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The whole body cryostimulation modifies irisin concentration and reduces inflammation in middle aged, obese men

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ABSTRACT

The anti-inflammatory effect induced by exposure to low temperature might trigger the endocrine function of muscle and fat tissue. Thus, the aim of this study was to investigate the influence of the whole body cryostimulation (CRY) on irisin, a myokine which activates oxygen consumption in fat cells as well as thermogenesis. In addition, the relationship between hepcidin (Hpc) – hormone regulating iron metabolism, and inflammation was studied.

A group of middle aged men (n = 12, 38 ± 9 years old, BMI > 30 kg m^{-2}) participated in the study. Subjects were exposed to a series of 10 sessions in a cryogenic chamber (once a day at 9:30 am, for 3 min, at temperature −110 °C). Blood samples were collected before the first cryostimulation and after completing the last one. Prior to treatment body composition and fitness level were determined. The applied protocol of cryostimulation lead to rise the blood irisin in obese non-active men (338.8 ± 42.2 vs 407.6 ± 118.5 ng mL^{-1}), whereas has no effect in obese active men (371.5 ± 30.0 vs 343.3 ± 47.6 ng mL^{-1}). Values recorded 24 h after the last cryo-session correlated significantly with the fat tissue, yet inversely with the skeletal muscle mass. Therefore, we concluded the subcutaneous fat tissue to be the main source of irisin in response to cold exposures. The applied cold treatment reduced the high sensitivity C-reactive protein (hsCRP) and Hpc concentration confirming its anti-inflammatory effect.

1. Introduction

Obesity is a serious health problem in developed countries, and the prevalence of obesity has increased dramatically for several decades [43]. Wide range of co-morbidities such as: insulin resistance, metabolic syndrome, type 2 diabetes, hypertension, chronic kidney disease, cardiovascular disease, heart failure, cancer, and dementia are associated to obesity through the low-grade inflammation [26]. This stage is characterized by an enhancement of the pro-inflammatory cytokines' concentrations, particularly through their release from adipose cells and macrophages [41]. The elevated pro-inflammatory cytokines might stimulate the synthesis of hepcidin (Hpc), which is a disulfide-rich peptide produced by hepatocytes and an iron-regulating hormone [35]. Hepcidin synthesis is also stimulated by high blood iron concentration [12]. An increased level of blood hepcidin inhibits iron transport from the duodenum, thus limiting iron absorption [33]. The rise of hepcidin concentration found in a chronic state of inflammation can be treated as a non-specific host defense strategy, aimed at limiting the availability of iron for microorganisms [32]. Conversely, in overweight and obese people with a low-grade systemic inflammation, a positive association was found between the C-reactive protein and ferritin [4]. Unexpectedly, obese subjects exhibited low iron concentrations, even anemia, in response to elevated hepcidin concentrations [1].

There are few strategies decreasing chronic low-grade inflammation in obese people. Since low grade inflammation can be associated with physical inactivity in healthy subjects [38], physical

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exercise is one of the mechanisms that may be stimulate the anti-inflammatory response and protection against chronic medical disorders induced by this stage [36]. However, in some cases physical activity was almost completely ineffective in reducing systemic inflammation [25]. Thus, there is a need to look for additional methods supporting the anti-inflammatory strategies. Last papers revealed that the whole-body cryostimulation (CRY) is considered as the an alternative and complementary method to both above mentioned strategies for reducing inflammation [16,29,45]. Nonetheless, still physical fitness level might modify the effect of CRY [45].

