FULL TITLE: Brown Adipose Tissue Improves Whole Body Glucose Homeostasis and Insulin Sensitivity in Humans

SHORT TITLE: Brown Adipose Tissue and Glucose Metabolism

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ABSTRACT

Brown adipose tissue (BAT) has attracted scientific interest as an anti-diabetic tissue owing to its ability to dissipate energy as heat. Despite a plethora of data concerning the role of BAT in glucose metabolism in rodents, the role of BAT (if any) in glucose metabolism in humans remains unclear. To investigate whether BAT activation alters whole-body glucose homeostasis and insulin sensitivity in humans, we studied 7 BAT positive (BAT+) men and 5 BAT negative (BAT-) men under thermoneutral conditions and after prolonged (5-8 h) cold exposure (CE). The two groups were similar in age, body mass index, and adiposity. CE significantly increased resting energy expenditure, whole-body glucose disposal, plasma glucose oxidation, and insulin sensitivity in the BAT+ group only. These results demonstrate a physiologically significant role of BAT in whole-body energy expenditure, glucose homeostasis, and insulin sensitivity in humans and support the notion that BAT may function as an anti-diabetic tissue in humans.
Diabetes mellitus currently affects 25.8 million Americans (1). Obesity, which typically precedes diabetes, results in altered glucose control. Indeed, adiposity has been implicated in the etiology of insulin resistance, where lipid moieties from the white adipose tissue (WAT) depots disrupt insulin signaling.

Adult humans have recently been shown to have brown adipose tissue (BAT) (2-4). Further, BAT is associated with leanness (5) and euglycemia (4; 6; 7), suggesting that, unlike other adipose tissue depots, BAT may be protective against obesity and diabetes. The unique feature of BAT that explains the proposed effects on obesity and glucose control is the abundance of mitochondria, which contain uncoupling protein 1 (UCP1). UCP1 uncouples oxidative phosphorylation by allowing protons to re-enter the mitochondrial matrix independent of ATP-synthase producing heat instead of chemical energy (8). Rodents studies show that upon stimulation, BAT uses glucose and free fatty acids (FFA) for thermogenesis (8), confirming the regulatory role of BAT in adiposity, glucose homeostasis, and insulin sensitivity (9; 10).

The physiological significance of BAT activation in whole-body substrate metabolism in humans remains unclear. Orava et al. reported that cold exposure (CE) resulted in a 12-fold increase in glucose disposal in BAT (11; 12), but not in other tissues. This suggests that upon activation, BAT clears plasma glucose. However, these investigators did not show differences in whole-body insulin-stimulated glucose disposal between individuals with detectable BAT (BAT+) or non-detectable BAT (BAT-), questioning the ability of BAT to modulate whole-body glucose metabolism. Similarly, Ouellet et al. recently showed that under acute CE, BAT oxidizes primarily intracellular fatty acids; plasma substrate oxidation by BAT was minimal (12).

The results from the aforementioned studies question the role of BAT in whole-body metabolism in humans. However, Orava et al. (11; 13) compared whole-body insulin-stimulated
glucose disposal between BAT+ and BAT- groups under thermoneutral conditions. The role of BAT in whole-body glucose metabolism is likely more readily discerned when BAT is activated. Furthermore, Ouellet et al. demonstrated that BAT minimally contributed to whole-body plasma glucose utilization, which might be due to the shorter duration of CE and the presence of mild shivering (12). Animal studies suggest that BAT first consumes intracellular energy stores before utilizing plasma-borne substrates to support its energy needs (8). Subsequently, longer CE may be necessary to elucidate the role of BAT activation on plasma glucose disposal and oxidation in humans.

The aim of this study was to investigate the effect of prolonged (5-8h) cold-induced BAT activation on the whole-body glucose metabolism. Twelve men (7 BAT+, and 5 BAT-) were studied under CE and thermoneutral (TN) conditions. CE increased resting energy expenditure (REE), plasma glucose oxidation, and whole-body insulin-stimulated glucose disposal in the BAT+ individuals only. The current data support a physiologically significant role for BAT in whole-body glucose homeostasis and insulin sensitivity in humans.
RESEARCH DESIGN AND METHODS

Subjects

Twelve males participated in the study. The participants were screened for health status, smoking, alcohol or drug use and recent medication or supplement use. Only healthy individuals qualified to participate in the study. Informed written consent was obtained prior to inclusion to the study from all participants in accordance with the Declaration of Helsinki. The University of Texas Medical Branch and Institute for Translational Sciences Institutional Review Boards approved the experimental protocol.

Experimental protocol

Each participant completed one CE study and one TN study, two weeks apart (Figure 1). The CE trial was performed first to determine BAT volume and anatomical location using 2-deoxy-2-[\(^{18}\text{F}\)]-fluoro-D-glucose (\(^{18}\text{F-FDG}\)-PET/CT).

