Energy expenditure and macronutrient oxidation in response to an individualized non-shivering cooling protocol

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What is already known about this subject?

- A decade ago, brown adipose tissue (BAT) was reported to be present and metabolically active in adult humans. Since then, non-shivering mild cold exposure has been considered a potential therapeutic tool in obesity management.

- Chronic exposure to non-shivering cold stimulus appears to improve energy metabolism and metabolic health in healthy individuals and people with obesity or type 2 diabetes mellitus.

What are the new findings in your manuscript?

- A mild cold exposure at a temperature adjusted to elicit maximum non-shivering thermogenesis induces a modest increase in energy expenditure, which is maintained constant during an hour.

- A metabolic shift on macronutrient oxidation rates to sustain cold-induced thermogenesis (CIT) was observed by which fat oxidation increased in parallel to a decrease in carbohydrate oxidation at the beginning of cold exposure. Later, an inverse tendency appeared by which carbohydrate oxidation increased and fat oxidation slightly decreased.

How might your results change the direction of research or the focus of clinical practice?

- This study suggests that mild cold exposure is not a feasible tool to induce negative energy balance in humans. However, pharmacological or nutraceutical continuous induction of maximum non-shivering thermogenesis might be a feasible target for weight loss.

- The observed shift on macronutrient oxidation rates may indicate that tissues playing a key role on CIT (i.e. BAT and muscles) could be different during the first stages of cold exposure than after 30 minutes of cold exposure.

ABSTRACT

Objective: To describe the energy expenditure (EE) and macronutrient oxidation response to an individualized non-shivering cold exposure in young healthy adults.

Methods*:* Two different groups of 44 (Study 1: 22.1±2.1 years old, 25.6±5.2 kg/m^2, 34% men) and 13 young healthy adults (Study 2: 25.6±3.0 years old, 23.6±2.4 kg/m^2, 54% men) participated in this study. We measured resting EE (RMR) and macronutrient oxidation rates, by indirect calorimetry under fasting conditions, in a warm environment (30-minutes) and in mild cold conditions (65-minutes, wearing a water perfused cooling vest set at an individualized temperature adjusted to the individuals' shivering threshold).

Results: In study 1, EE increased in the initial stage of cold exposure and remained stable for the whole cold exposure (P<0.001). Mean CIT (9.56±7.9 Kcal/h) was 13.9±11.6 %RMR (Range: -14.8/39.9 %RMR). Carbohydrate oxidation decreased during the first 30 minutes of the cold exposure and later recovered up to the baseline values (P<0.01) in parallel to opposite changes in fat oxidation (P<0.01). Results were replicated in study 2.

Conclusions: A 1-hour mild cold exposure individually adjusted to elicit maximum non-shivering thermogenesis induces a very modest increase in EE and a shift of macronutrient oxidation that may underlie a shift in thermogenic tissues activity.

INTRODUCTION

Since brown adipose tissue (BAT) was shown to be present and metabolically active in adult humans in 2009 (1–4), cold-induced thermogenesis (CIT) has received considerable attention as a possible target for stimulating BAT and energy expenditure (EE), helping weight control and counteracting obesity (5). Indeed, several independent groups have reported that chronic exposure to non-shivering cold stimulus seems to enhance energy metabolism and metabolic health in healthy individuals (6) and people with obesity (7) or type 2 diabetes mellitus (8). Noteworthy, it remains unclear whether human BAT-related health-benefits are explained by increases in EE or by other mechanisms beyond energy balance (9, 10). Moreover, there is an open debate regarding the relative contribution of BAT to CIT, in comparison with other thermogenic tissues such as skeletal muscle or white adipose tissue (11).

CIT is produced by both non-shivering and shivering mechanisms (12). Shivering thermogenesis is the EE necessary to cover involuntary skeletal muscle contractions in response to cold, while non-shivering thermogenesis refers to the energy consuming process not depending on muscle contraction such as uncoupling respiration in brown adipocytes mitochondria (13). Although some have reported that shivering thermogenesis can increase EE up 5-fold above resting metabolic rate (RMR), its uncomfortable nature, together with the loss of motor coordination, makes it a poorly tolerated stimuli, and therefore, a non-plausible option to be used in clinical settings (13). On the other hand, non-shivering thermogenesis produces moderate increases of EE, and estimations report a range between 0 and 30% of the RMR in young healthy adults (13). Moreover, it has been argued that non-shivering thermogenesis may be comfortable enough to be considered a possible tool in the prevention and treatment of obesity (13). Of note, shivering and non-shivering thermogenesis do not appear to occur in sequential phases

but more as parallel phenomenon (i.e. even at light cold stimulation shivering thermogenesis seems to contribute to CIT) (14), and its relative contribution to CIT can vary within-individuals (15).

