CLINICAL STUDY

Minimal changes in environmental temperature result in a significant increase in energy expenditure and changes in the hormonal homeostasis in healthy adults

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Abstract

Objective: Resting energy expenditure (EE) is a major contributor to the total EE and thus plays an important role in body weight regulation. Adaptive thermogenesis is a major component of EE in rodents, but little is known on the effects of exposure of humans to mild and sustainable reduction in environmental temperature.

Design: To characterize the dynamic changes in continuously measured resting EE, substrate utilization, and hormonal axes simultaneously in response to mild reduction in environmental temperature, we performed a cross-over intervention.

Methods: Twenty-five volunteers underwent two 12-h recordings of EE in whole room indirect calorimeters at 24 and 19 °C with simultaneous measurement of spontaneous movements and hormonal axes.

Results: Exposure to 19 °C resulted in an increase in plasma and urine norepinephrine levels ($P < 0.0001$), and a 5.96% ($P < 0.001$) increase in EE without significant changes in spontaneous physical activity. Exposure to the lower temperature resulted in a significant increase in free fatty acid levels ($P < 0.01$), fasting insulin levels ($P < 0.05$), and a marginal decrease in postprandial glucose levels. A small but significant ($P < 0.002$) increase in serum free thyroxine and urinary free cortisol ($P < 0.05$) was observed at 19 °C.

Conclusions: Our observations indicate that exposure to 19 °C, a mild and tolerable cold temperature, results in a predictable increase in EE driven by a sustained rise in catecholamine and the activation of counter-regulatory mechanisms.

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Introduction

Obesity, which is the result of a sustained imbalance between energy intake and energy expenditure (EE), has reached epidemic proportions. As a result, obesity-related complications contribute substantially to mortality and healthcare costs (1). Interestingly, even small but prolonged changes in the energy balance equation (energy gap), as low as 100 kcal/day, may play a significant role in the gradual weight gain observed in the adult population (2). Most obesity-directed interventions, pharmacological and behavioral, are aimed at reducing energy intake. No intervention, except increased physical activity, is successful at increasing EE safely and effectively. Besides physical activity (voluntary, spontaneous, and shivering), the other components of total EE are the thermic effect of food

and resting EE (3). In humans, resting EE accounts for 65–85% of total EE and is mainly determined by body size and composition, but it can also be influenced by pathological states, such as hypo- or hyper-thyroidism (4), fever, and drugs (5).

Exposure to cold temperature (16 °C) results in a predictable increase in resting EE (6) (non-shivering thermogenesis), which may be partly explained by an activation of brown adipose tissue (7–9). However, such a level of cold exposure is not sustainable over a long period of time. Furthermore, to our knowledge, no study aimed at characterizing the changes in EE upon temperature manipulation within the range observed in climate-controlled buildings has been performed. Interestingly, the recent trend in regulating indoor climates has been proposed as a significant contributor to the increase in obesity prevalence (10). In this study,

we have accurately characterized the changes in EE, substrate utilization, heart rate variability, temperature, and hormonal homeostasis in healthy volunteers of both sexes exposed to 19 °C, well within the range of tolerability.