Three days before each study, subjects followed a weight-maintaining diet and refrained from physical activity and alcohol and caffeine. The evening before the study, subjects were admitted to the Institute for Translational Sciences-Clinical Research Center at UTMB. Patients were fed a standardized evening meal. During CE and TN studies, subjects were fasted overnight and remained rested in bed for the duration of the study. Subjects wore standardized clothing (T-shirt and shorts).

CE protocol and TN conditions

We used the following individualized CE protocol to maximize non-shivering thermogenesis; subjects were studied in a room with an ambient temperature of \(~19^\circ\text{C}\) and
wearing garments cooled by liquid circulation (Cool Flow® vest and blanket, Pleasant Hill, IA).

The temperature of the vest was initially set at 20°C, then decreased at 1°C intervals through an air-conditioned temperature control bath (Chiller Reservoir System, Polar Products Inc., Pleasant Hill, IA) until subjects reported shivering. Then, the temperature was increased by 1°C and was maintained constant. We visually inspected the subjects for shivering. In a subset of individuals (n=3) we performed electromyography (Bagnoli 8, Delsys, Boston, MA) at 5 min intervals to verify muscle activity (14). Core temperature and skin temperature was measured using a telemetric pill (Core-Temp, HQ Inc., Palmetto, FL) and wireless probes (iButtons, Maxim, Dallas, TX)(15), respectively.

**Stable isotope infusion**

After 3h of either CE or the TN study, blood was sampled to determine background enrichment, prior to the administration of the following stable isotope tracers (Cambridge Isotope Laboratories, Andover, MA) through a catheter in the forearm vein: a) a primed (17.6 µmol.kg⁻¹) constant (0.22 µmol.kg⁻¹.min⁻¹) 2h infusion of [6,6-²H₂]-glucose to assess glucose kinetics (16); b) a constant 2h infusion of potassium [U-¹³C₁₆]-palmitate (0.02 µmol.kg⁻¹.min⁻¹) to assess FFA kinetics (17; 18); c) a bolus of NaH¹³CO₃ (55 µmol.kg⁻¹ dissolved in 0.9% NaCl) was also given to prime the body’s bicarbonate pool. Blood and breath samples were obtained at 1h 50 min, 1h 55 min, and 2h after the initiation of the stable isotope infusion to determine isotopic enrichments of plasma glucose and palmitate, breath CO₂, and to measure substrate concentrations. The same regimen, including only [6,6-²H₂]-glucose, was repeated during the hyperinsulinemic-euglycemic clamp. Whole-body glucose disposal and FFA oxidation were calculated as previously described (19; 20). Changes between CE and TN in glucose uptake were calculated as the difference between the glucose uptake in CE and TN conditions.
Hyperinsulinemic Euglycemic Clamp

Hyperinsulinemic-euglycemic clamps were conducted during the last 2h of each trial, as previously described (21). Insulin (Lilly, Indianapolis, IN), was given at a rate of 20 mU.m\(^{-2}\).min\(^{-1}\), dissolved in sterile NaCl 0.9% (19). We used a low insulin infusion rate to avoid stimulation of muscle plasma glucose disposal (22). Whole-body insulin-stimulated glucose disposal was evaluated using the insulin sensitivity index (23) and the formula: 
\[
\text{glucose disposal (µmol.kg}^{-1}\cdot\text{min}^{-1})/\text{steady-state plasma glucose (mg.dl}^{-1})
\]

Positron Emission Tomography/Computerized Tomography

After 5h, subjects were injected with a bolus of 185 MBq of \(^{18}\)F-FDG. We performed a PET/CT (GE Discovery ST 4 Slice, General, Electric, Milwaukee, WI) scan an hour later to assess BAT volume and BAT glucose disposal \([\text{BAT volume (ml)}\times \text{mean standardized disposal value, g.ml}^{-1} (SUV)]\). An independent blinded investigator assessed the PET/CT scans for BAT, as previously described (12). Subjects with pronounced \(^{18}\)F-FDG disposal were assigned to the BAT+ group (BAT+; n=7; BAT volume=69±18 ml, Figure S1), while subjects with no or minimal \(^{18}\)F-FDG disposal were assigned to the BAT negative group (BAT-; n=5; BAT volume=4.4±2.3 ml, p<0.05, Figure S2).

Body Composition

Dual X-Ray Absorptiometry (Lunar iDXA*, GE Healthcare, Mickleton, NJ) was used to evaluate the lean and fat body mass of the participants. CT was used to estimate total, subcutaneous, and visceral abdominal tissue area (25-27).
Indirect calorimetry

REE was calculated using whole body oxygen consumption and carbon dioxide production rates measured by indirect calorimetry (Vmax Encore™, Care Fusion Inc., San Diego, CA) (28). Indirect calorimetry equipment failed on two occasions (one BAT+ and one BAT- subject).

