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Title: Brown adipose tissue activation as measured by infrared thermography by mild anticipatory psychological stress in lean healthy females

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Running Title: Brown adipose tissue is activated by stress

Abstract: Brown adipose tissue (BAT) has been implicated in the pathogenesis of obesity, type-2 diabetes and the metabolic syndrome, and is a potential therapeutic target. BAT can have a significant impact on energy balance and glucose homeostasis through the action of uncoupling protein (UCP)1, dissipating chemical energy as heat following neuro-endocrine stimulation. We hypothesised that psychological stress, which is known to promote cortisol secretion, would simultaneously activate BAT at thermoneutrality. BAT activity was measured using infrared thermography to determine changes in temperature of the skin overlying supraclavicular BAT (TSCR). A mild psychological stress was induced in five healthy, lean, female, Caucasian volunteers using a short mental arithmetic (MA) test. TSCR was compared to a repeated assessment, where the MA test was substituted for a period of relaxation. Although MA did not elicit an acute stress response, anticipation of MA testing led to an increased in salivary cortisol, indicative of an anticipatory stress response, that was associated with a trend towards higher absolute and relative TSCR. A positive correlation between TSCR and cortisol was found during the

anticipatory phase, a relationship that was enhanced by raised cortisol linked to MA. Our findings suggest that subtle changes in the level of psychological stress can stimulate BAT, findings that may account for the high variability and inconsistency in reported BAT prevalence and activity measured by other modalities. Consistent assessment of this uniquely metabolic tissue is fundamental to the discovery of potential therapeutic strategies against metabolic disease.

New Findings: What is the central question of this study? Does psychological stress, which is known to promote cortisol secretion, simultaneously activate brown adipose tissue function in healthy adult females. What is the main finding and its importance?

One explanation for the pronounced differences in brown adipose tissue function between individuals lies in their responsiveness to psychological stress and as such should be taken into account when examining it's in vivo stimulation.

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**Brown adipose tissue activation as measured by infrared
thermography by mild anticipatory psychological stress in lean
healthy females**

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ABSTRACT

Brown adipose tissue (BAT) has been implicated in the pathogenesis of obesity, type-2 diabetes and the metabolic syndrome, and is a potential therapeutic target. BAT can have a significant impact on energy balance and glucose homeostasis through the action of uncoupling protein (UCP)1, dissipating chemical energy as heat following neuro-endocrine stimulation. We hypothesised that psychological stress, which is known to promote cortisol secretion, would simultaneously activate BAT at thermoneutrality. BAT activity was measured using infrared thermography to determine changes in temperature of the skin overlying supraclavicular BAT (T_{SCR}). A mild psychological stress was induced in five healthy, lean, female, Caucasian volunteers using a short mental arithmetic (MA) test. T_{SCR} was compared to a repeated assessment, where the MA test was substituted for a period of relaxation. Although MA did not elicit an acute stress response, anticipation of MA testing led to an increased in salivary cortisol, indicative of an anticipatory stress response, that was associated with a trend towards higher absolute and relative T_{SCR} . A positive correlation between T_{SCR} and cortisol was found during the anticipatory phase, a relationship that was enhanced by raised cortisol linked to MA. Our findings suggest that subtle changes in the level of psychological stress can stimulate BAT, findings that may account for the high variability and inconsistency in reported BAT prevalence and activity measured by other modalities. Consistent assessment of this uniquely metabolic tissue is fundamental to the discovery of potential therapeutic strategies against metabolic disease.

INTRODUCTION

There is increasing evidence that brown adipose tissue (BAT) has an important physiological role beyond that of thermoregulation in newborn infants and rodents (Ouellet *et al.*, 2012; Cypess *et al.*, 2014; Sidossis & Kajimura, 2015). Adult humans thus have significant amounts of BAT (Cypess *et al.*, 2009; van Marken Lichtenbelt *et al.*, 2009; Virtanen *et al.*, 2009) and as a highly metabolic tissue with the capacity to oxidise both glucose and lipid, attention has turned to its involvement in the pathogenesis of obesity, type 2 diabetes and the metabolic syndrome (Nedergaard & Cannon, 2010).

