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The effects of underfeeding on whole-body carbohydrate partitioning, thermogenesis and uncoupling protein 3 expression in human skeletal muscle

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Aim: Underfeeding is known to reduce resting energy expenditure (REE) as an energy-conserving mechanism and may also reduce insulin sensitivity. Uncoupling protein 1 is known to have a significant role in energy expenditure (EE) in small mammals, but the role of UCPs in humans is unclear. UCP3 is primarily expressed in human skeletal muscle, a significant site of whole-body EE in lean individuals and therefore has a potential role in human metabolism. Here, we examine the effects of short-term underfeeding on UCP3 skeletal muscle expression, and on whole-body insulin sensitivity, substrate utilization and thermogenesis.

Methods: Eleven non-obese men [age 22.8 ± 1.34 years, body mass index 23.4 ± 0.71 kg/m^2, mean ± s.e.m.] were fed for two periods of 6 days, an underfeeding diet (UF) (50% predicted requirements for weight maintenance) and an eucaloric diet (EU), with the same macronutrient composition, in random order. Subjects visited the laboratory on four separate occasions, before and after each dietary period. REE, metabolites and muscle biopsies (vastus lateralis) were taken and the thermogenic response to a hyperinsulinaemic euglycaemic clamp was measured over a 2-h period. UCP3 mRNA levels were measured using Taqman.

Results: After underfeeding for 6 days, REE fell by 0.43 ± 0.17 kJ/min ($p = 0.032$), with weight loss of 2.05 ± 0.34 kg ($p < 0.001$). Baseline fasting glucose was significantly lower at 4.26 ± 0.07 mmol/l ($p = 0.005$), with a corresponding fall in carbohydrate oxidation (0.08 ± 0.03 g/min; $p = 0.04$). Fasting free fatty acids (FFA) increased by 0.13 ± 0.03 mmol/l ($p < 0.001$), with an increase in β-hydroxybutyrate concentrations of 0.41 ± 0.07 mM ($p < 0.001$) compared with post-EU. There was no significant change in UCP3 mRNA levels pre- and post-UF [10.4 ± 6.8 arbitrary units (au); $p = 0.16$] compared with pre- and post-EU (3.2 ± 7.3 au; $p = 0.67$).

There was no thermogenic response to the clamp after 6 days of underfeeding and a significant reduction in glucose disposal rates (from 46.35 ± 2.15 to 39.46 ± 1.12 μmol/min/kg; $p = 0.003$). Carbohydrate oxidation rates were lower by 0.08 ± 0.03 g/min ($p = 0.011$) compared with pre-UF, with no change in glucose storage rates (28.2 ± 2.4 μmol/min/kg pre-UF; 27.0 ± 2.3 μmol/min/kg post-UF; $p = 0.7$). EU resulted in a mildly underfed state with marginal weight loss (0.55 ± 0.28 kg; $p = 0.08$), and fasting FFA increased by 0.13 ± 0.03 mmol/l ($p < 0.001$) and β-hydroxybutyrate concentrations by 0.05 ± 0.02 mM ($p = 0.03$) compared with pre-EU. There was no change in glucose disposal or storage rates compared with pre-EU.

Conclusions: Underfeeding for 6 days has no significant effect on UCP3 mRNA expression in skeletal muscle in non-obese men but is associated with changes in carbohydrate fuel partitioning, REE and the thermogenic response to the glucose clamp. Mild underfeeding had no effect on insulin sensitivity, but more severe energy restriction reduced insulin-stimulated glucose oxidation without affecting glucose storage.

Keywords: energy restriction, euglycaemic clamp, hyperinsulinaemic, thermogenesis, uncoupling protein

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Introduction

The human body is capable of adapting its energy expenditure (EE) to changes in dietary intake in order to reduce fluctuations in body weight, but there is a great individual variability in these responses. This variability may in part be genetic, resulting in some individuals being more susceptible to weight gain and obesity.

Underfeeding is known to reduce resting energy expenditure (REE) as an energy-conserving mechanism [1] and is accompanied by weight loss, with a reduction in metabolically active tissue. Seven days of underfeeding has previously been shown to lead to insulin resistance, with a reduction in insulin-stimulated glucose oxidation and glucose-induced thermogenesis in response to a glucose clamp [2], but the mechanisms of this reduction are not fully understood.

Uncoupling protein 3 (UCP3), discovered in 1997 along with UCP2, has a 57% homology with UCP1, a mitochondrial protein that uncouples respiration from phosphorylation and dissipates energy as heat. UCP1 is known to have an important role in cold-induced thermogenesis and hence EE in brown adipose tissue (BAT) in small mammals, but its role in adult humans is limited in view of the small amounts of BAT present. UCP2 is expressed ubiquitously but UCP3 is primarily expressed in skeletal muscle, the largest tissue mass in non-obese humans and an important contributor to whole-body REE.

