Exercise counteracts the effects of short-term overfeeding and reduced physical activity independent of energy imbalance in healthy young men

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Key points summary:

- Physical exercise significantly improves health but to what extent these benefits depend on altered energy balance remains unclear.

- In a human experimental model, we investigated whether daily exercise could counteract the effects of short-term overfeeding and under-activity independent of its impact on energy imbalance in healthy young men.

- Short-term positive energy balance from overfeeding and under-activity resulted in impaired metabolic outcomes and alterations in the expression of several key genes within adipose tissue involved in nutritional balance, metabolism and insulin action.

- These changes were mostly prevented by the addition of a daily vigorous- intensity exercise bout even in the face of a standardised energy surplus.

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ABSTRACT

Physical activity can affect many aspects of metabolism but it is unclear to what extent this relies on manipulation of energy balance. Twenty-six active men (age 25 ± 7 years) were randomly-assigned either to consume 50 % more energy than normal by over-consuming their habitual diet for 7 days whilst simultaneously restricting their physical activity below 4000 steps·day^{-1} to induce an energy surplus (SUR group; n=14) or to the same regimen but with 45 min of daily treadmill running at 70 % of maximum oxygen uptake (SUR+EX group; n=12). Critically, the SUR+EX group received additional dietary energy intake to account for the energy expended during exercise; thus maintaining a matched energy surplus. At baseline and follow-up, fasted blood samples and abdominal subcutaneous adipose tissue biopsies were obtained and oral glucose tolerance tests conducted. Insulinaemic responses to a standard glucose load increased 2-fold from baseline to follow-up in the SUR group ($\Delta 17 \pm 16$ nmol·120min·l^{-1}; *P*=0.002) whereas there was no change in the SUR+EX group ($\Delta 1 \pm 6$ nmol·120min·l^{-1}). Seven of 17 genes within adipose tissue were differentially-expressed in the SUR group; expression of SREBP1c, FAS and GLUT4 was significantly up-regulated and expression of PDK4, IRS2, HSL and VISFATIN was significantly down-regulated (P≤0.05). The pAMPK/AMPK protein ratio in adipose was significantly down-regulated in the SUR group (P=0.005). Vigorous-intensity exercise counteracted most of the effects from short-

term overfeeding and under-activity at the whole-body level and in adipose tissue, even in the face of a standardised energy surplus.

Abbreviations list

AKT1: RAC-alpha serine/threonine-protein kinase

AKT2: RAC-beta serine/threonine-protein kinase

ALT: Alanine transaminase

AMPK: 5' AMP-activated protein kinase

BMI: Body mass index

cDNA: Complementary DNA

CI: Confidence interval

CRP: C-Reactive protein

Ct: Threshold cycle

DEXA: Dual-energy X-ray absorptiometry

DIT: Diet induced thermogenesis

DNL: De novo lipogenesis

EDTA: Ethylenediaminetetraacetic acid

ELISA: Enzyme-linked immunosorbent assay

EPOC: Excess post-exercise oxygen consumption

FAS: Fatty acid synthase

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GLUT4: Glucose transporter type 4

HDL: High-density lipoprotein

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HOMA-IR: Homeostasis model assessment for insulin resistance

HOMA-β: Homeostasis model assessment for β-cell function

HSL: Hormone-sensitive lipase

iAUC: Incremental area under the curve

IL-18: Interleukin-18

IL-6: Interleukin-6

IRS1: Insulin receptor substrate 1

IRS2: Insulin receptor substrate 2

LDL: Low-density lipoprotein

LPL: Lipoprotein lipase

NEFA: Non-esterified fatty acid

OGTT: Oral glucose tolerance test

PAEE: Physical activity energy expenditure

pAKT: Phospho protein kinase B

PAL: Physical activity level

pAMPK: Phospho 5' AMP-activated protein kinase

PAR-Q: Physical activity readiness questionnaire

PCR: Polymerase chain reaction

PDK4: Pyruvate dehydrogenase kinase isozyme

PPARγ: Peroxisome proliferator-activated receptor gamma

PPIA: Peptidylpropyl isomerase A

RER: Respiratory exchange ratio

RMR: Resting metabolic rate

SD: Standard deviation

SREBP1c: Sterol regulatory element binding protein 1c

TAG: Triacylglycerol

TNFa: Tumor necrosis factor alpha

V O$_{2max}$: Maximal oxygen uptake

WBC: White blood cell

Introduction

Western lifestyles are typified by chronically low levels of physical activity and excessive caloric intake, resulting in positive energy balance (Hill & Wyatt, 2005; Uauy & Diaz, 2005) and an accumulation of triacylglycerol within adipocytes. Adipose tissue is not just a site of energy storage but also acts as an endocrine organ capable of secreting growth factors and adipokines (Rajala & Scherer, 2003), with implications for metabolic control via adipocyte insulin resistance and subsequent elevations in blood lipids (Khan *et al*., 2006). This provides one mechanism for the established links between energy imbalance, obesity and insulin resistance (Horowitz, 2007).

Intermittent and/or sustained periods of overfeeding and/or relative physical inactivity are necessarily responsible for net positive energy balance. Numerous studies have examined overfeeding of varied duration and composition, with the resultant positive energy balance associated with impaired insulin sensitivity (Wang *et al*., 2001; Cornier *et al*., 2006; Brons *et al*., 2009) and marked alterations in adipose tissue gene expression (Shea *et al*., 2009; Alligier *et al*., 2012). This in turn impacts the systemic concentration of key adipokines involved in the regulation of energy balance, such as leptin (Lammert *et al*., 2000; Wang *et al*., 2001; Brons *et al*., 2009), adiponectin (Brons *et al*., 2009) and visfatin (Sun *et al*., 2007). Equally, enforced physical inactivity results in impaired insulin sensitivity and other negative

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health outcomes in healthy, active individuals (Vukovich *et al*., 1996; Arciero *et al.,* 1998; Olsen *et al*., 2008).

In contrast to the relative wealth of information in relation to the separate influences of overfeeding or physical inactivity, it is remarkable that only two studies have explored the combined impact of both factors applied simultaneously. A recent study by Knudsen *et al*. (2012) demonstrated impaired insulin sensitivity after just 3 days of overfeeding (150 % habitual intake) combined with restricted step count (≤1500 steps per day), an effect that preceded any measurable change in body composition. Earlier, Hagobian & Braun (2006) reported similarly rapid metabolic dysregulation in a cohort of habitually-active participants provided with 125 % of their habitual intake for 3 days whilst abstaining from structured exercise. Importantly, however, insulin responses were subsequently restored to baseline levels by a single bout of moderate-intensity exercise, despite additional overfeeding on that final day to match for the energy expended during exercise. This finding presents the interesting possibility that prescribed daily exercise may offset the negative health effects of combined overfeeding plus reduced physical activity independent of any impact on energy (im)balance.