Materials and methods

Participants and study design

The study was approved by the NIDDK-NIAMS IRB and conducted at the NIH Clinical Center in Bethesda, MD, USA. The protocol was designed as a randomized, single blind, cross-over intervention study (ClinicalTrials.gov identifier number NCT00521729). Inclusion criteria for the enrollment in the study were an age range of 18–60 years and body mass index (BMI) 20–27 kg/m². Exclusion criteria were thyroid disease (by history or TSH <0.4 or >5.0 mIU/l at screening); hypertension (by history or blood pressure values >140/90 mmHg) or cardiovascular disease; pregnancy or use of hormonal contraception; diabetes mellitus; serum cholesterol ≥240 mg/dl; and/or serum triglycerides ≥220 mg/dl. Female participants were studied in the early follicular phase of the menstrual cycle (days 2–10). After enrollment, the study volunteers were admitted to the Metabolic Clinical Research Unit, and after a 2-day equilibration diet (see below), they were randomly assigned to a 12-h stay in a whole room indirect calorimeter (respiration chamber) at either 24 °C (75 °F) or 19 °C (68 °F). After a 36-h recovery period, the volunteers repeated the 12-h stay at the second study temperature (Fig. 1A). Two days prior to testing and throughout the duration of the study, the volunteers received a caffeine-free weight maintenance diet providing 50% carbohydrate, 20% protein, and 30% fat. Resting energy requirements for the equilibration diet were calculated from the subject's height, weight, and age using the Mifflin–St Jeor equation (11). An activity factor associated with the subjects' self-reported physical activity was used to calculate their total daily energy needs (12). The day prior to the respiration chamber recording, the study volunteers finished their dinner by 1900 h, i.e. 12 h before the study. After a 6-h stay in the respiration chamber, the volunteers received one standard liquid meal (Boost Plus, Nestle Healthcare Nutrition, Inc., Vevey, Switzerland) representing 30% of their calculated energy needs as part of the equilibration diet. After the completion of the 12-h chamber recording, the volunteers were offered a meal containing the remaining 70% calorie content of the estimated daily energy needs. Throughout the recordings, the volunteers wore standard clothing (hospital scrubs), and no blanket was provided. The volunteers were asked to limit their physical activity to a minimum and were encouraged to watch TV or to use a computer while sitting on a

Figure 1 (A) Study protocol overview. The study was designed as a randomized, single blind, cross-over intervention. After enrollment, the volunteers received a weight maintenance diet for 2 days before undergoing 12-h respiration chamber and hormonal axes recording. After 36 h of recovery, the study volunteers underwent the same procedure at the second experimental temperature (see text for details). (B) Blood samples collection and procedures during a 12-h respiration chamber stay. Blood sampling was performed through an airtight sampling port every hour except during the first 2 h of the recording and the first 2 h after the meal when the sampling was performed every 30 min.

recliner or staying in bed. Blood samples were collected at –15, 0, 30, 60, 90, 120, 180, 240, 300, 360, 390, 420, 450, 480, 540, 600, 660, and 720 min via one of the airtight sampling ports while the volunteer was sitting in the recliner. Samples for microdialysis (15 µl) were collected at baseline and every 30 min throughout the recordings (Fig. 1B); microdialysis vials were passed via one of the airtight sampling ports and stored at –20 °C until processed.

Respiration chamber

Each study volunteer underwent continuous recordings of EE and respiratory quotient in a whole room indirect calorimeter. A similar calorimeter was described

previously (13, 14), which can be operated at any stable interior (room) temperature ranging $16.5\text{--}25.2 \pm 0.5^\circ\text{C}$ and at a relative humidity of 35–55%. All temperature, humidity, and barometric pressure were continuously measured (Optica, GE Sensing, Billerica, MA, USA). Airtight sampling ports and a four-way air-locking food and specimen passage were designed to allow blood draws and specimen retrievals with minimal disturbance to the chamber environment. The accuracy and precision of the respiration chambers were evaluated initially using a series of propane calibration tests at 19 and 24°C . The results of the tests demonstrated no significant difference in the accuracy of the VO_2 and VCO_2 measurements between 19 and 24°C when the temperature was maintained within 0.2°C of the set-point temperature. Each recording started at 0800 h and ended at 2000 h. Although the chambers have a small window exposed to the south, most of the lighting was provided by fluorescent bulbs.

Weight, height, and body composition

Each volunteer was weighed daily using a digital balance (Scale-Tronix 5702, Carol Stream, IL, USA) under fasting conditions. Height was measured using a stadiometer (Seca 242, Hanover, MD, USA). Body composition was recorded by a dual-energy X-ray absorptiometry scanner (Lunar iDXA, GE Healthcare, Madison, WI, USA) and analyzed with proprietary software (15).

Physiological monitoring

Vital signs Blood pressure and heart rate values were recorded in the morning of the study upon awakening and immediately after the completion of the respiration chamber stay.

Heart rate variability A Holter monitor (Del Mar-Reynolds, Irvine, CA, USA) was used to record ECG waveform data throughout each study. The inter-beat interval data were analyzed in a custom program created in Matlab (The Mathworks, Natick, MA, USA). Heart rate variability determination was performed using established methods (16). Thirty minute segments overlapping by 50% were used to compute the power in the very low frequency, low frequency, and high frequency bands, representing autonomic influence on the heart via hormonal regulation, the sympathetic system, and the parasympathetic system respectively (17).

