Title: Molecular Adaptations of Adipose Tissue to 6 weeks of Morning Fasting vs Daily Breakfast Consumption in Lean and Obese Adults

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In lean individuals, six weeks of extended morning fasting increases the expression of genes involved in lipid turnover (ACADM) and insulin signalling (IRS2) in subcutaneous abdominal adipose tissue.

In obese individuals, six weeks of extended morning fasting increases IRS2 expression in subcutaneous abdominal adipose tissue.

The content and activation status of key proteins involved in insulin signalling and glucose transport (GLUT4, Akt1 and Akt2) were unaffected by extended morning fasting. Therefore, any observations of altered adipose tissue insulin sensitivity with extended morning fasting do not necessarily require changes in insulin signalling proximal to Akt.

Insulin-stimulated adipose tissue glucose uptake rates are lower in obese versus lean individuals, but this difference is abolished when values are normalised to whole-body fat mass. This suggests a novel hypothesis which proposes that the reduced adipose glucose uptake in obesity is a physiological downregulation to prevent excessive de novo lipogenesis.

ABSTRACT

This experiment assessed molecular responses of human subcutaneous abdominal adipose tissue (SCAT) to 6 weeks of morning fasting. Forty-eight, healthy lean (n = 29) and obese (n = 20) adults provided SCAT biopsies before and after six weeks of morning fasting (FAST; 0 kcal until 1200 h) or daily breakfast consumption (BFAST; ≥700 kcal before 1100 h). Biopsies were analysed for mRNA levels of selected
genes, and GLUT4 and Akt protein content. Basal and insulin-stimulated Akt activation and tissue glucose uptake rates were also determined. In lean individuals, lipid turnover and insulin signalling genes (ACADM and IRS2) were upregulated with FAST versus BFAST [1.14 (95%CI: 0.97 to 1.30) versus 0.80 (95%CI: 0.64 to 0.96), p=0.007 and 1.75 (95%CI: 1.33 to 2.16) versus 1.09 (95%CI: 0.67 to 1.51), p=0.03, respectively). In obese individuals, no differential (FAST versus BFAST) expression was observed in genes involved in lipid turnover (all p>0.1). GLUT4, Akt protein content and insulin-stimulated Akt phosphorylation were unaffected by FAST versus BFAST in both lean and obese cohorts (all p>0.1). Lower insulin-stimulated glucose uptake rates in obese versus lean individuals were eradicated when normalised to whole-body fat mass (P=0.416). We conclude that morning fasting upregulates lipid turnover genes in SCAT of lean individuals. Secondly, altered SCAT insulin sensitivity with morning fasting is unlikely to be explained by signalling proximal to Akt. Finally, lower insulin-stimulated SCAT glucose uptake rates in obese individuals are proportional to whole-body fat mass, suggesting a compensatory downregulation, presumably to prevent excessive de novo lipogenesis in adipose tissue. This trial was registered as ISRCTN31521726.

INTRODUCTION
Once considered an inert storage depot, an understanding of the active roles of adipose tissue in metabolic health is gaining momentum (Thompson et al., 2012). Adipose tissue is dynamic; buffering daily lipid flux, and regulating glucose and lipid metabolism (Garg, 2011; Stanford et al., 2015), inflammation and hormone production (Thompson et al., 2012). Importantly, insulin action in adipocytes plays a
major role in whole body insulin sensitivity (Abel et al., 2001) and thus adipose tissue is viewed as a key target for metabolic control.

Subcutaneous adipose tissue is the primary site of storage and release of fatty acids to the systemic circulation (Koutsari & Jensen, 2006). Adipose tissue physiology is highly responsive to acute alterations in energy flux, such as fasting, feeding and physical activity (Ruge et al., 2009). The acute switch in subcutaneous abdominal adipose tissue (SCAT) metabolism from fasting to feeding results in large changes in glucose and fatty acid flux (Ruge et al., 2009). This is partly co-ordinated by insulin signalling and subsequent translocation of glucose transport proteins and lipoprotein lipase activation (Ruge et al., 2009; Chen et al., 2017). Notably, the SCAT response to fasting versus feeding is profoundly blunted in obesity. The postprandial increase in glucose and fatty acid uptake by SCAT in obese individuals is less than half that seen in lean individuals (McQuaid et al., 2011). This dysregulation of glucose and fatty acid uptake by adipose tissue in obesity is also accompanied by a lower mRNA expression of genes involved in insulin signalling (IRS2), glucose (GLUT4) (Travers et al., 2015b) and fatty acid metabolism (ACLS1, LPL, AGPAT9, PLIN, HSL, PNPL2, DGAT1 and DGAT2) (McQuaid et al., 2011; Travers et al., 2015b), with a concomitant increase in mRNA expression of genes relating to inflammation/cytokine signalling (IL1RA, IL-18 and MCP-1) (Travers et al., 2015b). While much is known about the distinct metabolic and molecular responses of SCAT to an acute feeding stimulus in both lean and obese individuals, the chronic molecular adaptations of SCAT to modified fasting and feeding patterns have never been assessed in either lean or obese individuals.
The *Bath Breakfast Project* was a randomised controlled trial comparing extended morning fasting (0 kcal until 1200 h) with daily breakfast consumption (≥700 kcal before 1100 h) in healthy lean and obese adults (Betts *et al.*, 2011). Compared to daily breakfast consumption, extended morning fasting impaired glucose control in SCAT in lean, but not obese individuals (Betts *et al.*, 2014; Chowdhury *et al.*, 2016a). Specifically, extended morning fasting in lean individuals increased *in vivo* SCAT interstitial glucose variability in the afternoon and evening (Betts *et al.*, 2014) and *ex vivo*, an index of SCAT insulin sensitivity increased from baseline to follow-up in the breakfast group, but not in the fasting group (Betts *et al.*, 2014). Importantly, these observations were not observed in an obese cohort (Chowdhury *et al.*, 2016a). Furthermore SCAT from obese individuals displayed quantitatively lower rates of insulin-stimulated glucose uptake (expressed per mg lipid) compared to that from lean individuals both at physiological, and supraphysiological insulin concentrations (Chowdhury *et al.*, 2016a).

