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Short-term Cold Acclimation Recruits Brown Adipose Tissue in Obese Humans

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Recruitment of brown adipose tissue (BAT) has emerged as a potential tool to combat obesity and associated metabolic complications. Short-term cold acclimation has been shown not only to enhance the presence and activity of BAT in lean humans but also to improve the metabolic profile of skeletal muscle to benefit glucose uptake in patients with type 2 diabetes. Here we examined whether short-term cold acclimation also induced such adaptations in 10 metabolically healthy obese male subjects. A 10-day cold acclimation period resulted in increased cold-induced glucose uptake in BAT, as assessed by [^{18}F]fluorodeoxyglucose positron emission tomography/computed tomography. BAT activity was negatively related to age, with a similar trend for body fat percentage. In addition, cold-induced glucose uptake in BAT was positively related to glucose uptake in visceral white adipose tissue, although glucose uptake in visceral and subcutaneous white adipose tissue depots was unchanged upon cold acclimation. Cold-induced skeletal muscle glucose uptake tended to increase upon cold acclimation, which was paralleled by increased basal GLUT4 localization in the sarcolemma, as assessed through muscle biopsies. Proximal skin temperature was increased and subjective responses to cold were slightly improved at the end of the acclimation period. These metabolic adaptations to prolonged exposure to mild cold may lead to improved glucose metabolism or prevent the development of obesity-associated insulin resistance and hyperglycemia.

Because of the ever-growing prevalence of global obesity, there is an ongoing search for effective strategies to enhance energy expenditure (EE) and subsequently counteract obesity and its negative metabolic consequences. Adaptive thermogenesis—that is, an increased capacity for heat production upon prolonged exposure to cold (1)—has gained considerable renewed interest in this context, especially because of the recent appreciation of considerable amounts of metabolically active brown adipose tissue (BAT) in adult humans (2–4). BAT is highly specialized to convert energy from substrate oxidation directly into heat through the action of uncoupling protein 1, thereby contributing to the maintenance of a constant internal body temperature upon exposure to cold. Because of this "energy-wasting" process, BAT activation results in increased EE, which could reverse or protect against obesity. Indeed, several rodent studies have demonstrated that BAT activation and recruitment by prolonged exposure to cold or β-adrenergic stimulation ameliorates diet-induced obesity and improves its associated complications, such as disturbed glucose and lipid homeostasis (5,6). In animals, prolonged cold stimulation also induces browning of distinct white adipose tissue (WAT) depots, likely contributing to improved substrate metabolism (7). This phenomenon also has recently been observed in human subcutaneous WAT upon severe and prolonged adrenergic stress (8).

In humans, as in mice, exposure to cold is one of the most powerful physiological stimuli for activation of BAT

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(9). As such, using dedicated cooling protocols to activate BAT, we have shown that BAT is present and active upon acute exposure to cold in 90–100% of lean, young adults and that BAT activity is related to nonshivering thermogenesis (NST) (10,11). In addition, several studies have reported negative relations between BAT activity and age and body fatness in humans (3,12), likely the result of the "whitening" of classical BAT depots (13), most notably the supraclavicular BAT depot. Interestingly, both older age (14) and obesity (15) are also associated with a blunted NST response upon exposure to mild cold. This has led to the hypothesis that recruitment of BAT by cold acclimatization could enhance cold-induced EE, thereby counteracting WAT accumulation and its possible negative metabolic consequences. We (11) and others (16–18) recently showed that BAT can be effectively recruited in lean individuals by means of prolonged intermittent exposure to cold, that is, cold acclimation. Interestingly, BAT recruitment was indeed paralleled by enhanced cold-induced NST (11,16). Moreover, Yoneshiro et al. (16) showed a negative relation between changes in BAT activity and whole-body fat mass after daily 2-h exposure to cold (17°C) for 6 weeks, suggesting an antiobesity effect of BAT recruitment in humans. Importantly, these studies were all performed in young, lean, and healthy subjects. A 10-day cold acclimation period in patients with type 2 diabetes resulted in only a minor increase in metabolic activity of the supraclavicular BAT region (19). However, in addition to being overweight, these patients were also older and presented already very low activity of this BAT region at baseline. Therefore, it remains to be established whether significant amounts of BAT can be recruited in obese subjects within a wide age range and whether this is associated with enhanced NST.

In addition to BAT, skeletal muscle (SM) and visceral and subcutaneous adipose tissue metabolism also may be affected by prolonged exposure to cold, as has been suggested by animal studies (7,20,21) and our recent observation of enhanced basal GLUT4 translocation upon cold acclimation in patients with type 2 diabetes (19). However, it is not known whether prolonged, intermittent exposure to cold can elicit such improvements in metabolically healthy obese subjects as well. Therefore, we studied here changes in NST, BAT presence and activity, browning of WAT, and SM metabolic parameters after a 10-day cold acclimation period in healthy obese males.

RESEARCH DESIGN AND METHODS

Subjects

Ten overweight/obese healthy male participants were included in this study (Table 1). All participants were screened for medical history and status. Exclusion criteria included diabetes mellitus, history of cardiovascular disease, use of β-blockers, liver or kidney dysfunction, asthma or any other obstructive pulmonary disease, and severe physical activity more than twice per week. Studies were performed between March 2014 and February 2015.

Table 1—Subject characteristics

Characteristics	Average	Range
Age (years)	36.0 ± 13.0	19–59
Weight (kg)	108.4 ± 18.3	78.9–129.4
Height (m)	1.81 ± 0.07	1.68–1.89
BMI (kg/m^2)	32.9 ± 3.5	28.1–36.8
Fat mass (kg)	32.6 ± 10.2	20.1–44.9
Fat (%)	29.2 ± 5.3	19.4–33.8

Data are expressed as mean ± SD unless otherwise indicated.

Study Approval

The Ethics Committee of Maastricht University Medical Center approved the study protocol, and all participants provided written informed consent. Procedures were conducted according to the principles of the Declaration of Helsinki.

Study Design

A 10-day cold acclimation intervention was performed as previously described (11,19). Before the cold acclimation period, body composition was determined by means of DXA (Discovery A; Hologic, Bedford, MA). An individualized cooling protocol was subsequently performed, followed by [^{18}F]fluorodeoxyglucose (FDG) positron emission tomography (PET)/computed tomography (CT) (Gemini TF PET/CT; Philips, Eindhoven, the Netherlands) for quantification of cold-induced BAT activity. In addition, on a separate day and under thermoneutral conditions, a biopsy was taken from the vastus lateralis muscle in the morning after an overnight fast. During the subsequent cold acclimation period, subjects were exposed to an environmental temperature of 14–15°C for 10 consecutive days, with exposure to cold for 2 h on the 1st day, 4 h on the 2nd day, and 6 h on the 3rd through the 10th days. At the beginning of the 10th day a second muscle biopsy was taken (at thermoneutral conditions), and the individualized cooling protocol and [^{18}F]FDG-PET/CT were repeated on day 11. Body weight was measured before and after cold acclimation.