Core body and skin temperatures Core body and skin temperatures were measured continuously using ingestible capsules and dermal patches (Philips Respironics, Bend, OR, USA). The dermal patches were placed on the

posterior aspect of the neck, abdomen (1 inch above the navel), and dominant thigh.

Activity monitors Actical accelerometers (Philips Respironics) worn on the wrist, ankle, and hip were used to record the subjects' physical activity every 15 s.

Data processing Data recorded by the physiological monitors (heart rate, core body and skin temperatures), physical activity monitors, and respiration chamber were processed in identical fashion for the 24 and 19°C studies. The first hour of the recording was excluded from the analysis to allow for equilibration of the respiration chamber equipment and for the volunteer to be acclimated to the chamber environment. The last hour of the recording was also excluded from the analysis to prevent contamination of the data from anticipatory activity. The intervening 10-h recording from minute 61 to 660 was analyzed. The hours preceding and following the meal were excluded from the pre- and postprandial analyses, only to prevent feeding-related anticipation or activity from confounding the data.

Microdialysis

Microdialysis of subcutaneous adipose tissue was performed using a CMA 60 (CMA Microdialysis, Stockholm, Sweden) catheter with a flow rate of $0.5\ \mu\text{l}/\text{min}$ using Ringer's solution. The study volunteers were instructed in changing the vials every 30 min. Glucose and glycerol were measured by a microanalyzer (CMA Microdialysis).

Subcutaneous adipose tissue biopsy

Seven volunteers consented for subcutaneous adipose tissue biopsy from the periumbilical area within 30 min of the completion of each respiration chamber recording.

Laboratory assessment

Plasma specimens collected in chilled tubes were separated within 30 min, whereas serum specimens were separated within 1 h of collection. For the measurement of urine analytes (urea, myoglobin, cortisol, and catecholamines), 12-h specimens were collected by splitting them in non-preservative and preservative (acetic acid) containers as appropriate for the test. Urinary myoglobin and urinary free cortisol concentrations were measured at the Mayo Medical Laboratories (Rochester, MN, USA). Plasma catecholamine concentrations were determined in the laboratories of the Reproductive Biology and Medicine Branch of NICHD (18), whereas all other tests were performed in the NIH Clinical Center Department of Laboratory Medicine.

Real-time PCR

Total RNA was extracted from the subcutaneous adipose tissue samples using the RNeasy method and converted to cDNA using a reverse transcriptase kit from Marligen (Ijamsville, MD, USA). The cDNA was then amplified in a TaqMan 7000 real-time PCR apparatus using cyclophilin, *UCP1*, *PRDM16*, *PGC1* (*PPARGC1A*), and *D2* TaqMan gene expression assays (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Statistical analysis was performed using Prism 5 (GraphPad, La Jolla, CA, USA) using two-tailed paired *t*-test. Non-parametric data were analyzed using Wilcoxon signed rank sum test. Where indicated, data were log transformed. The area under the curve (AUC) was calculated using the trapezoidal rule. Results are expressed as mean $\pm$ 1 s.d.; an α error of 0.05 was considered the threshold for statistical significance. A power calculation was performed *a priori* based on the primary outcome of EE. Assuming a delta value of 7% in EE for a 6 °C difference in environmental temperature (19), a sample size of ten subjects from each gender would provide an 80% statistical power at a significance of $P < 0.05$.

Results

Study volunteers' characteristics

Twenty-five eligible individuals (Table 1) completed the study (Fig. 2).

Physiological parameters

A summary of the physiological recordings is reported in Table 2.

Table 1 Subject characteristics (mean $\pm$ s.d.).