Differences in insulin-stimulated (physiological and/or maximal) glucose uptake by adipose tissue could be explained by differences in GLUT4 content and/or alterations in insulin signalling and GLUT4 translocation (Tan *et al.*, 2015; Travers *et al.*, 2015a). For example, adipose tissue from individuals who are obese or have type 2 diabetes displays reduced insulin-stimulated glucose uptake, alongside a 40-85% reduction in GLUT4 protein content (Garvey *et al.*, 1991). The lower GLUT4 protein content is thought to be regulated at the mRNA level, as GLUT4 mRNA expression shows a progressive decrease across the range from lean to obese (Travers *et al.*, 2015a). Akt (PKB) is a key insulin signalling protein that is essential for most metabolic actions of insulin, including phosphorylation of TBC1D4/AS160...
leading to GLUT4 translocation (Kane et al., 2002; Tan et al., 2012). Defects in signalling proximal to Akt have been identified as potential contributors to insulin resistance in various models (Tan et al., 2015). We therefore focussed on GLUT4 content, Akt signalling and gene expression in SCAT to elucidate the mechanisms that regulate adipose tissue adaptations over 6-weeks of extended morning fasting compared to regular breakfast consumption in lean and obese individuals.

**METHODS**

**Ethical approval**

This study adhered to the standards set by the Declaration of Helsinki and the procedures followed were in accordance with the protocol approved by the National Health Service South-West 3 Research Ethics Committee (10/H0106/13). All participants provided informed written consent prior to participation in the study.

**Experimental design**

The Bath Breakfast Project is a randomised controlled trial registered with Current Controlled Trials [ISRCTN31521726] and this protocol has since been published in full (Betts et al., 2011), with trial enrolment, baseline/eligibility testing, allocation and follow-up all conducted in accordance with CONSORT guidelines (Schulz et al., 2010). A CONSORT flow diagram, along with precise details of this protocol and the rationale for our approach/methods have previously been published (Betts et al., 2011; Betts et al., 2014). Demographic, anthropometric and physiological characteristics of those who completed the trial are presented in Table 1. This cohort
completed intensive laboratory-based assessments at baseline and we have previously reported
resting metabolic rate (via indirect calorimetry from gaseous exchange), anthropometric
characteristics [i.e. body mass and DXA-derived body composition (Hologic Discovery W)],
and some physiological characteristics (specifically, fasting plasma glucose and insulin
concentrations; C-ISI Matsuda, adipose tissue glucose uptake rates expressed per mg lipid)
(Betts et al., 2014; Chowdhury et al., 2015; Chowdhury et al., 2016a; Chowdhury et al.,
2016b). We repeat these data here for context in relation to the novel molecular outcomes,
and clearly indicate whenever data were previously published.

Participants all met the following inclusion criteria: aged 21-60; record of regular
menstrual cycle/contraceptive use (if relevant); no anticipated changes in diet and/or
physical activity habits during the study period; weight stable (within 2 % over past 6
months); non-shift workers; not pregnant or breastfeeding; and were free from any
other condition or behaviour deemed either to pose undue personal risk or introduce
bias into the experiment.

While all participants in both groups remained in a 10-h overnight fasted state
(i.e. 0900 ± 1 h), a small (~1 g) subcutaneous adipose tissue biopsy (~1 g) was sampled
from the abdomen, ~5 cm lateral to the umbilicus, - by needle aspiration (14 gauge)
after local anaesthesia (4 mL, 1% lidocaine) as previously described (Walhin et al., 2013;
Travers et al., 2015a). These were used to provide measures of the post-absorptive
protein content of GLUT4, Akt1 and Akt2, in addition to the mRNA
expression of key selected genes involved in adipose tissue metabolism and inflammation. Furthermore, measures of adipose tissue glucose uptake and Akt phosphorylation were performed under basal and insulin-stimulated conditions.

These measures were followed-up 6 weeks later, along with continuous (5-min sampling interval) monitoring of interstitial glucose concentrations via a subcutaneous abdominal catheter (iPro, Medtronic) both to document chronic glycemic responses and to verify compliance, as reported previously (Betts et al., 2014; Chowdhury et al., 2016a). Eumenorrheic women provided baseline samples 2 weeks prior to the start of the 6-week intervention so that follow-up samples could be acquired 3-10 days after the onset of menses (i.e. follicular phase). During the 6-week intervention, participants were randomised (1:1 allocation ratio) into either a group prescribed a self-selected energy intake of ≥700 kcal before 1100 h daily, with at least half consumed within 2 h of waking (BFAST group) or a group to extend their overnight fast by abstaining from ingestion of energy-providing nutrients (i.e. plain water only) until 1200 h each day (FAST group). The randomisation scheme was generated by the principal investigator (JAB) using a computer-based random number generator and was stratified according to baseline breakfast habits (block size = 4), with frequent breakfast consumption defined as the ingestion of ≥50 kcal within two hours of waking on most days of the week. Investigators enrolling participants (JDR & EAC) were unaware of these details and independently requested group assignments to prevent deciphering of the allocation sequence. Due to the self-administered nature of the treatments, it was not possible to blind participants to group allocation. The intervention was applied under free-living
conditions and all other lifestyle choices were allowed to vary naturally. Compliance was confirmed via self-report and verified via continuous glucose monitoring; data reported herein are therefore only for those individuals for whom baseline and follow-up measurements are available (i.e. a completers-only analysis).