Uncoupling activity stimulates glucose uptake [3], and UCP3 mRNA overexpression in cell cultures results in stimulation of glucose transport and increased GLUT4 recruitment [4]. In animal studies, overexpression of UCP3 mRNA (>20-fold human UCP3) increases EE. Transgenic UCP3-overexpressed mice are hyperphagic, remain lean, weigh 30% less than their wild-type controls and have lower fasting glucose concentrations [5], suggesting that overexpression of UCP3 may protect against diet-induced obesity and diabetes. However, studies in UCP3 knockout mice show that they do not become obese and have normal REE, thermoregulation and glucose homeostasis [6].

Studies on energy restriction have shown variable results on UCP3 mRNA expression in skeletal muscle. Five days of energy restriction to approximately 10–15% of voluntary intake in obese and lean subjects increased muscle UCP3 mRNA expression [7], whereas a week of 50% food restriction decreased UCP3 mRNA expression in rodent muscles [8].

Studies reflecting longer term energy restriction have shown downregulation of UCP3 mRNA.

Weight-stable weight loss in type 2 diabetic subjects on a 10-week energy-restricted diet was associated with a reduction in UCP3 mRNA expression [9]. This was also associated with decreased REE, plasma free fatty acid (FFA) concentrations and fat oxidation. In obese patients, similar findings were reported after prolonged underfeeding for 9 months after gastric banding [10]. One study has shown that UCP3 expression is lower in obese women who are diet resistant compared with those who are diet responsive, suggesting that the greater the level of expression, the greater the success of weight loss in response to energy-restricted diets [11], providing a possible therapeutic target in managing obesity.

Animal studies have shown that 12–18 months of underfeeding increases UCP3 expression [12], whereas 2 years after bariatric surgery in 11 obese subjects, who were weight stable, there was a decrease in UCP3 expression, which correlated with decreased intramyocytic triglyceride content [13].

Hyperinsulinaemic euglycaemic clamps are a well-recognized technique for assessing insulin sensitivity as high levels of insulin suppress endogenous hepatic gluconeogenesis, and glucose disposal rates can be estimated [14]. Combining this technique with indirect calorimetry enables the determination of whole-body carbohydrate partitioning (i.e. oxidative and non-oxidative glucose disposal) and thermogenic responses to glucose and insulin. Insulin sensitivity is known to improve in weight-stable obese subjects who have lost weight [15], while 7 days of underfeeding in the non-obese produces little change in insulin-stimulated glucose disposal rates [2], and acute starvation reduces insulin sensitivity [16]. Hyperinsulinaemia, in itself, has no effect on UCP3 expression [7]. Given that underfeeding affects both REE and substrate utilization, one would hypothesize that if skeletal muscle UCP3 is related to either thermogenesis or fat utilization, then underfeeding would be associated with a change in UCP3 expression. Moreover, the effect of underfeeding on insulin sensitivity and carbohydrate partitioning may also be related to changes in UCP3.

The present study was designed to examine the effects of 6 days of underfeeding (50% predicted requirements) on carbohydrate partitioning, thermogenesis and UCP3 expression in human skeletal muscle in lean male subjects, relating any changes in UCP3 to the effects of underfeeding on substrate utilization and insulin sensitivity.

Materials and Methods

Human Subjects

Eleven healthy normal-weight men participated in these studies. Subjects were aged 22.8 ± 1.34 years, and body

mass index was 23.4 ± 0.71 kg/m^2. Body composition was determined by both bioimpedance (Quadscan 4000, Bodystat, Douglas, Isle of Man) (473.73 ± 11.48 Ω) and skinfold thickness measurements (Harpenden Skinfold Calipers, Baty International, West Sussex, UK) after a 12-h fast [17], and the mean of the two used to estimate body fat and fat-free mass (FFM) (12.35 ± 1.21% total body fat; 67.7 ± 1.7 kg FFM). Exclusion criteria included any disease, condition or drug that would affect metabolic rate.

Informed written consent was obtained from all subjects after explanation of the protocol, and the study was approved by the University of Nottingham Medical School Ethics Committee prior to commencing.

Study Protocol

For each subject, a 6-day diet was designed based on the subject's individual 3-day food and daily activity diary. Using Schofield's equation [18], basal metabolic rate was calculated and total EE estimated according to the physical activity ratio score determined from the activity diary. Two diets were individually designed taking into account the dietary preferences. An eucaloric diet (EU) was based on current consensus information of an average diet in young men comprising 35% fat, 45% carbohydrate and 20% protein. These were similar to the analysis of the 3-day diaries (34.2% fat, 46.4% carbohydrate and 19.4% protein). Underfeeding diets (UF) were based on 50% predicted requirements for weight maintenance with the same protein, fat and carbohydrate composition as for EU. Subjects visited the laboratory after a 12-h overnight fast on four separate occasions, before and after completing the 6-day EU or UF in random order. Subjects were asked to abstain from alcohol during the diets but to maintain physical activity. There was a re-equilibration period of at least 2 weeks on an *ad libitum* normal diet between the controlled dietary periods.

Studies took place in a temperature-controlled room (23 °C), with subjects wearing a T-shirt and shorts only. On arrival, subjects rested supine for 30 min, while one intravenous cannula was inserted into the antecubital vein for infusion of insulin and glucose and another was inserted retrogradely into a vein on the dorsal aspect of the hand for blood sampling. A slow-running infusion of 0.9% saline was used to keep this patent. The hand was placed in a warm air box (50–55 °C) to obtain arterialized blood samples [19]. The first 2-ml sample was discarded to avoid contamination with saline.