There is a large inter-individual variation in non-shivering thermogenesis and cold tolerance. Therefore, to study the responses to mild cold exposure, there is a need to individualize the cold stimulus to the individuals' cold tolerance (16, 17). van der Lans et al. (16) proposed to first assess a shivering threshold (i.e. lowest external temperature without evoking externally observable and perceived shivering) as a reference point for adjusting the temperature at which an individual should be exposed. Although other methods have been proposed such as skin temperature clamping (18) or cold perception adjusting (19), the lowest tolerable temperature above the shivering threshold has been broadly accepted and used as a valid approach to induce BAT activation and CIT (17, 20– 23). Some studies have however shown that skeletal muscle thermogenesis takes place with very mild cold stimuli before observing shivering (14). As a result, the shivering threshold approach may not fully exclude the skeletal muscle shivering thermogenesis.

Research has traditionally spent more attention on the study of shivering thermogenesis (24), and important gaps remain regarding the human physiological responses to mild cold exposure in terms of EE and metabolic fuels selection (12). We hypothesized that a mild cold exposure designed to elicit non-shivering thermogenesis would exert light, but clinically meaningful if maintained in time, modifications on EE and metabolic fuels selection. Thus, the present study aimed to describe the EE and macronutrient oxidation response to an individualized non-shivering cold exposure in young healthy adults.

METHODS

Participants

Two different cohorts took part in this study (table 1). For the first cohort (thereinafter called study 1), 63 participants (45 female) were included in the study, all of them being part of the ACTIBATE study (25). However, only 44 out of 63 were included in the statistical analyses (Figure 1, see the reasons below). Study 1 was conducted in October and November 2016. For study 2, 13 participants were recruited and evaluated between December 2017 and January 2018. Inclusion criteria in both studies were: <35 years old, report being healthy, non-smoker and not taking any medication, have had a stable body weight (<3 Kg change) during the past 3 months, and not regularly exposed to cold. The study and written informed consent considered the last revision of the Declaration of Helsinki and were approved by the Human Research Ethics Committee of the University of Granada (nº924) and of the Servicio Andaluz de Salud (Centro de Granada, CEI-Granada).

We used a dual X-ray absorptiometry scan (Discovery Wi, Hologic, Inc., Bedford, MA, USA) to assess body composition, while a Seca scale and a stadiometer (model 799, Electronic Column Scale, Hamburg, Germany) were used to measure weight and height, respectively.

Previous conditions to the study days

Participants attended to the research center on two separate occasions. During the first visit, the individual's shivering threshold was assessed while during the second visit CIT was measured. They were asked to sleep as usual, to avoid both moderate (24 hours) and vigorous (48 hours) physical activity prior to the testing days, and to commute by motorized vehicle.

In study 1, participants fasted for at least 6 hours; 9±3.7 hours. Only those participants strictly following the fasting time indications (i.e. between 6 and 8 hours; n=18) were included in the macronutrient oxidation rate analyses (26, 27) (table 1). In study 2, participants were instructed to consume a standardized meal (35% of estimated total EE; boiled rice, tomato sauce, and omelet) 10 hours before the CIT assessment. Moreover, they were instructed to collect all the urine from the standardized meal to the CIT assessment start (i.e. 10 hours urine production). Another urine sample was collected immediately after the CIT assessment.

Shivering threshold test (day 1)

The shivering threshold assessment methodology has been extensively described elsewhere (23, 28). In brief, after voiding, the participants dressed-up with standardized clothes (clothing insulation value: 0.20). They then entered a warm room (22.1±1.6ºC) where they remained seated for 30 minutes, before entering a cold room (19.8±0.5ºC), where they dressed-up with a temperature-controlled water perfused cooling vest (Polar Products Inc., Ohio, USA). The vest circulating-water temperature was initially set at 16.6ºC and was decreased every 10 minutes until 3.8ºC was reached or until shivering occurred. Shivering was determined visually and self-reported. The vest circulating-water temperature when shivering started was considered as the shivering threshold.