We therefore hypothesised that short-term overfeeding and reduced physical activity would significantly impair metabolic function but that the incorporation of daily physical exercise within the same experimental model would prevent these changes (independent of any net effect on energy balance). Furthermore, given the potential independent effects of energy balance and exercise on adipose tissue function given its role in energy storage (Thompson *et al*., 2012); we hypothesised that changes in the expression of key genes within adipose tissue

in response to a standardised energy surplus would be offset by daily vigorous-intensity exercise.

Methods

Participants

Twenty-six healthy, habitually active male volunteers aged 25 ± 7 years, with a maximal oxygen uptake (V̇O_{2max}) of 56.9 ± 5.6 ml$^.$kg$^{-1.}$min^{-1}, body mass index (BMI) of 23.8 ± 2.5 kg$^.$m^{-2} and physical activity level (PAL; total energy expenditure / resting energy expenditure) of 1.9 ± 0.4 were recruited from the local community. Participants had been weight stable ($\pm$ 3 kg) for at least 6 months. Only volunteers who undertook structured vigorous-intensity exercise for thirty minutes or more, at least three times a week were eligible for the study, assessed via a self-report questionnaire. Participants completed a health questionnaire and Physical Activity Readiness Questionnaire (PAR-Q) to further verify eligibility and provided written and verbal consent prior to taking part. The protocol was approved by the Bath NHS Research Ethics Committee (REC reference number: 07/H0101/234) in accordance with the *Declaration of Helsinki.* Exclusion criteria encompassed all known behaviours or conditions that either posed undue personal risk to participants or could introduce bias into the experiment.

Experimental design and protocol

A randomised parallel group design was used for this trial (registration number: ISRCTN59822195). Participants were randomly-allocated by a third party to experience either a fixed energy surplus via 7 days of overfeeding and restricted physical activity (SUR) or the same energy surplus with a daily bout of physical exercise (SUR+EX). The SUR group reduced their daily activity to ≤4000 steps while increasing their habitual energy intake by 50

%. The SUR+EX group adhered to a matched model of overfeeding and reduced physical activity except for the inclusion of a daily bout of vigorous-intensity treadmill running (5 min warm-up at 60 % V̇O_{2max} then 45 min at 70 % V̇O_{2max}), with an additional overfeed individually prescribed to account for the energy expended during each exercise bout (i.e. habitual energy intake was increased by 75 ± 3 % in this group in order to standardise energy surplus). The magnitude of the overfeeding was chosen based on previous overfeeding studies while the intensity/duration of the exercise was selected following pilot work which indicated this was a demanding but achievable target for this population. During 3 days leading up to the trial, volunteers adhered to their habitual lifestyle (i.e. diet and physical activity/exercise), whilst also recording their step count using a pedometer (Yamax, Japan) that was also worn throughout the trial period. Participants also abstained from tea/coffee and alcohol the day immediately before each trial. On Day 1 participants arrived at the laboratory at 0700 ± 0.5 h following an overnight fast (≥ 10 h). Body mass was measured to the nearest 0.1 kg using electronic scales (Tanita Corporation, Japan). Anthropometric measurements were made in triplicate using a metallic tape measure (Lufkin, US) before lean and fat mass were determined using Dual-Energy X-ray absorptiometry (DEXA; Discovery, Hologic, Bedford, UK). Resting Metabolic Rate (RMR) was determined via indirect calorimetry based on four 5 minute expired gas collections accepted as stable within 100 kcal·day^{-1} (Compher *et al*., 2006; Betts *et al*., 2011); the lowest of these was accepted as RMR. Following application of a topical local anaesthetic, a cannula was inserted into an antecubital vein and a baseline blood sample collected. A subcutaneous abdominal adipose tissue sample was then obtained before an Oral Glucose Tolerance Test (OGTT). Blood pressure was measured in triplicate at the end of each trial. This entire protocol was then repeated at follow-up (Day 8).

Preliminary measurements

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Participants recorded their food and fluid intake for a typical 7-day period prior to taking part in the study using a set of digital weighing scales (Model 3001, Salter, Kent, UK). Dietary records were analysed using the software CompEat Pro Version 5.8.0 (Nutrition Systems, UK), which is based on food composition tables for UK foods. Energy intake was estimated using this software and Diet Induced Thermogenesis (DIT) was estimated pre-intervention as 10 % of energy intake (Westerterp 2004). Combined heart-rate/accelerometry (Actiheart™) was used to determine habitual physical activity thermogenesis at baseline, whereas pedometer step counts were used to estimate activity during the intervention (the impact of profound overfeeding on heart rate responses confounds energy expenditure estimates using combined heart rate/accelerometry).

Participants' sub-maximal and maximal oxygen uptake (V O_{2max}) were assessed on a treadmill prior to the intervention (Woodway, ELG 70, Weiss, Germany) following a method adapted from Taylor *et al.* (1955). The percentage of O_2 and CO_2 in expired air samples was determined using paramagnetic and infrared gas analysers, respectively (Series 1400, Servomex Ltd., Sussex, UK). All participants performed a 30 min bout of treadmill running before 1400 h the day before they were due to start the first trial to ensure that this was standardised.

Intervention description and assumptions

The SUR group and the SUR+EX group reduced their daily activity to ≤4000 steps but volunteers in the SUR+EX group were also required to perform 45 min of treadmill running each day of the intervention at 70 % of their maximum oxygen uptake (i.e. in addition to their permitted 4000 steps). Importantly, the SUR+EX group was prescribed additional energy intake to account for the energy expended during the exercise. The Excess Post-exercise

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Oxygen Consumption (EPOC) associated with each exercise bout was estimated as 6.6 % of energy expended during the run (LaForgia *et al.* 2006). The DIT associated with this extra food prescribed to the SUR+EX group (compared to the SUR group) was calculated as 10 % of the energy expended during the run (including EPOC; Westerterp 2004). The estimated EPOC and DIT associated with the exercise bout were then added to the prescribed energy intake. Finally, the contribution of RMR towards total energy expenditure during each run (50 min) was subtracted from the overfeeding calculation as this was already taken into account when prescribing the 50 % overfeed based on a 24 h RMR. The final exercise bout prescribed under the SUR+EX treatment was performed at a standardised time of day specific to each participant. This took place at the same time as the running bout each individual had performed the day before baseline measures were taken and before 1400 h (i.e. to facilitate meaningful comparisons between baseline and follow-up). The first exercise bout in the SUR+EX group was supervised and compliance to the following six exercise bouts was 100 % as confirmed by combined heart rate/accelerometry records (Actiheart™).