On each occasion, the response to a hyperinsulinaemic euglycaemic clamp was measured over a 2-h period [14]. The clamp was composed of a continuous intravenous infusion of insulin using 30 units of human soluble insulin (Human Actrapid, Novo Nordisk, Bagsvaerd, Denmark) mixed with 58 ml of 0.9% saline and 2 ml of the subject's venous blood to prevent the adherence of insulin to the syringe. The subject's surface area was calculated using a standard nomogram, using their height and weight, from which an infusion rate was calculated at 40 mU/m^2/min. Using a three-way tap via the same cannula, 20% glucose was infused at a variable rate to maintain a stable blood glucose concentration. In this study, blood glucose was clamped at 4.5 mmol/l and arterialized blood glucose was measured every 5 min and glucose infusion adjusted accordingly. This allowed whole-body glucose disposal rate to be estimated.

Throughout the study, brachial arterial blood pressure and heart rate were measured using an automated sphygmomanometer (Datascope, Datascope Medical, Huntingdon, UK).

Muscle biopsies, taken from vastus lateralis, were performed at each visit after the resting basal period.

Calculation of the REE and estimation of carbohydrate and lipid oxidation rates were done using indirect calorimetry.

Glucose disposal rates were calculated from the glucose infusion rates. Glucose storage (i.e. non-oxidative glucose disposal) rates were calculated by subtracting glucose oxidation rates from glucose disposal rates.

Muscle Biopsies

Using Bergstrom needles, muscle samples were obtained percutaneously from the vastus lateralis muscle under local anaesthesia. Muscle biopsies were immediately frozen in liquid nitrogen and stored at −80 °C for subsequent analysis of UCP3 mRNA.

Indirect Calorimetry

During the last 30 min of the basal and the infusion periods, respiratory exchange measurements were made by using a computerized flow-through canopy gas analyzer system [20]. This was used to estimate oxidation rates and to calculate REE by recordings of O_2 consumption and CO_2 production [21].

Analytical Procedures

Blood samples were drawn during the baseline period and every 30 min during the 2-h infusion and measured for hormones and intermediary metabolites. Glucose was measured every 5 min throughout the clamp using

a Yellow Springs analyser (YSI 2300 Stat plus, Yellow Springs Industries, Yellow Springs, OH, USA).

For the measurement of adrenaline and noradrenaline, 75μl EGTA glutathione was mixed with 2 ml of plasma and stored at −80 °C for subsequent analysis using high-performance liquid chromatography with electrochemical detection [22].

FFA, glycerol and triglycerides were measured by enzymatic methods (NEFA C, WAKO Chemicals GmbH, Osaka, Japan; Glycerol diagnostic kit, Sigma catalogue no. 337-B, Poole, Dorset; Serum triglyceride determination kit, Sigma Aldrich, Poole, Dorset).

Insulin concentrations were measured using radioimmunoassay (Coat-a Count, Diagnostic Products, Los Angeles, CA, USA), and blood lactate was analysed using a Yellow Springs analyser (YSI 2300 Stat plus; Yellow Springs Industries).

One hundred and fifty millilitres of blood was deproteinized in 300 μl of 10% perchloric acid and stored at −20 °C for analysis of β-hydroxybutyrate concentrations.

RNA Preparation and Quantification

The mRNA levels of UCP3 were quantified by Taqman real-time polymerase chain reaction. Briefly, total RNA was isolated from muscle biopsy samples using Trizol (Invitrogen, Carlsbad, CA, USA), and further purified using RNeasy RNA clean up columns (Qiagen GmbH, Hilden, Germany). RNA was reverse transcribed using Superscript reverse transcriptase (Invitrogen), and cDNA was quantified using the Oligreen ss DNA quantification kit (Molecular Probes). Primers and FAM/TAMRA labelled probe used to detect human UCP3 long form are as follows: forward primer, 5′-CTG GAC TAC CAC CTG CTC ACT-3′; reverse primer, 5′-AGG TTA CGA ACA TCA CCA CGT-3′; probe, 5′-AGG ATC CCA AAC GCA AAA AGG AGG-3′.

For the UCP3 long form, the protocol was optimized at 900 nM for both primers and 200 nM for the probe using 100 ng of cDNA in a 25-μl reaction volume. Quantification was achieved by using serial dilutions of the amplification product cloned into the pBluescript KS (Stratagene, La Jolla, CA, USA) vector to generate standard curves.

Statistical Analysis

Values are given as means ± s.e.m. Comparison of responses between the diets was assessed by analysis of variance (ANOVA), with subsequent pairwise comparisons using *t*-tests. Simple regression analysis was also performed. Data were analysed using SPSS (SPSS Inc., Chicago, IL, USA). The threshold for significance was $p < 0.05$.

Results

The Effect of Diet (Basal Data)

Body Weight

There was no significant difference in baseline weights prior to each diet ($p = 0.12$) (table 1).