Characterised by the presence of uncoupling protein (UCP)1, this unique mitochondrial protein uncouples the respiratory chain from the production of ATP, allowing the dissipation of excess chemical energy as heat (Cannon & Nedergaard, 2004). In adult humans, UCP1 containing adipose tissues have been identified primarily in the neck and upper thorax (Virtanen *et al.*, 2009; Zingaretti *et al.*, 2009; Jespersen *et al.*, 2013), around the kidneys (Svensson *et al.*, 2014) and heart (Sacks *et al.*, 2009), and UCP1 also appears to be inducible in depots classically considered to be white (Sidossis *et al.*, 2015). Importantly, when the amount of BAT is reduced, or is dysfunctional, excess adiposity can occur (Feldmann *et al.*, 2009; Vijgen *et al.*, 2011). Several studies have demonstrated not only lower BAT activity in obese individuals as compared to those who are lean, but also reduced UCP1 (Vijgen *et al.*, 2011; Carey *et al.*, 2014), and a negative association between BAT activity and body mass index (BMI) (Saito *et al.*, 2009; Pfannenberg *et al.*, 2010; Robinson *et al.*, 2014).

BAT is under the control of the sympathetic nervous system, and is activated directly by catecholamines through β -adrenoreceptor signaling (Cannon & Nedergaard, 2004) and in humans this response can be mimicked using pharmacological stimulation with the β_3 -adrenergic agonist mirabegron (Cypess *et al.*, 2015). In addition, there is indirect evidence of active BAT under basal conditions from the observation that active noradrenaline uptake within supraclavicular BAT under warm conditions is correlated with core body temperature (Hwang *et al.*, 2015). The presence of BAT has largely been determined from the increased uptake of radiolabelled glucose during PET-CT during cold exposure of fasted subjects (van Marken Lichtenbelt *et al.*, 2009; Virtanen *et al.*, 2009). However, the extent to which BAT activity can be promoted under more normal physiological conditions has been less well studied, as this cannot be readily achieved using PET-CT. Alternative approaches are therefore needed to explore the regulation of BAT and investigate the effects of potential therapeutic agents. Infrared thermography is able to functionally assess BAT in adults and children by measuring changes in skin temperature overlying the main BAT depot in humans (Lee *et al.*, 2011; Symonds *et al.*, 2012) that has now been shown to appear similar to that shown by PET-CT (Salem *et al.*, 2015). Furthermore, due to the low variability in measurements between subjects (Symonds *et al.*, 2012) it can be used to undertake studies in comparatively small subject groups. Infrared thermography thus has the potential to elucidate the mechanisms by which BAT activation occurs and thus optimise metabolic health. On the other hand, thermography can be affected by cutaneous vasoconstriction, that can be modulated by the sympathetic nervous system, in addition to changes in ambient temperature (Greaney *et al.*, 2015).

FDG uptake in BAT has also been shown to be affected by psychological state prior to scanning, such that simple relaxation measures (exposure to audiovisual intervention) resulted in a significant reduction in BAT activity (Vogel *et al.*, 2012). The aim of our study was therefore to determine whether mild psychological stress would acutely promote supraclavicular BAT activity detectable using dynamic thermal imaging in subjects maintained within a warm environment. We hypothesised that exposure to a short mental arithmetic (MA) test would elicit an acute stress response, thereby activating BAT thermogenesis.

METHODS

Following University of Nottingham School of Medicine Ethics Committee approval, five lean Caucasian females aged between 21-29 years gave written informed consent to participate in the study. The study conformed to the standards set by the Declaration of Helsinki. The experimental protocol and study equipment were demonstrated to each study participant at least 24 hours prior to the initial study session to ensure familiarisation. All subjects were fasted from midnight, study sessions commenced between 0800 and 0900, and thermography commenced at least 60 minutes following waking. We aimed to induce mild psychological stress using a short MA test, and anticipated that in addition to our intervention this visit would engender more stress than the second due to the anticipation of the test. Therefore, we coupled the stressful intervention with the first visit, and the relaxing intervention with the second. By keeping the order of interventions identical between participants, we increased the internal consistency of the results, maximising the power to detect a difference due to stress.

