Glucagon Increases Energy Expenditure Independently of Brown Adipose Tissue Activation in Humans

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ABSTRACT

Obesity is a global health concern. An ideal drug would inhibit appetite and elevate energy expenditure (EE), but no currently available medication can safely do this. Glucagon elevates EE in humans, but the mechanism remains unknown.

AIMS: To investigate, for a given energy expenditure rise, the differential effects of glucagon infusion and cold exposure on brown adipose tissue (BAT) activation in humans.

METHODS: Indirect calorimetry and supraclavicular thermography was performed in 11 healthy male volunteers before and after cold exposure, glucagon infusion (at 23°C) and vehicle infusion (at 23°C). All volunteers underwent 18F-FDG PET/CT scanning with cold exposure. Subjects with cold-induced BAT activation on 18F-FDG PET/CT (n=8) underwent a randomly allocated second 18F-FDG PET/CT scan (at 23°C) either with glucagon infusion (n=4) or vehicle infusion (n=4).

RESULTS: EE increased by 14% following cold exposure and by 15% post-glucagon infusion (50 ng/kg/min) (P<0.05 vs control for both). Cold exposure produced an increase in neck temperature (+0.44°C; P<0.001 vs control), but glucagon infusion did not alter neck temperature. In subjects with cold-induced increase in the metabolic activity of supraclavicular BAT on 18F-FDG PET/CT, a significant rise in metabolic activity of BAT following glucagon infusion was not detected. Cold exposure increased sympathetic activation, as measured by circulating norepinephrine levels, but glucagon infusion did not.

CONCLUSIONS: Glucagon increases energy expenditure to a similar magnitude compared to cold activation, but independently of BAT thermogenesis. This finding is of importance for the development of safe treatments of obesity through upregulation of energy expenditure.
INTRODUCTION

Obesity results from an excess of caloric intake relative to energy expenditure (EE). There are now 600 million adults who are obese (body mass index, BMI >30 kg/m²) worldwide. Obesity is causally linked to the development of Type 2 diabetes, cardiovascular disease, some cancers (1) and early mortality. Lifestyle strategies for weight loss are based around calorie restriction alongside increasing EE via exercise, but these efforts are difficult to sustain in the long term. Currently licensed drugs, generally aimed at either reducing intestinal fat absorption (such as intestinal lipase inhibitors) or inhibiting appetite (such as phentermine), have limited weight loss efficacy on their own. Anti-obesity agents which elevate EE by stimulating the sympathetic nervous system have produced dangerous side effects, particularly cardiovascular morbidity (2-4). There is a great unmet need for drugs which can safely inhibit appetite and increase energy expenditure.

One possible solution is a pharmacological agent that acts as a co-agonist of both the glucagon-like peptide-1 (GLP-1) and glucagon receptors, harnessing the anorectic and glucose-lowering effects of the former with increased energy expenditure via the latter (5, 6). Glucagon infusion acutely increases EE in humans (7, 8) but the mechanism by which this occurs is not known. Rodents can increase their EE via activation of brown adipose tissue (BAT), which consumes fuel for thermogenesis using uncoupling protein-1 (UCP-1), and do so in response to both cold exposure and caloric excess (9). UCP-1 positive BAT deposits that can be activated by a variety of stimuli, most potently cold exposure, have been recently described in adult humans, particularly in the supraclavicular neck region (10-12).

Isolated brown fat cells from rats have been demonstrated to respond thermogenically to glucagon (13) and glucagon administration to rodents increases BAT mass and activity (14). Glucagon knockout mice have reduced thermogenic responses to cold exposure and pharmacological
adrenergic stimulation, which is restored by glucagon replacement (15), although the participation
of brown adipose tissue in the thermogenic response to glucagon administration is not firmly
established (16). Therefore glucagon may increase EE in humans via activation of BAT, but studies
investigating this are lacking.

We carried out a series of experiments to investigate, for the first time, whether the increased EE
resulting from acute glucagon administration was mediated by BAT activation in adult humans.

MATERIALS and METHODS

Subjects

11 healthy male subjects (mean age 26.1 years, range 20.8–39.8 years, mean BMI 22.5 kg/m², range
20.5–25.2 kg/m²) were recruited through advertisement and assessed as healthy during a screening
visit with a medical history, routine blood tests and electrocardiogram. Exclusion criteria were
smoking, substance abuse, eating disorders, regular medication and medical or psychiatric illness.
The study was approved by the London Central Ethics and Research Committee (13/LO/0925),
registered with ClinicalTrials.gov (NCT01935791) and performed in accordance with the Declaration
of Helsinki. Informed consent was obtained from subjects prior to enrolment in the study.

Study Visits

Cold exposure and glucagon infusion have both been shown to acutely elevate energy expenditure
(EE) in humans but have never been directly compared in the same cohort. We performed this
comparison in 11 healthy young males. Each subject underwent measurement of resting metabolic
rate (RMR) using an indirect calorimeter (Gas Exchange Monitor, GEM Nutrition, UK) at the start and
end of three separate interventions: cold exposure, glucagon infusion in a warm room and vehicle
infusion in a warm room. During these visits, we also utilised infra-red thermography of the neck, which has recently been reported as a reliable method of measuring BAT thermogenesis (17, 18). The calorimetry/thermal imaging visits were randomly assigned and are summarised in 1-3 below:

1. Calorimetry and thermal imaging wearing a Polar Products® cooling vest (19) with a 55-minute intravenous vehicle (Gelofusin®, Braun UK) infusion (cold visit).
2. Calorimetry and thermal imaging in a warm room (ambient temperature 22–25°C) with a 55-minute intravenous vehicle infusion (control, vehicle visit).
3. Calorimetry and thermal imaging in a warm room (ambient temperature 22–25°C) with a 55-minute intravenous glucagon infusion at 50 ng/kg/min (Novo Nordisk, UK) (glucagon visit).