Subjects consumed standardized meals (42%, 35%, and 23% energy from carbohydrates, fat, and protein, respectively) on the evenings before the experimental days. They were asked to refrain from heavy exercise for at least 48 h before the measurements.

Cold Acclimation

During cold acclimation, subjects were dressed in T-shirts and shorts and remained sedentary. Subjects were instructed not to change their dietary habits and food intake in the cold room throughout the 10-day cold acclimation period. At prescribed time points (t = 0, 20, 40, 60, 90, 120, 180, 240, 300, and 360 min), blood pressure was monitored and visual analog scales for sensation, thermal comfort, and shivering were completed. Incremental areas under the curve (iAUCs) of these visual

analog scales were calculated using the trapezoid rule. Wireless temperature sensors (iButton; Maxim Integrated Products, San Jose, CA) were placed on 14 International Organization for Standardization (ISO)–defined sites on days 3, 7, and 10 to measure skin temperature (22). Mean skin temperature was calculated as the average temperature of measurements at these 14 sites, distal skin temperature as the average temperature of the left foot and right hand, and proximal skin temperature as the average temperature of the chest, abdomen, scapula, and lower back. iAUCs from t = 20–350 min were calculated to determine physiological skin temperature responses during cold acclimation.

Individualized Cooling and PET/CT

An individualized cooling protocol was performed after a 4-h fasting period as previously described (11,19). Briefly, subjects were wrapped in a water-perfused suit (ThermaWrap Universal 3166; MTRE Advanced Technologies Ltd., Yavne, Israel) and were measured at thermoneutrality for 45 min. Thereafter, subjects were gradually cooled to a temperature just above the individual's shivering point and were measured for 30 min at this temperature. Core and skin temperatures, skin perfusion, heart rate, and EE (determined by means of indirect calorimetry using EZCAL; Maastricht Instruments, Maastricht, the Netherlands) were measured continuously, and blood pressure was monitored every 15 min during the cooling protocol. NST was calculated as the percent increase in EE upon exposure to mild cold above the basal metabolic rate (measured at thermoneutrality). At the end of the thermoneutral period and at the end of the cold period, blood was drawn from a catheter placed in an antecubital vein. A bolus of 74 MBq [^{18}F]FDG was subsequently injected intravenously. One hour after injection the PET/CT protocol started with a low-dose CT scan (120 kV, 30 mAs), immediately followed by a static PET scan (6 or 7 bed positions, 4 min/bed position) covering the skull to the abdomen.

PET/CT Analysis

PET/CT scans were analyzed using PMOD software (version 3.0; PMOD Technologies, Zurich, Switzerland) by two of the researchers (M.J.W.H. and A.A.J.J.v.d.L.) and an experienced nuclear medicine physician (B.B.). BAT activity was defined as [^{18}F]FDG uptake >1.5 standardized uptake value (SUV) in fat tissue (Hounsfield units between −10 and −180). SUV was calculated as ([^{18}F]FDG uptake [kBq/mL])/(injected dose [kBq]/patient weight [g]). Regions of interest were semiautomatically outlined for determination of mean (SUVmean) and maximal (SUVmax) [^{18}F]FDG uptake in BAT locations.

Since not all subjects showed pronounced BAT activity, defined by the SUV threshold of >1.5, fixed volumes of interest (VOIs) (2.67 cm^3) were also carefully selected in the supraclavicular adipose tissue region (Hounsfield units between −10 and −180). These VOIs were placed in the area with the highest [^{18}F]FDG uptake in the baseline PET scan and in the same anatomic position in the second PET scan to compare activity of this putative BAT region in the same anatomic location before and after cold acclimation (23). VOIs were also placed in the liver and brain and in seven SM groups (deltoid, biceps and triceps brachii, erector spinae, scalene, levator scapulae, and psoas major muscles), as previously described (19,24). Average activity of these seven muscle groups is presented as average SM [^{18}F]FDG uptake. Subcutaneous WAT was measured in the dorsolumbar region near vertebrae L3, as well as in the abdominal WAT region in the same transverse slices (both 8 cm^3). Visceral WAT was measured behind the xiphoid (1.3 cm^3), at the omental WAT region at the level of the spleen and stomach (4 cm^3), at the paracolic WAT region (4 cm^3), and at the infrarenal WAT region (8 cm^3) (Supplementary Fig. 1). Average activity of these regions is presented as average subcutaneous WAT and average visceral WAT [^{18}F]FDG uptake, respectively. The VOIs were used to compare [^{18}F]FDG uptake (calculated as SUVmean) between these tissues and between scans before and after cold acclimation.

Ex Vivo SM Respiration

After the biopsy, a portion of the muscle tissue was immediately frozen in melting isopentane and stored at −80°C until assayed. Another portion (~30 mg) was instantly placed in ice-cold preservation medium (BIOPS; OROBOROS Instruments, Innsbruck, Austria) and used for the preparation of permeabilized muscle fibers (25). These were subsequently analyzed for mitochondrial oxidative capacity using an oxygraph (OROBOROS Instruments) according to the method described by Hoeks et al. (26).

In separate measurements, mitochondrial leak respiration was determined as the residual respiration upon addition of 1 μg/mL of the ATP synthase–inhibitor oligomycin, using pyruvate (5 mmol/L) as a substrate (in the presence of 4 mmol/L malate). All oxygen consumption measurements were performed in quadruplicate. Respiration data were normalized for mitochondrial DNA copy number (ratio of ND1 to lipoprotein lipase), as described previously (25). One muscle biopsy failed before cold acclimation; therefore, values for nine subjects are presented.

Muscle Biopsy Analysis

Protein levels were determined by Western blotting according to standard procedures. Primary antibodies directed against GLUT4 (sc-1608; Santa Cruz Biotechnology Bio-Connect, Huissen, the Netherlands), total OXPHOS human Western blot antibody cocktail (ab110411; Abcam, Cambridge, U.K.), and α-sarcomeric actin (loading control; A2172; Sigma, Zwijndrecht, the Netherlands) were detected using appropriate secondary antibodies conjugated with IRDye680 or IRDye800 and detected with the Odyssey Near Infrared System (LI-COR, Westburg, Leusden, the Netherlands).