At the start of the first study session, subjects were informed that they would be undertaking a short two minute MA test (Memon *et al.*, 2013) during which they would receive real time verbal feedback regarding their performance. Subjects were unaware, however, that those answering more than two successive questions correctly would be told that their next answer was incorrect regardless of the answer given. The second study session was undertaken at least 48 hours after the first and was identical in every respect to the MA session, other than the MA test was substituted for a two minute relaxation video (R), participants were aware of this from the beginning of the second study session.

Both study sessions consisted of an acclimatisation period of 30 minutes where subjects were asked to sit comfortably at rest with their supraclavicular region exposed, following which a 30 minute baseline period was recorded in which supraclavicular skin temperature and mean skin temperature were measured and samples of saliva taken for cortisol measurement. On completion of baseline measurements, either two minutes of MA or R was undertaken followed by a further period of 50 minutes consisting of thermography and salivary sampling as summarised in Figure 1.

T_{SCR} was measured using infrared thermography (FLIR B425; FLIR Systems, Danderyd, Sweden), and utilised as an indicator of BAT activity as described previously (Symonds *et al.*, 2012; Robinson *et al.*, 2014). A region of interest (ROI) was defined as that bound by the left sternocleidomastoid muscle, clavicle and lateral contour of the neck using ThermaCAM Researcher Pro 2.10. These were then exported into Excel to calculate the 97.5th percentile temperature value of the ROI. Images were taken in triplicate at each designated time point, and at intervals prior to and following MA/R testing (Figure 1). T_{SCR} at each time point was calculated as the average value derived from the triplicate images. Baseline was defined as mean temperature of the 10 minutes preceding MA or R. Mean skin temperature (T_{MSK}) was evaluated using measurements of skin temperature obtained from wireless data loggers (iButton - model no. DS1219H-F50, Maxim, Sunnyvale, California) placed at seven body sites (i.e. forehead, trunk, arm, hand, lower leg, thigh, foot) and calculated using the Hardy-Du Bois formula (Hardy & Oppel, 1938). T_{SCR} was adjusted for the

local effect of ambient temperature by calculating the difference between T_{SCR} and T_{MSK} .

Salivary cortisol samples were taken using small cotton swabs (Salivette®, Sarstedt, Germany) 10 minutes, and immediately, prior to MA or R and at 10, 20, 30, 40 and 50 minutes post intervention. These were stored at -80°C until analysis using a commercially available ELISA kit designed for use on human saliva samples (Kit no. 1-3002, Salimetrics, UK). A total of 70 samples were analysed and inter-assay and intra-assay precisions were less than 10%.

Statistical Analysis

Data was assessed for normality using the Kolmogorov-Smirnov normality test and is presented as mean \pm standard error (SEM) unless otherwise stated. Comparisons between data obtained during MA and R testing at single time points were made using a paired t-test. Pearson product moment correlation coefficients were calculated to determine the correlation between salivary cortisol levels and T_{SCR} . To determine the effect of MA and R over time, two-way repeated measures ANOVA was undertaken with Sidak's post-hoc multiple comparisons test.

RESULTS

All subjects were lean Caucasian females, with an average height, weight and BMI of $1.70\pm 0.04\text{m}$, $65.6\pm 5.05\text{ kg}$ and $22.2\pm 0.95\text{ kg/m}^2$ respectively. Ambient room temperature did not differ between the MA and R study sessions and was on average $22.8\pm 0.2^\circ\text{C}$. A summary of these anthropometric measures and room conditions are shown in Table 1.