18F-fluorodeoxyglucose (18F-FDG) PET/CT scanning is the most widely accepted method of identifying metabolically active BAT. All 11 subjects attended an initial “cold” 18F-FDG PET/CT scan study visit (described in 4 below), to ascertain if they were BAT positive or BAT negative:

4. 18F-FDG PET/CT wearing the cooling vest with a 55-minute intravenous vehicle infusion.

Eight of the 11 subjects were confirmed as having supraclavicular BAT deposits on their initial 18F-FDG PET/CT scan based on standardised uptake value (SUV) characteristics previously defined (20). This BAT positive cohort then underwent a fifth study day, when a second 18F-FDG PET/CT scan was performed, randomised to either:

5. a. 18F-FDG PET/CT with a 55-minute glucagon infusion at 50 ng/kg/min at 23°C (n=4) OR
   b. 18F-FDG PET/CT with a 55-minute vehicle infusion at 23°C (n=4).
Subjects fasted and drank only water from 22:00h the night before each study visit. They ate the same meals the day before every study visit, abstained from alcohol, caffeine and avoided strenuous exercise for 24h prior to each visit. Each visit was separated by at least 3 days. On arrival, peripheral venous cannulae were inserted in both forearms (one for infusion and one for blood sampling). Volunteers were blinded to the contents of the intravenous infusions. RMR was measured at the start and end of each thermal imaging visit using an indirect calorimeter (Gas Exchange Monitor, GEM Nutrition, UK). At the start of each study visit the calorimeter was calibrated with “zero” (0.00% O\textsubscript{2} and 0.00% CO\textsubscript{2}) and “span” gases (20.00% O\textsubscript{2} and 1.00% CO\textsubscript{2}) gases (BOC, UK). Human glucagon was purchased from Novo Nordisk (UK). 1mg was diluted into 50ml Gelofusin and flow rate through the Alaris\textsuperscript{®} syringe pump was adjusted to a maintenance intravenous delivery rate of 50 ng/kg/min. This dose was chosen based on previous work in our department to produce an acute rise in energy expenditure comparable with other studies examining the effects of cold exposure on energy expenditure and BAT activity (21). To ensure plateau plasma levels were quickly attained when glucagon was delivered, infusions were ramped at 4x maintenance rate (MR) for the first 5 minutes, 2x MR for the next 5 minutes and 1x MR for the remainder of the 55 minute infusion. The Polar Products\textsuperscript{®} cooling vest used in this experiment has been previously confirmed to successfully induce cold-activated BAT without needing to change the ambient temperature of the experimental room(19). The waistcoat-shaped vest was worn by the participants directly over a thin cotton hospital gown. Cold water (maintained at 8°C by attaching to a temperature-controlled tank) was continuously pumped through the vest. The subjects felt cold but none of them reported (or were observed to be) shivering (this was checked every 15 minutes).

**Thermal Imaging Protocol**
Volunteers wore a pair of light cotton trousers only. On the cold visit day the cooling jacket was wrapped around the volunteer’s torso leaving the supraclavicular regions uncovered. After a 30-minute acclimation period, baseline thermal images were recorded for 10 min using a Flir T440bx infrared camera (Flir Systems, UK) mounted on a tripod placed 1 m from the subject. The subjects always sat fully upright in the same chair, with head and arm rests maintained in the same positions. Volunteers were asked to remain as still as possible, with their shoulders held un-rotated against the back of the chair to minimise movement within the image frames during thermal recordings. Immediately after the baseline thermal imaging session, subjects were placed under the hood of the calorimeter for the baseline RMR recording (for 15 minutes).

At $t = -10$ minutes the infusion (vehicle or glucagon) was started and on the cold visit day, cold water was pumped through the vest to begin the cooling process. The infusions were stopped at $t = 45$ minutes and the cold vest maintained for a further 45 minutes. Thermal imaging recordings were taken at baseline (Run A), just after the start of the intervention (Run B), and the final run (Run C) coincided with the end of infusion or cold vest exposure. Immediately after, a final RMR measurement with the hood calorimeter was also recorded (Figures S1a and S1b). Blood samples were taken at $t = -55, -10, 0, 10, 15, 30, 45$ and 60 minutes (see below).

**18F-FDG PET/CT protocol**

On arrival peripheral venous cannulae were inserted in both forearms. Volunteers wore light cotton trousers. After 30 minutes acclimation, at $t = -75$ minutes, the cooling vest was put on and cold water circulated through for 60 minutes prior to the start of the scan. The vest was later draped over the volunteers’ legs whilst in the scanner. On the second visit (vehicle or glucagon infusion) no cooling step was taken. At $t = -10$ minutes the volunteers entered the scanner and the infusion was
started (ramped for the first 10 minutes as previously described). Prior to the PET scan, an anatomical CT scan of neck and thorax was performed. At t=0 minutes, 180 MBq of $^{18}$F-FDG was injected IV and a 60 minute dynamic emission scan was performed, with an axial field of view from mandible to mid-thorax (Figure S1c). Blood samples were taken at t= −55, −10, 0, 1, 3, 5, 10, 15, 30, 50 and 60 minutes.