Blood Samples Analysis

Plasma glucose concentrations were determined using an automated glucose analyzer (Stat 2300; Yellow Spring Instruments). Gas chromatography mass spectrometry was used to quantify plasma enrichments of glucose and palmitate (Agilent, Santa Clara, CA). Plasma insulin and FT3 concentrations were measured using chemiluminescence (Dxi 600, Beckman Coulter, Pasadena, CA). Free thyroxine (FT4) was measured via a quantitative electrochemiluminescent immunoassay (ARUP, Salt Lake City, UT). Plasma norepinephrine was determined using quantitative high-performance liquid chromatography (ARUP, Salt Lake City, UT). Further, serum interleukin 6 (IL6) (R&D System, Minneapolis, MN), plasma irisin (Phoenix Pharmaceuticals, Burlingame, CA) and FGF21 (Biovendor, Asheville, NC) concentrations were determined using commercially available ELISA kits.

Adipose Tissue Sampling

During CE, adipose tissue samples from the supraclavicular and abdominal areas were obtained using a Bergström needle. In a subset of individuals (3 BAT+ and 1 BAT-), biopsies were obtained during both the CE and TN trials. The location for the supraclavicular biopsy was
determined by the $^{18}$F-FDGPET/CT images showing cold-activated BAT. In BAT- individuals, the supraclavicular depot was sampled 2-3 cm from the skin surface area—the same depth that the BAT depot has been localized in BAT+ individuals. Each sample was divided into pieces for the analyses below:

a. **Immunohistochemistry:** Adipose tissue, preserved in 10% formalin, was embedded in paraffin wax. Immunohistochemistry was performed using a standard protocol (29). Briefly, after blocking (Rabbit IgG kit, Vector laboratory, CA) for 45 min, sections were incubated overnight with primary antibody (Rabbit anti-UCP1 antibody, Sigma-Aldrich U6382, 1:1600) at 4°C. Sections were incubated with secondary antibody (Rabbit IgG, Vector laboratory, CA), ABC solution and then a diaminobenzidine solution (DAB peroxidase substrate kit, Vector laboratory, CA) at room temperature.

b. **Mitochondrial Respiration:** Mitochondrial function was determined by high-resolution respirometry (30). Briefly, maximal uncoupled mitochondrial respiration was measured in permeabilized (5 µM digitonin) WAT (~50 mg) and BAT (~10 mg) following the addition of substrates (1.5 mM octanoyl-carnitine, 5 mM pyruvate, 10 mM glutamate, 2 mM malate, 5 mM ADP and 10 mM succinate) and the ATP-synthase inhibitor oligomycin (5 µM).

c. **Gene expression.** Total RNA was isolated and cDNA synthesized and analyzed by quantitative real-time PCR as previously described (2), using gene-specific primers at a final concentration of 300 nM (Table S2). 18S was used as the normalizing gene and an established supraclavicular BAT sample was included as a positive control for BAT (2).

**Statistical analysis**
Results are presented as means±SEM/SD. Differences between CE and TN trials were evaluated using paired $t$-tests for normally distributed data. Non-normally distributed data were evaluated using Wilcoxon’s rank test. One sample $t$-test was used to evaluate whether CE induced changes in the various metabolic measurements compared to TN. Differences between BAT- and BAT+ subjects were evaluated using a Mann-Whitney $U$-test.

To evaluate differences in the circulating levels of the various hormones/cytokines, we used a mixed analysis of variance model. Treatment differences (CE-TN) in the levels of hormones/cytokines were determined for each subject at each time point, accounting for the paired within-subject correlation between treatments. A mixed analysis of variance modeled the differences as a function of time and BAT status; an AR-1 correlation structure accounted for by the within-subject repeated measures over time, and a weighting structure adjusted for heterogeneity of variance over time. Differences were transformed as needed to better approximate normal distributions, with results inverse-transformed appropriately. Statistical analyses were performed using Graph Pad version 5 for Mac OS X (La Jolla, CA) and R statistical software (R Core Team, 2013, version 3.0.1). All statistical tests assumed a 95% level of confidence.
RESULTS

By design, the BAT+ group had higher BAT volume and activity (p<0.05) than the BAT-group, but they were similar in age and anthropometric characteristics (Table 1). BAT+ individuals required slightly, but not significantly, lower ambient and garment temperatures to reach their non-shivering threshold (Table S1). CE decreased skin temperature in both groups (p<0.05). The BAT- group had a marginally lower core temperature during CE compared to TN (p=0.06).