Calculation of energy surplus (post-intervention)

The energy surplus induced by the overfeeding and restricted physical activity model was quantified retrospectively post-intervention based on the following assumptions. For each macronutrient, we assumed that the amount entering the system was equal to the amount ingested (i.e. assuming negligible urinary or faecal losses). The factors 16.76, 29.33, 37.29 and 15.71 $kJ^.g^{-1}$ (4, 7, 8.9 and 3.75 $kcal^.g^{-1}$) were therefore used to calculate the metabolised energy contents of protein, alcohol, fat and carbohydrate, respectively (Bender, 2006). We estimated DIT to be 21 % for protein, 15 % for alcohol, 2 % for fat and 8 % for carbohydrate based on Westerterp (2004). Based on these values, DIT was calculated to be 8.3 ± 0.6 % (post-intervention) of energy intake for the population who took part in this study. Resting

carbohydrate and fat oxidation were based on indirect calorimetry measurements collected in a fasted state at baseline and follow-up. Where Respiratory Exchange Ratio (RER) was >1, the apparently negative rates of lipid oxidation were assumed to quantitatively reflect proportional lipid synthesis (Frayn, 1983). The amount of lipid synthesised was added to the fat intake of each participant for calculations of substrate balance. It was assumed that 1 g of glucose was required to produce 0.52 g of lipid based on Ferrannini (1988). Any minor Physical Activity Energy Expenditure (PAEE) during the intervention was calculated as the product of average daily step count and an assumed stride length of 0.825 m (Auvinet *et al.*, 2002) based on an oxygen cost of 0.125 $ml^.m^{-1}.kg^{-1}$ (Dill, 1965). Exclusive reliance on carbohydrates as a fuel source during physical activity was assumed as participants mean RER during overfeeding was 0.98 even under fasted and resting conditions at follow-up. The overall energy surplus was estimated by calculating the sum of the surpluses associated with each macronutrient.

Blood Sampling & Analysis

Following the application of a topical local anaesthetic (1.5 ml Ametop gel, Smith & Nephew, Hull, England) an 18-gauge 1.3 x 45 mm cannula (BD Venflon Pro) was inserted into an antecubital vein. For the OGTT, participants ingested 113 ml (75 g) of glucose (maltodextrin) solution (Polycal, Nutricia, UK) within 5 min. Once ingested, blood samples were collected every 15 min for 2 hours. Blood was dispensed into collection tubes (Sarstedt Ltd., Leicester, UK) containing ethylenediaminetetraacetic acid (EDTA) as the anticoagulant for plasma samples or serum-separator tubes for serum samples. Plasma tubes were centrifuged immediately whereas serum tubes were left to clot for 15 minutes at room temperature before being centrifuged (3500 g for 10 min at 4^oC). All blood analyses were performed in duplicate. Immunoassays for serum triacylglycerol (TAG), total and high-density lipoprotein (HDL)

cholesterol, alanine transaminase (ALT), plasma glucose (Randox, Crumlin, Co. Antrim, UK) and non-esterified fatty acids (NEFA; Wakochemicals GmbH, Germany) were performed using a Cobas Mira (Cobas, Roche Diagnostics Limited, UK). Low-density lipoprotein (LDL) cholesterol was calculated using the Friedewald equation (Friedewald *et al*., 1972). Homeostasis model assessment for insulin resistance (HOMA-IR) was calculated as fasting glucose (mmol$\cdot$l^{-1}) x fasting insulin (mU$\cdot$l^{-1}) / 22.5 (Turner *et al*., 1979). Homeostasis model assessment for β-cell function (HOMA-β) was calculated as fasting insulin (mU$\cdot$l^{-1}) x 20 / fasting glucose (mmol$\cdot$l^{-1}) - 3.5 (Matthews *et al*., 1985). The Insulin Sensitivity Index (ISI comp/Matsuda Index) was calculated as 10000 / square root (fasting glucose (mg$\cdot$dl^{-1}) x fasting insulin (μU$\cdot$ml^{-1}) x mean OGTT glucose value x mean OGTT insulin value (Matsuda & DeFronzo, 1999). Commercially available enzyme-linked immunosorbent assays (ELISA) were used to measure serum adiponectin, leptin, C-reactive protein (CRP) (Quantikine, R&D Systems Inc., Abingdon, UK), interleukin 6 (IL-6; Quantikine HS, R&D Systems Inc., Abingdon, UK),insulin and C-peptide (Mercodia AB, Uppsala, Sweden). Absorption was determined using a microplate reader (HTIII, Anthos Labtec Instruments Ltd., Ringer, East Sussex) at the wavelengths specified by the kit manufacturer.

Adipose Tissue Biopsy

Adipose tissue biopsies were performed under local anesthesia (1 % lidocaine) after insertion of the cannula and before the OGTT. Subcutaneous abdominal adipose tissue was biopsied 4-7cm lateral of the umbilicus with a 14-gauge needle using the needle aspiration technique, with follow-up biopsies sampled from the opposite side.

Adipose Tissue Sample Processing

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The sample was cleaned with isotonic saline and any clot was manually removed. After weighing the sample, it was homogenised in 5 ml of Trizol (Invitrogen, UK) and placed on dry ice before being stored at -80°C. Subsequently, samples were defrosted and spun at 2500 g for 5 min at 4°C. The top layer and pellet were removed and 200 µl of chloroform was added per 1ml of Trizol. After shaking the mixture vigorously for 15 s samples were incubated at room temperature for 3 min and then centrifuged at 2500 g for 5 min at 4°C. The aqueous phase was removed and used for gene expression while the organic phase was used for western blot analysis.