**Blood Sampling**

Samples for analysis of plasma glucagon were collected in lithium heparin tubes containing 0.15ml aprotinin (Trasylol, Bayer Schering Pharma, Berlin, Germany) and immediately underwent centrifugation, following which plasma was promptly separated and stored at −20°C until analysis using a radioimmunoassay (22). Glucagon and glucose was measured at all time points and catecholamines (plasma epinephrine and norepinephrine) were measured on the start and end samples, by the Department of Chemical Pathology, Imperial College Healthcare National Health Service Trust. Human FGF-21 levels in the start and end samples were measured using an ELISA manufactured by Millipore (Merck Millipore, Darmstadt, Germany).

**Image Analysis**

For each subject, the second visit CT scan was non-linearly registered and warped to the first visit CT scan using a multi-resolution free-form deformation technique, measuring the mutual information as a similarity measure between CTs. The 3D warp field estimate was subsequently applied to the dynamic PET of the second visit. Adipose tissue region of interest (ROI) was defined using tissue density on CT (−150 < Hounsfield Units < −5). A BAT ROI was then defined as adipose tissue with a standardised uptake value (SUV) of ≥2 on the cold scan. Time activity curves (TACs) for BAT were generated by applying the ROIs to the dynamic PET data from both visits. Arterial plasma radioactivity was estimated using a whole blood TAC derived from an aorta ROI scaled by the plasma
to blood ratio derived from the discrete blood samples. Using the graphical Patlak model, estimates of the metabolic rate (K) of FDG were calculated. Finally, the metabolic rate of glucose [MRgluc] was calculated by dividing the metabolic rate of FDG by the lump constant (LC=1.14 in adipose tissue, (23) and multiplying by the concentration of glucose in the plasma. Representative images are shown in Figure 2.

Thermal recordings taken with Flir Tools Plus software (Flir Systems, UK) were saved as stills every 30 seconds. Supraclavicular ROIs defined by a triangle limited by the acromioclavicular joint, cricoid prominence and the sternoclavicular joint were drawn on the first image using Thermacam Pro software (Flir Systems UK) and repositioned on all subsequent stills from that run. Data from each ROI were exported into Excel, where each cell recorded a camera pixel temperature reading, and a macro was created to calculate the mean temperature of the top 10% hottest pixels. Visual comparison of which pixels within the thermal image ROI were being included in the temperature analysis (by highlighting those falling in the top 10% of pixel temperatures) confirmed in all cases that the hottest areas in the supraclavicular ROI formed a contiguous cluster located on skin overlying a depot of BAT (Figure 3). As a comparator (control) region, we also measured the temperature changes in a region of interest defined over the deltoid region for every thermal image. The deltoid ROI (an area known to be devoid of underlying BAT) was defined as a right angled triangle extending from the acromioclavicular joint to lateral extremity of the deltoid.

**Statistical Analysis**

Based on previous studies of the hormonal and cold exposure effects on human brown adipose tissue activity (24, 25), we calculated that, for an equivalent rise in energy expenditure, a sample size of 4 per group (glucagon versus control) would have a 95% power to detect a significant difference
between the effects of cold exposure and glucagon using $^{18}$F-FDG PET/CT in confirmed BAT-positive
volunteers. Due to safety considerations, as PET/CT scans expose healthy patients in this study to
ionising radiation, we limited this study to males only and to the smallest possible sample size to
answer our initial question about the differential effects of cold exposure and glucagon
administration on human BAT activity for a given rise in EE.

All data was analysed using GraphPad Prism 6 software (GraphPad Software, Inc., San Diego,
California). Results are presented as means±SEM. One way repeated measures ANOVA with Tukey’s
test was used to compare differences between the three intervention groups. A p value<0.05 was
considered to be statistically significant.

RESULTS

Cold exposure and glucagon infusion produce a comparable acute rise in energy expenditure in
adult humans

Cold exposure with a cooling vest and 50 ng/kg/min glucagon infusion cause a similar rise in RMR
(approximately 14% and 15% from baseline respectively) compared with vehicle: 193.0±27.2
kcal/day with cold, 41.1±7 kcal/day with vehicle and 230.8±30.1 kcal/day with glucagon (Figure 4a).
Baseline respiratory quotients (RQ: $V_{CO_2}/V_{O_2}$) in each group were not significantly different to one
another: 0.797±0.016 (cold), 0.788±0.017 (vehicle) and 0.765±0.021 (glucagon), but rose
significantly with glucagon infusion [change from baseline in RQ by end of intervention: –
0.012±0.009 (cold), –0.003±0.001 (vehicle), and +0.055±0.016 (glucagon, p<0.01)] indicative of the expectedResult in rate of carbohydrate oxidation with glucagon, in line with its glucose liberating
effects and hence the relative substrate availabilities of glucose versus free fatty acid, and consistent
with our previous data (7, 8). Plasma levels of glucagon rose only during the glucagon infusion,
which is shown in Supplemental Figure S2 alongside plasma glucose and insulin levels. Previous studies have suggested that glucagon stimulates FGF-21 secretion in humans (26) and that FGF-21 stimulates BAT. We found a non-significant rise in circulating FGF-21 levels in our cohort in response to glucagon infusion, also detailed in Supplemental Figure S2.