**BAT activation increases energy expenditure and plasma substrate oxidation**

Indirect calorimetry and stable isotopes were used to determine REE and substrate oxidation rates. CE increased REE (15%) in the BAT+ group only (p<0.05, Figure 2A), suggesting that BAT activation increases REE during non-shivering CE. The cold-induced increase in REE in the BAT+ group was fueled primarily by plasma-derived glucose and FFA oxidation (p<0.05 for both; Figures 2B-C). Plasma glucose and FFA contributed ~30% and 70%, respectively, to the observed increase in REE in the BAT+ group. These data suggest that upon prolonged stimulation, BAT largely oxidizes plasma substrates to support its energy demands. This finding supports a role for BAT in the regulation of systemic glucose and FFA metabolism.

**Glucose disposal during cold exposure occurs primarily in BAT**

$^{18}$F-FDG uptake during CE increased significantly in BAT (p<0.05), but not in the liver, abdominal visceral adipose tissue, abdominal subcutaneous adipose tissue, or skeletal muscle (vastus lateralis and pectoralis) (Figure 3). Interestingly, the mean heart SUV decreased
significantly with CE in BAT+ subjects (p<0.05). In agreement with previous reports (11; 12), our data suggest that mild CE results in increased disposal of labeled glucose by BAT only, with minimal or no effects in other tissues.

**BAT activation increases basal whole-body glucose disposal**

To determine the physiological significance of BAT activation on whole-body glucose metabolism, we assessed glucose kinetics during TN conditions and during prolonged (5h) CE. CE significantly increased whole-body glucose disposal only in the BAT+ group (p<0.05) (Table 2, Figure 4A). Theoretically, if BAT remained chronically active, it could dispose ~23g of glucose in 24 hours. Combined with the PET/CT data, showing that 

*18*F-FDG uptake increased only in BAT (Figure 3), these data suggests that when activated BAT has the ability to take up significant amounts of glucose from the circulation, and therefore can play a significant role in glycemic control.

**BAT activation increases insulin-stimulated whole-body glucose disposal**

Under thermoneutrality, insulin infusion significantly increased whole-body glucose disposal in both groups (p<0.05 for both) (Table 2). This increase reflects increased glucose disposal in many tissues and indicates that the two groups were sensitive to insulin in peripheral tissues (i.e. skeletal muscle, WAT), even though the BAT- group needed higher insulin to achieve the same result (i.e. slightly less insulin sensitive). Further, insulin infusion suppressed endogenous glucose production similarly in both groups (p<0.05) indicating that both groups were sensitive to insulin at the level of the liver.
After establishing that both groups were responsive to insulin, we determined the effect of cold-induced BAT activation on glucose disposal during hyperinsulinemia (resembling the post-prandial state). To this end, we infused insulin after 6h of CE. Compared to the insulin-induced increase in glucose disposal in thermoneutrality, CE further increased glucose disposal in the BAT+ group only (p<0.05) (Table 2, Figure 4B). The results were similar when the whole-body glucose disposal rates were corrected for glucose levels (whole-body insulin sensitivity index, Figure 4C) or in combination with insulin concentration (insulin sensitivity index, Figure 4D). Finally, hyperinsulinemia equally suppressed endogenous glucose production in both groups under CE (p<0.05). These findings suggest that BAT activation may play a functional role in peripheral glucose disposal, both in the post-absorptive and postprandial state.

**BAT status and hormonal/cytokine response to cold**

Our study was not designed to examine the mechanisms regulating the effect of BAT activation on glucose metabolism. Nevertheless, we measured the levels of FT3, FT4, FGF21, cytokines (irisin, leptin, IL6), and norepinephrine, which have been previously associated with BAT activation (8; 10; 30; 31). The BAT+ group demonstrated higher increases in the circulating concentrations of norepinephrine, FGF21, and FT3, when compared to the BAT- group (Figures 5A-C). FT4, irisin and IL6 levels were not significantly different between the two groups (Figures 5D-F).

**Molecular and functional characterization of BAT**

The assignment of subjects into the BAT+ and BAT- groups was based on the cold-induced glucose uptake in the supraclavicular region as assessed by PET/CT. The reason for the
low supraclavicular glucose uptake in the BAT- group could be due to the fact that this group had less BAT. Alternatively, the BAT- group might have had comparable amounts of BAT, relative to the BAT+ group, but was unable to activate it in response to the cold stimulus used, i.e. their threshold for cold-induced activation of BAT may have not been reached. To address the question whether BAT- subjects had BAT or not, we performed molecular and functional studies on adipose tissue samples collected from the supraclavicular depot by the guidance of the PET/CT images, and compared them with abdominal subcutaneous adipose tissue samples.