	Male (n = 15)	Female (n = 10)	Total (n = 25)
Height (cm)	174.7 $\pm$ 7.6	165.8 $\pm$ 6.3	171.1 $\pm$ 8.3
Weight (kg)	73.1 $\pm$ 9.6	61.0 $\pm$ 5.3	68.2 $\pm$ 10.0
BMI (kg/m ²)	23.9 $\pm$ 2.2	22.2 $\pm$ 1.9	23.3 $\pm$ 2.2
Age (years)	26.1 $\pm$ 5.8	32.3 $\pm$ 10.6	28.6 $\pm$ 8.5
Body fat (%) ^a	19.7 $\pm$ 6.2	26.4 $\pm$ 3.5	22.7 $\pm$ 6.1
Calorie content of meal in respiration chamber (kcal) ^b	837 $\pm$ 163	630 $\pm$ 78	754 $\pm$ 169
Ethnicity	8 Caucasian 2 Black 4 Asian 1 Hispanic	6 Caucasian 3 Black 1 Asian	14 Caucasian 5 Black 5 Asian 1 Hispanic

^an = 22 (ten females).

^bThirty-percent of calculated daily energy needs.

Figure 2 CONSORT chart. Study volunteers screening, accrual, temperature sequence allocation, and completion rate.

Respiration chamber Exposure to 19 °C resulted in an increase in EE in 18 of 21 subjects (Fig. 3), with a group mean of 5.96% ($P < 0.001$) and a trend toward a decrease in preprandial respiratory quotient ($P = 0.09$). At 19 °C, the increase in EE was observed both pre- and postprandially (Fig. 4 and Table 2). No differences were observed between the sexes.

Physical activity No difference in the physical activity was observed in the subjects at the two temperature exposures (Table 2).

Core body and skin temperatures There was no significant change in the core body temperature, but skin temperatures at the neck, abdomen, and thigh were reduced at 19 °C (all $P < 0.001$). The core body and thigh temperatures demonstrated a significant increase during the postprandial period at 24 and 19 °C (both $P < 0.01$).

Heart rate and heart rate variability Heart rate was significantly reduced at 19 °C ($P = 0.004$). The magnitude of all three heart rate variability components significantly increased at 19 °C ($P < 0.001$).

Table 2 Energy expenditure, activity, body temperature, and heart rate variability.

	n	12-h average		Preprandial period			Postprandial period			
		24 °C	19 °C	24 °C	19 °C	24 °C	19 °C			
Calorimetry										
Energy expenditure (kcal/h)	21	82.09 ± 8.61	86.98 ± 9.93	P < 0.001	77.94 ± 8.58	83.38 ± 9.67	P < 0.001	86.70 ± 9.31 [†]	90.52 ± 11.23 [†]	P < 0.001
Respiratory quotient	21	0.821 ± 0.025	0.816 ± 0.022	P = 0.23	0.801 ± 0.025	0.796 ± 0.024	P = 0.09	0.850 ± 0.029 [†]	0.843 ± 0.026 [†]	P = 0.32
Activity										
Wrist activity (activity counts)	22	69 442 ± 35 806	72 434 ± 36 999	P = 0.97	27 402 ± 14 167	30 823 ± 18 040	P = 0.41	27 798 ± 16 436	26 884 ± 15 159	P = 0.39
Hip activity (activity counts)	21	3897 ± 3709	3609 ± 3248	P = 0.50	1554 ± 1247	1586 ± 1417	P = 0.82	1657 ± 1846	1412 ± 1464	P = 0.21
Ankle activity (activity counts)	22	11 662 ± 9563	10 690 ± 6541	P = 0.74	4324 ± 3638	4384 ± 2975	P = 0.74	5691 ± 7846	4917 ± 4238	P = 0.53
Body temperature										
Core body temperature (°C)	10	36.90 ± 0.24	36.87 ± 0.25	P = 0.56	36.79 ± 0.28	36.82 ± 0.22	P = 0.92	37.04 ± 0.22 [†]	36.96 ± 0.27	P = 0.06
Neck temperature (°C)	17	35.12 ± 0.59	34.05 ± 1.05	P < 0.001	34.96 ± 0.64	33.82 ± 1.15	P < 0.001	35.28 ± 0.63 [*]	34.32 ± 1.12 [†]	P < 0.001
Abdominal temperature (°C)	22	34.32 ± 0.75	32.99 ± 1.26	P < 0.001	34.12 ± 0.80	32.63 ± 1.37	P < 0.001	34.47 ± 0.83 [*]	33.40 ± 1.31 [†]	P = 0.0016
Thigh temperature (°C)	21	32.62 ± 0.74	30.63 ± 1.68	P < 0.001	32.39 ± 0.79	30.39 ± 1.58	P < 0.001	32.93 ± 0.90 [†]	30.98 ± 2.09	P < 0.001
Heart rate variability										
Heart rate (BPM)	20	65.26 ± 6.98	62.33 ± 8.72	P = 0.004	61.89 ± 7.17	59.52 ± 7.95	P = 0.029	68.78 ± 7.68 [†]	65.43 ± 9.71 [†]	P = 0.005
Very low frequency HRV (ms ²)	20	4146 ± 1808	5432 ± 3086	P = 0.0016	4803 ± 2044	6311 ± 3659	P = 0.0034	3487 ± 1826 [†]	4413 ± 2538 [†]	P = 0.14
Low frequency HRV (ms ²)	20	1737 ± 998	2233 ± 1402	P = 0.0038	1972 ± 1166	2491 ± 1599	P = 0.0048	1485 ± 777 [†]	1938 ± 1211 [†]	P = 0.021
High frequency HRV (ms ²)	20	663 ± 455	962 ± 648	P = 0.001	744 ± 451	1056 ± 702	P = 0.0043	575 ± 507 [*]	837 ± 653 [†]	P = 0.0043

