4*, HS01037712_m1), *PGC1A* (HS01016719_m1), PR domain containing 16 (*PRDM16*, HS09922674_m1), large ribosomal protein P0 (*RPLP0*, HS99999902_m1), TATA box binding protein (*TBP*, HS00427620_m1), T-box 1 (*TBX1*, HS00271949_m1), transmembrane protein 26 (*TMEM26*, HS00415619_m1) and UCP1 (HS00222453_m1). Relative target mRNA expression levels were calculated as $2^{-\Delta\Delta Ct}$ and normalized to endogenous control *RPLP0* in skeletal muscle and *TBP* in subcutaneous fat.

**Statistical analysis**

Statistical analyses were performed using SPSS 20.0 software. Effect measures are presented as means ± standard error of the means. Statistical evaluation was done by Student’s *t*-tests for paired or unpaired observations. The relation between variables was analysed by bivariate correlation (Pearson’s or Spearman’s test).

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher’s web site:

Fig. S1. Circulating irisin does not increase in response to 12 weeks of training.

Fig. S2. UCP1 expression is not correlated to circulating irisin or FNDC5 before 12 weeks of training.

Fig. S3. FNDC5 mRNA is not induced in human myotubes in response to ionomycin and forskolin incubation.