BAT+ subjects had UCP1-positive multilocular adipocytes in the supraclavicular tissue (Figure 6A), whereas the corresponding tissue of the BAT- subjects demonstrated minimal UCP1 staining and comprised of largely unilocular adipocytes (Figure 6B). The abdominal subcutaneous adipose tissue samples were similar between the two groups, comprising of unilocular adipocytes with no UCP1 staining (Figure 6C-D). Quantification of the UCP1 staining confirms these results (supraclavicular adipose tissue BAT+ vs BAT-, p=0.06, Figure 6E). In agreement with the results from the immunohistochemical analysis, UCP1 gene expression was higher in the supraclavicular adipose tissue from the BAT+ group relative to the BAT- group (p=0.06, Figure 6F) and at a similar level as in a well-documented BAT control sample included in the analysis. No UCP1 expression was detected in the abdominal subcutaneous adipose tissue samples. The molecular studies were complemented with functional studies showing that the maximal uncoupled mitochondrial respiration was significantly higher in the supraclavicular adipose tissue compared to the subcutaneous abdominal adipose tissue (p<0.05) only in the BAT+ group (Figure 6G). These results show that only the supraclavicular adipose tissue from the BAT+ group presents with features of BAT while this is not the case for
the BAT- group. Hence, the low glucose uptake in the BAT- group is due to a lower BAT content rather than a failure to activate BAT.

In order to further examine how CE affects human BAT on a molecular level, biopsies were taken both at TN and CE in a subset of BAT+ subjects. As predicted by animal studies on bona fide BAT, CE induced the expression of both UCP1 and DIO2 (type 2 deiodinase) (Figures 6H-I). In addition, CE induced the expression of ADRB3 (β3-adrenergic receptor) and PGC1α (Figures S3-4). Hence, CE appears to trigger the expression of essential thermogenic genes in humans. The expression of the glucose transporter 1 gene (GLUT1) increased with CE in three out of four subjects while GLUT4 gene expression increased in two out of four participants (Figure S5-6).
We have shown a physiologically significant effect of BAT activation on whole-body glucose disposal in humans, supporting a functional role of BAT in glucose homeostasis and insulin sensitivity in humans. Our data suggest that ~70ml of active BAT can clear significant amounts of glucose from the circulation. This glucose is used to support the cold induced increase in REE in the BAT+ group. Further, this was accompanied by an increase in peripheral insulin-stimulated glucose disposal in BAT+ individuals. Our data demonstrate a significant role for BAT activation in altering glucose control and insulin sensitivity in humans.

Plasma glucose oxidation accounted for ~30% of the estimated increase in REE during CE in BAT+ subjects. This estimate is consistent with results from animal studies (32). Plasma FFA oxidation was responsible for the remaining 70% of the increase in REE. This is also in agreement with animal data suggesting that FFA represent the primary substrate oxidized by UCP1-positive mitochondria (32). Our findings of significant plasma substrate utilization by BAT upon activation are in contrast to a recent report that BAT utilizes mainly intracellularly stored triacylglycerols, and very little, if any, plasma substrates (12). This may be due to the short (2.5h) duration of CE in this study (12) compared to our longer CE protocol (5h). This supposition is supported by rodent data which indicates that BAT first exhausts intracellular stores before relying on plasma-borne substrates to support its energy needs (8). Therefore we believe that our results differ from those previously reported (12) due to our CE protocol being two times longer. The duration of CE should be a consideration for future studies aimed at elucidating the role of BAT activation in human metabolism.

BAT is an insulin-sensitive tissue (11). Moreover, since humans spend a significant portion of the day in the post-prandial state, we examined the effect of cold-induced BAT
activation during a hyperinsulinemia–euglycemia clamp, in order to simulate the post-prandial state in a laboratory setting. Hyperinsulinemia significantly increased whole-body glucose disposal and insulin sensitivity in the BAT+ group only. These results suggest that BAT activation may modulate whole-body glucose metabolism in the postprandial state. Moreover, relative to the TN study, CE improved indexes of insulin sensitivity in the BAT+ group only, further supporting a role of BAT activation in whole-body insulin sensitivity in humans.

Although we cannot exclude the contribution of indirect pathways in the observed increase in the insulin-stimulated whole-body glucose uptake, we believe that the increase was due to BAT activation and not a systemic response to CE. This is supported by the fact that hyperinsulinemia during thermoneutrality increased whole-body glucose uptake equally in both groups, whereas hyperinsulinemia during CE increased glucose uptake only in the BAT+ group. Moreover, we performed a low-dose insulin clamp to avoid significant muscle glucose uptake. Further, it is unlikely that muscle glucose uptake was proceeding at a significant rate, as CE causes peripheral vasoconstriction (33), which has been shown to decrease peripheral insulin stimulated glucose uptake (34). Indeed, CE has been found to preferentially enhance insulin signaling in BAT and to perturb insulin signaling in muscle and WAT (35), suggesting that BAT is likely the tissue primarily responsible for the reported improvements in insulin sensitivity during CE.