**Glucose uptake and protein expression analysis in isolated primary adipocytes**

Adipose tissue biopsies were digested with collagenase before determination of [U-\(^{14}\)C]-D-glucose uptake as previously described (Foley *et al.*, 1983; Kashiwagi *et al.*, 1983; Betts *et al.*, 2011). The lipid content of adipocytes was determined by extraction and weighing of total lipids of a 50 \(\mu\)L aliquot of 20% adipocytes in suspension. The cell suspension was mixed with 2.7 mL of isopropanol:heptane:H\(\text{H}_2\text{SO}_4\) (40:10:1 ratio) followed by the addition of 1.8 mL heptane and 1.0 mL of ddH\(\text{H}_2\text{O}\) before vortexing and centrifugation. A 1.0-mL aliquot of the organic layer was evaporated and the lipid subsequently weighed.

Proteins were separated by SDS-PAGE and transferred using a semidy electro-transfer to a nitrocellulose membrane and were normalised to GAPDH and per mg lipid within each sample. Western blotting analysis was performed with the following antibodies: Akt1 (Millipore), Akt2 (Millipore), phosphoserine 473 Akt (Cell Signalling Technologies, USA), GLUT4 (Holman *et al.*, 1990, JBC), GAPDH (Proteintech, USA). The images were acquired in an EPI Chemi II darkroom (UVP) and bands quantified using VisionWorks LS analysis software (UVP).
RNA extraction and quantitative real-time PCR

Total RNA was extracted by the method of Chomczynski and Sacchi (Chomczynski & Sacchi, 1987) using TRIzol reagent (Invitrogen, Paisley, UK). Quantification and purity of RNA were assessed using Nanodrop ND-100 (Thermo Fisher Scientific, Wilmington, USA). Reverse transcription was carried out using 500ng of total RNA using the SuperScript III cDNA kit (Invitrogen, Paisley, UK). Taqman Low density Custom Array using Micro Fluidic cards (ABI Applied Biosystems, UK) was used for the relative quantification of expression of 48 genes as previously described (Tsintzas et al., 2013). Each card allowed for 8 samples to be run in parallel against 48 Taqman gene expression assay targets that were pre-loaded into each of the wells on the card. A control sample was loaded in each card to assess reproducibility of data between cards and normalise for variations in the expression of the target genes between cards. Briefly, 50 µL of Taqman Universal PCR master mix (2x) (ABI Applied Biosystems, UK) was added to 200 ng RNA equivalent of cDNA into an Eppendorf RNase-free tube. RNase-free water was added to make the total volume of the reaction mixture up to 100 µL. The reaction mixture was mixed, centrifuged and loaded into one of the fill reservoir of the Micro Fluidic card. The cards were centrifuged (MULTIFUGE 3 S-R, Heraeus) and ran on a 7900HT Fast Real-Time PCR System (ABI Applied Biosystems, UK). Relative quantification of the genes of interest was performed using the comparative CT method. The geometric mean of 3 housekeeping genes [18S ribosomal RNA (18S), PPIA and PGK1] was used to normalise the data, as previously described (Tsintzas et al., 2013; Tsintzas et al., 2017). These genes were selected a priori, based on their stability in multiple adipose tissue/adipocyte samples (Neville et al., 2011) and this was confirmed in the present study using an algorithm as previously described (Andersen et al., 2004).
The normalisation to multiple housekeeping genes was used to minimize variations in the expression of individual housekeeping genes (Vandesompele et al., 2002). One gene (UCP1; uncoupling protein 1) was undetectable and therefore data for 44 genes are presented (Table 2).

**Statistical analyses**

Statistical analyses were performed using SPSS v24 (IBM, New York, NY) and GraphPad Prism v7 (GraphPad Software, San Diego, CA). Values are means with 95% confidence intervals (CI) unless stated otherwise and were checked for normal distribution by the Shapiro-Wilk normality test prior to analysis. mRNA expression is presented as means ± 95%CI in the first instance (Figures 1 and 2), and secondly as effect sizes from baseline to follow-up in a heat-map (Figure 3). This combination was chosen in order to capture both the quantitative mRNA expression in addition to the relative change within the context of variability. The effect size (Cohen’s d) was calculated as:

\[ d = \frac{\text{mean}_{\text{follow-up}} - \text{mean}_{\text{baseline}}}{SD_{\text{pooled}}} \]

where:

\[ SD_{\text{pooled}} = \sqrt{\frac{SD_{\text{baseline}}^2 + SD_{\text{follow-up}}^2}{2}} \]

Differences between treatments (breakfast versus fasting) were assessed by ANCOVA with baseline scores as the covariate (Bland & Altman, 2015). The phosphorylation status of AKT at basal and physiological insulin concentrations was
assessed by ANCOVA with both treatment (breakfast versus fasting) and insulin concentration (basal versus 50 pM) as fixed factors and baseline scores as the covariate. All $p$-values have been adjusted for multiple comparisons by Bonferroni correction. Characteristics of lean and obese cohorts at baseline were compared by paired t-tests or mann-whitney tests as appropriate. Since the a priori plan of this study was to assess lean and obese cohorts separately (Betts et al., 2011), the responses of lean and obese cohorts to the intervention were not statistically assessed, and therefore inferences of each cohorts responses should be considered separately from one another. Relationships between glucose uptake and GLUT4 protein content were assessed via Pearson correlation coefficients.