For GLUT4 imaging, immunofluorescence assays were performed as previously described (19), using primary

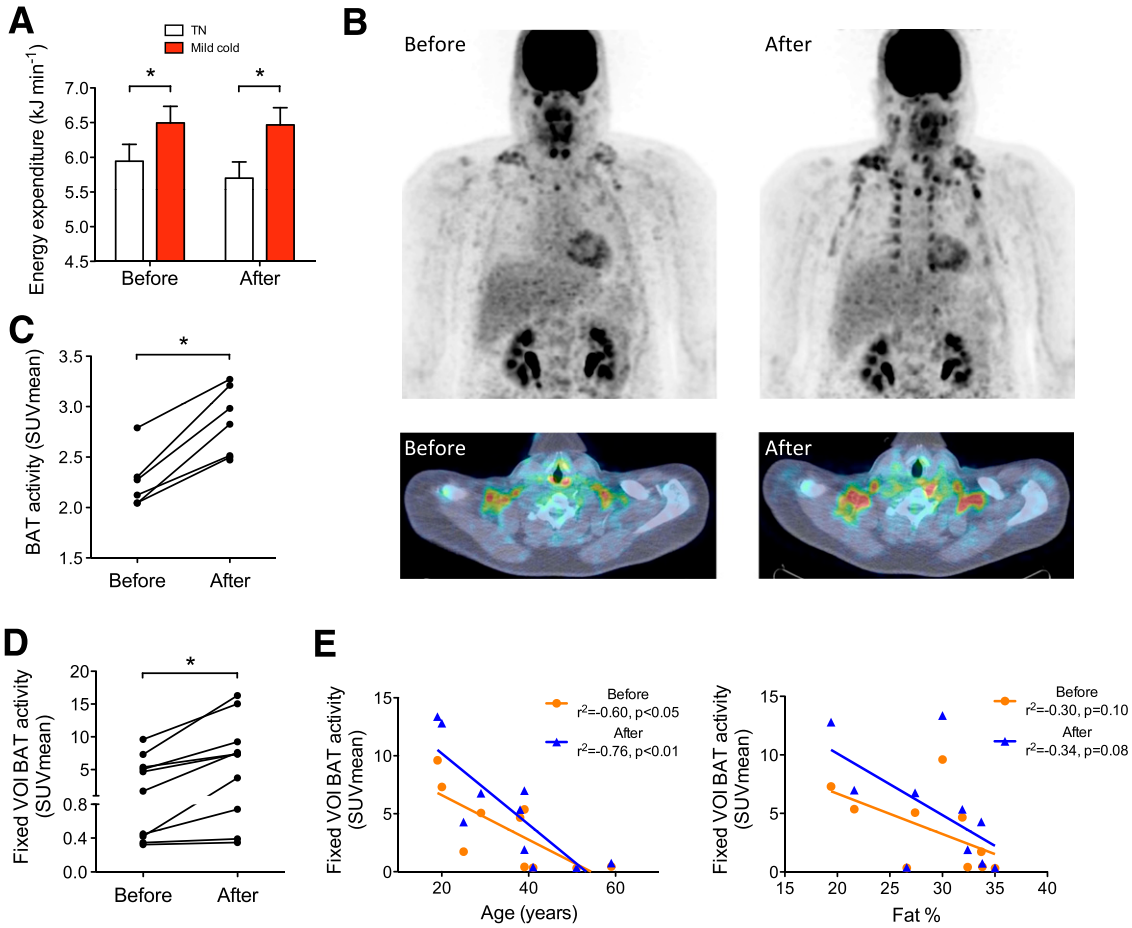

Figure 1—EE and BAT activity before and after exposure to cold in obese subjects, and comparison of BAT activity between obese and lean cold-acclimated subjects. *A*: EE during thermoneutral (TN) and mild cold conditions, before and after cold acclimation. *$P < 0.05$, TN vs. mild cold. *B*: Upper-body [^{18}F]FDG-PET/CT image of an obese subject upon exposure to mild cold, before (left) and after (right) cold acclimation (top); transverse PET/CT fusion slices of the supraclavicular region showing [^{18}F]FDG uptake in BAT locations upon exposure to mild cold, before (left) and after (right) cold acclimation (bottom). *C*: Individual data on BAT activity for the BAT-positive subjects. *$P < 0.05$, before vs. after cold acclimation. *D*: Individual data on activity of the supraclavicular BAT region measured by the fixed volume method in all subjects. *$P < 0.05$, before vs. after cold acclimation. *E*: Correlation between BAT activity and age (left) and body fat percentage (right), before and after cold acclimation. Data are expressed as mean ± SEM.

antibodies directed to GLUT4 (sc-1608; Santa Cruz Biotechnology) and laminin (L9393; Sigma), and Alexa Fluor 555– and Alexa Fluor 488–conjugated secondary antibodies. Slices were observed using a Nikon E800 fluorescence microscope with NIS-Elements imaging software (Nikon Europe BV, Amsterdam, the Netherlands) and were captured with identical exposure time and gain settings in paired ("before-and-after") samples. Without any adjustments with respect to color intensity, brightness, or contrast, RGB-stacked images were quantified using the Plot Profile tool in ImageJ software. Quantification was performed by a researcher (M.J.W.H.) who was blinded to subject identity and sample time (before vs. after). The intensity of GLUT4-dependent signals (16 bits) was measured throughout the sections, and measured data on intensity were used to generate overlying plots of GLUT4 and laminin. GLUT4-derived staining intensity at the sarcolemma (average of five pixels) was quantified at multiple locations per individual (21.4 ± 4.7 locations before and 20.4 ± 4.2 locations after cold acclimation) at randomly chosen cross-sections of muscle biopsies. This was divided over the average GLUT4 staining intensity in 15 pixels located in the cytosol of the same cell. Thus a GLUT4 intensity sarcolemma-to-cytosol ratio >1.0 indicates that relatively more GLUT4 was detected in the cell membrane than in cytosolic regions and hence reflects GLUT4 translocation.

Blood Analysis

Blood samples were analyzed according to standard laboratory procedures, as described previously (11).