Quantitative real time-PCR

The aqueous phase was mixed with an equal volume of 70 % Ethanol before being loaded on an RNeasy mini column for extraction (Qiagen, Crawley, UK). Each sample was quantified using spectrophotometry, with 2 µg of total RNA reverse transcribed using a high capacity cDNA Reverse Transcription kit (Applied Biosystems, Warrington, UK). Assays from Applied Biosystems were used: ADIPONECTIN (Hs00605917_m1), LEPTIN (Hs00174877_m1), SREBP-1c (Hs01088691_m1), PDK4(Hs00176875_m1), FAS (Hs00188012_m1), PPARγ (Hs01115513_m1), TNFa (Hs99999043_m1), GLUT4 (Hs00168966_m1), IRS2 (Hs00275843_s1), LPL (Hs01012567_m1), HSL (Hs00193510_m1), VISFATIN (Hs00237184_m1), IRS1 (Hs00178563_m1), IL18 (Hs00155517_m1), IL6 (Hs00985639_m1), AMPK (Hs01562315_m1 & Hs00178903_m1 combined), APELIN (Hs00175572_m1). Real-time PCR was performed using a StepOne^{TM} (Applied Biosystems, Warrington, UK). PPIA (Peptidylpropyl isomerase A) was used as an endogenous control. The comparative Ct method was used to process the data where ΔCt = Ct Target gene − Ct Endogenous control. Data was normalised to an internal calibrator and baseline.

Western Blot Analysis

The protein fraction was isolated from the adipose tissue biopsy sample and 1 ml of organic phase was mixed with 1.5 ml of isopropanol. The samples were incubated for 10 min at room temperature and centrifuged at 2500 g for 10 min at 4^oC. The pellets were washed with 0.3M Guanadine HCl in 95 % Ethanol, incubated with rotation for 20 min at room temperature and pelleted. This wash procedure was repeated 3 times. After the final wash, pellets were mixed with 2 ml of 100 % Ethanol and left for 20 min at room temperature before being centrifuged. The pellets were left to air dry and resuspended in 400 µl 1 % SDS before being briefly sonicated (18 µm) on ice. The protein content of the samples was determined by BCA protein assay (Thermo Scientific). Protein (20 µg) was separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked using 5 % (w/v) Marvel (dried skimmed milk powder) in TBS-T (0.1 % Tween 20 in Tris-Buffered Saline) for 60 min before the primary antibody was incubated overnight at 4^oC. The antibody was diluted in 1 % BSA in TBS-T for Akt1 (Millipore), Akt2 (Cell Signaling Technology), GLUT4 (Holman *et al*., 1990), AMPK (Cell Signaling Technology), GAPDH (Millipore) or 5 % BSA TBS-T for pAKT (Cell Signaling Technology), pAMPK (Cell Signaling Technology). The nitrocellulose was washed and incubated with the secondary antibody for 1 h at room temperature. The secondary antibodies (Goat anti-rabbit IgG HRP conjugate (Millipore, USA) or Goat anti-mouse IgG HRP conjugate (Thermo scientific, USA)) were diluted 1:4000 in 5 % Marvel-TBS-T. The nitrocellulose was washed and developed using ECL or ECL Advance following the manufacturer's guidelines. The image was developed in an EPI Chemi II darkroom and bands quantified using Labworks analysis software (UVP). A subset of samples representative of the overall group insulin response to the OGTT were analysed (SUR group, n=7; SUR+EX, n=8).

Statistical analysis

To simplify data analysis and facilitate a more meaningful interpretation of an otherwise complex factorial research design (Hopkins *et al.,* 2009 & Matthews *et al*., 1990), serial measurements of glucose, insulin and C-peptide at baseline and follow-up were converted into simple summary statistics to illustrate the net response of each parameter (i.e. within-subject peak concentrations, time to peak and incremental area under curve; Wolever & Jenkins, 1986). Pre-planned contrasts (#) were conducted in relation to the absolute group differences both at final follow-up and the relative change from baseline. The precise time-course of responses within and between trials were analysed using factorial 2- and 3-way mixed-model analysis of variance (group✕day & group✕day✕time, respectively) irrespective of minor deviations from a normal distribution (Maxwell & Delaney, 1990, p. 109) but with the Greenhouse-Geisser correction applied to intra-individual contrasts for <0.75 and the Huynh-Feldt correction adopted for less severe asphericity (Atkinson, 2002). Where significant interactions were observed, multiple *t*-tests were applied to determine the location of variance both between treatments at level time-points and between time-points within each treatment relative to baseline, with both methods subject to a Holm-Bonferroni correction (Atkinson, 2002). For all the above statistical approaches, statistical significance was set at an alpha level of $P \leq 0.05$. Data are presented in text as means and standard deviations (SDs), whereas the variance bars on figures are confidence intervals (CIs) that have been corrected to remove inter-individual variation (Masson, 2003). For reference, the magnitude of these CIs illustrate the change at each time point relative to baseline such that, in general, plotted means whose CIs do not overlap by more than one-half of one side of an interval are likely to be deemed statistically different according to conventional significance testing (Masson, 2003). A main effect of day denotes an effect of energy surplus *per se* (†: Day 1 *vs*. Day 8 both groups), whereas a day✕group interaction means there is a mediating effect of exercise (*). Statistical analysis for the gene expression data was carried out on the logged

transformed data following normalisation to an internal calibrator and baseline (Livak &
Schittgen, 2001), whereas integrated optical density was used for the Western Blot data. The
Pearson rank correlation was employed to determine the strength of relationships between
parameters.

Results

Induced energy surplus

The estimated energy surplus induced by the overfeeding and restricted physical activity
model was successfully standardised between groups (Figure 1). At baseline (prior to the
intervention), energy intake was 13485 ± 2367 kJ·day^{-1} and energy expenditure was $14792 \pm$
2849 kJ·day^{-1}. Mean pedometer steps significantly decreased during the intervention within
the SUR and SUR+EX groups from 12562 ± 3520 to 3672 ± 860 steps·day^{-1} and from 10544
± 2756 to 3690 ± 400 steps·day^{-1}, respectively (day effect; $P<0.001$). There was a significant
increase in RMR at follow-up in the SUR group from 7777 ± 1282 to 8619 ± 1161 kJ·day^{-1}
but no change in the SUR+EX group (8087 ± 1127 to 8275 ± 1131 kJ·day^{-1}, day✗group
interaction; $P=0.009$). At follow-up, RER had significantly increased in both the SUR ($0.83 \pm$
0.05 to 0.98 ± 0.07) and SUR+EX groups (0.80 ± 0.06 to 0.90 ± 0.07; $P<0.001$).

[INSERT FIGURE 1 ABOUT HERE]

Anthropometric and physiological measures

Anthropometric and physiological measures pre- and post-intervention are summarised in Table 1. Body mass, waist and hip circumference and lean mass were significantly increased at follow-up in both groups ($P \leq 0.05$) but to a different extent (day×group interaction; $P \leq 0.05$). Fat mass, abdominal fat and systolic blood pressure significantly increased in both groups ($P \leq 0.05$).