CIT and cold-induced macronutrient oxidation rates (day 2)

Participants returned to the lab (the same time of day as before) 5-7 days (study 1) or 2 days (study 2) after the shivering threshold test. After voiding, they dressed in the same standardized clothes and entered a warm (23.2±0.7ºC) room. Before assessing EE, all participants reclined in a bed for 20 minutes. Later, their RMR was assessed over 30 minutes, following current methodological guidelines (29). Immediately after, the

participants walked into the cold room (19.7±0.4ºC) and dressed in the cooling vest set at 4ºC above the participant's shivering threshold. They laid in a reclined bed while the CIT assessment was carried out for two 30-minute periods, separated by a 5-minute pause. For assessing RMR and CIT, ventilatory gas exchange was collected by using a neoprene face-mask hooked up to a CCM Express or Ultima CardiO2 metabolic cart (Medgraphics Corp, Minnesota, USA) (30, 31). Flow, at the beginning of every test day, and gas analyzers, before every 30-minute assessment, were calibrated following the manufacturer´s instructions.

In study 1, despite of the careful assessment of shivering threshold, 17 (16 women) out of 63 participants shivered during the CIT assessment. Those individuals were excluded from further statistical analysis. Moreover, 2 males were also excluded from the analyses for presenting respiratory exchange ratio (RER) values higher than 1.1 or lower than 0.7 in any measure point (29) (Figure 1).

CIT and macronutrient oxidation rates estimation

Oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were downloaded from the metabolic cart software (Breeze Suite, 8.1.0.54 SP7) every minute. In all 30-minute record period we discarded the first 5-minute record. We then selected the most stable 5-minute period among the remaining records (25 minutes) to be considered as the RMR, as previously described (30, 32). For calculating CIT, data was averaged every 5 minutes. In addition, we divided the CIT record into 4 parts and selected the most stable 5-minute period within every part. Finally, we calculated the area under the curve (trapezoidal rule) using the 4 selected 5-minute periods and the RMR, expressing it as a percentage of RMR. We have previously published that this method for data analyses resulted in more plausible estimations when compared to other methods for data analyses (33). The Weir´s abbreviated equation was used to estimate EE (a) (34), while Frayn´s

equation (35) (not taking into account any urinary nitrogen in study 1) were used for estimating carbohydrates (CHOox) (b) and fat oxidation (FATox) (c).

(a) EE (Kcal/min) $= 3.941 \times VO_2$(l/min) $+ 1.106 \times VCO_2$ (l/min) $- 2.17$ x N(g/min)

(b) $CHOox$ (g/min) $= -3.21 \times VO_2$ (l/min) $+ 4.55 \times VCO_2$ (l/min) $- 2.87$ x N(g/min)

(c) $FATox$ (g/min) $= 1.67 \times VO_2$ (l/min) $- 1.67 \times VCO_2$ (l/min) $- 1.92$ x N(g/min)

In study 2, we measured total urine volume and urea concentration in both urine samples. Thereafter, we estimated nitrogen urine levels from the urea concentration (Spinreact, Catalogue No. 283-17) following the equation "N(g/l) = 0.0065* Urea(mg/dl) + 1.2598" obtained from a linear regression including urea and nitrogen measured concentrations (kjeldahl method (36)) from an independent cohort of young healthy adults (n=19, 16 women; 21.87±2.05 years old; 24.87±3.71 kg/m^2). Protein oxidation (g/min) was calculated as N(g/min) x 6.25 (37).

Statistical analysis

Repeated-measures analyses of variance (ANOVA), with Bonferroni post-hoc corrections, were used to test differences in EE, CHOox and FATox across time. Paired t-test were used to compare protein oxidation before and during the cold stimulus.

The analyses were conducted using the Statistical Package for Social Sciences (SPSS, v. 21.0, IBM SPSS Statistics, IBM Corporation). Figures were created using Graph Pad Prism (GraphPad Software, v. 8.3.1, CA, USA). The level of significance was set at <0.05.

RESULTS

Mild cold exposure significantly increased EE (P<0.001; Figure 2A) from the beginning of cold-exposure and remained stable from that moment till the end of the mild cold exposure. Mean overall CIT estimation was 13.9±11.6 %RMR (Range: -14.8/39.9 %RMR, Figure 2B). When translated to cumulative EE (i.e. the total amount of energy above the RMR expended during the whole 65-minute cold exposure), it resulted in 9.56±7.9 Kcal.