Norepinephrine also induces BAT activation by causing lipolysis (8). FFAs subsequently bind to and activate UCP1, leading to mitochondrial thermogenesis (8). We observed greater norepinephrine levels in response to CE in the BAT+ subjects, which was likely responsible for the increase in REE observed in the BAT+ group. Moreover, norepinephrine production can stimulate BAT to produce FGF21 (36). FGF21 improves glucose homeostasis and insulin
sensitivity by increasing GLUT1 expression in WAT. A number of animal studies (8; 10) and a few recent humans studies support a role for BAT as an endocrine tissue (37). In agreement with this, CE induced greater increases in circulating levels of FT3, norepinephrine, and FGF21 in the BAT+ compared to the BAT- group in the current study. Thus, the reported improvement in insulin sensitivity in BAT+ subjects may be attributable to circulating levels of FGF21 during CE. Adrenergic stimulation and FT3 are upstream regulators of FGF21 production. Therefore, the concomitant increase in FT3 and norepinephrine levels highlights a plausible pathway by which BAT may function as an endocrine organ. Specifically, CE increases DIO2 activity in BAT, resulting in an acceleration in the conversion of thyroxin to triodothyrinine, the active form of thyroid hormone (38). Indeed, our results also show increased DIO2 expression in BAT upon CE. In mice, triodothyrinine stimulates the sympathetic nervous system, leading to higher UCP1 expression in BAT (30).

Strengths of this study include the use of an individualized, prolonged non-shivering CE protocol to elicit maximal non-shivering thermogenesis and BAT activation, along with the use of gold standard direct measures of whole-body glucose metabolism in individuals with or without active BAT depots. Although the methodological approaches available and the restrictions governing human research limit our ability to provide more direct and mechanistic evidence, the results of this study strongly support that BAT activation plays a physiologically significant role in whole-body glucose and energy metabolism in humans. The use of invasive methodologies to directly measure substrate metabolism and collect supraclavicular biopsy samples from human volunteers allow physiologically meaningful conclusions to be made.

In conclusion, our results suggest that BAT activation increases whole-body glucose disposal and insulin sensitivity in humans. These results support a functional role of BAT in
whole-body glucose and energy homeostasis. Future research is needed to further investigate the
effect of chronic BAT activation and the mechanisms underlying BAT activation in humans in
order to identify safe and efficacious lifestyle or pharmaceutical interventions that may activate
BAT \textit{in vivo} or induce the “browning” of the more abundant WAT. If the potential of UCP1-
positive adipocytes to alter energy metabolism and expenditure in humans can be fully realized,
BAT will likely emerge as a therapeutic target in the battle against obesity and diabetes.
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LS designed the study in conjunction with EV, EB, MC and CY. MC, CY, and NMH performed the clinical trials. MC and CRA performed the statistical analysis. MS analyzed hormone measurements of blood samples. MC performed the stable isotope analysis. SML performed the PET/CT scan analysis. CP and TC performed mitochondrial respiration analysis. PA performed the adipose tissue biopsies and he was responsible for the medical coverage of the studies. FC performed and supervised the PET/CT scans. MEL and SE designed and performed the gene expression experiments. MC wrote the initial draft of the manuscript. All authors critically reviewed the manuscript.

The study was registered at clinicaltrials.gov (NCT01791114).

CONFLICT OF INTEREST STATEMENT
S.E. is a shareholder and consultant to Ember Therapeutics. The rest of the authors have no conflict of interest to report.

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**GUARANTOR NAME**

MC and LSS are the guarantors of this work and, as such, had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

**REFERENCE PRIOR TO PUBLICATION**
REFERENCES


### Table 1. Subjects’ characteristics

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<th>Parameters</th>
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<td>BAT activity [BAT volume (ml)*mean SUV (g ml⁻¹)]</td>
<td>9.3 ± 4.4</td>
<td>153.2 ± 42.6*</td>
</tr>
<tr>
<td>AUC_{glu} (mg dl⁻¹)</td>
<td>296.3 ± 15.6</td>
<td>248.5 ± 25.6</td>
</tr>
</tbody>
</table>