Data describing the influence of coldness exposure on irisin is limited [24] with the information about the impact of the whole body cryostimulation on this protein completely lacking. Irisin is a recently discovered myokine, secreted into the circulation following a proteolytic cleavage from its cellular form, fibronectin-type III domain-containing 5 (FNDC5) in response to exercise [6]. Its release is induced during exercise in mice and humans, causing an increase in energy expenditure in mice with no changes in movement or food intake. In addition, irisin activates oxygen consumption in white fat cells, and thermogenesis [22]. Consequently, irisin is considered to be beneficial in the treatment of obesity, diabetes, and a wide range of pathological conditions characterized by an imbalance between energy demand and expenditure [22,40]. Recent papers report that irisin negatively correlates with the BMI, waist-hip ratio, and fat mass in men as well as that circulating irisin is lower in non-diabetic overweight and obese men [17,30,39]. Lee and co-workers noted that cold treatment is one of the factors stimulating irisin secretion. Water-infused thermoblankets (from 27 °C cooled to 18 °C and further lowered by 2 °C every 3 min until 12 °C temperature) was found to have stimulated irisin secretion [24]. Hence, irisin seems to be an important link between cold exposure treatment and obesity.

Overall, basing on the current information about changes stimulated by cryotherapy and its efficiency in reducing low grade systemic inflammation in obese men, the aim of our study was to assess the impact of CRY on irisin concentration among obese men at various fitness level. The second purpose of our investigation was to assess the influence of the whole body cryostimulation on hepcidin concentration and iron status.

2. Materials and methods

2.1. Subjects

Twelve obese men participated in the experiment (38.4 ± 8.2 years of age, height of 179.0 ± 6.0 cm). The participants were categorised as obese based on their BMI according to the current guidelines (BMI >30 kg m^{-2}) and visceral fat area above 100 cm^2 [44]. Additionally, the group was divided into two subgroups according to the participants' cardiorespiratory fitness, which was measured in terms of the maximal oxygen consumption (VO_{2max}). Based on the classification proposed by Astrand [3], subjects with VO_{2max} above 35 mL kg^{-1} min^{-1} were assigned to the high fitness level (HFL) group (weight of 93.8 ± 11.5 kg), whereas those with the lower $VO_{2\ max}$ were enrolled in the low fitness level (LFL) group (weight of 116.5 ± 24.8 kg). All the subjects underwent a medical check-up before being submitted to cold exposure. They were also medication-free. The participants had never previously been subjected to any form of cryotherapy. Written, informed consent was obtained from all subjects. All procedures were approved by the Bioethical Committee of the Regional Medical Society in Gdansk NKEBN/245/2009. One week prior to the start of the experiment, body composition and aerobic capacity were determined for each participant. During the experiment, all participants were instructed not to change any aspect of their daily habits, such as diet, and to avoid any form of exercise.

2.2. Body composition assessment

Body mass (BM) and body composition were estimated using a multi-frequency impedance plethysmograph body composition analyser (In Body 720, Biospace Analyzer, Korea). This analyser accurately measured the amount of body water and body composition, including fat mass, free fat mass, skeletal muscle mass and soft lean mass. Additionally, the visceral fat area was determined and expressed in cm^2. The precision of the repeated measurements was expressed as the coefficient of variation, which was 0.6% for fat mass percentage on average [42].

2.3. Cardiorespiratory fitness measurement

To determine the VO_{2max}, the participants performed a graded cycle ergometry test on an electromagnetically braked, cycle ergometer (ER 900 Jaeger, Germany/Viasys Health Care). The participants were allowed a 5-min warm up period at an intensity of 1.5 W kg^{-1}, with a pedalling cadence of 60 rpm. Immediately following the warm-up, the participants began VO_{2max} testing by cycling at increasingly workloads by 25 W min^{-1} until the participant reached the point of volitional exhaustion [20,45]. The recovery was passive, with the participant in a seated position. Breath-by-breath pulmonary gas exchange was measured (Oxycon-Pro, Jaeger -Viasys Health Care, Germany) throughout the VO_{2max} test; the O_2 and CO_2 analysers were calibrated prior to each test using standard gases of known concentrations in accordance with manufacturer guidelines.