[INSERT TABLE 1 ABOUT HERE]

Insulin, C-peptide & Glucose responses to OGTT

The OGTT elicited a significantly greater insulinaemic response at follow-up for the SUR group, with a 2-fold increase in 2 h insulin iAUC. In contrast, the 2 h insulin iAUC of the SUR+EX group was unaffected (Figure 2; day×group interaction P=0.002). The C-peptide response to the OGTT was significantly greater at follow-up for the SUR group (n=8), while it remained unchanged for the SUR+EX group (n=8; day×group interaction P=0.002; see Supplemental data Figure 2a). The glycaemic response following the OGTT was not systematically affected in either group.

[INSERT FIGURE 2 ABOUT HERE]

The Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) increased at follow-up in both groups. The Homeostasis Model Assessment of β-cell function (HOMA-β) increased at follow-up in the SUR group (day×group interaction; P=0.04). The Matsuda Index or Composite Index (ISI comp) decreased at follow-up in the SUR group (day×group interaction; P=0.04; Table 2).

Fasted Blood measurements

Fasting serum levels of adiponectin, total cholesterol, LDL cholesterol and whole blood white blood cell count (WBC) significantly increased at follow up and more so in the SUR group (day✗group interaction $P \leq 0.05$; Table 2).

[INSERT TABLE 2 ABOUT HERE]

Adipose tissue Gene Expression

Expression of SREBP1c, FAS and GLUT4 was significantly up-regulated in the SUR group; there was a day✗group interaction ($P \leq 0.05$). Expression of IRS2 and VISFATIN was significantly down-regulated in the SUR group (day✗group interaction; $P < 0.05$). Expression of PDK4 and HSL was significantly down-regulated at follow-up in both groups ($P \leq 0.05$) but to a different extent (day✗group interaction; $P \leq 0.05$). There was a main effect of day in the expression changes of ADIPONECTIN, LEPTIN, PPARγ, IL-6, AMPK and Apelin ($P \leq 0.05$). Individuals with one or both samples outside the detectable limit (Ct>35) were excluded from the analysis. Four genes were expressed at very low levels; TNFα was undetectable in 9 % of samples; IL-18 was undetectable in 32 % of samples; IL-6 was undetectable in 39 % of samples and Apelin was undetectable in 50 % of samples. Gene expression data are presented in Figure 3.

[INSERT FIGURE 3 ABOUT HERE]

Western Blots Analysis

The ratio between pAMPK and AMPK was significantly down-regulated in the SUR group (dayxgroup interaction; P=0.005). GLUT4 and pAKT/AKT 1& 2 ratios were not impacted by the overfeeding and restricted physical activity model (Figure 4).

[INSERT FIGURE 4 ABOUT HERE]

Discussion

The combination of short-term overfeeding and reduced physical activity had a dramatic impact on insulin sensitivity and adipose tissue gene expression. We demonstrated that 7 days of overfeeding and restricted physical activity induced a reduction in insulin sensitivity in healthy individuals, with significantly altered expression patterns of several key genes and proteins within adipose tissue that are involved in nutritional homeostasis, metabolism and insulin action. Notably, the inclusion of a daily vigorous-intensity exercise bout attenuated or even prevented these changes independent of any net effect on energy imbalance.

Combining reduced physical activity with overfeeding represents an experimental model to investigate the benefits of exercise independent of energy status. We successfully maintained a comparable energy surplus between the SUR and SUR+EX groups. Importantly, whilst the SUR+EX group expended ~700 kcal through daily exercise (treadmill running), this was accounted for in the prescribed overfeeding. The SUR group experienced an increase in fasted insulin concentrations and a ~2-fold increase in the insulinaemic response to the OGTT whereas there was no such change in SUR+EX group. The greater insulin response to

the OGTT reflects greater insulin secretion as indicated by the C-peptide response to the OGTT, which provides a more accurate assessment of β-cell function than peripheral insulin levels as it overcomes the issue of hepatic insulin clearance (Cobelli *et al.*, 2007). Brief periods of positive energy balance have been shown to lead to impaired insulin sensitivity whether this is achieved through decreased physical activity (Vukovich *et al.*, 1996; Arciero *et al*., 1998; Hamburg *et al*., 2007; Sonne *et al*., 2010), overfeeding (Wang *et al*., 2001; Cornier *et al*., 2006; Brons *et al*., 2009) or both (Knudsen *et al*., 2012; Hagobian and Braun, 2006). Hagobian and Braun (2006) showed that single 60-minute bout of exercise performed *after* 3 days of overfeeding and detraining partially *restored* the insulinaemic response to feeding. To our knowledge, the present study is the first to demonstrate that daily vigorous-intensity exercise has the ability to preserve or restore metabolic function during an acute bout of energy surplus.

Few studies have examined the impact of overfeeding and/or reduced physical activity on adipose tissue and this is surprising given its role in energy storage and metabolic control. AMPK plays a role in cellular energy homeostasis and increases glucose transport and fatty acid oxidation while inhibiting other pathways such as lipogenesis and gluconeogenesis. In the current study, AMPK mRNA in adipose tissue was down-regulated in both groups but more so in the SUR group. This was confirmed at the protein level and the ratio between pAMPK and AMPK was significantly down-regulated in the SUR group. This would be likely to reduce glucose uptake plus decrease fatty acid oxidation; both fasting and exercise have been shown to activate AMPK in adipose tissue (Daval *et al*., 2006). Recently, AMPK has become a potential therapeutic target for the treatment of obesity and Type 2 Diabetes (Pedersen, 2007) and thus it is particularly noteworthy that exercise prevented the changes to AMPK in adipose tissue even in the context of a profound energy surplus.

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Expression of PDK4 in adipose tissue decreased in both groups but more so in the SUR group, highlighting a switch of oxidative fuel from fatty acids to glucose. PDK4 is a mitochondrial protein and a member of the pyruvate dehydrogenase kinase family, a group of enzymes that inhibit the pyruvate dehydrogenase complex by phosphorylating one of its subunits (Rowles *et al.,* 1996). Expression of this gene is regulated by glucocorticoids, retinoic acid and insulin (Kwon & Harris, 2004). Our data support the role of insulin since there was an inverse correlation between changes in insulin iAUC and changes in the expression of PDK4 (r=-0.62; *P*=0.002). There was also an inverse correlation between changes in insulin iAUC and changes in the expression of IRS2 (r=-0.56; *P*=0.007). IRS2 mRNA was down-regulated in the SUR group in response to the intervention but was not affected in the SUR+EX group. IRS2 is a cytoplasmic protein that mediates the effects of insulin and various cytokines (Sun *et al*., 1995). The changes in PDK4 and IRS2 reported here are in agreement with transcriptome analysis conducted on abdominal subcutaneous fat samples from lean and obese men following short-term overfeeding (Shea *et al*., 2009). We suggest that these changes at the mRNA level within adipose tissue may be a secondary response to the marked hyperinsulinaemia induced by overfeeding and restricted physical activity.