Statistical Analysis

Statistical analyses were performed with PASW Statistics 22.0 for Mac (IBM, Armonk, NY). The Shapiro-Wilk test was used to test for normal distribution of all parameters. For normally distributed data, two-sided paired-sample

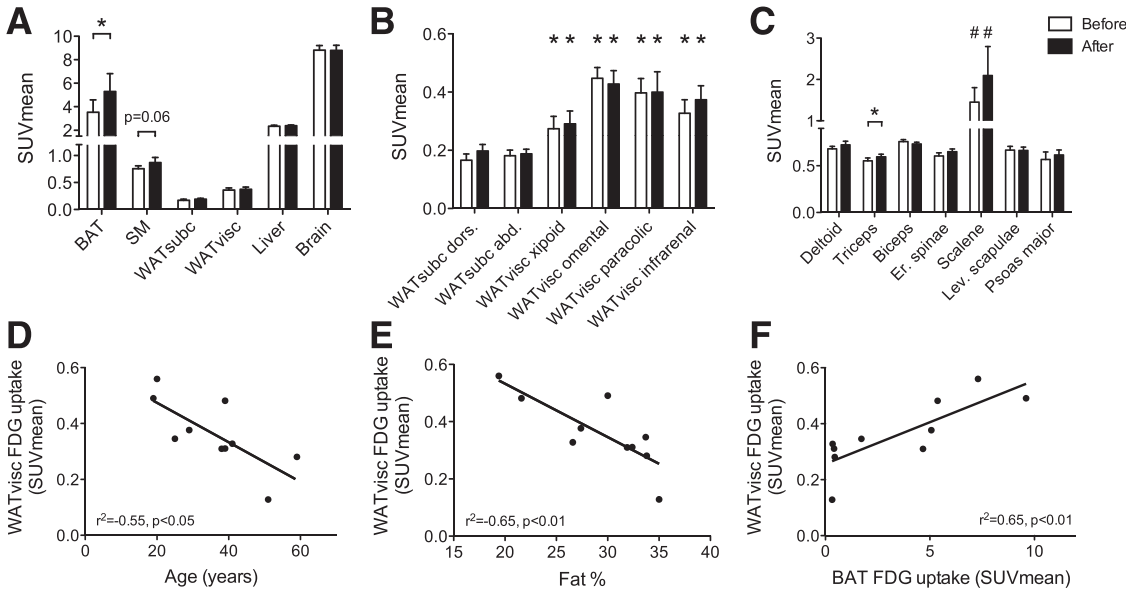

Figure 2—Cold-induced [^{18}F]FDG uptake in WAT and SM before and after cold acclimation. *A*: [^{18}F]FDG uptake in BAT, liver, brain, and average SM, subcutaneous WAT (WATsubc), and visceral WAT (WATvisc) before and after cold acclimation. *P < 0.05, before vs. after cold acclimation. Data are expressed as mean ± SEM. *B*: [^{18}F]FDG uptake in individual subcutaneous and visceral WAT regions used to calculate average WATsubc and WATvisc activity, respectively, in *A*. Abd., abdominal; dors., dorsal. *P < 0.05, compared with average subcutaneous WAT [^{18}F]FDG uptake. Data are expressed as mean ± SEM. *C*: [^{18}F]FDG uptake in individual SM groups used to calculate average SM [^{18}F]FDG uptake in *A*. Er. spinae, erector spinae; Lev. scapulae, levator scapulae. *P < 0.05, before vs. after cold acclimation; #P < 0.05 compared with [^{18}F]FDG uptake in all other muscle groups. Data are expressed as mean ± SEM. *D*: Correlation between visceral WAT [^{18}F]FDG uptake and age. *E*: Correlation between visceral WAT [^{18}F]FDG uptake and body fat percentage. *F*: Correlation between [^{18}F]FDG uptake in visceral WAT and supraclavicular BAT.

t tests were used to compare findings before and after cold acclimation and between thermoneutral and mild cold conditions; Wilcoxon signed rank tests were used for nonnormally distributed data. Pearson correlations were used to identify correlations between variables. A P value <0.05 was considered statistically significant.

RESULTS

Energy Expenditure

Before and after cold acclimation, subjects were evaluated for metabolic responses upon acute exposure to mild cold using an individualized cooling protocol. Compared with thermoneutrality, acute exposure to mild cold caused a significant increase in EE before (from 5.9 ± 0.8 to 6.5 ± 0.8 kJ min^{-1}; $P < 0.01$) as well as after (from 5.7 ± 0.7 to 6.5 ± 0.8 kJ min^{-1}; $P <$ 0.01) the cold acclimation period. NST was not significantly different upon cold acclimation (9.7 ± 9.1% before vs. 13.8 ± 8.9% after; P = 0.38). Although EE during exposure to cold was similar, the basal metabolic rate was slightly lower after cold acclimation (5.9 ± 0.8 before vs. 5.7 ± 0.7 kJ/min after; P = 0.06) (Fig. 1*A*). Respiratory quotient did not change upon acute exposure to cold, neither before (from 0.79 ± 0.06 to 0.80 ± 0.05; P = 0.32) nor after (from 0.77 ± 0.04 to 0.77 ± 0.06; P = 0.31) cold acclimation, and was similar between measurements in both thermoneutral (P = 0.46) and mild cold conditions (P = 0.52).

Increased BAT Activity Upon Cold Acclimation

Before cold acclimation, pronounced BAT activity (SUV >1.5) was observed in 6 of 10 subjects. Of note, the age range of these BAT-positive subjects was 19–39 years, whereas the age range of the BAT-negative subjects was 39–59 years. In the six BAT-positive subjects, BAT regions were semiautomatically outlined: cold acclimation resulted in increased mean (SUVmean from 2.3 ± 0.3 to 2.9 ± 0.3; $P < 0.01$) (Fig. 1*B* and *C*) and maximal (SUVmax from 8.8 ± 3.7 to 18.9 ± 5.5; $P < 0.01$) upper-body BAT activity in these subjects. Interestingly, one of the subjects who did not show pronounced BAT activity before did present detectable BAT after cold acclimation. Because BAT activity (defined by SUV >1.5) could not be detected in all subjects before cold acclimation, we also used the fixed volume method to determine BAT activity in predetermined VOIs in the supraclavicular adipose tissue depot. This method allows comparison of [^{18}F]FDG uptake in this putative BAT region in the same anatomic location before versus after cold acclimation and in subjects with low amounts of BAT. This fixed VOI method revealed that activity of the supraclavicular BAT region was increased in all subjects upon cold acclimation ($P < 0.05$) (Fig. 1*D*). Of note, SUVs using this method (Fig. 1*D*) are generally higher than mean SUVs when total BAT volume is outlined (Fig. 1*C*), since these VOIs are placed only in the area of maximal BAT activity.