There was a significant decrease in weight after a 6-day UF ($p < 0.005$ ANOVA). Mean weight decrease was 2.05 ± 0.34 kg post-UF ($p < 0.001$) and 0.55 ± 0.28 kg post-EU ($p = 0.078$).

Plasma Hormones and Metabolites

There was no significant difference in baseline fasting hormone or metabolite levels between pre-diet visits (table 2).

Baseline fasting plasma FFA concentrations were not significantly different between pre-diet visits, but fasting concentrations after a 6-day diet were significantly different ($p < 0.001$ ANOVA). FFA increased by 0.15 ± 0.05 mmol/l ($p = 0.01$) after the UF and by 0.08 ± 0.03 mmol/l ($p = 0.04$) after EU. The absolute values after 6-day UF were 0.13 ± 0.03 mmol/l greater than after EU ($p < 0.001$) (figure 1).

There was no significant difference in fasting glycerol levels between each of the four visits before and after each dietary period. On the contrary, fasting triglyceride levels were different after EU compared with UF ($p < 0.005$, ANOVA), having fallen significantly after underfeeding by 0.19 ± 0.05 mmol/l ($p = 0.003$).

Fasting glucose, after the dietary period, differed ($p < 0.01$, ANOVA) and fell after underfeeding by 0.33 ± 0.09 mmol/l ($p = 0.005$), with a corresponding fall in fasting insulin levels after 6 days of underfeeding by 1.46 ± 0.44 μU/ml ($p = 0.005$).

Baseline lactate concentrations fell after both diets also ($p < 0.005$, ANOVA), by 0.19 ± 0.06 mmol/l ($p = 0.016$) after UF and 0.18 ± 0.07 mmol/l ($p = 0.021$) after EU.

Fasting β-hydroxybutyrate levels at baseline were significantly different ($p < 0.001$, ANOVA).

After 6 days of underfeeding, fasting plasma β-hydroxybutyrate levels were dramatically increased by 0.45 ± 0.09 mM ($p < 0.001$) compared with pre-UF and increased by 0.41 ± 0.07 mM ($p < 0.001$) compared with the post-EU (figure 1). There was a slight rise in β-hydroxybutyrate levels after the EU (by 0.05 ± 0.02 mM; $p = 0.03$).

There was no significant difference in baseline plasma adrenaline and noradrenaline concentrations between all four visits.

Table 1 Baseline and post 2 hour hyperinsulinaemic clamp (mean values with standard errors for eleven subjects). Post values are averaged over time between 90 and 120 minutes of the clamp.

	Pre Eucaloric	Post Eucaloric	Pre Underfeeding	Post Underfeeding
Weight (kg)				
Basal	77.5 ± 2.3	76.9 ± 2.1	78.4 ± 2.3	76.4 ± 2.2‡‡‡
Systolic Blood Pressure (mmHg)				
Basal	122 ± 3	123 ± 3	125 ± 3	120 ± 3‡
Post	127 ± 2††	127 ± 3†	130 ± 3†	121 ± 2
Diastolic Blood Pressure (mmHg)				
Basal	64 ± 3	61 ± 3	66 ± 3	62 ± 2
Post	62 ± 2	63 ± 3	65 ± 2	60 ± 2
Heart Rate (beats per minute)				
Basal	59 ± 2	57 ± 3	63 ± 2	60 ± 2
Post	61 ± 3	59 ± 3	63 ± 3	57 ± 3
Lipid Oxidation (g.min^{-1})				
Basal	0.053 ± 0.008	0.082 ± 0.011	0.065 ± 0.011	0.087 ± 0.011
Post	0.027 ± 0.010	0.063 ± 0.028	0.022 ± 0.009†	0.044 ± 0.009†††
Carbohydrate Oxidation (g.min^{-1})				
Basal	0.157 ± 0.018	0.086 ± 0.025	0.135 ± 0.023	0.054 ± 0.024‡
Post	0.239 ± 0.029†	0.167 ± 0.065	0.255 ± 0.027††	0.171 ± 0.015†††
REE (kJmin^{-1})				
Basal	5.42 ± 0.18	5.47 ± 0.13	5.57 ± 0.25	5.15 ± 0.15‡
Post	5.73 ± 0.19††	6.04 ± 0.18†	5.75 ± 0.17	5.23 ± 0.22
UCP3mRNA (arbitrary units)				
Basal	52.4 ± 2.6	49.1 ± 6.3	72.8 ± 15.8	62.3 ± 15.0

Pre versus Post diet : ‡ $p \leq 0.05$; ‡‡ $p \leq 0.01$; ‡‡‡ $p \leq 0.001$
Post EU versus Post UF: *$p \leq 0.05$;** $p \leq 0.01$; *** $p \leq 0.001$
Pre versus Post clamp : $^{†}p \leq 0.05$; †† $p \leq 0.01$; ††† $p \leq 0.001$

EE and Substrate Oxidation

There was no significant difference between REE and substrate oxidation between both pre-diet visits (table 1).