Data are mean ± SEM.* = p<0.05. BMI: body mass index; BAT+: detectable BAT; BAT-: no detectable BAT; BSA: Body Surface Area, SUV: Standardized Disposal Value; AUC_{glu}: Glucose Area Under the Curve.
Table 2. Indices of whole-body glucose homeostasis under cold exposure (CE) and thermoneutral (TN) conditions.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>BAT- (n = 5)</th>
<th>BAT+ (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Basal conditions</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (mg.ml(^{-1}))</td>
<td>90.0±7.9</td>
<td>94.0±3.4</td>
</tr>
<tr>
<td>Whole-Body Glucose disposal (µmol.kg(^{-1}).min(^{-1}))</td>
<td>9.4±0.7</td>
<td>9.7±0.6</td>
</tr>
<tr>
<td>Insulin (µU.ml(^{-1}))</td>
<td>8.6±4.0</td>
<td>8.2±1.3</td>
</tr>
<tr>
<td>Hyperinsulinemic euglycemic conditions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose (mg.ml(^{-1}))</td>
<td>83.8±3.1</td>
<td>85.8±3.5</td>
</tr>
<tr>
<td>Whole Body Glucose disposal (µmol.kg(^{-1}).min(^{-1}))</td>
<td>12.4±2.2‡</td>
<td>12.8±1.8</td>
</tr>
<tr>
<td>Endogenous glucose production (µmol.kg(^{-1}).min(^{-1}))</td>
<td>5.6±1.6</td>
<td>7.5±1.1</td>
</tr>
<tr>
<td>Suppression of endogenous glucose production (%)</td>
<td>-59.7 ± 9.4</td>
<td>-48.2 ± 6.9</td>
</tr>
<tr>
<td>Insulin (µU.ml(^{-1}))</td>
<td>33.5±4.3</td>
<td>31.0±6.7</td>
</tr>
</tbody>
</table>

Data are mean ± SEM. BAT+: detectable BAT; BAT-: no detectable BAT* = p<0.05 between CE and TN conditions. †p<0.05 between BAT- and BAT+ subjects, ‡ p<0.05 between basal and hyperinsulinemic euglycemic conditions.
FIGURES LEGENDS

Figure 1. Study design. Tracers infused: [6,6-²H₂]-glucose, [U-¹³C₁₆] palmitate, ¹³C sodium bicarbonate. CE: cold exposure, IC: indirect calorimetry, PET/CT: positron emission tomography/computed tomography, TN: thermoneutral.

Figure 2. The role of brown adipose tissue (BAT) in resting energy expenditure (REE) and substrate oxidation. (A) REE upon cold exposure (CE) and thermoneutral (TN) conditions in subjects with detectable BAT (BAT+) and non-detectable BAT (BAT-). (B) Plasma glucose oxidation under CE and TN conditions in BAT+ and BAT- subjects. (C) Plasma free fatty acid (FFA) oxidation under CE and TN conditions in BAT+ and BAT- subjects. White bars= thermoneutral, black bars=cold exposure. Data are mean ± SEM. * = p<0.05 between CE and TN.

Figure 3. Mean standardized disposal value (SUV) for glucose of various tissues during cold exposure (CE) and thermoneutral (TN) conditions. BAT+: Subjects with detectable BAT, BAT-: subjects with non-detectable BAT, SQAT: subcutaneous adipose tissue, VAT: visceral adipose tissue. White bars= thermoneutral, black bars=cold exposure. Data are mean ± SEM. *=p<0.05 and ** p<0.01 between CE and TN.

Figure 4. Effect of brown adipose tissue (BAT) on whole-body glucose disposal and insulin sensitivity. (A-B) Change in whole-body glucose disposal under cold exposure (CE) relative to thermoneutrality (TN) in basal condition (A) and during hyperinsulinemic–euglycemic clamp (B). (C) Change in the insulin sensitivity index (ISI) for CE and TN. (D) Change in whole-body...
insulin sensitivity for CE relative to TN. Data are means ± SEM. White bars=BAT-, black bars=BAT+. * = p<0.05 and ** = p< 0.01 indicate a significant change with CE compared to TN conditions. †= p<0.05 between BAT+ and BAT-.

Figure 5. Change in plasma concentrations of hormones and cytokines with cold exposure in subjects with detectable or non-detectable brown adipose tissue activity (BAT+ and BAT-, respectively). (A) Change in plasma norepinephrine levels with cold exposure in BAT+ and BAT- individuals. (B) Change in plasma fibroblast growth factor 21 (FGF21) concentrations with cold exposure in BAT+ and BAT- individuals. (C) Change in plasma free triiodothyronine (FT3) and (D) plasma free thyroxine (FT4) concentration with cold exposure in BAT+ and BAT- individuals. (E) Change in plasma irisin concentration with cold exposure in BAT+ and BAT- individuals. (F) Change in serum interleukin 6 (IL6) concentrations with cold exposure in BAT+ and BAT- individuals. Data are mean ± SEM.* = p<0.05 and ** = p< 0.01 indicates a significant difference in response to CE between BAT+ and BAT- subjects.