2.4. Whole-body cryostimulation

All of the participants were subjected to a series of coldness exposures (once per day at 9:30 a.m.), including 10 sessions in a cryogenic chamber at the Pomeranian Rheumatologic Centre in Sopot, Poland, which were conducted by highly qualified medical staff. Each cryostimulation session lasted 3 min at a temperature of −110 °C. Entry into the cryo-chamber was preceded by a 20–30 s adaptation period in the vestibule at a temperature of −60 °C. The subjects were dressed in shorts, socks, gloves, and a hat covering their auricles. The examined individuals did not participate in any other treatment after whole-body cryostimulation to avoid obscuring the interpretation of the cryogenic effect. Each exposure was preceded by a light breakfast between 07 and 07:30 a.m. according to the instructions given to the subjects [45].

2.5. Blood analysis and collection

Blood samples were taken from the antecubital vein into the vacutainer tubes with EDTAK $_2$, before, 30 min and 24 h after the first and last cryo-session. Immediately following the blood collection one portion of the sample was transferred to centrifuge tubes containing aprotinin (catalog no RK-APRO) from Phoenix Pharmaceuticals Inc. The final concentration of aprotinin was 0.6 Trypsin Inhibitor Unit/1 mL of blood. The samples were centrifuged at 2000 g for 10 min at 4 °C. The separated plasma samples were frozen and kept at −70 °C until later analysis.

Plasma interleukin IL-6 and high sensitivity C-reactive protein (hsCRP) levels were determined by enzyme immunoassay methods using commercial kits (R&D Systems, USA, catalog no. HS600B, DCRP00 respectively). The average intra-assay CV was 8.0% for IL-6 and hsCRP.

Quantification of plasma irisin was based on a competitive

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enzyme immunoassay and the assay kits were purchased from Phoenix Pharmaceuticals Inc (catalog no EK 067-16). Details on the ELISA assay have been described elsewhere [18]. The dilution of sample was applied 1:5. The intra-assay coefficients of variability (CVs) and inter-assay CVs reported by the manufacturer were 4%–6% and 8%–10%, respectively.

The changes in hepcidin concentration and iron status have been determined in the blood samples collected at the baseline and 24 h after last (10 th) cold exposure session had ended. The serum hepcidin levels were determined using a DRG Elisa kit. The analytical sensitivity of the hepcidin kit was calculated by subtracting 2 standard deviations from the mean of 20 replicate analyses of the Zero Standard (SO) and was established to be 0.9 ng·ml^{-1} kit. All data were corrected according to the changes in hematocrit.

Serum ferritin was measured by (SYSMEX XE 2100, Architect ci 8200, and Test 1 SDL). Iron was measured on the Roche Modular System (Roche Diagnostics, Switzerland).

2.6. Statistical calculations

Statistical calculations were performed using STATISTICA 10.0. Results are expressed as the mean, standard deviation (x ± SD). The normality of data was tested using the Shapiro–Wilks W-test. The level of significance was set at 0.05 for all analyses. In order determining the differences in of physical performance and body composition between active and non-active groups U-Mann Whitney test was applied. The differences between obtained data before and after the cryotherapy intervention were analysed using repeated measures of variance (ANOVA) for parametric variables and U-Mann Whitney test for nonparametric ones. Associations among measured parameters were analysed using Pearson's linear regression (coefficient, r).

3. Results

3.1. Baseline data

All participants completed the study, with no adverse events being reported. The basic anthropometric and physiological characteristics of the subjects are summarised in Table 1. The characteristic is presented according to the cardiorespiratory fitness level. All participants were categorised as obese, based on the average BMI of the HFL (30.1 ± 4.0 kg m^{-2}) and LFL groups (35.7 ± 4.5 kg m^{-2}). Nonetheless the free fat mass (FFM kg) and skeletal muscle mass (SMM kg) in HFL group did not differ to LFL group. Interestingly, statistical differences were observed in percentage of skeletal muscle mass between HFL and LFL groups. Also, the absolute fat mass and percentage of fat tissue values exceeded the recommended ranges for subject age. Moreover an elevated amount of visceral fat area (VFA) was observed. HFL group was characterised by significantly lower fat content than LFL group (120.9 ± 13.3 vs 181.5 ± 30.0; p < 0.05).