RESULTS

Relative post-absorptive mRNA expression pre- and post-intervention

In lean individuals, the post-absorptive adipose tissue mRNA expression of genes involved in lipid turnover and the proximal components of the insulin signalling pathway (ACADM and IRS2) were upregulated with extended morning fasting relative to regular breakfast consumption in the lean cohort ($p = 0.007$ and $p = 0.03$, respectively; Figures 1 and 3). However, there was no differential (breakfast versus fasting) mRNA expression of four key genes further downstream of PIK3R, in lean individuals (Figure 3; all $p > 0.1$). The mRNA expression of genes involved in AMPK signalling, Inflammation/Cytokine signalling, and those involved in mitochondrial signalling were not affected by extended morning fasting, compared to regular breakfast consumption (all $p > 0.1$; Figure 3).

In obese individuals, the post-absorptive adipose tissue mRNA expression of genes involved in lipid turnover were not affected by extended morning fasting versus regular breakfast consumption (all $p > 0.1$; Figures 2 and 3). IRS2 mRNA expression was increased with extended morning fasting versus regular breakfast consumption ($p = 0.049$; Figure 3), however, genes involved in insulin signalling proximal to IRS2, displayed no differential regulation by extended morning fasting versus regular breakfast consumption in obese individuals (all $p > 0.1$; Figure 3).
The mRNA expression of genes involved in AMPK signalling and Inflammation/Cytokine signalling were unaffected by extended morning fasting, compared to regular breakfast consumption (all $p > 0.1$; Figure 3).

Figure 1. mRNA expression of 44 selected genes in subcutaneous abdominal adipose tissue (SCAT) of lean humans randomised to extended morning fasting [FAST; $n = 13$ (A)] or regular breakfast consumption [BFAST; $n = 13$ (B)] for 6 weeks. Values represent the ratios of the mRNA content of target genes to the geometric mean content of housekeeping genes expressed as means ± 95%CI.
Figure 2. mRNA expression of 44 selected genes in subcutaneous abdominal adipose tissue (SCAT) of obese humans randomised to extended morning fasting [FAST; n = 10 (A)] or regular breakfast consumption [BFAST; n = 10 (B)] for 6 weeks. Values represent the ratios of the mRNA content of target genes to the geometric mean content of housekeeping genes expressed as means ± 95%CI.
Figure 3. mRNA expression of 44 selected genes in subcutaneous abdominal adipose tissue of lean and obese humans randomised to extended morning fasting (FAST; \( n = 13 \) lean; \( n = 10 \) obese) or regular breakfast consumption (BFAST; \( n = 13 \) lean; \( n = 10 \) obese) for 6 weeks. The intensity of colour represents the effect sizes (Cohen’s \( d \)) of the change from pre-to-post intervention and \( p \) values represent the FAST versus BFAST comparison of mRNA expression levels from ANCOVA with baseline mRNA levels as the covariate.
GLUT4, Akt1 and Akt2 protein content

In lean individuals, the protein content of GLUT4, Akt1 and Akt2 were unaffected by extended morning fasting compared to regular breakfast consumption, whether expressed either per mg lipid, or per mg lipid multiplied by adipose tissue mass (Figures 4A and 4C; all $p > 0.1$).

In obese individuals, the protein content of GLUT4, Akt1 and Akt2 were unaffected by extended morning fasting compared to regular breakfast consumption, whether expressed either per mg lipid, or per mg lipid multiplied by adipose tissue mass (Figures 4B and 4D; all $p > 0.1$).

**Figure 4.** Protein content of glucose transporter 4 (GLUT4) Akt1 and Akt2 in adipose tissue of lean (A, C) and obese (B, D) humans randomised to extended morning fasting (FAST; $n = 5$ lean; $n = 4$ obese) or regular breakfast consumption (BFAST; $n = 4$ lean; $n = 8$ obese) for 6 weeks. Data are expressed per mg lipid (A, B) and per mg lipid multiplied by DXA-derived adipose tissue mass (C, D) and are presented as means ± 95% CI.
Akt activation

In the lean cohort, Ser 473 phosphorylation of Akt increased up to ~50% of a maximal response with physiological concentrations of insulin (Figure 5A, $p = 0.011$). But there was no treatment (FAST versus BFAST), nor any insulin x treatment interaction effect ($p = 0.441$ and $p = 0.725$, respectively).

**Figure 5.** Phosphorylation of Akt expressed as a percentage of maximal phosphorylation (at 20 nM Insulin) in isolated adipocytes of lean (A) and obese (B) humans randomised to extended morning fasting (FAST; $n = 5$ lean; $n = 4$ obese) or regular breakfast consumption (BFAST; $n = 4$ lean; $n = 8$ obese) for 6 weeks. Data are presented from measures under basal and physiologic (50 pM) insulin concentrations as means ± 95%CI. PRE, pre-intervention; POST, post-intervention.
In the obese cohort, Ser 473 phosphorylation of Akt increased with physiological concentrations of insulin (Figure 5B, \( p = 0.003 \). But there was no treatment (FAST versus BFAST), nor any insulin x treatment interaction effect (\( p = 0.627 \) and \( p = 0.909 \), respectively).