A change in insulin sensitivity using the ISI index (Matsuda & DeFronzo, 1999) could reflect a decrease in insulin sensitivity at the tissue level or a relative increase in hyperinsulinaemia, or a combination of the two. The decrease in IRS2 and AMPK at gene expression level and decrease in pAMPK/AMPK ratio point towards a decrease in adipose tissue insulin sensitivity in the SUR group – but more direct measures are needed to confirm that the change in ISI score reflects a change within adipose tissue.

SREBP-1c is a transcription factor that regulates expression of the lipogenic enzyme FAS (Minehira *et al*., 2003). Overfeeding and reduced activity significantly increased the expression of both SREBP-1c and FAS transcripts in the SUR group and changes in SREBP-1c and FAS were correlated (r=0.63; *P*=0.002). Minehira *et al.* (2003) made a similar finding in humans using a carbohydrate overfeeding protocol. Our participants consumed a diet rich in carbohydrates during the intervention (~700g·day^{-1}) and so it is likely that this would have rapidly saturated liver and muscle glycogen stores. The SUR group had little capacity to further increase carbohydrate oxidation as a result of restricted physical activity thermogenesis and thus it is unsurprising that SREBP-1c and FAS are up-regulated in the SUR group as SREBP-1c has been linked with de novo lipogenesis (DNL) which has been shown to take place in adipose tissue (Strawford *et al*., 2004; Roberts *et al*., 2009; Collins *et al*., 2011), thus providing a route for disposal of excess glucose. Our calculations are consistent with the possibility that the increase in RMR in the SUR group may be explained by DNL, as removing the estimated energy cost of this process (Acheson *et al*., 1988) results in a similar RMR at follow-up in each group (8112 ± 1173 kJ·day^{-1} and 8166 ± 1135 kJ·day^{-1} in SUR and SUR+EX groups, respectively). Collectively, our results indicate that combined overfeeding and reduced physical activity favours net lipogenesis (as indicated by changes in the following transcripts: ↑SREBP-1c, ↑FAS, ↓HSL) but not when the same energy surplus occurs alongside daily vigorous intensity exercise.

Our post-intervention calculations show that carbohydrate and fat contributed approximately 20 % and 50 % towards the overall energy surplus, respectively. There was a non-significant tendency for positive carbohydrate balance (i.e. intake – oxidation – conversion to lipid) to be greater in the SUR than SUR+EX group (1092 ± 797 g·week^{-1} and 708 ± 809 g·week^{-1} in SUR and SUR+EX groups, respectively; *P*=0.24). However, the current experiment was designed

to induce a matched energy surplus with or without exercise and not to account for the route of disposal for each macronutrient. Inevitably, the SUR+EX oxidised more carbohydrate as a result of daily vigorous-intensity exercise where carbohydrate is the primary substrate but, because the overfeeding was based on their usual diet, the surplus for individual macronutrients was not matched. It is possible that the tendency for approximately 50 g·d^{-1} greater carbohydrate balance between groups could explain differences in insulin sensitivity at follow-up. However, exercise is known to enhance insulin action even when expended energy and carbohydrate are replaced (Stephens *et al*., 2007). Based on our observations, we cannot know the fate of the surplus carbohydrate. Some possibilities for disposal of surplus carbohydrate not stored as glycogen include: excretion (although this is likely to be minor; Acheson *et al*., 1988), adaptive thermogenesis (e.g., fidgeting), and/or an under-estimation of DNL. Recent work by Hodson *et al.* (2013) indicates that DNL takes place after meals within the adipose tissue of healthy humans whereas we have based our calculations on only fasting measures, so it is likely that our calculations of DNL will have been under-estimated the amount of excess macronutrients converted into fat. Future studies replacing individual macronutrients and accounting for their fate would help understand the importance of each macronutrient in regulating insulin sensitivity and adipose tissue metabolism. Future studies might also explore whether exercise of a different intensity and duration (with different metabolic demands) elicits a similar response.

Profound short-term macronutrient imbalances also have the capacity to confound prediction of energy imbalances based on observed changes in mass. In the present study, the SUR group gained 2.7 kg and the SUR+EX group gained 1.6 kg. This difference may be related to differences in glycogen storage. Glycogen is stored alongside water but the ratio is unclear and is likely to vary under different conditions (Olsson & Saltin,1970; Sherman *et al*., 1982;

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Piehl Aulin *et al*., 2000). Interestingly, a study by Horton *et al.* (1995) showed that 7 days of CHO overfeeding led to a ~30 % greater weight gain than matched overfeeding with fat, which is very similar to the greater weight gain observed in the SUR group in the present study. Thus, in the short-term, a given energy or nutrient imbalance will not necessarily translate into a proportional change in mass.

In summary, our study shows that short-term overfeeding combined with reduced physical activity induced a reduction in insulin sensitivity, hyperinsulinaemia and altered expression of several key genes within adipose tissue. The addition of daily vigorous-intensity exercise mostly prevented these changes independent of any net effect on energy imbalance. Whether this is facilitated by regular glycogen turnover or some other consequence of muscle contraction *per se* remains to be explored. These results demonstrate that exercise has a profound effect on physiological function even in the face of a considerable energy surplus.

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Author contributions

Jean-Philippe Walhin was responsible for study design and conduct, data collection, data interpretation, statistical analysis, and manuscript revision; James Betts was responsible for study design, data interpretation, and manuscript revision; Judith Richardson was responsible for western blotting data collection and interpretation; Dylan Thompson was responsible for funding, study design, data interpretation, and manuscript revision.