The average values of irisin recorded in the whole group at the baseline was equal 352.4 ± 40.0 ng mL^{-1}. Any statistical correlations were noted between body composition and irisin concentration before the exposure to CRY. Taking into account cardiorespiratory level, HFL group was characterized by 8.8% elevated irisin concentration at baseline but this was not significant difference to LFL group.

Obtained data confirmed our initial assumption that obese men experienced the low-grade systemic inflammation. Although the values of hsCRP were still in reference range, the average concentration of hsCRP in HFL group amounted to 2.2 ± 0.6 and in LFL 4.4 ± 1.6 mg L^{-1} (p = 0.003) (Fig. 2). All positive correlations between fat expressed in kg and percentage, VFA and hsCRP were statistically significant (r = 0.88, r = 0.83, r = 0.77; p < 0.05

Table 1
Anthropometric and physiological characteristics of participants.

Variable	HFL-group	LFL-group	*P* Value
BMI [kg m^{-2}]	30.1 (4.0)	35.7 (4.5)*	0.04
FFM [kg]	72.9 (±6.4)	69.8 (±7.0)	ns
SMM [kg]	41.7 (±3.8)	39.6 (±4.1)	ns
SMM [%]	41.7 (±3.8)	39.6 (±4.1)	0.05
Fat [kg]	20.9 (±6.2)	46.7 (±18.8)*	0.05
Fat [%]	22.0 (±4.6)	38.9 (±6.4)*	0.05
VFA [cm^2]	120.9 (±13.3)	181.5 (±30.0)*	0.05
VO_{2max} [mL kg^{-1} min^{-1}]	43.2 (±3.4)	26.1 (±1.2)*	0.05

Values are means (±SD); BMI –body mass index, FFM – free fat mass, SMM – skeletal muscle mass, % SMM – percentage of skeletal muscle mass, Fat – fat mass, Fat % – percentage of body fat.
VFA – visceral fat area, VO_2max – maximal oxygen uptake expressed in relatively values HFL(n = 5) high fitness level, LFL(n = 7) low fitness level, * significantly different from HFL group.
ns: non-significant differences.