Circulating norepinephrine levels rose significantly only with cold exposure but not with vehicle or glucagon infusion (cold 2.33±0.14 to 2.84±0.33 mmol/L [p<0.05], vehicle 1.95±0.18 to 1.74±0.18 mmol/L and glucagon infusion 2.19±0.18 to 2.02±0.26 mmol/L). Circulating epinephrine levels did not change (data not shown) in any group. Heart rate and blood pressure (BP) data are presented in Supplemental Figure S3. There was a small rise in heart rate with glucagon infusion, consistent with its well-described chronotropic effects. In comparison, cold-induced peripheral vasoconstriction and hence a rise in peripheral vascular resistance may account for the measured rise in mean arterial pressure (MAP) with cold exposure. In general, these findings support the conclusion that for a similar rise in RMR between glucagon infusion and cold exposure, glucagon infusion induces less sympathetic outflow.

Cold exposure but not glucagon infusion activates human BAT despite a comparable rise in energy expenditure

We tested the hypothesis that the increased EE resulting from acute glucagon administration to adult humans was mediated by BAT activation utilising both 18F-FDG PET/CT and thermal imaging.

1. Cold exposure but not glucagon infusion activates human BAT as measured using 18F-FDG PET/CT
In the 8 subjects confirmed as having active supraclavicular BAT deposits, BAT mass ranged from 4.8 to 92.9 g, mean 36.7±11.1 g. In our cohort of BAT positive volunteers, the metabolic rate of glucose uptake [MR(gluc)] in BAT was significantly higher with cold exposure than both vehicle and glucagon infusion. Although there was a small trend in BAT activation following glucagon infusion, there was no statistical difference in BAT MR(gluc) between vehicle and glucagon infusion (Figure 4b): mean BAT [MR(gluc)] with cold exposure 0.074±0.007 µmol/kg/min, vehicle 0.010±0.003 µmol/kg/min and with glucagon 0.029±0.008 µmol/kg/min. In line with this, a breakdown of the EE rise between the BAT positive and BAT negative groups (Supplemental Figure S4) revealed that the rise in EE induced by glucagon in BAT negative subjects was not less than that in the BAT positive group, confirming that glucagon has an effect on RMR independent of BAT activity.

2. Cold exposure but not glucagon infusion activates human BAT as measured using thermal imaging

All 11 subjects (8 BAT positive and 3 BAT negative, as defined by their ¹⁸F-FDG PET/CT characteristics on cold exposure, described above) also underwent thermography of their neck during the three study visits when calorimetry was performed (Figure 1 and Supplemental Figures 1a and 1b). A rise in supraclavicular skin surface temperature has previously been shown to indicate underlying BAT thermogenic activity (17, 27). In the BAT positive group of 8, there was a significant temperature rise in the neck with cold exposure, with a mean increase in temperature between Run A (baseline) and Run C (end exposure) of 0.44±0.08°C. In contrast there was no change in supraclavicular neck temperature with cold exposure in the BAT negative group (Figure 5a). These findings confirm that thermal imaging was able to independently identify the same individuals who had cold-induced BAT activation as shown by ¹⁸F-FDG-PET/CT. In both the BAT positive and BAT negative groups, thermal imaging revealed no change in neck temperature when subjects received either vehicle (Figure 5b) or glucagon infusion (Figure 5c). As a comparator (control) region, we plotted the temperature
changes in a region of interest defined over the deltoid region (an area known to be devoid of underlying BAT) for all of the thermal images (Supplemental Figure S4). All of the conditions (vehicle in a warm room, glucagon in a warm room and cold exposure) were associated with a fall in the skin temperature over the deltoid, since the experimental protocol required them to sit with the torso/shoulders exposed. However, the fall in deltoid temperature in the vehicle and glucagon visits was very similar (-1.05°C and -1.09°C respectively) whereas the fall in temperature in response to the cold exposure was much greater (-1.90°C). This data suggests that the rise in BAT positive supraclavicular temperature with cold exposure is even more pronounced (relative to the drop in skin temperature elsewhere).

Taken together, these data demonstrate that cold exposure and glucagon infusion cause a similar rise in EE, but only cold exposure is associated with significant thermogenic activation of BAT in the acute setting.

DISCUSSION

This study is the first to measure the effects of glucagon infusion on brown adipose activity in humans. We found that glucagon and cold exposure induced a comparable acute rise in energy expenditure in man, but in this setting only cold exposure, and not glucagon, induced the activity of supraclavicular BAT in adult humans measured using either $^{18}$F-FDG-PET/CT or thermal imaging. We also found that although cold exposure was capable of increasing sympathetic activation (as determined by steady-state norepinephrine levels), glucagon infusion did not significantly alter circulating norepinephrine levels. These findings support the conclusion that, whilst acute cold exposure and acute glucagon infusion produce a very similar rise in RMR, the effector mechanisms through which EE is elevated are different. These findings are of importance to drug development
based on glucagon receptor agonism, as the dose of glucagon used in this study increased energy expenditure significantly without sympathetic system activation, thus potentially avoiding the deleterious effects on the cardiovascular system that have been encountered with some weight-loss medications.