CHOox decreased after 30 minutes of mild cold exposure (P=0.001), although its level returned to the baseline after 40 minutes of mild cold exposure (Figure 2F). The peak FATox was observed at 30 minutes of cold exposure (P<0.001; Figure 2F). FATox progressively increased until minute 30, and later on, there was a decrease. All results were similar in men and women, and in individuals with BMI<25kg/m^2 and BMI>25kg/m^2 (data not shown). Mean overall CI CHOox was -7.3±22.5%basalCHOox (Range: -36.4/34.31 % basalCHOox) and mean overall CI FATox was 53.6±90.9 %basalFATox (Range: -33.9/382.1 % basalFATox).

Figure 3 shows EE and macronutrient oxidation rates during a mild cold exposure in the study 2. Cold exposure increased EE as in study 1 (P>0.001; Figure 3A). The effect of cold exposure on both CHOox and FATox (Figure 3F) was also similar to study 1. Finally, Figure 4 shows the estimated protein oxidation before (10 hours) and during the mild cold exposure in study 2. Mild cold exposure significantly increased protein oxidation (0.91±0.38 g/min during the cold exposure vs. 0.54±0.29 g/min for the 10h preceding cold exposure; P=0.005).

DISCUSSION

We show that a 1 hour individualized mild cold exposure produced a moderate increase in energy expenditure (9.56±7.9 Kcal/h) in young healthy adults. Interestingly, we observed a metabolic shift in time for sustaining CIT. CHOox decreased during the first 30 minutes of the cold exposure and recovered up to the baseline values at the end of the cold exposure. In contrast, FATox continuously increased during the first 30 minutes, and decreased in the second part of the cold exposure. Of note is that these results were replicated in two independent studies which further reinforce the findings. Our results show a modest EE increase during mild cold exposure which concurs with previous studies (12). Our data also show a previously unreported shift in the nutrient oxidation rates for sustaining the CIT during the first phase of mild cold exposure (first 30 minutes). This finding might have important implications for the understanding of physiological processes underlying human non-shivering thermogenesis.

Mild cold exposure induces moderate increases in energy expenditure

In study 1, some individuals showed negative values of CIT (i.e. lower EE in cold than in RMR) while others experienced a 39% increase over RMR, which concurs with the huge inter-individual variability (38, 39) reported in previous studies. Whether non-shivering cold exposure induces a stable energy expenditure change from the beginning of the exposure is unclear (40). We observed that when applying an individualized cooling protocol at a temperature near to the shivering threshold, maximum non-shivering thermogenesis appears to be elicited from the beginning of the cold exposure. This constant thermogenic rate concurs with other studies analyzing the EE elicited by shivering (41, 42). On the other hand, it is not clear whether the magnitude of non-shivering thermogenesis is able to induce a significant negative energy balance (14, 43). We observed that even when applying a protocol designed to elicit maximum non-

shivering thermogenesis, all participants presented an EE below 1.4 metabolic equivalent (MET, i.e. 40% of RMR increase). Taking into account that even a very low intensity exercise, such as walking, can elicit 2-3 METs, together with the fact that cold exposure may induce an hyperphagic response (44), it seems that cold exposure at temperatures eliciting low shivering and maximum non-shivering thermogenesis are not an efficient stimuli to induce negative energy balance. It should also be noted that this mild cold stimulus produces a considerable burden and discomfort for participants, despite only 9.56±7.9 Kcal were burned (above the RMR) after an hour at this uncomfortable situation. Nonetheless, if maximum non-shivering thermogenesis were induced by pharmacological or nutraceutical agents (45, 46), instead of cold exposure, and were continuously elicited during 24h, our data would translate into 212±175 Kcal/day. Importantly, recent studies using radiotracers for the quantification of BAT energy expenditure have estimated that, if maximally and continually activated, human BAT could account for as much as ≈15 Kcal/day (20, 47, 48). This is less than 10% of the 24h CIT estimation made in our study, which suggest that tissues other than BAT (e.g. skeletal muscle) may represent better targets for stimulating non-shivering thermogenesis in humans (5). Nonetheless, alternative mechanisms beyond energy balance (9, 10) have been suggested to mediate the health promoting effects of BAT and chronic cold exposure (6–8). Therefore, mild cold exposure and/or pharmacological BAT activation or recruitment might not be discarded as possible therapeutic target including insulin sensitizing effects.