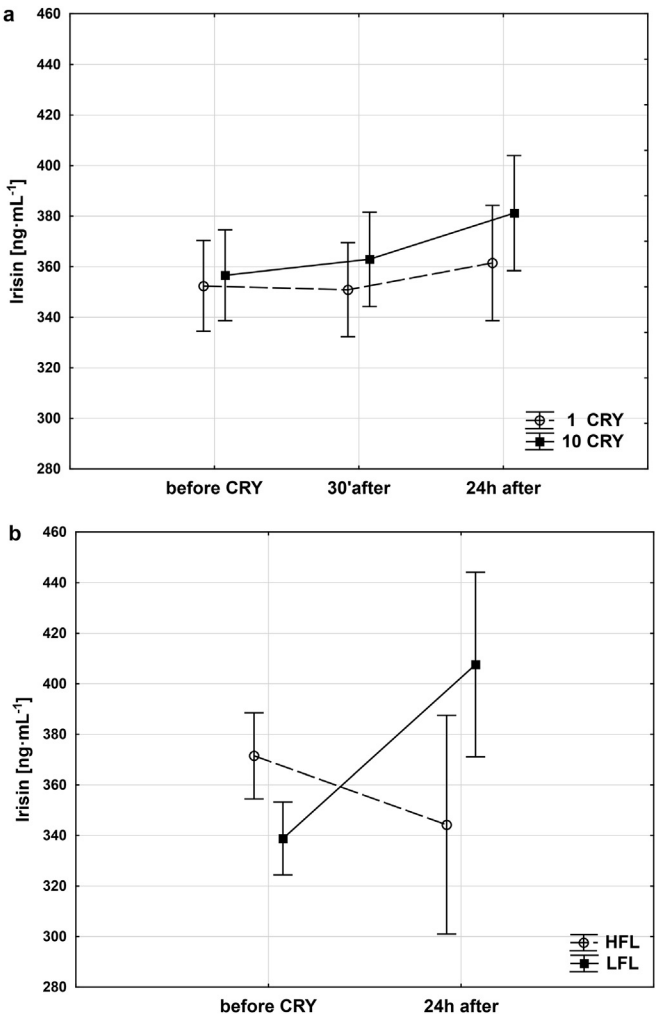

Fig. 1. Changes in irisin concentration in response to the first and last session of whole body cryostimulation (CRY): a. In the whole group of participants (the mean total effect of I CRY vs X CRY intervention is significantly different at p level = 0.04). b. In HFL and LFL group (for LFL group the differences were statistically significant p < 0.05, for HFL group the changes after 10 sessions of WBC were not differ to the baseline).

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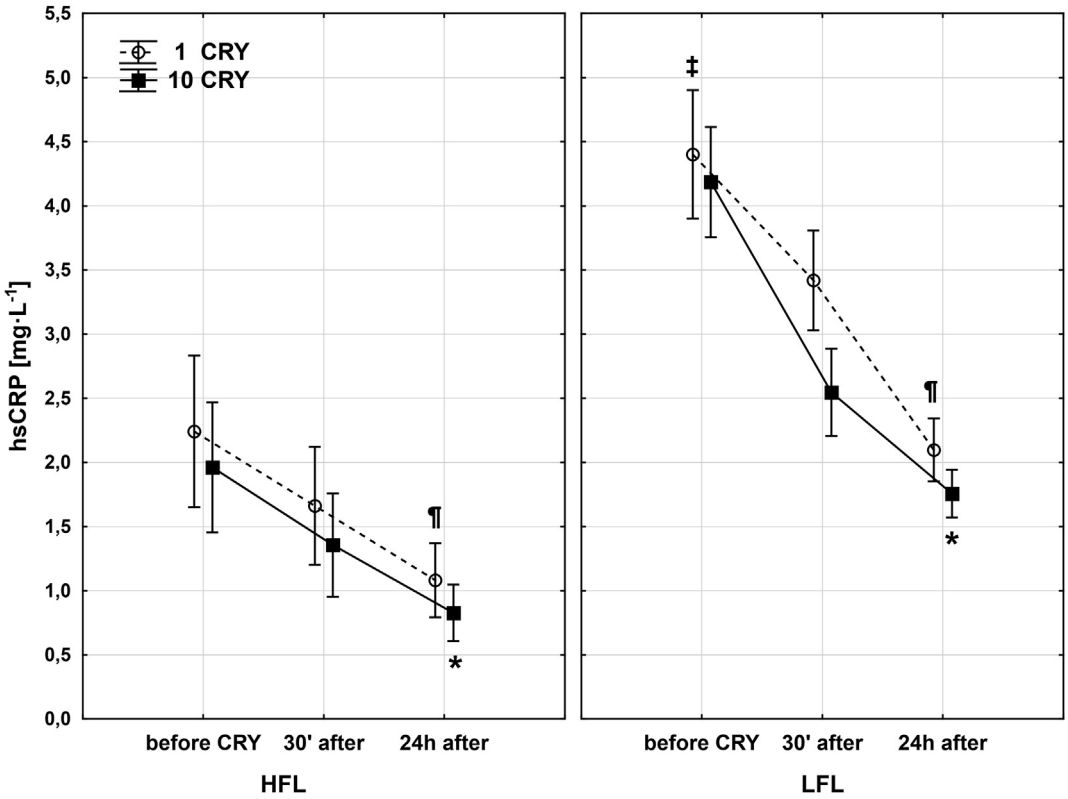