**Adipose tissue glucose uptake**

At baseline, GLUT4 levels modestly positively correlated with adipose tissue glucose uptake at physiological insulin concentrations (i.e. 50 pM; Figure 6A). However, the baseline-to-follow-up change in GLUT4 protein content did not correlate with the change in insulin-stimulated glucose uptake (Figure 6B).

**Figure 6.** Relationships between protein content of glucose transporter 4 (GLUT4) and glucose uptake in adipose tissue of lean and obese humans randomised to extended morning fasting (FAST; \( n = 5 \) lean; \( n = 4 \) obese) or regular breakfast consumption (BFAST; \( n = 4 \) lean; \( n = 8 \) obese) for 6 weeks expressed as absolute values at baseline (A) or the pre-to-post intervention change (B).
At baseline, adipose tissue glucose uptake under physiological concentrations of insulin were ~2.6-fold higher in lean, compared to obese individuals when expressed per mg lipid [Figure 7A; difference between lean and obese: 0.038 pmol·min⁻¹·mg lipid⁻¹; P < 0.0001], as previously reported without direct comparison between lean and obese cohorts (Chowdhury et al., 2016a). Furthermore, adipose tissue glucose uptake expressed per mg lipid negatively correlated with DXA-derived whole-body fat mass (r = -0.480, p < 0.001). However, once normalised for whole-body adipose tissue mass, the difference in adipose tissue glucose uptake rates between lean and obese cohorts was abolished [Figure 7B; difference between lean and obese: 0.148 pmol·min⁻¹·mg lipid⁻¹ x adipose tissue mass; P = 0.416].
Figure 7. Baseline adipose tissue glucose uptake at physiological concentrations (50 pM) of insulin in lean (n = 29) and obese (n = 18) humans expressed per mg lipid (A), or per mg lipid multiplied by DXA-derived whole-body fat mass (B). Horizontal lines represent means ± 95%CI. Differences between lean and obese cohorts were compared by Mann-Whitney tests.
DISCUSSION

The present work is the first to examine the molecular responses of human adipose tissue to extended morning fasting. Six weeks of extended morning fasting alters the post-absorptive mRNA expression of a number of metabolic genes in human adipose tissue of lean individuals. In particular, extended morning fasting increases the post-absorptive expression of genes involved in lipid turnover compared to daily breakfast consumption in lean individuals. Furthermore, important regulators of insulin sensitivity (e.g. IRS2) were also upregulated by fasting versus breakfast consumption in lean individuals. Extended morning fasting did not, however, alter the protein content of GLUT4, Akt1, Akt2, nor insulin-induced Akt phosphorylation in either lean or obese individuals. Whilst some previous studies have characterised the response of lean and obese adipose tissue to an acute feeding stimulus (McQuaid et al., 2011), the chronic molecular responses of human adipose tissue to different feeding and fasting patterns had never previously been assessed in either lean, nor in obese individuals.

There is strong evidence that the protein content of GLUT4 is an important contributory factor to the reduction in human adipose tissue insulin sensitivity in obesity and type 2 diabetes. GLUT4 content is typically >40% lower in adipose tissue from individuals with obesity and type 2 diabetes, when compared to lean individuals, seemingly regulated at the mRNA level (Garvey et al., 1991). In line with this, we observed that the content of GLUT4 positively correlated with adipose tissue glucose uptake at physiological concentrations of insulin, thereby supporting the role of GLUT4 content in adipose tissue insulin sensitivity. These reductions in protein content may therefore contribute to the lower rates of glucose uptake in adipose tissue.
tissue from obese individuals compared to lean individuals at both physiological and maximal (Chowdhury et al., 2016a) insulin-stimulated conditions.

Whilst GLUT4 content is of importance in explaining the alterations in insulin sensitivity with chronic changes in fat mass, we did not observe changes in protein content of GLUT4, Akt1 or Akt2 with extended morning fasting compared to regular breakfast consumption, at the level of either mRNA or protein. Therefore, changes in the content of these proteins may not be necessary to explain differences in adipose tissue glucose control with extended morning fasting versus breakfast consumption in lean individuals, when energy balance is maintained (Betts et al., 2014). In support of this, the pre-to-post intervention change in GLUT4 content did not correlate with the change in insulin-stimulated glucose uptake. The lack of an increase in post-absorptive GLUT4 and Akt mRNA expression, and in GLUT4, Akt1 and Akt2 protein content, suggests that phosphoprotein signalling leading to increases in GLUT4 translocation and/or activity are likely to be primarily responsible for the alterations in adipose tissue glucose control previously observed with extended morning fasting versus breakfast consumption (Betts et al., 2014), rather than de novo synthesis of proximal signalling proteins or GLUT4 proteins.

In order to assess whether activation of Akt could explain any adaptations in adipose tissue glucose control with altered meal patterns, we assessed the phosphorylation status of Akt in human adipocytes under basal and physiological (50 pM) insulin stimulation ex vivo. In neither lean nor obese individuals did extended morning fasting alter insulin-stimulated Akt phosphorylation compared to regular breakfast consumption. This is consistent with murine data demonstrating that 28 days of high-
fat diet induced-insulin resistance in adipose tissue does not coincide with a reduction in Akt phosphorylation or in GLUT4 protein content, but rather with a reduction in AS160 phosphorylation (Tan et al., 2015). Therefore, the effects of fasting/feeding patterns on adipose tissue glucose control in lean individuals are likely to be explained by signalling downstream of Akt, but the precise location of this regulation remains to be determined.