Mild cold exposure induced a shift on fuel oxidation

We observed a marked shift in macronutrient oxidation to sustain the thermogenic rate. Previous studies have reported similar metabolic shifts at a constant thermogenic rate in response to cold (in shivering conditions) (12, 41, 42), however, we observed a decrease in CHOox, which has not been reported before. Preserving muscle glycogen is considered

to have a profound impact on cold endurance, and therefore on cold survival (12). Consequently, fatty acids are the most sustainable fuel for thermogenic purposes. FATox is the predominant substrate for both non-shivering and shivering thermogenesis (12). Therefore, it is biologically plausible that a shift to FATox is produced when thermogenic needs are not maximized, such as at the beginning of the cold exposure.

BAT is not the only contributor responsible for non-shivering thermogenesis in humans. Skeletal muscle possibly contributes to non-shivering thermogenesis even to a larger extent (14, 20). Although both BAT and skeletal muscle preferentially use fatty acids as energy fuel in non-shivering situations, BAT is probably more FATox-preferential, as more than 90% of its energy consumption relies on fat oxidation (49, 50), while skeletal muscle presents a more balanced nutrient uptake (12). Therefore, it is plausible to speculate with the possibility of BAT being the main contributor to CIT at initial stages of mild cold exposure while muscle contribution to CIT would increase progressively, therefore balancing the contribution of CHOox and FATox. This hypothesis is supported by recent studies showing that BAT is rapidly activated upon cold exposure and seems to stabilize after 35 minutes of cold exposure (51–53). Moreover, we previously reported a high prevalence of BAT in study 1 (88% BAT positive, BAT volume=94.4±59.6 ml), measured by static ^{18}F-Flurodeoxyglucose PET/CT scan after 2 hours of personalized cold exposure, in study 1 participants (23, 54); therefore BAT thermogenesis was likely induced during the mild cold exposure. New studies examining the contribution of both tissues, BAT and skeletal muscle, from the very beginning of the cold exposure are needed to confirm such hypothesis.

Protein oxidation was only assessed in study 2, in which we observed a significant increase in response to mild cold exposure. A recent study in both mice and humans showed that upon cold exposure, BAT significantly increases the uptake and oxidation of

branched-chain amino acids (BCAA) (55). Importantly, blunting BCAA oxidation in BAT significantly affected BAT thermogenic capacity in mice (55). Therefore, the increase of protein oxidation observed in our study might be explained by the increased uptake and oxidation of BCAA in BAT. Such statement is quite speculative since we did not specifically measure BCAA oxidation in study 2. Further studies are needed to test whether there is an association between BAT activity and cold-induced protein oxidation in humans.

In agreement with previous studies (12), we observed a considerable high inter-individual variability in cold-induced macronutrient oxidation rates. In human studies, different patterns of shivering (i.e. muscle recruitment) explain most of the inter-individual variability in cold-induced macronutrient oxidation rates (12, 41, 42). Since the protocol we applied is considered to result in low shivering contribution to CIT, different patterns of shivering may not explain such a large inter-individual variability. Alternatively, inter-individual differences on tissues (BAT, skeletal muscle, and white adipose tissue) relative contribution to CIT (i.e. proportion of CIT being produce by each tissue) might partially explain such a high inter-individual difference (5).

Limitations

In study 1, carbohydrates and fat oxidation rates were calculated without considering protein oxidation, since we did not measure urine nitrogen excretion. In addition, in study 1, we did not strictly control fasting period and previous meal content. However, we performed a confirmatory study (i.e. study 2) analyzing protein oxidation and strictly controlling fasting time, previous meal content and similar results were found. Secondly, the visually detected or self-reported shivering is likely to occur after electrical muscle activity (40). Therefore, the absence of electromyographical recording might have underestimated the shivering threshold temperature. Moreover, despite we excluded

participants presenting detectable shivering during the CIT assessment, we cannot discard that non-visually detectable nor self-noted shivering thermogenesis was present (14). Nonetheless, it is probable that non-shivering thermogenesis was the predominant form of CIT in the included participants. In addition, it should be noted that the study sample was entirely composed of young (18-32 years) healthy individuals, and thus the results cannot be generalized to older or unhealthy individuals. Finally, it should be noted that the reliability of the indirect calorimeters used in this study was not optimal (31). This probably explains the presence of non-physiological values obtained in 2 out of our 63 participants enrolled in study 1.