Fig. 2. Alternations in hsCRP in response to the first and last session of whole body cryostimulation in HFL and LFL group (‡ significantly different from baseline (before 1 CRY) HFL group ($p = 0.003$), ¶ significantly different: baseline (before 1 CRY) vs 24 h after 1 CRY (HFL $p = 0.005$; LFL $p = 0.001$), * significantly different: baseline (before 10 CRY) vs 24 h after 10 CRY (HFL $p = 0.004$; LFL $p = 0,0005$).

respectively) among all subjects. In addition, the negative correlation between maximal oxygen uptake and hsCRP concentration was noted ($r = -0.68$; $p < 0.05$) for the whole group. In HFL group the concentration of IL-6 was lower (1.3 ± 0.5 pg mL^{-1}) than in LFL participants (Table 2). The average values of IL-6 in LFL group was 69% higher (2.2 ± 0.8 pg mL^{-1}) in comparison with those who characterized by elevated fitness level. Moreover, the group of obese subjects experienced the relatively high level of hepcidin [19]. Iron and ferritin level among our subjects were in reference range (Table 2). Irisin concentration recorded before CRY correlated inversely with the ferritin level. For the whole group, the association between irisin and ferritin before CRY was equal $r = -0.60$ ($p < 0.05$). Interestingly, among LFL group this relationship was even stronger $r = -0.83$ ($p < 0.05$).

3.2. Effect of 10 sessions of whole-body cryostimulation

Although comparison of single measurements did not revealed statistical differences between each values, analysis of means calculated at first and last sessions of CRY in all points of blood collection (Fig. 1a) indicated that the series of 10 cryostimulation exposures altered irisin concentration among all subjects (the

Table 2
The hepcidin concentration and iron status in response to 10 sessions of whole body cryostimulation.

Variable	HFL-group		LFL-group		Differences	*P* Value
	Before first CRY	24 h after last CRY	Before first CRY	24 h after last CRY		
Serum iron [μg dL^{-1}]	105.3 (±5.3)	94.9 (±12.7)	117.7 (±37.5)	104.8 (±21.7)	Time	ns
					Group	ns
					Time × group	ns
Serum ferritin [ng mL^{-1}]	104.0 (±16.8)	101.0 (±9.9)	108.7 (±41.4)	105.0 (±41.8)	Time	ns
					Group	ns
					Time × group	ns
Hepcidin [ng ml^{-1}]	86.9 (±8.58)	72.2 (±3.08)	103.8 (±42.4)	96.0 (±17.0)	Time	0.03[a]
					Group	ns
					Time × group	ns
IL-6 [pg ml^{-1}]	1.3 (±0.5)	1.3 (±0.4)	2.2 (±0.8)	2.0 (±1.0)	Time	ns
					Group	ns
					Time × group	ns

Values are means (±SD), ns – no statistical differences for group and time before and after cryostimulation HFL – high fitness level, LFL – low fitness level. CRY the whole body cryostimulation, 24 h after last 10 session of cryostimulation, differences: time-comparison before and after cryostimulation.
[a] Significantly different group-differences between HFL and LFL, time × group – interactions between group and time, ns-non significant differences.

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mean total effect value of 1st CRY vs 10th CRY intervention is significantly different, $p = 0.04$) Interestingly, exposure to coldness in HFL group caused a small drop of irisin (371.5 ± 30.0 ng mL^{-1} at baseline vs 343.3 ± 47.6 ng mL^{-1} 24 h after last session of CRY). In contrast, the tendency in LFL group was opposite. The irisin increased in LFL group from 338.8 ± 42.2 ng mL^{-1} to 407.6 ± 118.5 ng mL^{-1} (Fig. 1b). The body composition modified the rise of irisin in our group. The increase of irisin concentration recorded directly before, 30 min and 24 h after 10th sessions of cryostimulation positively correlated with body fat percentage of fat mass and visceral fat area. Additionally, the significant relations were observed between percentage of skeletal muscle mass and irisin concentration. In measurements, which were performed before, 30 min and 24 h after 10th session, subjects with higher skeletal muscle mass had lower irisin concentration (Table 3).