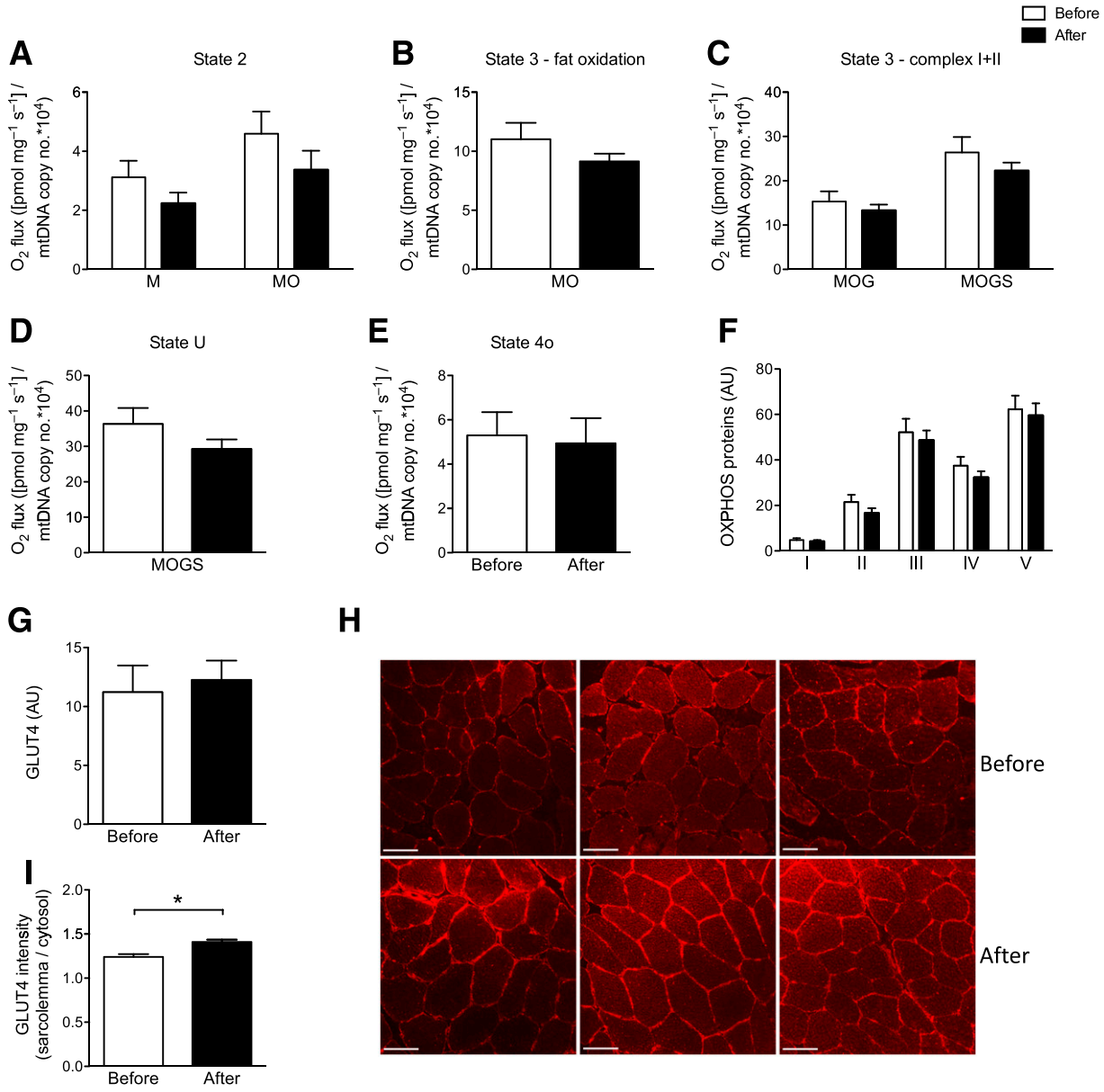

Figure 3—SM mitochondrial respiration, OXPHOS, and GLUT4 protein content and GLUT4 localization, before and after cold acclimation. *A–E*: Respiration measurements in permeabilized muscle fibers. M, malate; O, octanoyl carnitine; G, glutamate; S, succinate. *A*: Respiration upon the addition of substrates only (state 2). *B* and *C*: ADP-stimulated respiration fueled by several mitochondrial complex I– and complex II–linked substrates (state 3). *D*: Maximally uncoupled respiration upon the addition of the chemical uncoupler FCCP (state U; i.e., maximal electron transport chain capacity). *E*: Respiration not linked to ATP synthesis upon the addition of the ATPase inhibitor oligomycin (state 4o, mitochondrial leak respiration). *F*: Expression of oxidative phosphorylation (OXPHOS) protein complexes I through V. *G*: GLUT4 protein expression. *H*: Three representative images of GLUT4 immunostaining on 5-μm-thick fresh-frozen SM tissue sections from fasted muscle biopsies taken before (top) and after (bottom) cold acclimation. Scale bars = 60 μm. *I*: Quantification of the ratio of GLUT4 staining intensity at the sarcolemma and cytosol. Data are expressed as mean ± SEM. *$P < 0.05$.

BAT activity was negatively related to age. A similar trend was observed for fat percentage (Fig. 1*E*) both before and after cold acclimation.

WAT and SM [^{18}F]FDG Uptake

Cold-induced [^{18}F]FDG uptake in WAT was measured in VOIs placed in several subcutaneous and visceral white fat depots (Supplementary Fig. 1). Average visceral WAT [^{18}F]FDG uptake, as well as [^{18}F]FDG uptake by all individual visceral WAT regions, was significantly higher than subcutaneous WAT [^{18}F]FDG uptake ($P < 0.05$ for all visceral WAT regions) (Fig. 2*B*). None of the WAT depots showed significant changes in activity upon cold acclimation. Compared with the supraclavicular BAT region, [^{18}F]FDG uptake in both visceral and subcutaneous WAT was significantly lower both before and after cold acclimation ($P < 0.05$ for both) (Fig. 2*A*). Interestingly, next to BAT, [^{18}F]FDG uptake in visceral WAT was also negatively

related to age ($r^2 = -0.55$; $P < 0.05$) (Fig. 2*D*) and body fat percentage ($r^2 = -0.65$; $P < 0.010$) (Fig. 2*E*). Activity of all fat depots was strongly interrelated, with significant correlations between [^{18}F]FDG uptake in BAT and visceral WAT ($r^2 = 0.65$, $P < 0.01$ before and $r^2 = 0.72$, $P < 0.01$ after cold acclimation) (Fig. 2*F*) and between visceral and subcutaneous WAT ($r^2 = 0.65$, $P < 0.01$ before and $r^2 = 0.49$, $P < 0.05$ after cold acclimation).