*P < 0.05 compared with preprandial period of the same day. [†]P < 0.01 compared with preprandial period of the same day.

Blood pressure No significant difference was observed at baseline in either systolic (24 °C: 116.1 ± 11.8 vs 19 °C: 118.3 ± 10.0 mmHg, P = NS) or diastolic (24 °C: 66.8 ± 7.3 vs 19 °C: 68.8 ± 7.9 mmHg, P = NS) blood pressure. Conversely, at 19 °C upon completion of the study, both systolic (24 °C: 125.8 ± 12.7 vs 19 °C: 130.6 ± 13.5 mmHg, P < 0.05) and diastolic (24 °C: 70.4 ± 6.7 vs 19 °C: 76.4 ± 11.9 mmHg, P < 0.02) blood pressure values were significantly increased.

Laboratory data

The laboratory data obtained are reported in Table 3.

Catecholamines While baseline plasma nor-epinephrine levels were similar between the two temperature exposures, a significant increase in the AUC was observed at 19 °C (P < 0.0001; Fig. 4). Concordantly, plasma dihydroxyphenylglycine (DHPG) and 3,4-dihydroxyphenylacetic acid (DOPAC), and 12-h urinary catecholamine showed significant increases (all P ≤ 0.05). The observed changes were similar in men and women.

Glucocorticoid axis While no significant changes were observed in both plasma ACTH and serum cortisol AUCs, a small but significant increase in urinary free cortisol was observed at 19 °C (P < 0.05). When the analysis was performed according to the gender, a small but significant increase in serum cortisol AUC (P < 0.04) was observed in males but not in females. Conversely, the increase in urinary free cortisol excretion at 19 °C was significant in females (P < 0.02), but not in males.

Thyroid hormones axis Compared with exposure to 24 °C, exposure to 19 °C resulted in small, non-significant increases in total triiodothyronine (T₃) and TSH AUCs and a significant increase in serum free thyroxine (T₄; P = 0.03). When the analysis was performed according to the gender, a small but significant increase in serum T₃ AUC was observed in males (P < 0.05) but not in females. Similarly, while the change in free T₄ was highly significant in males (P < 0.002), no significant change was observed in females.

Glucose and insulin While no statistical difference was observed in the 12-h AUCs for plasma glucose and the analysis was limited to 3 h following the meal (time 360–540 min), a significant reduction in the AUC was observed at 19 °C (P < 0.05). Conversely, although no significant change was observed in postprandial insulin AUC, exposure to 19 °C resulted in a small but significant increase in fasting (time – 15 to 300 min) insulin AUC (P = 0.04) without differences between the sexes.

Figure 3 Mean energy expenditure rate in 21 participants over 12 h spent at 24 °C (left) and 19 °C (right). Data are presented as individual recordings and as box-whisker plots for all the 21 participants, 14 males (filled circles) and 7 females (open circles).

Free fatty acids and β -hydroxybutyrate Exposure to 19 °C resulted in a significant increase in free fatty acids ($P < 0.01$) and a non-significant increase in β -hydroxybutyrate AUCs without differences between the sexes.