Salivary cortisol response

Anticipation of MA was associated with significantly higher salivary cortisol concentrations at 10 minutes prior to the test compared to the relaxation film (i.e. 0.62 ± 0.17 versus $0.25\pm 0.05\text{ }\mu\text{g/dL}$, $p=0.004$), confirming that anticipation alone was sufficient to act as a mild psychological stressor. Then after administration of either MA or R, salivary cortisol decreased (Figure 2). Although there was a continued trend towards higher levels prior to MA (0.41 ± 0.11 versus $0.21\pm 0.05\text{ }\mu\text{g/dL}$), this difference was no longer statistically significant ($p=0.09$).

Anticipation of MA is associated with increased supraclavicular temperature

A trend for higher absolute value of T_{SCR} prior to MA (as compared to R) was also observed at 20 minutes ($T_{\text{SCR}}\ 34.9\pm 0.2^\circ\text{C}$ versus $34.6\pm 0.1^\circ\text{C}$, $p=0.09$). Furthermore, following adjustment for ambient temperature by calculating the difference between T_{SCR} and T_{MSK} ($T_{\text{Diff}} = T_{\text{SCR}} - T_{\text{MSK}}$), a significantly greater T_{Diff} was observed at both 20 minutes and 10 minutes prior to MA testing (Figure 3) indicating that anticipation of MA testing was associated with a relatively higher T_{SCR} .

Supraclavicular temperature increases following MA and R and is associated with baseline T_{SCR} and salivary cortisol concentration

Unexpectedly, T_{SCR} rose significantly from baseline in the subjects attending for R (Figure 4). Two-way repeated measures ANOVA identified a significant effect of time ($p=0.004$) with no effect of psychological stress. Sidak's post hoc multiple comparisons test revealed the effects of time were observed at 30, 40 and 50 minutes and were limited to the R session (Figure 4C and 4D). In contrast to the changes in T_{SCR} , T_{MSK} were seen to fall significantly over time in both study sessions by 30 minutes in the response period, suggestive of a drop in overall body skin temperature (Figure 4E and 4F). Figures 4A and 4B further demonstrate representative changes in T_{SCR} visualised as thermograms between baseline and post MA or R testing at 50 minutes with no change with MA but an increase in R.

Furthermore, when the maximal change in T_{SCR} (ΔT_{SCRmax}) was considered, although small significant increases in T_{SCR} were observed following in both the MA and R testing, no significant difference was observed between the two sets of experimental conditions (ΔT_{SCRmax} – R; $0.54 \pm 0.15^\circ\text{C}$ versus ΔT_{SCRmax} – MA; $0.44 \pm 0.05^\circ\text{C}$, $p=0.5$). A negative correlation was observed between baseline T_{SCR} and ΔT_{SCRmax} (Pearson's $r=-0.72$, $p=0.017$) indicating that the degree of maximal temperature change over time was inversely related to the baseline starting value of T_{SCR} (Figure 5). A significant positive correlation was observed between paired measures of T_{SCR} and salivary cortisol at 10 minutes prior to MA testing, but not prior to R (Figure 6).

DISCUSSION

We have shown that mild anticipatory psychological stress induced by the expectation of MA testing increased supraclavicular skin temperature that is indicative of raised BAT activity (Jang *et al.*, 2014; Salem *et al.*, 2015). This finding is therefore supportive of a significant impact of anxiety on BAT, as suggested previously from studies in both humans (Hwang *et al.*, 2015) and rodents (Lkhagvasuren *et al.*, 2011; Mohammed *et al.*, 2014). Furthermore, we have shown that irrespective of the cause elevated cortisol is strongly correlated with supraclavicular temperature. This finding is in accord with the previously suggested role of the hypothalamic-pituitary-adrenal axis in sensing energy balance and caloric flow (Kirschbaum *et al.*, 1997). It has also been recently shown in rodents that the dorsomedial hypothalamic node bifurcates stress signaling to the sympathetic outflow to promote BAT thermogenesis as well as to the endocrine outflow thereby promoting stress hormone release (including corticoids) through the HPA-axis (Kataoka *et al.*, 2014)..