Figure 6. Molecular and functional characterization of supraclavicular and abdominal subcutaneous adipose tissue samples in subjects with (BAT+) or without (BAT-) cold-induced brown adipose tissue. Uncoupling protein 1 (UCP1) staining (40x) in the supraclavicular (A, B) and abdominal subcutaneous (C, D) adipose tissue samples from representative BAT+ (A, C) and BAT- (B, D) participants. (E) Quantification of UCP1 staining in supraclavicular (SC) and abdominal (ABD) subcutaneous adipose tissue. (F) UCP1 gene expression in SC and ABD subcutaneous adipose tissue. (G) Uncoupled mitochondrial respiration in SC and ABD subcutaneous adipose tissue. Data are means ± SEM. White
circles=BAT-, black circles BAT+. * = p<0.05 between CE and TN. & = p<0.05 between SC and ABD. (H) UCP1 gene expression in BAT under thermoneutral (TN) and cold exposure (CE) conditions. Data are mean ± SD. *** = p<0.005, **** = p<0.001. (I) Type II iodothyronine deiodinase (DIO2) gene expression in BAT under TN and CE conditions. Data are mean ± SD. *** = p<0.005.
Change in Insulin-stimulated Whole Body Glucose Disposal ($\mu$mol kg$^{-1}$ min$^{-1}$)

**BAT-**

**BAT+**

Change in ISI ($10^{-3}$ dl kg$^{-1}$ min$^{-1}$ (μU ml$^{-1}$))

**BAT-**

**BAT+**

Change in Whole Body Insulin Sensitivity

**BAT-**

**BAT+**

Change in Basal Whole Body Glucose Disposal ($\mu$mol kg$^{-1}$ min$^{-1}$)

**BAT-**

**BAT+**

Change in ISI ($10^{-3}$ dl kg$^{-1}$ min$^{-1}$ (μU ml$^{-1}$))
Uncoupled Mitochondrial Respiration (pmol sec$^{-1}$ mg$^{-1}$)

E

UCP1 Expression in adipose tissue (% Strong Positive Pixel Count)

F

UCP1 mRNA (fold of ctrl BAT)

G

Uncoupled Mitochondrial Respiration (pmol sec$^{-1}$ mg$^{-1}$)

H

Subject 1

Subject 2

Subject 3

Subject 4

DIO2 (fold of ctrl BAT)

UCP1 (fold of ctrl BAT)

DIO2 (fold of ctrl BAT)
Online Appendix

Brown Adipose Tissue Improves Whole Body Glucose Homeostasis and Insulin Sensitivity in Humans
Supplemental Figure Legends

Figure S1. 2-deoxy-2-[18F]fluoro-D-glucose (18FDG)- Positron Emission Tomography (PET) and/or Computed Tomography (CT) images from participants in the brown adipose tissue positive (BAT+) group.

Figure S2. 2-deoxy-2-[18F]fluoro-D-glucose (18FDG)- Positron Emission Tomography (PET) and/or Computed Tomography (CT) images from participants in the brown adipose tissue negative (BAT-) group.

Figure S3. β3-adrenergic receptor (ADRB3) gene expression in brown adipose tissue under thermoneutral (TN) and cold exposure (CE) conditions. Data are mean ± SD. ** = p<0.01, *** = p<0.005, **** = p<0.001.

Figure S4. Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α) gene expression in brown adipose tissue under thermoneutral (TN) and cold exposure (CE) conditions. Data are mean ± SD. *= p<0.05, ** = p<0.01, **** = p<0.001.

Figure S5. Glucose Transporter 1 (GLUT1) gene expression in brown adipose tissue under thermoneutral (TN) and cold exposure (CE) conditions. Data are mean ± SD. *= p<0.05, ** = p<0.005, **** = p<0.001.

Figure S6. Glucose Transporter 4 (GLUT4) gene expression in brown adipose tissue under thermoneutral (TN) and cold exposure (CE) conditions. Data are mean ± SD. **** = p<0.001.
Subject 1

**Diabetes**

Subject 2

Subject 3

Subject 4
Table S1. Body and environmental temperatures

<table>
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<tr>
<th></th>
<th>BAT- (n=5)</th>
<th>BAT+ (n=7)</th>
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<tr>
<td></td>
<td>TN</td>
<td>CE</td>
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<tr>
<td>Core Temperature (°C)</td>
<td>36.9±0.2</td>
<td>36.4 ±0.2</td>
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<tr>
<td>Skin Temperature (°C)</td>
<td>34.0±0.4</td>
<td>30.7±0.4*</td>
</tr>
<tr>
<td>SupracaIcular Adipose Tissue Skin Temperature (°C)</td>
<td>33.5±0.6</td>
<td>34.5±0.3</td>
</tr>
<tr>
<td>Cooling Garment Temperature (°C)</td>
<td>19.8±1.3</td>
<td>19.3±0.5</td>
</tr>
<tr>
<td>Ambient Temperature (°C)</td>
<td>26.2±0.5</td>
<td>19.9±0.5*</td>
</tr>
</tbody>
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Data are mean ± SEM. *p<0.05 by Wilcoxon’s test between CE and TN. BAT+: detectable BAT; BAT-: no detectable BAT; TN: thermoneutral conditions; CE: cold exposure.
<table>
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<td>18S</td>
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<td>GCTGGAATTACCGCGGCT</td>
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