Cold-induced [^{18}F]FDG uptake into SM was determined in several different upper-body muscle groups. Before cold acclimation, [^{18}F]FDG uptake was highest in the scalene muscles ($P < 0.05$ compared with all other muscles). Average SM [^{18}F]FDG uptake was not related to either age ($r^2 = 0.05$; $P = 0.53$) or body fat percentage ($r^2 = 0.00$; $P = 0.94$). Upon cold acclimation, triceps brachii showed a small but significant increase in [^{18}F]FDG uptake (SUVmean from 0.55 ± 0.10 to 0.59 ± 0.09; $P < 0.05$); a similar trend was observed for the scalene muscles ($P = 0.07$) (Fig. 2*C*). The resulting average SM [^{18}F]FDG uptake tended to be higher after cold acclimation (SUVmean 0.76 ± 0.17 before vs. 0.87 ± 0.30 after cold acclimation; $P = 0.06$) (Fig. 2*A*).

SM Respiration and Protein Expression

SM biopsies were taken from vastus lateralis muscle before and after cold acclimation and analyzed for mitochondrial respiration. No effects of cold acclimation on mitochondrial oxygen consumption were found in any of the mitochondrial respiration states analyzed (Fig. 3*A–E*). Thus, neither maximally coupled, ADP-stimulated respiration (Fig. 3*B* and *C*) nor maximally uncoupled, FCCP-stimulated respiration (Fig. 3*D*) nor mitochondrial leak respiration (Fig. 3*E*) were affected by the cold acclimation period. In addition, protein levels of oxidative phosphorylation complexes I through V were not significantly affected by cold acclimation (Fig. 3*F*).

Total GLUT4 protein level was also unchanged upon cold acclimation (Fig. 3*G*). However, the subcellular distribution pattern of GLUT4 was clearly altered after cold acclimation, with enhanced GLUT4 enrichment at the cell membrane (ratio of GLUT4 staining intensity in the sarcolemma and cytosol changed from 1.24 ± 0.09 before to 1.41 ± 0.08 after cold acclimation; $P < 0.01$) (Fig. 3*H* and *I*), indicating increased GLUT4 translocation to the sarcolemma in the basal state. This enhanced GLUT4 localization at the sarcolemma is consistent with the observation of increased [^{18}F]FDG uptake in SM upon cold acclimation.

Plasma Biochemistry

No changes in fasting thermoneutral plasma glucose, insulin, nonesterified fatty acid, triglyceride, thyroid hormone, and catecholamine concentrations were observed upon cold acclimation (Table 2). Acute exposure to mild cold resulted in decreased glucose concentrations both before and after cold acclimation, whereas only after cold acclimation was this accompanied by decreased insulin concentrations (Table 2).

Table 2—Plasma hormone and metabolite concentrations at a thermoneutral temperature and during exposure to mild cold, before and after cold acclimation

Variable	Before cold acclimation			After cold acclimation		
	Thermoneutral	Mild cold	Δ	Thermoneutral	Mild cold	Δ
NEFA (μmol/L)	501 ± 145	539 ± 178	34 ± 89	477 ± 155	517 ± 174	40 ± 123
Triglycerides (μmol/L)	1,585 ± 653	1,966 ± 824	381 ± 600	1,644 ± 932	1,924 ± 1,033*	280 ± 177
Glucose (mmol/L)	5.0 ± 0.3	4.7 ± 0.4*	−0.30 ± 0.2	5.2 ± 0.5	4.8 ± 0.3*	−0.4 ± 0.3
Insulin (μU/mL)	12.2 ± 3.3	12.4 ± 3.5	0.2 ± 2.1	14.2 ± 4.3	12.9 ± 4.9†	−1.3 ± 1.4†
TSH (units/mL)	1.5 ± 0.8	1.4 ± 0.7	−0.1 ± 0.2	1.5 ± 0.7	1.4 ± 0.6	−0.1 ± 0.1
Free T4 (pmol/L)	13.3 ± 2.2	13.2 ± 1.7	−0.1 ± 1.1	12.7 ± 1.7	12.8 ± 1.8	0.0 ± 0.7
Norepinephrine (nmol/L)	1.7 ± 0.4	3.2 ± 1.0*	1.5 ± 0.9	2.0 ± 1.1	3.3 ± 1.5†	1.2 ± 1.5
Epinephrine (nmol/L)	0.12 ± 0.04	0.11 ± 0.04	−0.01 ± 0.06	0.13 ± 0.05	0.10 ± 0.05†	−0.03 ± 0.05

Data are expressed as mean ± SD. NEFA, nonesterified fatty acid; TSH, thyroid-stimulating hormone; T4, thyroxine. *$P < 0.01$, †$P < 0.05$, mild cold vs. thermoneutral conditions.

Norepinephrine concentration increased upon acute exposure to mild cold in both measurements, whereas epinephrine concentration decreased after cold acclimation (Table 2). Interestingly, the change in norepinephrine concentration upon acute exposure to cold showed a nonsignificant correlation with BAT activity before cold acclimation (r^2 = 0.42; P = 0.08), but this correlation was significant after cold acclimation (r^2 = 0.53; $P < 0.05$).

Body Temperatures, Blood Pressure, Skin Perfusion

Temperature of the water-perfused suit used during the individualized cooling protocol was similar at thermoneutral (30.2 ± 0.6 vs. 30.4 ± 0.5°C; P = 0.30) and mild cold conditions (24.1 ± 2.0 vs. 24.4 ± 2.2°C; P = 0.59) before versus after cold acclimation, respectively. Consequently, mean skin temperature was also lowered to the same extent before (−5.1 ± 2.0°C) and after (−5.2 ± 2.3°C) cold acclimation upon acute cooling ($P > 0.05$) (Table 3). In addition, core temperature, blood pressure, and heart rate were similar during thermoneutral conditions and showed similar responses upon acute cooling (Table 3). Skin perfusion at the hand was also unaffected by cold acclimation, whereas skin perfusion at the underarm was reduced to a larger extent upon acute cooling after cold acclimation (−43 ± 23.5% before vs. −67.1 ± 22.3% after cold acclimation; P = 0.050) (Table 3), indicating enhanced vasoconstriction.