In 2009, metabolically active BAT in adult humans was described in detail (10, 11). This has led to a resurgence of interest in the utility of BAT as a tantalising target for safely increasing EE to aid weight loss. Enhanced BAT activity may also induce favourable effects on glucose homeostasis and circulating lipids (28). Exposure to cold temperatures is the most potent stimulator of BAT metabolic activity, which has been correlated with the associated rise in EE that occurs in the cold [although not so in this particular study (Supplemental Figure 6)]. More recently, the concept of repeated cold exposure as a means of recruiting or upregulating BAT for therapeutic purposes has also been reported (29), although the feasibility of such an approach in a clinical setting remains to be seen. The effector mechanism for cold-induced thermogenesis is the sympathetic nervous system, which richly innervates BAT, via the β3 adrenoceptor. In line with this, patients with catecholamine-secreting tumours have evidence of increased BAT activity, which abates once the tumour is removed, and thyrotoxic patients show a similar upregulation of BAT activity, since thyroid hormone potentiates β-adrenergic signalling (25, 30). Conversely, a very similar study to ours described the effects of nonselective β-adrenergic stimulation by isoprenaline in healthy young men on BAT activity. Vosselman et al found that, although isoprenaline infusion increased energy expenditure to the same extent as cold exposure, it did not significantly activate BAT, indicating that other tissues are responsible for generalised β-adrenergic thermogenesis (24). Indeed, early pre-clinical trials of β3 agonists as anti-obesity agents did not progress due to poor efficacy (31). However a more recent study using newer and more highly specific β3 agonists (licensed for other conditions) suggest that pharmacological manipulation of BAT remains a viable option. Cypess et al described significant BAT activation in response to a single dose of the selective β agonist
mirabegron, associated with a 200 kcal/day rise in EE, although this was not reported against the degree of measured cold-induced BAT activity in the cohort (32). More recently, Broeders et al described the effect of bile acids on human BAT activation, reporting a small but significant rise in BAT SUV max, from $1.0 \pm 0.4$ to $1.6 \pm 0.4$, compared with $7.2 \pm 5.4$ in acute cold exposure (33). They therefore concluded that that the maximal achievable BAT activation in these subjects far exceeded the effects of chenodeoxycholic acid, but that more potent bile acid BAT activators may have traction as anti-obesity drugs.

$^{18}$F-FDG-PET/CT, the imaging modality chosen for all of these BAT studies, is the currently accepted gold standard method for measuring BAT volume and activity. In rodent studies, thermographic measurement of BAT activity has been validated against biochemical markers of BAT thermogenesis, such as UCP-1 upregulation (34). In human studies, Jang et al reported that a temperature difference between the neck and sternum of 0.9°C had a positive predictive value of 85% for the presence of supraclavicular BAT (17), Symonds et al recorded rises of 0.5°C in supraclavicular temperature in children following mild cold exposure (18), and Boon et al reported a 0.4°C cold-induced supraclavicular skin temperature rise, which positively correlated with $^{18}$F-FDG-PET/CT quantification of underlying BAT (27). In our study all 8 subjects who had BAT activation following cold exposure as determined by $^{18}$F-FDG-PET/CT also showed a rise in neck temperature as determined by thermal imaging during cold exposure, although the correlation between PET-quantified BAT mass and cold-induced neck temperature rise was not significant in our cohort (data not shown). We also observed that the rise in neck temperature occurs within the first ten minutes following cold exposure and remains elevated for the duration of the exposure. In contrast, the subjects with an absence of cold-activated BAT activity on their PET/CT scan displayed no change or a fall in neck temperature under cold conditions. In all cases, both saline and glucagon infusions (in a warm room) produced no rise in neck temperature on thermal imaging, supporting the conclusion that glucagon produces at best only a very modest effect on BAT activity in this experimental setting.
To further check the validity of our thermal findings, we manually overlaid all thermal images on the corresponding PET/CT images. In all 8 BAT positive subjects, the 10% hottest pixels from the thermal image (that were used for our data analysis) consistently formed a contiguous cluster overlying the largest depot of BAT defined on that subject’s corresponding PET/CT. Furthermore, when we analysed the temperature change in the pixels that overlaid BAT on the corresponding PET/CT, the same temperature rise was found as produced by the (blinded) whole ROI analysis – confirming that analysis of the entire ROI accurately reflected skin temperature changes directly overlying a confirmed BAT depot. In the 3 BAT negative subjects, the 10% hottest pixels were more sparsely distributed across the entire ROI. These findings support the use of thermal imaging to investigate BAT activity by directly measuring the output of the uncoupling process, namely heat. Development of such novel methods for quantifying and measuring the function of human BAT is important, as the restoration or upregulation of BAT activity in humans may provide a non-surgical means of treating obesity. Indeed, whilst $^{18}$F-FDG PET/CT is still generally regarded as the gold standard for measuring BAT activity, the technique is expensive, exposes research participants to ionising radiation and may not be the best measure of BAT metabolism since circulating glucose is used as a substrate for BAT thermogenesis only after internal fatty acid stores and then circulating FFAs have been utilised.