The whole body cryostimulation reduced low grade inflammation, thus the hsCRP concentration in both groups significantly dropped (Fig. 2). The range of decline was similar in HFL and in LFL group: from at the baseline 2.2 ± 0.9 to 0.8 ± 0.4 mg L^{-1} 24 h after last session of CRY (HFL) and respectively 4.4 ± 1.6 to 1.8 ± 0.5 mg L^{-1} (LFL). Thus the interaction between groups was not statistically significant. Nonetheless the drop was essential. Moreover, hepcidin concentration was also reduced but this decline was not accompanied by changes in iron and ferritin concentration. The exposure to coldness did not adjust IL-6 concentrations significantly (Table 2). However, recorded small alternations in IL-6 concentration corresponded with changes in hepcidin ($r = 0.61$, $p < 0.05$).

4. Discussion

The study demonstrates that 10 sessions of the whole body cryostimulation caused the rise of irisin concentration in obese men. The obtained data confirm our initial hypothesis that exposure to extremely cold environment might induce muscle shivering and consequently irisin release from the muscle. Still, diverse tendency in irisin concentration among physically active and non-active subjects following 10 sessions, might suggest that fat tissue has also contributed to the irisin secretion. Thermodynamically cryotherapy works on the principle of an unidirectional heat transfer from high heat to low heat areas and tissue temperature heat loss to the external cooling modality [28]. In the process, depth of subcutaneous fat is a significant factor affecting the magnitude and rate of intramuscular cooling [31]. Roca-Rivada and co-workers showed that human subcutaneous as well as visceral fat tissue express and secret FNDC5/irisin [37]. Contrary to the previous observation [17,30,39] in the present study, no correlations were observed at the baseline between body composition and/or cardiorespiratory fitness level and circulating irisin levels. Conversely, our data showed that after 10 sessions of CRY, the rise in blood irisin correlated with the fat tissue amount. These data suggests that the subcutaneous fat tissue, rather than skeletal muscle, is the main source of irisin in response to extreme coldness. Such suggestion is supported by the fact that the rise in irisin was inversely proportional to the muscle mass.

There is limited number of works investigating the influence of coldness on irisin concentration. Lee and co-workers observed the irisin concentration to be increased in response to graduate cold water immersions [24]. Also, Costello noticed that compared to water immersions, the whole body cryostimulation is more effective in lowering skin temperature [8]. Still, even the exposure to −110 $^{\circ C}$ (extreme coldness) may not have impacted the muscle severely enough to induce shivering, especially in obese individuals with an excess of fat tissue. Hence, the impact of CRY in the protocol of 3-min exposure to low temperature used in our study may have been insufficient to induce changes in irisin blood level through muscle shivering. The observed rise of irisin stimulated by coldness may have either been a short-lasting effect or the irisin has been taken up very quickly. Similar response was observed by Loffer and co-workers, who recorded the irisin concentration to have increased in adults after a short-term acute exercise. The rise was detectable only immediately after the exercise and significantly diminished in 30 min time following the exercise [27]. This may explain the smallest correlation observed 24 h after the last session in our study. Nonetheless, the irisin concentration grew only in the group of obese non-active subjects following the series of cryostimulation sessions, while in the group of active obese man we did not record this tendency.

Data concerning the influence of exercise on irisin concentration are also unambiguous. A one year long lifestyle intervention program was associated with an improvement in anthropometric and metabolic parameters, resulting in higher irisin levels in obese children [10]. Other data indicated that single sessions of intense endurance exercise and heavy strength training lead to transient increases in irisin concentrations in blood [34]. In contrary, some data demonstrated that neither endurance training nor resistance training increases irisin concentration [14]. In addition, no significant changes in irisin concentration following a 10-week endurance training program were noted in a recent research on habitual runners vs non-runners, where runners were characterized by a significantly lower body weight and total body fat along with higher aerobic fitness compared to non-runners [15]. Instead, when compared with pre-training levels, a decreasing tendency in the irisin concentration was observed following the training program, independently of each subject's fitness level. Additionally, the mean irisin value tended to be lower in runners compared to non-runners, both before and after single bout of exercise [15].