Real-time PCR analysis and microdialysis of subcutaneous adipose tissue No transcript was observed for *UCP1* and *D2*, and a weak signal was observed for *PRDM16* and *PGC1*. No significant change was observed in the expression levels of these two genes (data not shown). A trend toward an increase in the glycerol AUC was observed in the microdialysate ($P = 0.07$) at 19 °C. Conversely, a non-significant reduction in the glucose AUC was observed in the same group ($P = 0.07$), without differences between the sexes.

Discussion

The primary aim of this study was to quantify the dynamic changes in EE and to investigate their associations with changes in hormonal axes, substrate utilization, and autonomic nervous system parameters in healthy subjects of both sexes exposed to a sustainable reduction in environmental temperature. We thus targeted whole-body adaptive thermogenesis, irrespective of the anatomical or tissue distribution. The two experimental conditions (19 and 24 °C) were chosen because they are tolerable and closely represent the range of temperatures in climate-controlled buildings and homes. To accurately measure the changes in hormonal homeostasis and substrate utilization, we performed multiple blood sampling concomitant to the EE measurement. We further explored the acute effects of a standard meal on these physiological parameters.

Our data demonstrate that a 5 °C reduction in the environmental temperature resulted in a consistent increase of about 6% in EE without differences between the sexes. The observed changes are due to an increase in non-shivering thermogenesis without any measurable increase in spontaneous physical activity. The relative magnitude of this increase in EE

was generally similar to that measured by others (20) upon exposure to colder temperatures for longer periods.

Not surprisingly, the exposure to 19 °C resulted in a decrease in skin temperature due to superficial vasoconstriction, which was consistent with previous findings (20). Contrary to earlier experiments (20) performed at a colder temperature (16 °C), we did not observe changes in the core body temperature. A postmeal increase in temperature, indicating a measurable thermic effect of food, was present in both the abdomen and the neck regions at 24 and 19 °C. However, this response was not observed at 19 °C in either the thigh or the core body temperatures, suggesting that the thermic effect of food is somewhat blunted upon exposure to 19 °C.

We postulate that the increase in sympathetic nervous system activity, as evidenced by the relative elevation of plasma and urinary catecholamine and by the increase in the low frequency heart rate variability, is a major effector for the increase in EE in response to 19 °C. The dynamic changes in plasma concentrations of DOPAC and DHPG mirrored that of norepinephrine, indicating that the overall catecholamine turnover was up-regulated. Consistent with previous findings (21), the observed reduction in the heart rate and the increase in blood pressure are most likely secondary to peripheral vasoconstriction.

Figure 4 Mean and s.d. of 21 participants for energy expenditure (top panel), respiratory quotient (middle panel), and plasma norepinephrine levels (bottom panel) at 24 °C (gray line) and 19 °C (black line) over the 12-h time course of the study. The dotted line indicates meal consumption at 360 min.

Table 3 Laboratory data.