Ouellet et al have reported on the utility of using other PET tracers, such as $^{11}$C-acetate (to determine tissue oxidative activity) and the fatty acid $^{18}$F-fluoro-thiaheptadecanoic acid ($^{18}$FTHA) to measure NEFA uptake in human BAT (35). Others have focused on other methods of imaging without the need for exposure to ionising radiation, such as fat-fraction MRI to quantify BAT mass coupled with functional MRI to investigate BAT activation, measuring blood flow as a marker of thermogenesis (29).
Our work has important therapeutic implications. Modern estimates suggest that BAT activity could account for 5% basal metabolic rate in humans (36), although its maximal inducible potential remains unknown. Two previous studies have reported on the addition of chronic glucagon receptor agonism to the anti-diabetic properties of GLP-1 receptor (GLP-1R) agonism in diet-induced obese (DIO) mice (5, 6). Co-agonism of glucagon and GLP-1 receptors induced superior body weight loss and improvements in glucose homeostasis than the GLP-1R agonist alone, and caused a significant rise in EE. These findings have opened up intense interest in the concept of balanced co-agonism of both the glucagon and GLP-1 receptors to achieve superior weight loss due to the additional energy expenditure effects of glucagon receptor activation. We confirmed the applicability of this finding to humans, demonstrating that glucagon acutely increases energy expenditure in humans (7), and this study builds on our understanding of the mechanism by which it does so.

The dose of glucagon used in this experiment was based on our previous knowledge of the dose required to significantly increase energy expenditure in humans. We have previously shown that a dose of 50 ng/kg/min (i.e. 14 pmol/kg/min) IV glucagon over 45 mins increased resting EE significantly by a mean of 146.99 kcal/day in healthy overweight individuals (7). This dose, administered over 60 minutes in our study (notably on a leaner and younger cohort) produced a 206.8±29.6 kcal/day rise in EE. In another recent publication we showed that a dose of 2.8 pmol/kg/min IV glucagon over 130 minutes (i.e. a fifth of the dose used here but over twice the time) produced a non-significant rise (n=13) in EE of 66.8 kcal/day in overweight volunteers (8). The plateau plasma levels measured in this experiment following glucagon infusion (~300 pM) are also in line with those previous reports, although higher than those typically expected when fasting (~50 pM). However, this would not be unexpected in the setting of a pharmacological (weight-loss) agent containing a glucagon agonist element. Notably, we have chosen to present our EE data in units of Kcal/day, thereby extrapolating our acute measurement of EE rise with glucagon to the 24
hour period. Whilst there is strong animal data that chronic GLP-1R and glucagon-receptor co-
agonism produces long lasting weight loss effects and increased energy expenditure, the effects of
chronic glucagon receptor agonism in humans are not known. Thus, whilst a 24 hr extrapolated
value for the EE effects of glucagon infusion may not necessarily be reflective of what would happen
with prolonged glucagon agonism over that period (or longer), it gives a sense of the potential
therapeutic effect of this strategy.

We powered this study to detect a significant difference between the effects of cold exposure and
glucagon using 18F-FDG PET/CT in confirmed BAT-positive volunteers. With the data presented in
this study, we can confidently state that, for the same rise in EE, glucagon has less effect on BAT
than cold, although it is not possible to exclude a small effect of glucagon on human BAT activity
with this study. Furthermore, although BAT activity was measured on PET/CT during the 55 minute
intravenous infusion of glucagon when plasma levels had reached a plateau, cold exposure was
initiated one hour before the scan, which may also have militated against detecting an effect of
glucagon on BAT activity. To reduce the contribution of cold-activated BAT on the placebo or
glucagon study days, we maintained the scanner room temperature at 23°C throughout the study.
Indeed, it would be interesting to further investigate the interplay between constitutive BAT activity
(as occurs in mild cold stress) and its responsiveness to glucagon, which was not answerable with
this particular study. Since we wished to limit radiation exposure to women of childbearing
potential, the data presented here is for young, healthy males only. Nevertheless, our findings imply
the existence of an as yet unidentified, and potentially targetable, glucagon-driven pathway to
enhanced energy expenditure. Other mechanisms by which glucagon may be exerting its thermic
effects have still to be explored in humans in greater detail. Futeile substrate cycling induced by non-
physiologic glucagon receptor activation provides a means to increasing the metabolic rate through
stimulation of energy-consuming cyclical metabolic pathways, with no net change in product
formation, in the liver and other tissues. Tracer studies have indicated a significant effect on glucose
cycling in response to glucagon infusion, an effect that occurs predominantly at low insulin levels and is abolished with high-dose insulin infusion. The contribution of this to whole body EE remains to be adequately explored (37, 38). Glucagon also has well described positive inotropic and chronotropic effects on the myocardium, although the small contribution of the heart to total RMR could not account for the rise in EE that we have measured following glucagon infusion (39). Finally, we have not excluded a small element of BAT activation in humans in response to glucagon administration and the effects of chronic glucagon receptor activation on brown adipose tissue mass and function in man also remain unknown. In line with animal data, this may include upregulation of classical BAT deposits versus the possibility that chronic glucagon receptor agonism may promote WAT transdifferentiation to a more brown-like phenotype. This study has highlighted these important areas for future research into the hormonal upregulation of human energy expenditure as a safe therapeutic option for obesity.
Author Contributions

VS, CI-E, ER, RG, SRB, TT and WSD designed the experiment; VS, CI-E, CC, AC, and AB carried out the experiments; VS, CI-E, CC, DT, ZW, NAN, ER and RG performed the image analysis and performed the statistical analyses. VS, CI-E and WSD wrote the manuscript. All authors contributed to editing the final manuscript and had final approval of the submitted article. WSD is the guarantor of this work, had full access to all the data, and takes full responsibility for the integrity of data and the accuracy of data analysis.