In our study, MA testing per se did not elicit the acute stress response we had expected. However, all but one subject was educated to post-graduate level, and scored highly on the MA test, suggesting it was not sufficiently challenging. Interestingly, we also observed an increase in T_{SCR} over the 50 minutes following MA and R, whilst the maximal change from baseline did not differ between the two experimental conditions. Consequently, these effects appear to be more pronounced following R. T_{SCR} when first recorded was also lower prior to R, which is indicative of lower baseline BAT thermogenesis. As T_{SCR} increased during both MA and R, and salivary cortisol measures did not increase in line with an MA induced acute stress response, further BAT stimulation must have occurred by an alternative mechanism.

It should be noted that for successful thermography subjects must remain still, such that despite imaging being undertaken fully clothed in a warm comfortable room, subjects may gradually cool down due to a reduction in non-exercise activity thermogenesis (NEAT). In support of this theory is the reduction in mean skin temperature we observed particularly in the R intervention. The MA group, with their higher T_{SCR} at baseline seemed better able to maintain their overall body temperature, such that BAT thermogenesis recruited during the anticipatory phase was sufficient to offset any reduction in NEAT, or represents maximally stimulated BAT at baseline. Further studies conducted at cooler ambient temperatures could now be undertaken to investigate this further.

Whilst the effect of catecholamines on BAT thermogenesis has been well characterised in rodents (Cannon & Nedergaard, 2004), and has been observed in humans with pathological adrenergic excess (Nagano *et al.*, 2015), and subjects exposed to therapeutic intervention (Cypess *et al.*, 2015), our understanding of the endocrine control of human BAT in the physiological state is less well understood. A role for anxiety, or anticipatory stress has also been suggested from the finding that administration of anxiolytic pre-medications such as propranolol diminishes the “unwanted” BAT glucose uptake signal in patients undergoing clinical PET-CT scanning (Gelfand *et al.*, 2005; Jacobsson *et al.*, 2005) as does exposure to relaxing audio-visual imagery (Vogel *et al.*, 2012). A potential role for cortisol in BAT function has not been previously shown in adults in vivo, and whilst the increase in cortisol observed in our study most likely represents the overall neuroendocrine response to acute stress (hypothalamic-pituitary-adrenal activation), including a concomitant increase in systemic circulating catecholamines. Our novel observation

that cortisol was positively correlated with T_{SCR} , in conjunction with in vitro studies in humans, (Barclay *et al.*, 2015) highlight the effect of cortisol on human BAT as an interesting avenue for future investigation.

Rodent studies, both in vitro (Soumano *et al.*, 2000; Viengchareun *et al.*, 2001) and in vivo have reported that glucocorticoids have an inhibitory effect on classical BAT, suppressing thermogenesis (Strack *et al.*, 1995), although these must be interpreted with caution as they were not undertaken at thermoneutrality (Celi *et al.*, 2015). It should be noted that endocrine factors suggested to stimulate BAT function in rodents may not necessarily have the same role in humans. For example, it has recently been shown that the 10 fold rise in plasma FGF21 with prolonged fasting in humans is accompanied with reduced BAT function (Fazeli *et al.*, 2015). Moreover, in rodents a dose 10 times higher than this is required to induce changes in BAT activity (Kwon *et al.*).

Cortisol is, however, known to have an important role in promoting maximal BAT function around the time of birth, although whether this is a direct effect or mediated by changes in thyroid hormones remains to be fully established (Mostyn *et al.*, 2003). Direct effects of exogenous cortisol administration on human BAT activity have yet to be shown, but hypercortisolaemia induced by exogenous administration of hydrocortisone results in a significant increase in energy expenditure (Brillon *et al.*, 1995). Whether this is mediated by BAT remains to be elucidated although we have recently shown that a 24h infusion of hydrocortisone into adult men of normal weight stimulates BAT function (H Scotney, ME Symonds, J Law, H Budge, D Sharkey and K Manolopoulos 2015, unpublished results). Dexamethasone stimulates the

proliferation and differentiation and function of human brown adipocytes in vitro (Barclay *et al.*, 2015). This suggests that metabolic manifestations of Cushing's syndrome could be related, in part, to the effect of hypercortisolaemia on BAT, such that the characteristic of regional excess adiposity (e.g. the buffalo hump) represent hyperproliferation of BAT as recently suggested from the response to trauma in subcutaneous adipose tissue following severe burning (Sidossis *et al.*, 2015).