REE differed in response to the diets ($p < 0.05$, ANOVA) and fell after 6 days of energy restriction by 0.43 ± 0.17 kJ/min ($p = 0.032$), with no significant change after EU (+0.05 ± 0.23 kJ/min; $p = 0.82$).

Baseline carbohydrate oxidation rates were different ($p < 0.01$, ANOVA) and fell after underfeeding by 0.08 ± 0.03 g/min ($p = 0.04$), with a trend for an increase in fat oxidation by 0.022 ± 0.12 g/min ($p = 0.09$). There was a trend for a fall in carbohydrate oxidation after 6 days of the EU (0.07 ± 0.03 g/min; $p = 0.06$).

Heart Rate and Blood Pressure

Baseline systolic blood pressure differed between diets ($p < 0.05$, ANOVA) and was reduced after 6 days of underfeeding by 5.2 ± 1.7 mmHg ($p = 0.013$). No significant changes were seen in diastolic blood pressure and heart rate.

UCP3 mRNA Levels

There was no significant change in UCP3 mRNA expression in skeletal muscle between each visit. The UCP3 mRNA post-UF was 10.4 ± 6.8 arbitrary units (au) lower ($p = 0.16$) and post-EU 3.2 7.3 au lower ($p = 0.67$), but neither was statistically significant (figure 2).

There was no correlation between change in UCP3 mRNA and change in REE, weight or other metabolites.

There was a correlation between weight loss and change in carbohydrate/fat oxidation (Pearson $r = 0.60$, $p = 0.003$; $r = -0.45$, $p = 0.036$ respectively), change in glucose ($r = 0.45$, $p = 0.036$), β-hydroxybutyrate ($r = -0.76$, $p < 0.001$) and triglycerides ($r = 0.72$, $p < 0.001$).

Response to the Clamp (Post-data)

Plasma Hormones and Metabolites

As expected, plasma glycerol and FFA concentrations fell significantly in all four visits in response to a hyperinsulinaemic euglycaemic clamp ($p < 0.001$, ANOVA).

Steady-state glucose infusion rate was achieved after 90 min of glucose and insulin infusion on each occasion. Mean blood glucose levels during the glucose clamps at each visit were similar: 4.46 ± 0.04 mmol/l (pre-EU), 4.47 ± 0.03 mmol/l (post-EU), 4.48 ± 0.03 mmol/l (pre-UF) and 4.45 ± 0.02 mmol/l (post-UF). The mean coefficients of variation were 2.7, 1.9, 2.5 and 1.5% respectively.

Table 2 Baseline and post 2 hour hyperinsulinaemic clamp plasma hormone and metabolite concentrations (mean values with standard errors for eleven subjects). Post values are averaged over time between 90 and 120 minutes of the clamp.

	Pre Eucaloric	Post Eucaloric	Pre Underfeeding	Post Underfeeding
FFA (mmol/l)				
Basal	0.28 ± 0.03	0.36 ± 0.02‡	0.35 ± 0.04	0.49 ± 0.03‡***
Post	0.05 ± 0.01†††	0.05 ± 0.01†††	0.06 ± 0.01†††	0.05 ± 0.01†††
Glycerol (mM)				
Basal	0.039 ± 0.007	0.035 ± 0.006	0.040 ± 0.006	0.040 ± 0.004
Post	0.022 ± 0.002	0.016 ± 0.002†††	0.022 ± 0.003††	0.016 ± 0.002†††
Triglycerides (mM)				
Basal	0.38 ± 0.07	0.36 ± 0.05	0.46 ± 0.08	0.28 ± 0.04‡‡*
Lactate (mmol/l)				
Basal	0.56 ± 0.06	0.38 ± 0.03‡	0.55 ± 0.07	0.36 ± 0.02‡
Post	0.69 ± 0.03	0.65 ± 0.04†††	0.68 ± 0.04	0.57 ± 0.02†††
Glucose (mmol/l)				
Basal	4.48 ± 0.10	4.45 ± 0.10	4.59 ± 0.08	4.26 ± 0.07‡‡
Insulin (μU/ml)				
Basal	5.94 ± 0.66	5.43 ± 0.55	5.82 ± 0.52	4.36 ± 0.17‡‡
Post	58.37 ± 2.51†††	58.99 ± 2.51†††	59.36 ± 2.62†††	56.12 ± 2.01†††
β-Hydroxybutyrate (mM)				
Basal	0.10 ± 0.04	0.16 ± 0.04‡	0.12 ± 0.02	0.57 ± 0.08‡‡‡***
Post	0.03 ± 0.00	0.04 ± 0.00††	0.03 ± 0.00††	0.05 ± 0.01†††
Adrenaline (nmol/l)				
Basal	0.13 ± 0.01	0.13 ± 0.02	0.12 ± 0.02	0.11 ± 0.02
Post	0.15 ± 0.02	0.18 ± 0.02	0.16 ± 0.04	0.16 ± 0.02††
Noradrenaline (nmol/l)				
Basal	0.86 ± 0.12	0.84 ± 0.12	0.93 ± 0.16	0.73 ± 0.08
Post	1.15 ± 0.16††	1.13 ± 0.14††	1.41 ± 0.17††	1.07 ± 0.13†††