	24 °C	19 °C	P
Catecholamines			
Plasma norepinephrine AUC ($\times 10^5$ pg min/ml)	2.74 $\pm$ 1.11	4.68 $\pm$ 2.27	<0.0001
Plasma norepinephrine 420' (pg/ml)	464.0 $\pm$ 264.7	895.7 $\pm$ 565.9	<0.001
Urine epinephrine (μ g/12 h)	5.10 $\pm$ 2.415	6.41 $\pm$ 3.636	0.05
Urine norepinephrine (μ g/12 h)	20.70 $\pm$ 8.659	29.44 $\pm$ 11.29	<0.001
Urinary DOPAC (μ g/12 h)	118.1 $\pm$ 47.80	136.3 $\pm$ 42.08	0.03
DOPAC AUC ($\times 10^5$ pg min/ml)	0.97 $\pm$ 0.45	1.14 $\pm$ 0.69	0.04
DHPG AUC ($\times 10^5$ pg min/ml)	8.12 $\pm$ 3.59	9.35 $\pm$ 4.25	<0.01
Glucocorticoid axis			
ACTH AUC ($\times 10^4$ pg min/ml)	1.25 $\pm$ 0.42	1.29 $\pm$ 0.54	0.49
Serum cortisol AUC ($\times 10^3$ μ g min/dl)	6.69 $\pm$ 1.26	7.03 $\pm$ 1.26	0.21
Urinary cortisol (μ g/g creatinine)	28.5 $\pm$ 17.4	38.5 $\pm$ 33.2	<0.02
Thyroid hormone axis			
TSH AUC ($\times 10^3$ μ UI min/ml)	0.98 $\pm$ 0.57	1.04 $\pm$ 0.53	0.22
T ₃ AUC ($\times 10^4$ ng min/dl)	7.02 $\pm$ 1.14	7.22 $\pm$ 1.31	0.07
Free T ₄ AUC ($\times 10^3$ ng min/dl)	0.98 $\pm$ 0.13	1.02 $\pm$ 0.15	0.02
Serum glucose and insulin			
Glucose AUC ($\times 10^3$ mg min/dl)	65.90 $\pm$ 4.32	65.03 $\pm$ 4.20	0.29
Fasting glucose AUC (–15' to 300') ($\times 10^3$ mg min/dl)	26.63 $\pm$ 1.95	26.62 $\pm$ 2.04	0.99
Postmeal glucose AUC (360'–540') ($\times 10^3$ mg min/dl)	19.71 $\pm$ 2.55	18.69 $\pm$ 2.61	0.04
Insulin AUC ($\times 10^3$ mIU min/ml)	8.53 $\pm$ 3.44	10.77 $\pm$ 6.34	0.07
Fasting insulin AUC (–15' to 300') ($\times 10^3$ mIU min/ml)	0.65 $\pm$ 0.86	0.83 $\pm$ 0.98	0.04
Postmeal insulin AUC (360'–540') ($\times 10^3$ mIU min/ml)	5.78 $\pm$ 2.19	7.39 $\pm$ 5.11	0.13
Microdialysis of subcutaneous adipose tissue			
Glycerol AUC ($\times 10^5$ μ mol min/ml)	1.12 $\pm$ 0.50	1.35 $\pm$ 0.62	0.07
Glucose AUC ($\times 10^3$ mmol min/ml)	2.84 $\pm$ 0.60	2.56 $\pm$ 0.60	0.07
Other measurements			
Free fatty acids AUC ($\times 10^5$ μ Eq min/l)	2.88 $\pm$ 0.93	3.24 $\pm$ 1.14	<0.01
β -Hydroxybutyrate AUC ($\times 10^4$ μ mol min/l)	7.70 $\pm$ 4.27	9.58 $\pm$ 6.95	0.50
Creatinine kinase AUC ($\times 10^5$ U min/l)	1.02 $\pm$ 0.77	1.08 $\pm$ 0.82	0.62
Urinary myoglobin	Not detected	Not detected	

Contrary to previous observations (7, 20), we did not observe any substantial difference in substrate utilization, although we observed a trend toward a reduction in fasting respiratory quotient (RQ) at 19 °C. This discrepancy could be explained by differences in experimental conditions, since the lower temperature was milder in this study. The increase in plasma free fatty acids (FFA) is consistent with catecholamine-stimulated lipolysis. The marginal increase in glycerol levels in the subcutaneous adipose tissue further supports this explanation.

The effects of catecholamines on glucose metabolism are more complex. The increase in fasting insulin observed at 19 °C is likely secondary to catecholamine-mediated hepatic gluconeogenesis (22); however, it did not extend into the postprandial period and the glucose AUC was lower during this part of the study. Furthermore, the raise in FFA likely contributes to the state of relative insulin resistance observed during fasting. Taken together, these findings can be interpreted as a state of relative insulin resistance and catecholamine-stimulated hepatic gluconeogenesis, with an overall increase in total consumption of metabolic fuels. The marginal decrease in glucose levels in the subcutaneous adipose tissue further strengthens this explanation.