Table 1. Anthropometric and physiological characteristics measured before and after 7 days of overfeeding and reduced physical activity with (SUR+EX; n=12) or without (SUR; n=14) an exercise prescription. (n.b. for DEXA measurements n=11 & 10, respectively). Mean ± SD. Change scores shown with 95 % confidence intervals. * $P \leq 0.05$ day×group interaction. # $P \leq 0.05$ baseline *versus* follow-up. † main effect of day (i.e.: Day 1 *vs*. Day 8 both groups; $P \leq 0.05$).

|	SUR			SUR+EX		
	Baseline	**Follow-up**	**Δ (95 % CI)**	**Baseline**	**Follow-up**	**Δ (95 % CI)**
Body Mass (kg)	75.5 ± 8.9	78.2 ± 9.2 #	2.7 (2.0 to 3.3)	78.2 ± 11.0	79.9 ± 11.3 #	1.6 (1.0 to 2.3) *
Waist Circumference (cm)	81.7 ± 5.2	84.1 ± 5.0 #	2.4 (1.6 to 3.2)	83.7 ± 7.1	84.8 ± 6.9 #	1.1 (0.6 to 1.6) *
Hip Circumference (cm)	97.0 ± 6.0	98.7 ± 6.0 #	1.7 (1.2 to 2.3)	98.6 ± 5.5	99.1 ± 5.4 #	0.5 (0.0 to 0.9) *
Fat Mass (kg; DEXA)	10.5 ± 3.6	10.7 ± 3.4	0.1 (-0.5 to 0.7)	12.4 ± 4.1	13.0 ± 4.5	0.6 (0.1 to 1.1) †
Lean Mass (kg; DEXA)	58.2 ± 5.8	60.8 ± 6.4 #	2.6 (1.9 to 3.4)	63.4 ± 7.2	64.5 ± 7.0 #	1.0 (0.1 to 2.0) *
Abdominal Fat (g; DEXA)	900 ± 416	932 ± 360	32 (-56 to 119)	1206 ± 477	1261 ± 479	55 (25 to 85) †
Systolic BP (mmHg)	122 ± 10	128 ± 8	6 (1 to 11)	126 ± 9	128 ± 10	2 (-2 to 6) †
Diastolic BP (mmHg)	69 ± 9	71 ± 4	2 (-2 to 6)	70 ± 7	70 ± 7	0 (-4 to 4)

Table 2. HOMA-IR, HOMA-β, Composite Index values and fasted blood measurements measured before and after 7 days of overfeeding and reduced physical activity with (SUR+EX; n=12) or without (SUR; n=14) an exercise prescription. Mean ± SD. Change scores shown with 95 % confidence intervals. * $P \leq 0.05$ dayxgroup interaction. # $P \leq 0.05$ baseline *versus* follow-up. † main effect of day (i.e.: Day 1 *vs*. Day 8 both groups; $P \leq 0.05$).

	SUR			SUR+EX		
	Baseline	Follow-up	Δ (95 % CI)	Baseline	Follow-up	Δ (95 % CI)
HOMA-IR	0.79 ± 0.67	1.54 ± 0.85	0.75 (0.30 to 1.20)	0.95 ± 0.58	1.20 ± 0.61	0.25 (-0.08 to 0.59) †
HOMA-β (%)	63± 53	119± 73 #	57 (23 to 91)	60± 33	74± 33	14 (-9to 37) *
Insulin Sensitivity Index (comp)	11.9 ± 4.2	6.2 ± 2.5 #	5.6 (3.4 to 7.8)	10.8 ± 5.9	8.9 ± 4.2	1.8 (-1.4 to 5.0) *
Adiponectin (µg·ml^{-1})	7.4 ± 2.7	9.6 ± 3.0 #	2.3 (1.6 to 3.0)	6.7 ± 4.3	7.9 ± 4.3 #	1.2 (0.2 to 2.2) *
Leptin (pg·ml^{-1})	1636 ± 1218	2706 ± 1827	1070 (568 to 1572)	1650 ± 1084	2212 ± 1233	562 (-2 to 11250) †
Total Cholesterol (mmol·l^{-1})	4.1 ± 0.8	4.6 ± 1.0 #	0.6 (0.2 to 0.9)	4.4 ± 0.9	4.5 ± 0.8	0.1 (-0.1 to 0.3) *
HDL Cholesterol (mmol·l^{-1})	1.3 ± 0.3	1.5 ± 0.3	0.1 (0.1 to 0.2)	1.4 ± 0.3	1.4 ± 0.3	0.1 (-0.0 to 0.2) †
LDL Cholesterol (mmol·l^{-1})	2.5 ± 0.8	2.8 ± 0.8	0.2 (-0.0 to 0.5)	2.9 ± 0.7	2.8 ± 0.6	0.1 (-0.2 to 0.1) *
NEFA (mmol·l^{-1})	0.5 ± 0.2	0.2 ± 0.1	0.3 (0.1 to 0.4)	0.5 ± 0.2	0.3 ± 0.1	0.2 (0.0 to 0.4) †
TAG (mmol·l^{-1})	0.9 ± 0.4	1.9 ± 1.4	1.0 (0.2 to 1.7)	0.9 ± 0.3	1.2 ± 0.6	0.3 (0.0 to 0.7) †
ALT (U·l^{-1})	22 ± 10	40 ± 24	17 (9 to 31)	25 ± 12	31 ± 17	6 (-6 to 18) †

|						
CRP (mg$\cdot$l^{-1})	0.8 ± 0.9	0.6 ± 0.5	0.1 (-0.6 to 0.3)	0.7 ± 0.9	0.8 ± 0.8	0.1 (-0.1 to 0.3)
IL-6 (pg$\cdot$ml^{-1})	1.3 ± 1.3	1.5 ± 1.4	0.2 (0.0 to 0.4)	0.5 ± 0.4	0.6 ± 0.3	0.1 (-0.2 to 0.4)
Whole blood WBC (x 10^{9}l^{-1})	5.3 ± 1.2	6.7 ± 1.4 #	1.5 (0.5 to 2.5)	5.2 ± 1.3	5.4 ± 0.8	0.1 (-0.6 to 0.8) *