Pre versus Post diet : $^{‡}p \leq 0.05$; $^{‡‡}p \leq 0.01$; $^{‡‡‡}p \leq 0.001$
Post EU versus Post UF: *$p \leq 0.05$;** $p \leq 0.01$; *** $p \leq 0.001$
Pre versus Post clamp : $^{†}p \leq 0.05$; $^{††}p \leq 0.01$; $^{†††}p \leq 0.001$

Mean glucose disposal rates were 45.56 ± 3.19 μmol/min/kg pre-EU, 44.69 ± 2.31 μmol/min/kg post-EU (pre- vs. post-$p = 0.68$), 46.35 ± 2.15 μmol/min/kg pre-UF and 39.46 ± 1.12 μmol/min/kg post-UF (pre- vs. post-$p = 0.003$) (figure 3). Glucose disposal rates were different between visits ($p < 0.05$, ANOVA), with a significant reduction in glucose disposal rates post-UF compared with post-EU ($p = 0.045$).

In response to the clamp, carbohydrate oxidation rates increased by similar amounts before and after both the EU

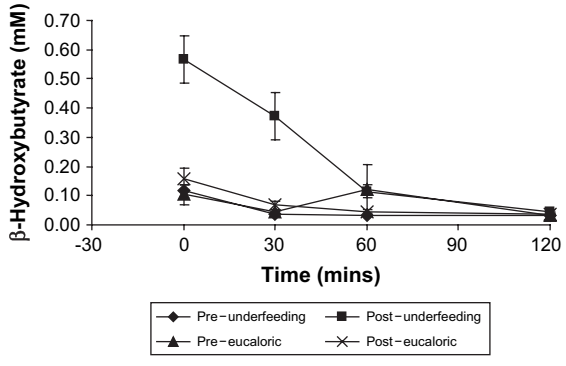

Fig. 1 Plasma free fatty acid and β-hydroxybutyrate concentrations at baseline and during each hyperinsulinaemic clamp. Values are means for 11 subjects, with the standard errors indicated by vertical bars.

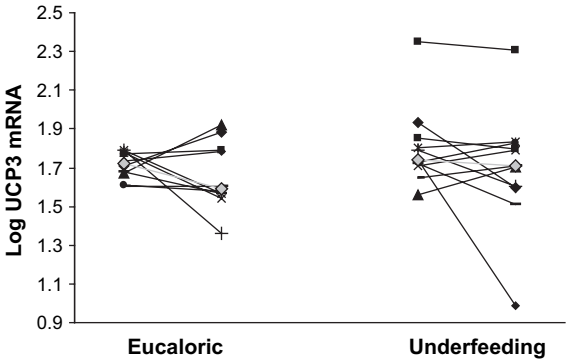

Fig. 2 Changes in skeletal muscle expression levels of uncoupling protein 3 mRNA before and after each diet. Median and values for 11 subjects.

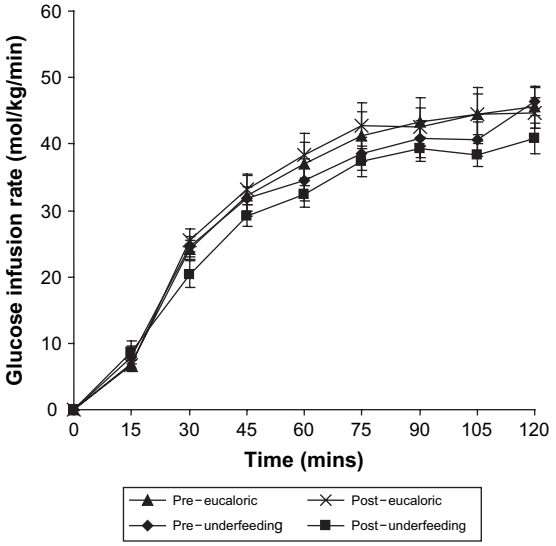

Fig. 3 Glucose infusion rates (μmol/min/kg) before and after 6-day diets. Values are means for 11 subjects, with the standard errors indicated by vertical bars.

period (0.08 ± 0.03 g/min and 0.08 ± 0.06 g/min) and the UF period (0.12 ± 0.03 g/min and 0.12 ± 0.02 g/min; non-significant, ANOVA). Carbohydrate oxidation rates, at the end of the 2-h clamp, were significantly lower at the post-UF visit compared with the pre-UF visit by 0.08 ± 0.03 g/min; $p = 0.011$ (post-clamp rates: 18.18 ± 1.89 μmol/min/kg pre-UF vs. 12.46 ± 1.26 μmol/min/kg post-UF). There was no significant difference in post-clamp carbohydrate oxidation rates between pre-EU and post-EU visit (post-clamp rates: 17.06 ± 1.97 μmol/min/kg pre-EU vs. 11.56 ± 4.97 μmol/min/kg post-EU; $p = 0.254$).

There was no significant difference in glucose storage rates between visits (28.5 ± 3.2 μmol/min/kg pre-EU; 33.1 ± 4.6 μmol/min/kg post-EU; 28.2 ± 2.4 μmol/min/kg pre-UF; 27.0 ± 2.3 μmol/min/kg post-UF).