At 19 °C, we also observed an increase in free T₄ (and serum T₃ in men), consistent with a shift toward the

activation of the pituitary–thyroid axis. Alternatively, one could speculate that the increase in free T₄ (as measured by analog assay) is at least in part secondary to an interference due to the relative increase in FFA levels (23). Nonetheless, it is worth noting that sustained exposure to β -adrenergic stimulation generates an increase in serum T₃ levels (24), in keeping with an activation of the type-2 deiodinase-mediated T₄ to T₃ conversion (25, 26). It is thus conceivable that prolonged exposure to mild cold temperature will result in a significant activation of the thyroid axis (with a further increase in EE) as a long-term compensatory response to cold exposure. Finally, although no changes were demonstrated in the ACTH levels, the small but significant increase in urinary free cortisol (and serum cortisol in men) implies activation of the hypothalamic–pituitary–adrenal axis consistent with a stress response. The changes in the glucocorticoid and thyroid axes were particularly evident in males. Although we cannot rule out gender as a primary cause of these differences, it is possible that the observed changes are at least in part attributable to the higher proportion of fat-free mass in males. Conversely, the greater increase in urinary cortisol observed in females is probably related to the lower fat-free mass (and hence relatively lower urinary cortisol/creatinine ratio).

Our data indicate that subtle modulations in the environmental temperature, well within the limits of tolerability, result in significant changes in EE and in many hormonal axes. The magnitude of the observed increase in EE is remarkable, since it is similar or superior to pharmacological interventions aimed at increasing EE and ultimately to weight loss (27–30). Indeed, projecting the point estimate increase in EE from this study over the 24-h period would represent 20% of the negative energy balance commonly prescribed in weight loss interventions (31, 32). It is worth noting that the strict adherence to such regimens is uncommon, and that the self-assessment of the dietary intake is usually biased toward under-reporting (33); hence the actual deficit in energy balance required to achieve a sustained weight loss would probably be significantly less than what is commonly prescribed, remarkably similar to the one we observed (2).

It is possible that compensatory mechanisms, such as an increase in energy intake (appetite), would prevent weight loss (34). Furthermore, our data indicate that exposure to 19 °C leads to a mild increase in blood pressure, a state of relative insulin resistance, and a marginal increase in cortisol whose long-term consequences could potentially trump the beneficial effects of increasing resting EE.

This study is a proof-of-concept that manipulations in the environmental temperature, well within the range of tolerability, result in a significant increase in EE and in measurable changes in hormonal homeostasis. One plausible mechanism is catecholamine induction of brown adipose tissue (BAT)-like activity. While no changes were observed in the BAT-specific transcripts in the subcutaneous adipose tissue, it is possible that longer duration of exposure to 19 °C could stimulate transcription of BAT-specific genes (35, 36). However, the actual contribution of BAT to the maintenance of energy balance in humans has not been empirically demonstrated, and it is possible that other tissues such as skeletal muscle may play a major role in adaptive thermogenesis in adult humans (37).

The findings of this study are particularly robust since, to the best of our knowledge, this is the first to study the simultaneous characterization of the changes in EE, sympathetic nervous system activity, and hormonal axes by frequent blood sampling in response to minimal perturbations of the environmental temperature in a relatively large number of volunteers of both the sexes. The study design and the use of a diet-controlled run-in period virtually eliminate the possibility of bias due to carry-over effect, and any confounder due to anticipation. Furthermore, we carefully calibrated our whole room indirect calorimeters at each temperature to ensure accurate measurements of physiological changes in EE and substrate oxidation. One obvious limitation of the study is represented by the brevity of the intervention; we were thus unable to demonstrate any difference in clinically significant

endpoints, such as BMI, body composition, and carbohydrate metabolism parameters. Furthermore, we could not evaluate the effects of counter-regulatory mechanisms. Since our study population was limited to non-obese, relatively young individuals, it is also possible that subjects with a higher percentage of fat mass, such as overweight and elderly individuals, may have a blunted response to this intervention (38). When we explored the individual changes in EE with respect to fat mass and changes in plasma norepinephrine, we observed a non-significant negative trend that supports this hypothesis (data not shown). Although we performed multiple comparisons on our dataset, one should consider that the primary endpoint was defined *a priori* in the setting of the statistical power analysis and that the findings in the various secondary endpoints are in keeping with the study hypothesis and primary endpoint findings. Thus, the possibility of type-1 error appears extremely unlikely.

In conclusion, our study demonstrates that minimal modulation in the environmental temperature results in a significant and potentially clinically relevant increase in EE. Further studies are needed to investigate the long-term effects of mild cold exposure on clinically relevant end-points and its applicability as an intervention aimed to promote weight loss.

Declaration 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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