Skin temperatures and blood pressure, in addition to subjective responses to exposure to cold, were also recorded at several time points during the cold acclimation days, and iAUCs for these parameters were calculated. The iAUC for thermal sensation was significantly decreased (−28.0%; $P < 0.05$; i.e., a sensation closer to neutral), and self-reported shivering was also lowered, albeit not significantly (−15.0%; P = 0.099), on day 10 compared with day 3 (first day of 6 h of exposure to cold) of the cold acclimation period (Fig. 4*A*), indicating subjective habituation to the colder environment. Interestingly, while mean and distal skin temperature responses were unchanged, proximal skin temperature was markedly higher upon cold acclimation (Fig. 4*B*). Blood pressure was higher at t = 20 min after entering the cold room at day 10 ($P < 0.05$, compared with day 3), after which it showed a similar pattern for the remaining time points (Fig. 4*C*).

DISCUSSION

This is the first study to show that in obese subjects significant amounts of BAT can be recruited by short-term cold acclimation. In addition, we show that glucose uptake of several adipose tissue depots is strongly interrelated, although BAT is the only fat depot that shows increased glucose uptake upon cold acclimation. In SM, cold acclimation leads to enhanced GLUT4 localization at the sarcolemma, which is paralleled by increased glucose uptake upon acute exposure to cold.

Table 3—Blood pressure, body temperatures, and skin perfusion at thermoneutral temperature and during exposure to mild cold, before and after cold acclimation

Variable	Before cold acclimation			After cold acclimation		
	Thermoneutral	Mild cold	Δ	Thermoneutral	Mild cold	Δ
Systolic blood pressure (mmHg)	117.4 ± 7.9	130.8 ± 5.9*	13.4 ± 7.6	117.2 ± 5.8	133.8 ± 7.3*	16.7 ± 8.5
Diastolic blood pressure (mmHg)	71.4 ± 8.0	85.9 ± 9.7*	14.5 ± 9.6	73.8 ± 10.0	87.1 ± 8.6*	13.4 ± 4.9
Mean arterial pressure (mmHg)	88.7 ± 7.5	102.7 ± 7.3*	13.9 ± 7.1	88.9 ± 8.6	102.9 ± 7.6*	14.0 ± 5.9
Heart rate (bpm)	64.2 ± 8.9	59.8 ± 6.6*	−4.3 ± 3.9	68.4 ± 8.3	62.7 ± 6.8*	−5.8 ± 5.5
Suit temperature (°C)	33.2 ± 0.6	24.1 ± 2.0*	−6.2 ± 1.9	30.4 ± 0.5	24.4 ± 2.2*	−5.9 ± 2.2
Core temperature (°C)	37.0 ± 0.3	37.1 ± 0.6	0.1 ± 0.5	36.9 ± 0.2	36.9 ± 0.3	0.0 ± 0.2
Mean skin temperature (°C)	34.7 ± 1.3	29.5 ± 1.5*	−5.1 ± 2.0	35.3 ± 0.3	30.0 ± 2.1*	−5.2 ± 2.3
Skin perfusion (relative %)						
Hand	100	17.5 ± 15.7*	−82.5 ± 15.7	100	13.2 ± 6.1*	−86.8 ± 6.1
Underarm	100	57.0 ± 23.5*	−43.0 ± 23.5	100	32.9 ± 22.3*	−67.1 ± 22.3

Data are expressed as mean ± SD. *$P < 0.01$, mild cold vs. thermoneutral conditions.

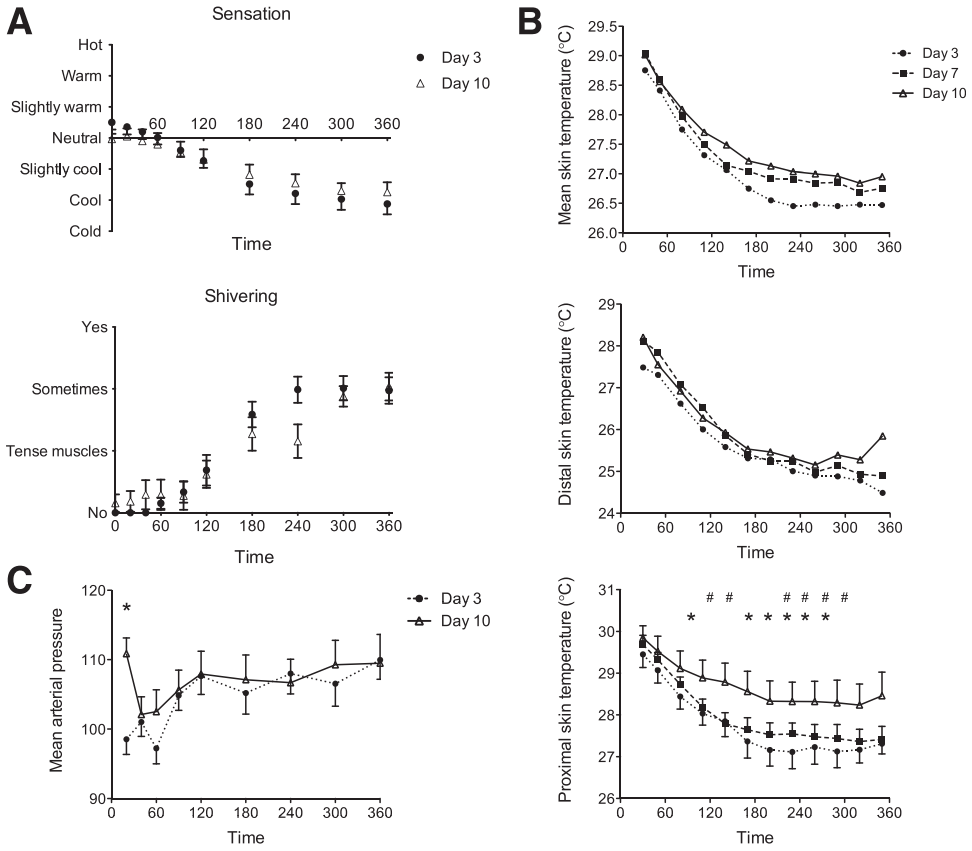

Figure 4—Subjective responses, skin temperatures, and blood pressure during cold acclimation. *A*: Changes in thermal sensation (top) and self-reported shivering (bottom) after entering the cold room on day 10 compared with day 3 of the cold acclimation period. *B*: Changes in mean (top), distal (middle), and proximal (bottom) skin temperatures after entering the cold room on day 10 compared with days 3 and 7. *C*: Change in mean arterial pressure after entering the cold room on day 10 compared with day 3. Data are expressed as mean ± SEM. **P* < 0.05, at a specific time point on day 10 vs. day 3; #*P* < 0.05 at a specific time point on day 10 vs. day 7.