In response to the clamp, lactate concentrations differed between diets ($p < 0.01$, ANOVA).

Lactate concentrations increased after the hyperinsulinaemic euglycaemic clamp in both diet groups (0.27 ± 0.04 mmol/l; $p < 0.001$ UF and 0.21 ± 0.03 mmol/l; $p < 0.001$ EU).

As expected, similar hyperinsulinaemic levels were achieved at all visits in response to the clamp.

β-Hydroxybutyrate levels fell during glucose and insulin infusion to similar concentrations in all four visits.

In response to the clamp, catecholamine concentrations increased ($p < 0.05$, ANOVA).

Adrenaline and noradrenaline concentrations increased in response to the clamp post-UF, but only noradrenaline was increased during the clamp at the other visits. Post-clamp noradrenaline levels were significantly lower in the post-UF visit compared with pre-UF visit (0.34 ± 0.13 nmol/l; $p = 0.022$).

EE and Substrate Oxidation

The thermogenic response to the clamp differed between diets ($p < 0.05$, ANOVA).

In response to a hyperinsulinaemic euglycaemic clamp, REE increased significantly in the pre-EU and post-EU visits (0.30 ± 0.08 kJ/min; $p = 0.005$; 0.57 ± 0.26 kJ/min; $p = 0.05$ respectively). No significant increase was seen in the pre-UF visit (0.17 ± 0.13 kJ/min; $p = 0.23$) or post-UF visit (0.08 ± 0.13 kJ/min; $p = 0.52$). During the last 30 min of the clamp, REE was significantly greater (by 0.51 ± 0.12 kJ/min; $p = 0.002$) in the pre-UF than in the post-UF visit.

There was no significant decrease in lipid oxidation during the clamp at either pre-EU (−0.025 ± 0.012 g/min; $p = 0.06$) or post-EU visit (−0.019 ± 0.027 g/min; $p = 0.51$). Lipid oxidation fell by 0.043 ± 0.014 g/min ($p = 0.011$) and 0.043 ± 0.008 g/min ($p < 0.001$) in pre- and post-UF visits following the clamp respectively.

Heart Rate and Blood Pressure

In response to the clamp, systolic blood pressure differed between dietary visits ($p < 0.005$, ANOVA).

Except in the post-UF visit, systolic blood pressure rose in all other visits during the hyperinsulinaemic euglycaemic clamp (5.0 ± 1.3 mmHg; $p = 0.003$ pre-EU; 3.8 ± 1.6 mmHg; $p = 0.042$ post-EU; 4.6 ± 2.0 mmHg; $p = 0.045$ pre-UF).

No significant changes were seen in diastolic blood pressure and heart rate.

Discussion

Individual variability in REE may make some people more prone to developing obesity than others [23]. Skeletal muscle, where UCP3 is almost exclusively expressed, is an important site of REE and therefore may influence energy metabolism and obesity. Underfeeding is known to reduce EE [1], and the body adapts in order to utilize macronutrient stores as efficiently as possible. Short-term studies demonstrate that energy restriction has varying effects on UCP3 expression and are confounded by the effects of fasting and the varying effects on metabolites. Certainly, UCP3 has been found to be elevated in weight loss induced by starvation [7,24]. This study was designed to investigate the effect of 50% energy

restriction for 6 days on carbohydrate partitioning, thermogenesis and UCP3 expression in non-obese individuals and to examine its effects on the substrate utilization and thermogenic responses to glucose plus insulin.

In this study, a 0.43 ± 0.17 kJ/min reduction in REE was observed after 6 days of 50% energy restriction, presumably in part related to the 2.05-kg mean weight loss. We did not measure body composition after each dietary period, as the techniques available are unlikely to be sufficiently sensitive to accurately measure the changes likely after 6 days. However, if one assumes that the 2.05-kg weight loss comprised 25–50% FFM and 75–50% fat mass, the likely reduction in FFM would have been between 0.5 and 1.0 kg, a maximum reduction of 1.5%. As REE fell by 8%, the REE per kilogram FFM was clearly reduced after 6 days of underfeeding, which may have been the result of some energy-conserving mechanisms. Obviously, energy intake was reduced by much more than this small decrease in REE, so there was a substantial negative energy balance and weight loss. Despite this marked negative energy balance and likely reduction in REE per kilogram FFM, skeletal muscle UCP3 mRNA expression was not different between UF and EU. There was a greater difference between pre- and post-UF UCP3 mRNA expression compared with post-EU, with a reduction of 10.4 ± 6.8 au (17%), but this did not reach statistical significance. On power calculations, double the number of subjects would have been required for this to be a significant reduction in UCP3 mRNA in skeletal muscle, assuming a constant variance. However, there was no correlation between weight loss and change in UCP3 expression.

In a previous study, 5 days of energy restriction, in obese and lean subjects, upregulated UCP3 mRNA expression by 2.5-fold [7]. Energy restriction was greater at an intake of 1045 kJ/day, with an average weight loss of 2.8 kg, reduced glucose, insulin and REE, but FFA were raised by almost twofold. It is possible that it was the elevation in FFA that mediated the upregulation of UCP3, while in our study, the weight loss was not as great nor was there as great an increase in FFA (1.4-fold).