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REFERENCES


Figure 1: Study visit summary

11 male subjects attended for 4 or 5 study visits and underwent (in random order)

- Thermal imaging and indirect calorimetry wearing a cooling vest with a 55-minute vehicle infusion (cold visit, see also Supplemental Figure 1a)

- Thermal imaging indirect calorimetry in a warm room (ambient temperature 22–25°C) with a 55-minute vehicle infusion (control visit, Supplemental Figure 1b)

- Thermal imaging indirect calorimetry in a warm room with a 55-minute glucagon infusion at 50 ng/kg/min (glucagon visit, Supplemental Figure 1b)

All 11 subjects also underwent an $^{18}$F-FDG PET/CT scan (Supplemental Figure 1c). The first PET/CT scan was always wearing the cooling vest with a 55-minute vehicle infusion. If this scan revealed cold-induced BAT activity, then the second PET/CT visit occurred, randomised to be either:

- $^{18}$F-FDG PET/CT in a warm room (ambient temperature 23°C) with a 55-minute glucagon infusion at 50 ng/kg/min OR

- $^{18}$F-FDG PET/CT in a warm room with a 55-minute vehicle infusion

Figure 2: Example PET/CT images

Example CT (top row), PET (middle row) and fusion PET/CT (bottom row) images. Column 1 is a subject who displays BAT activation during cold stimulation (one of the BAT positive cohort). Column 2 is of the same subject as Column 1, but when he received glucagon infusion on his second scan. Column 3 is a different subject who also displayed BAT activation on cold exposure, but showing his
scan in response to vehicle in a warm room. Column 4 shows images from a volunteer who did not demonstrate BAT activation during cold stimulation (one of the BAT negative cohort). PET images are average activity between 30 and 60 minutes normalized to injected dose and body weight. HU: Hounsfield units, SUV: standardized uptake value.

**Figure 3:** Examples of PET/CT and thermal images from the same subject at the end of a cooling vest visit.

Figure 3a is the fused $^{18}$F-FDG PET/CT image (yellow areas represent increased metabolic activity) of a BAT positive subject during cold stimulation. Figure 3b is a thermal image taken at the end of the same cold exposure from the same subject. The stars on the thermal image were used to define the anatomical landmarks used that defined the region of interest (ROI) for temperature analysis.

**Figure 4:** Effects of cold exposure, vehicle infusion and glucagon infusion in a warm room on energy expenditure (EE) (Figure 4a) and metabolic rate of glucose uptake (MR(gluc)) in BAT during $^{18}$F-FDG PET/CT (Figure 4b).

Resting metabolic rate was measured with an indirect calorimeter at the start and end of each intervention and the mean change from baseline are shown in Figure 4a. Baseline resting EEs were as follows 1279±59 kcal/day (cold), 1353±52 kcal/day (vehicle) and 1315±39 kcal/day (glucagon), and were not significantly different from one another. Data is presented for all n=11 volunteers (separated into BAT positive and BAT negative cohorts in Supplemental Figure S4). Figure 4b shows metabolic rate of glucose uptake (MR(gluc)) in BAT during $^{18}$F-FDG PET/CT determined under the same experimental conditions: cold exposure (blue bar), vehicle only (ambient temperature 23°C) (red bar) and glucagon infusion (green bar). Results are expressed as means ± SEM, ** P<0.01, *** p<0.001.
Figure 5 (a-c): Average supraclavicular region of interest (ROI) temperature (°C).

Results are shown for baseline (Run A), mid-intervention (Run B) and end intervention (Run C) for each visit type. Each run represents a 10-minute thermal recording, with stills extracted every 30 seconds and an average reading of the upper 10% pixels calculated. These were then averaged across the cohort of BAT positive (as defined on PET/CT; n=8, hatched bars) and BAT negative (n=3, white bars). Figure 5a shows response to the cooling vest, 5b for vehicle infusion in a warm room and 5c glucagon infusion in a warm room. Results are expressed as means ± SEM, ***p<0.001.

FIGURES
Figure 2: Example PET/CT images

Figure 3 – examples of PET/CT and thermal images from the same subject at the end of a cooling vest visit.
Figure 4: Effects of cold exposure, vehicle infusion and glucagon infusion in a warm room on energy expenditure (EE) (Figure 4a) and metabolic rate of glucose uptake (MR(gluc)) in BAT during 18F-FDG PEI/CT (Figure 4b).

4a

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4b

**

Figure 5: Average supraclavicular region of interest (ROI) temperature (°C).

5a: Cold exposure

5b: Warm room, vehicle only

5c: Warm room, glucagon
Glucagon Increases Energy Expenditure Independently of Brown Adipose Tissue Activation in Humans

Supplemental Figures with Legends

Supplemental Figures 1a to 1c: Study visit timelines (related to Main Figure 1, study visit summary).