Adaptive thermogenesis by means of prolonged exposure to mild cold and other ways to activate and recruit BAT have emerged as potential tools to combat obesity and its associated metabolic complications, such as insulin resistance. So far, recruitment of significant amounts of BAT has only been shown in young, lean, healthy subjects upon cold acclimation (11,16–18), and in morbidly obese subjects following massive weight loss (27), whereas in older subjects with type 2 diabetes cold acclimation led to only a minor increase in BAT activity (19). Here we show that substantial amounts of BAT can be recruited in healthy obese subjects by cold acclimation and confirm the negative relations between age, body fat, and BAT activity that were reported earlier (12). In addition, when comparing our previously published BAT activity values for young, lean male subjects (age <30 years) (11) with those in the young obese subjects from this study (age 19–29 years; n = 4), SUVs are indeed lower in the obese group (SUVmean 2.35 ± 0.31 vs. 2.60 ± 0.32 and SUVmax 9.43 ± 4.49 vs. 15.90 ± 5.75 in obese vs. lean subjects, respectively). When comparing BAT activity after cold acclimation, however, SUVs become comparable between these obese and lean individuals (SUVmean 3.07 ± 0.71 vs. 2.89 ± 0.37 and SUVmax 22.26 ± 2.56 vs. 19.9 ± 6.32 in obese vs. lean subjects, respectively). Although these observations need to be confirmed in larger groups of subjects, this may indicate that the capacity to recruit BAT at a young age is not hampered by obesity per se. This is also consistent with data from rodent studies showing BAT "whitening" upon intake of a high-fat diet (28) and evidence that such whitened adipocytes can again be interconverted into brown/beige adipocytes with a high thermogenic capacity upon cold stimulation (29).

The intense cold acclimation protocol (i.e., 6 h of cold exposure per day) that we used in this proof-of-principle study may not be directly therapeutically applicable for obese subjects. However, other recent studies of lean subjects have shown that more prolonged, less intense exposures to cold can also enhance BAT (16,18). It would therefore be interesting to explore the effects of such prolonged, mild cold regimens on BAT and substrate metabolism in obese subjects.

BAT recruitment was not associated with enhanced NST. In previous studies we (11) and others (16) reported that BAT recruitment by cold acclimation in lean, young

subjects was paralleled by an increase in NST. In older, overweight patients with type 2 diabetes impaired NST upon acute exposure to cold has been observed (19,30), whereas NST improved upon cold acclimation (19). Pronounced interindividual differences in metabolic responses to exposure to cold and possible type 2 error might explain the absence of such an effect in the obese subjects in our current study. In fact, the increased proximal skin temperatures measured on the final cold acclimation day actually point toward increased heat production during cold exposure, assuming that core temperatures were unaffected, as also shown during the individualized cooling protocols. It should also be noted, however, that despite several reported associations between BAT activity and NST, it is still unclear to what extent BAT activity directly contributes to measured whole-body oxidative metabolism in humans (31,32); thus, an increase in BAT activity is not necessarily directly reflected in increased NST. Future studies should therefore focus on further unraveling the contribution of BAT activity to NST by using individualized cooling protocols similar to those used here.

Contrary to BAT, [^{18}F]FDG uptake in visceral and subcutaneous WAT depots did not change upon cold acclimation, which argues against the notion that browning occurred in these adipose depots. However, a possible subtle degree of browning may in fact not be detectable because of the limited resolution of the PET scanner. An alternative explanation may be that browning in the sense of recruitment of brite/beige adipocytes in these depots is without physiological significance with respect to glucose uptake. Of note, in two previous publications we did not find any thermogenic gene induction in subcutaneous WAT biopsies after 10 days of cold acclimation (11,19). It is likely that a greater or more prolonged adrenergic stimulus is required to induce browning within these white adipose depots. Sidossis et al. (8) recently showed that upon severe and prolonged adrenergic stress (i.e., in burn victims), browning of subcutaneous WAT became apparent, which was associated with increased whole-body EE.

The negative relation between body fat and visceral WAT activity within our obese cohort confirms recent observations of lower fasting visceral WAT activity per volume in obese versus lean subjects (33). This effect is likely caused by the presence of more insulin-resistant adipocytes and impaired perfusion and vascular function in obesity, despite associations of obesity with the infiltration of macrophages in visceral WAT, which could actually augment glucose uptake (33–35). Such visceral WAT dysfunction may be involved in impaired systemic glucose disposal and insulin resistance (36), although it has also been suggested that lower visceral WAT activity per volume is counterbalanced by an expanded total fat mass, thereby providing a larger "sink" for glucose disposal (37).

We previously examined a possible role of SM in cold-induced thermogenesis and found no effects of cold acclimation on mitochondrial oxidative capacity or markers for mitochondrial uncoupling in young lean subjects (11) and subjects with type 2 diabetes (19). Here, we further confirm these findings in healthy obese subjects. In addition, we showed pronounced improvements in insulin-induced glucose uptake into SM upon cold acclimation in these subjects with type 2 diabetes, which was accompanied by enhanced GLUT4 translocation from the cytosol to the sarcolemma in the basal state (19). Although we did not intend to assess insulin sensitivity when we planned the current study, we were able to repeat GLUT4 immunofluorescence staining and quantification in fasting SM biopsies. As such, we were able to show enhanced basal GLUT4 translocation in healthy normoglycemic obese subjects as well, indicating a greater capacity for glucose uptake. These findings coincided with an increased cold-induced [^{18}F]FDG uptake into SM after cold acclimation, as visualized by PET/CT. The underlying mechanisms for these effects in SM remain to be elucidated; a recently newly identified adrenergic signaling pathway involving MTORC2 (38) or signaling molecules secreted by brown adipocytes (39) might play a role in this phenomenon.

In conclusion, we show that significant amounts of BAT can be recruited in obese subjects by means of intermittent exposure to cold. In addition, cold acclimation has a pronounced effect on SM, displaying enhanced GLUT4 translocation to facilitate glucose uptake. These metabolic adaptations to prolonged exposure to cold may induce improvements in whole-body glucose metabolism. The long-term effects of cold acclimation on BAT, SM, and whole-body metabolism remain to be elucidated.

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Author Contributions. M.J.W.H. designed the study, acquired and analyzed data, and wrote the manuscript. A.A.J.J.v.d.L. designed the study and acquired and analyzed data. B.B. and J.H. analyzed data. K.M.C.J. acquired and analyzed data. G.S. acquired data. F.M.M., P.S., and W.D.v.M.L. designed the study and interpreted data. All authors critically revised the manuscript and approved the final version. M.J.W.H. is the guarantor of this work and, as such, has full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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