Figure 1. Schematic representation of the achieved energy surplus induced by the overfeeding and restricted physical activity model in SUR group (n=13) and SUR+EX group (n=12). Energy intake estimated from diet analysis. RMR estimated from indirect calorimetry. DIT was calculated post-intervention as 8.3 % of energy intake, this was done by calculating the specific DIT associated with each macronutrient before the overall DIT was determined. PAEE was based on step count and indirect calorimetry (exercise). Energy surplus was calculated as the difference between energy intake and energy expenditure. Values are means ± CI.
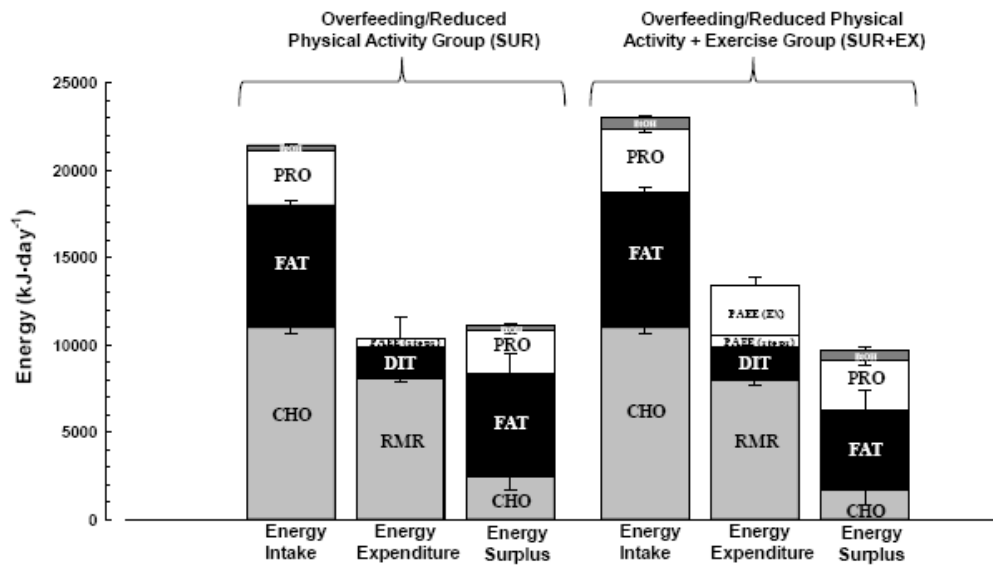
Figure 2. Serum insulin 2h iAUC (A), plasma glucose 2h iAUC (B; means ± CI) in response to the OGTT before and after a week of overfeeding and reduced physical activity (Panel A; * denotes a day×group interaction (*P*=0.002) # denotes values different pre-post within SUR group (*P*=0.001).
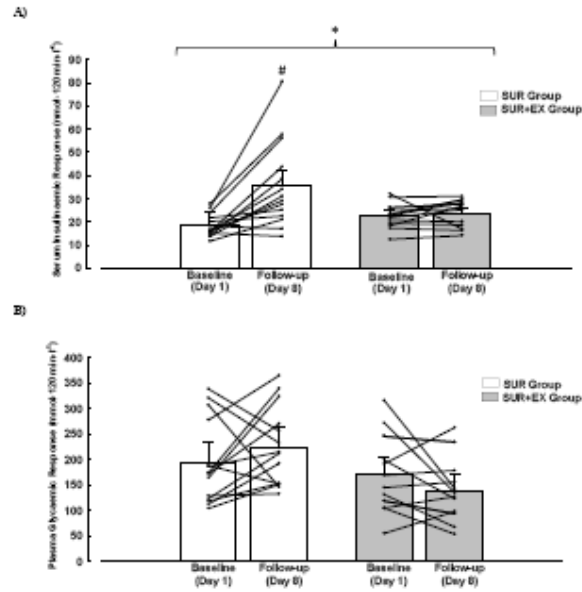
Figure 3. Relative gene expression of several key genes measured in adipose tissue at baseline and follow-up for the SUR group (n=10) and the SUR+EX group (n=12). Dashed line represents no change. Data normalised to PPIA, baseline and internal calibrator. Enough RNA could not be obtained from some smaller biopsies to carry out gene expression analysis on those samples, as a result n=10 in the SUR group and n=12 in the SUR+EX group. Any change score that was over 3 SD from the mean (n=14) were excluded from the presentation shown in this figure but were included in the statistical analysis which used the logged values. Samples outside the detectable limit (Ct>35; n=46) were excluded from the analysis. Leptin (SUR+EX, n=10), SREBP1c (SUR, n=9), PDK4 (SUR, n=8), FAS (SUR, n=9), TNFa (SUR, n=9; SUR+EX, n=9), IRS2 (SUR+EX, n=11), HSL (SUR+EX, n=11), VISFATIN (SUR+EX, n=11), IL18 (SUR, n=6; SUR+EX, n=7), IL6 (SUR, n=3; SUR+EX, n=6), AMPK (SUR+EX, n=11), APELIN (SUR, n=4, SUR+EX, n=6). Values are means ± SEM. * *P*≤0.05 day**x**group interaction. # *P*≤0.05 baseline *versus* follow-up. † main effect of day (i.e.: Day 1 *vs*. Day 8 both groups; *P*≤0.05).
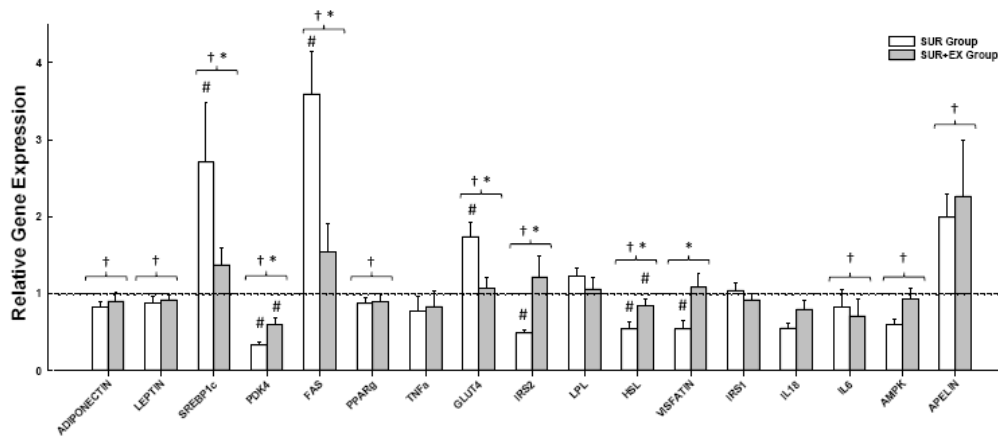
Figure 4. Mean fold change in the expression of several key proteins measured in adipose tissue at baseline and follow-up for the SUR group (n=7) and the SUR+EX group (n=8). Data normalised to GAPDH. Values are means ± CI. * *P*≤0.05 day×group interaction. # *P*≤0.05 baseline *versus* follow-up. † main effect of day (i.e.: Day 1 *vs*. Day 8 both groups; *P*≤0.05). Representative blots for each protein are shown in Supplemental data Figure 2b.