In retrospect, the EU did not quite meet energy requirements but resulted in very mild underfeeding and therefore the results reflect very mild and a more marked negative energy balance. This study therefore compared mild vs. severe undernutrition for 6-days as the post-EU resulted in a small reduction in weight of 0.55 kg (1%), a rise in fasting FFA and ketone concentrations, a reduction in fasting lactate levels and a trend for a reduction in glucose oxidation but with no change in REE.

The fall in blood glucose levels after underfeeding is likely to be related to the restriction in food intake and weight loss, as is the reduction in fasting triglycerides. This is reflected in a significantly lowered carbohydrate oxidation, with a shift towards fat oxidation suggesting a switch from glucose to fat metabolism. Significant underfeeding was achieved, confirmed by elevated β-hydroxybutyrate levels. This is consistent with raised FFA enhancing the hepatic formation of ketone bodies. After the EU, plasma β-hydroxybutyrate levels were also raised, which may reflect that subjects were slightly underfed, although there was neither a significant loss of weight nor a significant change in REE. Plasma insulin concentrations fell, albeit a small decrease by 1.46 ± 0.44 μU/ml, after 6 days of underfeeding corresponding to a lowered fasting glucose.

During the clamp, whole-body glucose disposal rates were approximated from glucose infusion rates as insulin at high concentrations suppresses hepatic gluconeogenesis [14].

The increase in thermogenesis in response to the clamp was expected at the pre- and post-EU visits as supported by previous studies [2,16]. An increase in REE occurred during the clamp at the pre-UF visit, but the variability was such that this rise was non-significant.

In this study, after underfeeding, there was a reduction in glucose disposal rates and no thermogenic response to the clamp, which may be related to a developing state of insulin resistance. This reduction in glucose-induced thermogenesis during the clamp has been supported by a previous study on 7 days of underfeeding [2]. Studies have suggested that 70% of glucose disposal, after an intravenous glucose load, occurs in skeletal muscle [25,2], which is therefore a major site of substrate utilization and requires the metabolic flexibility to adapt to changes in energy balance and fuel partitioning. Whether UCP3 has a role in this mechanism is unclear.

β-Hydroxybutyrate levels and lipid concentrations decreased, as expected during the hyperinsulinaemic euglycaemic clamp, with a fall in lipid oxidation and an increase in glucose oxidation representing a shift from fat to glucose metabolism. Similar increases in carbohydrate oxidation rates were observed between pre- and post-UF visits, but absolute oxidation rates were significantly lower after underfeeding than before. Despite the reduction in total glucose disposal rates after underfeeding, with a lower glucose oxidation rate in response to the clamp compared with the pre-UF visit, storage rates were similar between diets. This is confirmed by the previous studies that have shown that glucose storage is relatively unaffected by underfeeding, despite a reduction in insulin-stimulated glucose oxidation rates [2].

Interestingly, glucose oxidation was not significantly increased during the glucose clamp in the post-EU visit,

that is, in a mildly underfed state. While this may have been due to more variation in the data at this time-point, there was a trend for the absolute rate of glucose oxidation during the clamp to be lower after the eucaloric period than before. Thus, both mild and more severe underfeeding are associated with lower rates of insulin-mediated glucose oxidation, but only the more severe underfeeding is associated with a lower overall glucose disposal and the absence of a thermogenic response to the insulin and glucose infusion. Thus, it would appear that the effects of underfeeding on insulin sensitivity, insulin-stimulated glucose oxidation and thermogenesis are not uniform, with the initial effect being on glucose oxidation and more severe underfeeding than affecting glucose disposal and thermogenesis. Increasing energy restriction results in insulin resistance and reduced REE prior to any changes in glucose storage rates. Disturbances in fuel partitioning can compromise energy balance, and the shift from increased glucose oxidation may represent the beginnings of a change for energy conservation, induced by underfeeding, before REE and insulin sensitivity decline in response to increasing energy restriction. Shifts in fuel partitioning between oxidation and storage of carbohydrates may also ultimately have an effect on food intake, contributing to energy conservation [26].

There was a significant fall in systolic blood pressure with underfeeding, as seen in previous studies [2], which indirectly would be consistent with a decrease in sympathetic activity, but there was no change in catecholamine levels. In response to the clamp, noradrenaline levels increased in parallel to a rise in systolic blood pressure. Hyperinsulinaemia is proposed to increase sympathetic activity [27]. Despite the increase in adrenaline and noradrenaline levels in the underfed group, it neither did result in a significant increase in blood pressure after the clamp nor did it significantly increase EE.

Despite a reduction in weight, REE, basal glucose oxidation and insulin-stimulated glucose disposal in this study, UCP3 mRNA did not alter significantly in response to 6 days of underfeeding. These observations are not consistent with UCP3 contributing to the change in REE with underfeeding. However, it may have a role in more prolonged weight loss or more severe underfeeding/fasting, where it may be involved in handling high rates of fatty acid availability.

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