The lower insulin-stimulated adipose tissue glucose uptake per mg lipid from obese individuals reported previously (Garvey et al., 1991; Chowdhury et al., 2016a) could represent a physiological downregulation of insulin sensitivity. A physiological downregulation of adipose tissue non-esterified fatty acid release in obesity has previously been suggested whereby, with increasing adiposity, the downregulation of NEFA release per kilogram of adipose tissue preserves circulating NEFA homeostasis (Karpe et al., 2011). Consistent with this, obese participants in the present study did not display elevated circulating plasma NEFA concentrations relative to lean participants, in spite of more than two-fold greater average fat mass. To explore whether this may also be the case with insulin stimulated glucose uptake, we multiplied the rate of glucose uptake (originally expressed as pmol·min⁻¹·mg lipid⁻¹) by DXA-derived whole-body adipose tissue mass (kg). Once normalised for whole-body fat mass, no differences in insulin-stimulated glucose uptake were detected between lean and obese groups, and the spread of data were remarkably similar between these cohorts. It may therefore be that the reduction in insulin-stimulated glucose uptake in obese (but otherwise healthy) individuals is a physiological mechanism to limit the absolute rate of glucose uptake by adipose tissue, and thereby constrain the rate of de novo lipogenesis (DNL). In line with this, others have
demonstrated that individuals with obesity have a higher proportion of adipocytes that are refractory (rather than responsive) to insulin-stimulated glucose uptake in vitro (Lizunov et al., 2015), and have a lower adipose tissue expression of genes involved in lipogenesis (Roberts et al., 2009). However, since our finding was due to exploratory analysis, it should be confirmed in future studies.

In an attempt to explore transcriptional changes that could be chronically altered by changes in fasting and feeding patterns, we also assessed the mRNA expression of selected genes involved in a number of signalling pathways in the post-absorptive state before and after the intervention. The post-absorptive expression of a gene involved in lipid turnover (ACADM) was upregulated with 6 weeks of extended morning fasting relative to daily breakfast consumption, in lean individuals. These responses could be expected since extended morning fasting results in greater rates of whole-body lipid utilisation (Gonzalez et al., 2013) which, both at rest and during low intensity physical activity, is primarily supported by adipose tissue-derived non-esterified fatty acids (van Loon et al., 2001). Interestingly, these responses were not observed in the obese cohort. Whilst no statistical comparison was made between lean and obese responses to the intervention (due to a priori decisions on power), ACACA expression tended to respond in the opposite direction (i.e. downregulated with extended morning fasting versus daily breakfast consumption). It may be that the obese cohort was less metabolically flexible than the lean cohort, so were mobilising and oxidising lipids to a lesser extent than the lean cohort in the fasted state. Consistent with this, the fasting whole-body respiratory exchange ratio (RER) of the obese cohort at follow-up was higher than the lean cohort [lean versus obese difference: -0.03, p = 0.01]. Importantly, this difference in RER is not explained by
differences in dietary carbohydrate intake, since daily carbohydrate intake did not substantially differ between lean and obese individuals when expressed in absolute terms [lean: 292 (95%CI: 260 to 323) g·d⁻¹; obese: 283 (95%CI: 241 to 326) g·d⁻¹, \( P = 0.531 \)], and was therefore higher in lean versus obese individuals when expressed relative to body mass [lean: 4.4 (95%CI: 3.9 to 4.9 g·d⁻¹·kgBM⁻¹); obese: 2.9 (95%CI: 2.6 to 3.3) g·d⁻¹·kgBM⁻¹, \( p < 0.001 \)] (Betts et al., 2014; Chowdhury et al., 2016a).

Lean individuals demonstrated an upregulated post-absorptive mRNA expression of genes involved in the early phases of insulin signalling (e.g. \( IRS2 \)) with extended morning fasting, when compared to daily breakfast consumption. However, such changes were not evident in more distal components of the insulin signalling cascade (e.g. \( AKT \), \( FOXO1 \), nor \( TBC1D4 \)). Obese individuals also demonstrated an upregulation of \( IRS2 \). These findings are consistent with acute studies in humans, where adipose tissue \( IRS2 \) mRNA expression is upregulated with exercise performed in an overnight fasted state, compared to in the fed state (Chen et al., 2017) and skeletal muscle \( PI3KR1 \) mRNA expression robustly increases in response to metabolic challenges such as hyperinsulinaemia, hyperglycaemia and exercise (Tsintzas et al., 2013; Tsintzas et al., 2017). Since \( PI3KR \) is known to negatively correlate with proximal insulin signalling under specific metabolic conditions (Barbour et al., 2005; Brachmann et al., 2005), the trend for an increase in adipose tissue \( PI3KR1 \) expression with extended morning fasting in lean individuals is consistent with reduced adipose tissue glucose control. The finding that \( PI3KR1 \) did not respond to extended morning fasting compared to regular breakfast consumption in obese individuals, is likely to be explained by the presence of existing insulin
resistance in the adipose tissue of these individuals. This is also consistent with the findings from our previous work demonstrating that extended morning fasting did not alter adipose tissue glucose control in obese individuals (Chowdhury et al., 2016a).