Since obesity is associated with increased inflammation [23], the second aim of the present study was to investigate if CRY triggers a decline of hsCRP as a marker of inflammation. Previous research revealed that the protocol with the same number of CRY sessions induced an anti-inflammatory effect in obese man through attenuation of TNF-a concentration [45]. Baseline values of hsCRP and IL-6 in the present study indicated that our obese subjects had experienced a low grade inflammation, while the active, yet obese men exhibited lower concentrations of the inflammation markers.

Table 3
The correlations between body composition and irisin concentration after all series of the whole body cryostimulation among all participants.

Variable	Irisin BC [ng mL^{-1}]	Irisin 30 min [ng mL^{-1}]	Irisin 24 h [ng mL^{-1}]
Weight [kg]	0.65*	0.66*	0.46
BMI [kg m^2]	0.60*	0.62*	0.41
SMM [%]	−0.59*	−0.65*	−0.48
Fat [kg]	0.65*	0.68*	0.48
Fat [%]	0.57	0.63*	0.45
VFA [cm^2]	0.49	0.58*	0.43

Values are Person correlation, *values were significant at $P < 0.05$, BC-before X cryostimulation, 30 min after X cryostimulation, 24 h 24 h after X cryostimulation. SMM – skeletal muscle mass, Fat–fat mass, Fat%-percentage of body fat, VFA – visceral fat area.

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Such observations are backed by different research, where the anti-inflammatory effects of exercise was shown [36] and higher levels of physical activity associated with a lower concentration of serum hsCRP [2].

Despite the lack of differences between the groups (active vs non-active), the applied protocol of CRY significantly decreased hsCRP blood concentration in all subjects, thus, endorsing its anti-inflammatory action reported previously [16,45]. Among other, the inflammation might induced to changes in iron metabolism [13], hence, the effect of CRY on hepcidin was measured. As a result a significant reduction in hepcidin level was recorded 24 h after the last CRY session. As outlined above, hepcidin acts by blocking the ferroportin, which is the only know protein able to transport iron from the cells [11]. Rise in blood hepcidin leads to inhibition of ferroportin in many cells, including enterocytes in duodenum, hepatocytes, macrophages, adipocytes and many other what may result in decrease dietary iron absorption and blood iron but increase iron accumulation in rest of the cells [9]. Such iron redistribution induced by chronic hyper hepcidinemia may result in inflammation as iron acts as a pro inflammatory molecule [21]. Thus, in our opinion, the observed drop in blood hepcidin should be considered as an anti-inflammatory effect of CRY. It is worth noting that adipocytes in addition to liver, macrophages and pancreatic islets can also be the source of blood hepcidin [5]. In case of the obese individuals we observed both Hpc and BMI as well as the body fat % and Hpc to be correlated. These observations suggest that a decrease in blood Hpc may result from its declined release from the adipose tissue after CRY. Lowering of blood Hpc concentration along with IL-6 decrease supports the thesis that CRY reduces obesity-related low grade inflammation. Considering that iron deficiency is frequent in advanced stages of obesity possibly due to high Hpc concentration [7], CRY could be a good method to correct iron metabolism in those subjects.

Our data sets the basis for additional research since many questions remain to be answered concerning: the connection between body composition and irisin concentration, the number of cryostimulation sessions to stimulate irisin release and determinate if changes in hepcidin are also fat tissue dependent. The present study, thus, only partly addresses the question whether fat tissue serves as a source of irisin. Discussion and research are hence likely to continue, taking a careful account of the body temperature changes. In future projects the following limitations of this study should be addressed: a small number of subjects and time through which the positive changes are sustained.

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Conflict of interest

There are no professional relationships between any of the authors and manufacturers of equipment utilized in this study. No conflict of interest occurs.

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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