CONCLUSIONS

A mild cold exposure at a temperature adjusted to elicit maximum non-shivering thermogenesis induces a very modest increase in EE (<40% RMR; ≈1.4 METs), which is maintained constant during an hour. The cumulated cold-induced energy expenditure over an hour of mild cold exposure (9.6±7.9 Kcal/h) is probably insufficient to induce a negative energy balance in humans. However, we cannot exclude that sustained induction of maximum non-shivering cold-induced thermogenesis (212±175 Kcal/day), which might be achieved by pharmacological or nutraceutical agents, can be a feasible strategy for weight loss. Interestingly, we found a metabolic shift on macronutrient oxidation rates to sustain CIT by which FATox increased in parallel to a decrease in CHOox at the beginning of cold exposure. Later, an inverse tendency appeared by which CHOox increased and FATox slightly decreased. This may indicate that tissues playing a key role in CIT (i.e. BAT and muscles) could be different during the first stages of cold exposure than after 30 minutes of cold exposure.

ACKNOWLEDGMENT

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TABLES

Table 1. Participants' characteristic.

	Study 1 CIT analyses (n=44)		Study 1 NUTox analyses (n=18)		Confirmatory study (Study 2, n=13)	
Sex (n women. %)	29	(65.9)	13.0	(72.2)	6.0	(46.2)
Age (years)	22.2	(2.2)	21.9	(2.0)	25.6	(3.0)
BMI (kg/m^2)	25.6	(5.3)	24.3	(4.6)	23.6	(2.4)
Lean mass (Kg)	42.7	(10.4)	40.4	(8.0)	45.7	(13.3)
Fat mass (Kg)	27.2	(10.6)	25.0	(9.6)	18.4	(3.8)
Fat mass percentage (%)	37.0	(8.0)	36.1	(7.0)	28.4	(6.6)
RMR (Kcal/day)	1565	(278)	1554	(227)	1484	(286)
RER	0.862	(0.054)	0.842	(0.048)	0.833	(0.036)
Cooling vest water temperature (ºC)	8.4	(3.5)	9.2	(3.8)	12.1	(3.1)

Data are means (standard deviation) except for sex. CIT: Cold-induced thermogenesis; NUTox: Macronutrient oxidation rates; BMI: Body mass index; RMR: Resting Metabolic Rate; RER: resting respiratory exchange ratio.

FIGURE LEGENDS

Figure 1. Participant´s flow-chart. ACTIBATE: Activating brown adipose tissue through exercise; BMI: body mass index, CIT: Cold-induced thermogenesis; ECG: electrocardiogram; NUTox: Nutrient oxidation, RER: Respiratory exchange ratio.

Figure 2. Cold-induced thermogenesis (CIT) and macronutrient oxidation rates (study 1). Panels A (adapted from (33)) C and D: Minute 0 represents the resting metabolic rate (RMR). Values are mean and standard error of the mean. P values for the main effect of the repeated measures ANOVAs. * represent values significantly different than RMR. Panel B: histogram representing over-all CIT (each column representing one individual´s CIT). Dashed line represents mean CIT. CHOox: carbohydrates oxidation; FATox: fat oxidation; Kcal: Kilocalories; min: minutes; RER: respiratory exchange ratio; VO_2: oxygen consumption; VCO_2: carbon dioxide production.

Figure 3. Cold-induced thermogenesis (CIT) and macronutrient oxidation rates (study 2). Panels A, C and D: Minute 0 represents the resting metabolic rate (RMR). Values are mean and standard error of the mean. P values for the main effect of the repeated measures ANOVAs. Panel B: histogram representing over-all CIT (each column representing one individual´s CIT). Dashed line represents mean CIT. CHOox: carbohydrates oxidation; FATox: fat oxidation; Kcal: Kilocalories; min: minutes; RER: respiratory exchange ratio; VO_2: oxygen consumption; VCO_2: carbon dioxide production.

Figure 4. Protein oxidation before (10 hours) and during mild cold exposure in the study 2. P value is for paired t-test.