It could be considered surprising that mitochondrial genes did not display a differential expression with breakfast consumption versus fasting, given that the breakfast group had higher physical activity levels (Betts et al., 2014). Physical activity is known to acutely increase mRNA expression of various genes relating to mitochondrial signalling, such as PGC1α (PPARGC1A) in both skeletal muscle (Stephens et al., 2010) and adipose tissue (Chen et al., 2017). However, fasting per se can increase PGC1α mRNA expression in rodent muscle (de Lange et al., 2006) - although data in humans do not fully support this (Tsintzas et al., 2006) - and low carbohydrate availability augments the physical activity induced increase in PGC1α mRNA expression in human skeletal muscle (Camera et al., 2015). Therefore, it could be that any potential changes in PGC1α with our intermittent fasting protocol was offset by difference in the physical activity patterns between FAST and BFAST groups.

The large (>700 kcal) carbohydrate-rich breakfast employed in the present study may preclude generalisation to smaller breakfasts differing in composition. However the rationale for a large carbohydrate-rich breakfast was chosen to demonstrate proof-of-principle with a first study in this area. Furthermore, as an exploratory study of the molecular responses of adipose tissue to regular morning fasting versus breakfast consumption, there was insufficient published data to perform a power
calculation on the outcome variables described in the present study. Therefore, some outcomes variables may be underpowered and future work should aim to confirm and expand upon these findings, especially with breakfasts differing in macronutrient composition.

In conclusion, 6 weeks of extended morning fasting increases the post-absorptive expression of genes involved insulin signalling and lipid turnover, compared to daily breakfast consumption in lean individuals. Most of these responses are not observed in obese individuals. However, extended morning fasting did not alter the protein content or insulin-stimulated phosphorylation of Akt, nor the protein content of GLUT4. Therefore, any potential changes in adipose tissue glucose control with alterations in morning feeding patterns are likely to be due to proteins involved in signalling and GLUT4 translocation downstream of Akt. Finally, adipose tissue from obese individuals displays lower rates of insulin-stimulated glucose uptake than that from lean individuals, which appears to be proportional to whole-body fat mass and may represent an adaptive physiological downregulation of adipose tissue glucose uptake in obesity, thereby limiting the rate of de novo lipid storage.

Additional information

Competing interests

None of the authors declare any conflicts of interest in relation to this work.
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Author Contributions

This study was conducted at the University of Bath, UK, in collaboration with the University of Nottingham, UK. JAB, GDH, KT and DT designed the research; JAB, JDR, EAC and DT conducted the research; GDH and KT provided essential reagents and materials and provided access to protein and gene expression assays, respectively; JTG, JAB, EAC, JDR and FK analysed data and performed statistical analysis; JTG and JAB wrote the paper and have primary responsibility for final content. All authors read, edited and approved of the final manuscript. All authors also agree to be accountable for all aspects of the work, ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Finally, all persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Table 1. Whole-body characteristics at baseline in lean and obese individuals who donated adipose tissue biopsies. Data are mean ± SD unless otherwise stated. 

|                          | Lean Cohort (n = 29) | Obese Cohort (n = 20) | P value |
|--------------------------|----------------------|-----------------------|---------|
| Age, years               | 36 ± 11              | 43 ± 10               | P = 0.033 |
| Female, n (%)            | 21 (64%)             | 13 (65%)              | -       |
| Frequent Habitual Breakfast Consumer¹, n (%) | 26 (79%)             | 13 (65%)              | -       |
| Height, m                | 1.71 ± 0.07          | 1.71 ± 0.10           | p = 0.566 |

¹Frequent Habitual Breakfast Consumer: At least 4 days/week.
| Measure                                      | Phase | Value        | Value        | Significance |
|----------------------------------------------|-------|--------------|--------------|--------------|
| Body Mass, kg                                | PRE   | 66.2 ± 8.3   | 98.5 ± 20.1  | P < 0.001    |
| Lean Mass (DXA<sup>2</sup>)<sup>1</sup>, kg  | PRE   | 45.9 ± 8.4   | 53.5 ± 8.9   | P < 0.003    |
| Adipose Tissue Mass (DXA)<sup>7</sup>, kg    | PRE   | 17.3 ± 5.5   | 37.8 ± 10.0  | P < 0.001    |
| Body Mass Index, kg·m<sup>2</sup>            | PRE   | 22.6 ± 2.3   | 33.6 ± 5.0   | P < 0.001    |
| Fat Mass Index<sup>7</sup> (DXA)<sup>3</sup>, kg·m<sup>2</sup> | PRE   | 6.0 ± 2.1    | 13.3 ± 4.0   | P < 0.001    |
| Percent Body Fat<sup>7</sup> (DXA)           | PRE   | 26.4 ± 7.8   | 40.0 ± 7.3   | p < 0.001    |
| Resting Metabolic Rate<sup>4</sup>, kcal·d<sup>-1</sup> | PRE   | 1435 ± 187   | 1656 ± 296   | p = 0.006    |
| Fasting RER                                   | PRE   | 0.81 ± 0.06  | 0.83 ± 0.06  | p = 0.358    |
| Fasting Glucose<sup>7,9</sup>, mmol·L<sup>-1</sup> | PRE   | 5.35 ± 0.30  | 5.43 ± 0.38  | p = 0.589    |
| Fasting Insulin<sup>7,9</sup>, pmol·L<sup>-1</sup> | PRE   | 22.8 ± 9.6   | 59.1 ± 27.5  | P < 0.001    |
| C-ISI Matsuda index<sup>4,10</sup>           | PRE   | 9.9 ± 6.7    | 3.9 ± 1.7    | P < 0.001    |
| Fasting NEFA<sup>11</sup>, mmol·L<sup>-1</sup> | PRE   | 0.60 ± 0.25  | 0.53 ± 0.24  | P = 0.421    |