In conclusion, our findings have important implications in the study of BAT under supposedly basal conditions as it is clear that quite subtle changes in psychological stress, including anticipation, can stimulate the production of heat from BAT within the supraclavicular depot. This may explain why functional measurements of BAT measured using PET-CT are not directly confirmed with comparable changes in BAT temperature (Jang *et al.*, 2014) and further highlights the need for caution when interpreting such results. Our findings if repeated in larger subject groups could therefore enable novel interventions linked to mild stress in order to promote BAT function.

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Table 1: Summary of the anthropometric measures of the study participants and room temperature on each study day.

ID	Height (m)	Weight (Kg)	BMI	Age	Ambient room temperature - MA testing	Ambient room temperature – R testing
1	1.85	83.7	24.6	29 yr 2mths	22.5	23.0
2	1.70	59.1	20.5	26 yr 7 mths	23.4	23.3
3	1.65	53.9	19.8	21 yr 9 mths	23.1	22.9
4	1.72	64.3	21.8	24 yr 10mths	22.9	22.9
5	1.67	66.9	24.1	23 yr 2mths	23.0	22.1

FIGURE LEGENDS

Figure 1

Summary of the study protocol, demonstrating timings of thermal imaging and salivary cortisol assessment relative to mental arithmetic (MA) testing, or exposure to the audio-visual relaxation film (R) which were conducted at the same time on different days. T_{SCR} (supraclavicular temperature) = open triangle, and salivary cortisol = black square.

Figure 2

Salivary cortisol concentrations ($\mu\text{g/dL}$) at baseline (B) and following mental arithmetic testing (MA) or exposure to audio-visual relaxation film (R), in lean, Caucasian female subjects ($n = 5$). Data are shown as mean \pm SEM. MA = black square and R = white square.

Figure 3

A) Supraclavicular temperature (T_{SCR}) and B) Temperature difference between the supraclavicular region and mean skin temperature ($T_{Diff} = T_{SCR}$ minus T_{MSK}) 20 minutes and 10 minutes prior to mental arithmetic testing (black bars) and the audio-visual relaxation film (white bars) in lean, Caucasian white female subjects ($n = 5$). A: $p=0.048$ and B: $p=0.041$. Data are shown as mean \pm SEM.

Figure 4

A. Representative anterior thermograms of the supraclavicular region during baseline, and 50 minutes after a 2-minute mental arithmetic test.

B. Representative anterior thermograms of the supraclavicular region during baseline and 50 minutes after watching a 2-minute relaxation film.

C. and D. Demonstrate the changes in T_{SCR} from baseline up to 50 minutes following C) mental arithmetic testing or D) exposure to the relaxation video.

E. and F. Demonstrate the changes in T_{MSK} from baseline up to 50 minutes following E) mental arithmetic testing or F) exposure to the relaxation video.

Mental arithmetic testing = black symbols, Relaxation video = white symbols.

T_{SCR} = square and T_{MSK} = circle.

* $p < 0.05$ following two way repeated measures ANOVA. Data are shown as mean \pm SEM, $n = 5$.

Figure 5

Relationship between minimum baseline supraclavicular temperature (T_{SCR}) and maximal change in supraclavicular temperature (ΔT_{SCRmax}) throughout the duration of the study periods (Pearson's correlation coefficient $r = -0.72$, $p = 0.017$) in lean, Caucasian female subjects ($n = 5$). Mental arithmetic testing = black symbols, Relaxation video = white symbols.

Figure 6

Relationship between supraclavicular temperature (T_{SCR}) and salivary cortisol during the baseline period prior to mental arithmetic testing (Pearson's correlation coefficient, $r=0.97$, $p=0.008$) and prior to the audio-visual relaxation film (Pearson's correlation coefficient, $r=0.14$, $p=0.188$) in lean, Caucasian white female subjects ($n=5$). Mental arithmetic = black squares, relaxation film = white squares.