11 male subjects attended for 4 or 5 study visits (Main Figure 1) and underwent (in random order)

- Thermal imaging and indirect calorimetry wearing a cooling vest with a 55-minute vehicle infusion (cold visit, Supplemental Figure S1a)
- Thermal imaging indirect calorimetry in a warm room (ambient temperature 22–25°C) with a 55-minute vehicle infusion (control visit, Supplemental Figure S1b)
- Thermal imaging indirect calorimetry in a warm room with a 55-minute glucagon infusion at 50 ng/kg/min (glucagon visit, Supplemental Figure S1b)

All 11 subjects also underwent an $^{18}$F-FDG PET/CT scan (Supplemental Figure S1c). The first PET/CT scan was always wearing the cooling vest with a 55-minute vehicle infusion. If this scan revealed cold-induced BAT activity, then the second PET/CT visit occurred, randomised to be either:

- $^{18}$F-FDG PET/CT in a warm room (ambient temperature 23°C) with a 55-minute glucagon infusion at 50 ng/kg/min OR
- $^{18}$F-FDG PET/CT in a warm room with a 55-minute vehicle infusion
Figure S1a: Timeline for calorimetry and thermal imaging visit, cooling vest with vehicle infusion.

Figure S1b: Timeline for calorimetry and thermal imaging visit, warm room with vehicle or glucagon infusion.
Figure S1c: Timeline for PET/CT visit

Volunteer arrives, 2 forearm cannulae inserted

Cooling vest worn (visit 1 only; draped over legs in scanner)

t= -90  t= -60  t= -30  t= 0  t= 30  t= 60  t= 90  t= 120

FDG injected

Infusion (vehicle or glucagon)
Supplemental Figure 2: Plasma glucagon levels (S2a), glucose (S2b), insulin (S2c) and FGF-21 (S2d)

Plasma levels are plotted by study intervention for all 11 subjects during exposure to the cooling vest (blue legends), vehicle infusion in a warm room (red legends) and glucagon infusion in a warm room (green legends). All data points shown as mean ± SEM.

S2a: Plasma levels of glucagon peaked at 370 ± 87 pmol/L at 40 minutes following the start of the infusion and promptly fell back to baseline 10 minutes after discontinuation. Plasma levels of glucagon during the vehicle infusion and cooling vest protocols remained below 20 pmol/L and there were no differences between plasma (endogenous) glucagon levels measured in the cold exposure or (warm) vehicle visits.
S3b. Plasma glucose levels

S3c. Plasma insulin levels

S2d: Change in plasma FGF-21 levels between start (t=0) and end (t=60) intervention. There was a non-significant rise of $238 \pm 197$ pg/ml in circulating FGF-21 following glucagon infusion.
Supplemental Figure S3: Mean pulse rate and mean arterial pressure (MAP) by intervention

Figure S3a shows the mean pulse rate (measured every 15 minutes) of all 11 subjects during exposure to the cooling vest (blue bar), vehicle infusion in a warm room (red bar) and glucagon infusion in a warm room (green bar). S3b shows the change in MAP [estimated from diastolic (DBP) and systolic (SBP) measurements as DBP + 1/3(SBP-DBP)] between the start and end of the experimental exposures of cooling vest, vehicle and glucagon infusions. Results are expressed as means ± SEM, * p<0.05 and ***p<0.001 compared with vehicle.
Supplemental Figure S4: Effects of cold exposure and glucagon infusion in a warm room on energy expenditure (EE) separated into BAT positive (n=8) and BAT negative (n=3) groups.

Resting metabolic rate was measured with an indirect calorimeter at the start and end of each intervention and the mean change from baseline are shown.
Supplemental Figure S5: Average deltoid (control) region of interest (ROI) temperature (°C) (S5a-c) and neck (BAT positive) ROI temperature (°C) (S5d-f).

Results are shown for baseline (Run A, black bar) and end intervention (Run C, white bar) for each visit type. Each run represents a 10-minute thermal recording, with stills extracted every 30 seconds and an average reading of the upper 10% pixels calculated. Results are shown for n=8 BAT positive subjects (although there was no difference in the BAT positive versus BAT negative groups in the deltoid ROI, as expected for the control region which was chosen because it is known to be devoid of underlying BAT). Results are expressed as means ± SEM; ns not significant and ***p<0.001 compared with baseline.

DELTOID ROI THERMAL DATA

SUPRACLAVICULAR (NECK) ROI THERMAL DATA
Supplemental Figure 6: Scatter plots for BAT activity and EE rise.

S6a: BAT metabolic activity [MRgluc] (µmol/kg/min) averaged across the cold PET/CT scan against the % rise in cold-induced EE (as measured on the calorimetry day, under the same conditions of cold exposure).

![Scatter plot for BAT activity and EE rise against cold exposure](image1)

Pearson r 0.07295
95% confidence interval -0.6661 to 0.7397
P value (two-tailed) 0.8637

S6b: BAT metabolic activity [MRgluc] against glucagon-induced rise in EE

![Scatter plot for BAT activity and EE rise against glucagon](image2)

Pearson r -0.8575
95% confidence interval -0.9970 to 0.5892
P value (two-tailed) 0.1425