<sup>1</sup> defined as the ingestion of ≥50 kcal within two hours of waking on most days of the week
<sup>2</sup> Lean tissue mass excludes bone mineral content.
<sup>3</sup> DXA-derived fat mass index obese ranges (Kelly et al., 2009) = ≥13 kg·m<sup>-2</sup> (females) and ≥9 kg·m<sup>-2</sup> (males)
<sup>4</sup> C-ISI Matsuda index = 1,000/√[(fasted glucose (mg/dL) x fasted insulin (µIU/mL)) x [mean glucose over 120-min OGTT (mg/dL) x mean insulin over 120-min OGTT (µIU/mL)].
<sup>5</sup>Data presented are comparisons of lean and obese participants from Betts et al (Betts et al., 2014) and Chowdhury et al. (Chowdhury et al., 2016a).
<sup>7</sup>n = 19 for obese.
<sup>8</sup>n = 28 for lean.
<sup>9</sup>n = 24 for lean.
<sup>10</sup>n = 23 for lean and n = 16 for obese.
<sup>11</sup>n = 17 for obese.
<sup>12</sup>Capital P represents a Mann-Whitney test, and non-capital p represents unpaired t-test.
Abbreviations: DXA = Dual-Energy X-ray Absorptiometry; NEFA = Non-esterified fatty acid.
**Table 2.** Gene expression assay targets in human subcutaneous abdominal adipose tissue

| Gene     | Protein/Enzyme                                                                 |
|----------|-------------------------------------------------------------------------------|
| 1        | 18S rRNA 18S ribosomal RNA                                                   |
| 2        | ACACA Acetyl-CoA carboxylase alpha                                            |
| 3        | ACADM Medium-chain acyl-coenzyme A dehydrogenase                             |
| 4        | ADIPOQ Adiponectin                                                           |
| 5        | AKT1 Akt; Protein kinase B                                                    |
| 6        | AKT2 Akt; Protein kinase B                                                    |
| 7        | ANGPTL4 Angiopoietin-like 4                                                  |
| 8        | CCL2 Chemokine (C-C motif) ligand 2                                           |
| 9        | FABP4 Fatty acid binding protein 4                                            |
| 10       | FASN Fatty acid synthase                                                      |
| 11       | FOXO1 Forkhead box protein 01                                                 |
| 12       | G6PD Glucose-6-phosphate dehydrogenase                                        |
| 13       | HSD11B1 11β-hydroxysteroid dehydrogenase type 1                              |
| 14       | IL18 Interleukin 18                                                           |
| 15       | IL6 Interleukin 6                                                            |
| 16       | IRS1 Insulin receptor substrate 1                                             |
| 17       | IRS2 Insulin receptor substrate 2                                             |
| 18       | LEP Leptin                                                                   |
| 19       | LIPE Hormone sensitive lipase                                                 |
| 20       | LPL Lipoprotein lipase                                                        |
| 21       | MLXIPL Carbohydrate-response element-binding protein                          |
| 22       | NAMPT Nicotinamide phosphoribosyltransferase                                 |
| 23       | NFKB1 Nuclear factor NF-kappa-B p105 subunit                                 |
| 24       | NFKB2 Nuclear factor NF-kappa-B p100 subunit                                 |
| 25       | NR1H2 Liver X Receptor-Beta                                                   |
| 26       | NR1H3 Liver X Receptor-Alpa                                                   |
| 27       | PDK4 Pyruvate Dehydrogenase Kinase                                            |
| 28       | PER2 PER2                                                                    |
| 29       | PGK1 Phosphoglycerate kinase 1                                                |
| 30       | PIK3R1 Phosphatidylinositol 3-kinase regulatory subunit alpha                 |
| 31       | PNPLA2 Adipose triglyceride lipase/palatin-like phospholipase domain-containing protein 2 |
| 32       | PPARA Peroxisome proliferator-activated receptor alpha                         |
| 33       | PPARD Peroxisome proliferator-activated receptor delta                         |
| 34       | PPARGC1A Peroxisome proliferator-activated receptor gamma coactivator 1-alpha |
|   | Gene Symbol | Description                                                   |
|---|-------------|----------------------------------------------------------------|
|35| PPARG       | Peroxisome proliferator-activated receptor gamma               |
|36| PPIA        | Peptidylprolyl isomerase A/cyclophilin A/rotamase A            |
|37| PRKAA1      | 5’-AMP-activated protein kinase catalytic subunit alpha-1       |
|38| PRKAA2      | 5’-AMP-activated protein kinase catalytic subunit alpha-2       |
|39| RETN        | Resistin                                                       |
|40| SLC2A4      | GLUT4 (Glucose transporter type 4)                              |
|41| SREBF1      | Sterol regulatory element-binding transcription factor 1; sterol regulatory element-binding protein 1 |
|42| STAT5A      | Signal transducer and activator of transcription 5A            |
|43| STAT5B      | Signal transducer and activator of transcription 5B            |
|44| TBC1D4      | AS160 (Akt substrate of 160 kDa); TBC1D4 (TBC1 domain family member 4) |
|45| TNF         | Tumor necrosis factor                                          |
|46| UCP1        | Mitochondrial Uncoupling Protein 1                             |
|47| UCP2        | Mitochondrial Uncoupling Protein 2                             |
|48| UCP3        | Mitochondrial Uncoupling Protein 3                             |