24 h severe energy restriction impairs post-prandial glycaemic control in young, lean males

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Abstract

Intermittent energy restriction (IER) involves short periods of severe energy restriction interspersed with periods of adequate energy intake, and can induce weight loss. Insulin sensitivity is impaired by short-term, complete energy restriction, but the effects of IER are not well known. In randomised order, 14 lean men (age: 25 (SD 4) y; BMI: 24 (SD 2) $kg \cdot m^{-2}$; body fat: 17 (4) %) consumed 24 h diets providing 100% (10441 (SD 812) kJ; EB) or 25% (2622 (SD 204) kJ; ER) of estimated energy requirements, followed by an oral glucose tolerance test (OGTT; 75g glucose drink) overnight fasted. Plasma/ serum glucose, insulin, non-esterified fatty acids (NEFA), glucagon-like peptide-1 (GLP-1), glucose-dependant insulinotropic peptide (GIP) and fibroblast growth factor-21 (FGF21) were assessed before and after (0 h) each 24 h dietary intervention, and throughout the 2 h OGTT. Homeostatic model assessment of insulin resistance (HOMA2-IR) assessed the fasted response and incremental (iAUC) or total (tAUC) area under the curve were calculated during the OGTT. At 0 h, HOMA2-IR was 23% lower after ER compared to EB (P<0.05). During the OGTT, serum glucose iAUC (P<0.001) serum insulin iAUC (P<0.05) and plasma NEFA tAUC (P<0.01) were greater during ER, but GLP-1 (P=0.161), GIP (P=0.473) and FGF21 (P=0.497) tAUC were similar between trials. These results demonstrate that severe energy restriction acutely impairs postprandial glycaemic control in lean men, despite reducing HOMA2-IR. Chronic intervention studies are required to elucidate the long-term effects of IER on indices of insulin sensitivity, particularly in the absence of weight loss.

Introduction

Obesity is the result of chronic mismanagement of energy balance and is associated with several chronic diseases[1]. Recent analyses project the prevalence of obesity to continue to increase[2], with part of this increase attributable to a greater number of lean individuals gaining weight throughout adulthood[3]. Daily energy restriction of 20-50% of estimated energy requirements (EER) is frequently used as a method of managing energy balance[4], yet data suggests that only ~40% of individuals manage to achieve long term weight loss[5]. This may be due to the requirement for daily adherence to the diet in order to achieve a sufficiently large energy deficit for weight loss[6].

Intermittent energy restriction (IER), often termed 'intermittent fasting', has become the subject of considerable research attention as an alternative to continuous energy restriction[7]. Typically, IER permits consumption of an *ad-libitum* or adequate energy diet (i.e. ~100% EER) punctuated by short periods (24-48 h) of severe (~25% EER) or complete energy restriction. Previous studies have demonstrated 2-16 kg weight loss after 3-20 weeks of IER, which is comparable to losses induced with daily energy restriction[8]. With IER, this weight loss may be facilitated by a subjective and hormonal appetite response conducive to the maintenance of a negative energy balance[9,10,11]. As such, IER may be an effective alternative weight management strategy to traditional continuous moderate energy restriction.

By nature, IER requires individuals to undergo repeated cycles of acute severe energy restriction and refeeding. It has been demonstrated that a short (12-72 h) period of complete energy restriction (i.e. fasting) causes several metabolic alterations, including a reciprocal upregulation of lipolysis to provide non-esterified fatty acids (NEFA) for oxidation, and a downregulation of glycogenolysis to conserve glycogen stores[12]. This concurrently occurs with a decline in postprandial/ nutrient-stimulated insulin sensitivity and elevated plasma

glucose concentrations[13]. Typically IER protocols utilise partial (consuming ~25% EER) rather than complete (i.e. fasting) energy restriction, which may mitigate these effects[14]. It was recently shown in overweight/ obese individuals that partial energy restriction (~25% EER) produced a more favourable postprandial glycaemic response compared to complete energy restriction, but a degree of insulin resistance was still present[14]. However, metabolic regulation likely differs between lean and overweight/ obese individuals[15], as does the premise of IER (i.e. weight loss vs weight maintenance). Weight management is an integral part of reducing the prevalence of cardiometabolic disease. It has been well established that IER diets induce weight loss, which may in-itself impart a beneficial effect on risk markers for chronic disease. However, identifying whether there are specific metabolic effects of IER style diets, in lean individuals, will help determine whether IER might be used effectively as a tool for weight management[16].

Therefore, the aim of this study was to investigate the acute effects of 24 h severe energy restriction (~25% EER) in lean males, on indices of glycaemic control and metabolism; including fasting and postprandial measures of glucose, insulin, NEFA, glucagon-like peptide 1 (GLP-1), glucose-dependant insulinotropic peptide (GIP) and fibroblast growth factor-21 (FGF-21).

Methods

Subjects

This study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects/patients were approved by the Loughborough University Ethical Sub-committee for human participants (Reference number: R15-P032). Fourteen recreationally active, weight stable (>6 months), non-dieting males (age: 25 (SD 4) y; mass: 77.8 (SD 10.2) kg; height: 1.79 (SD 0.07) m; BMI: 24 (SD 2) $kg \cdot m^{-2}$; body fat: 17 (4) %) provided written informed consent to participate in the study. The sample size was based on the 2-h glucose area under the curve values for males from a previous study from our laboratory[11] that used a similar study design. Using an α of 0.05 and a β of 0.2, it was determined that 12 subjects would be required detect a 10% difference in glucose area under the curve.

Study design

Subject's height (Seca, Birmingham, UK), mass (Adam AFW-120K, Milton Keynes, UK), and body fat percentage[17] were determined during a preliminary visit to the laboratory. For inclusion, subjects were required to have a BMI<25 $kg \cdot m^{-2}$ and/ or a body fat percentage <25%[18]. Subjects completed two experimental trials in a randomised, counterbalanced order, with trials separated by ≥7 days. Each trial consisted of a 24 h period of either energy balance (EB) or energy restriction (ER), followed by an oral glucose tolerance test (OGTT).

Pre-trial standardisation

Dietary intake and physical activity in the 24 h preceding the first experimental trial were recorded, and replicated prior to the second trial. Alcohol and strenuous exercise were not permitted during this period, or during the study period.

Protocol

For each trial, subjects attended the laboratory on two consecutive mornings (~07:30), arriving via motorised transport after a >10 h overnight fast. Subjects were not permitted to consume food and drink additional to that provided during the study period.

Day 1: On arrival, subjects were seated for 30 min before a blood sample was collected by venepuncture from an antecubital forearm vein (-24 h). Before leaving the laboratory, subjects were provided with an individually standardised diet, and instructions on when to consume each item. Subjects were asked to perform minimal activity over the day. Diets were formulated to contain either 25% (ER) or 100% (EB) of EER, with EER calculated as the product of estimated resting metabolic rate[19] and a sedentary physical activity level of 1.4. Total energy was divided between four meals during EB and between two meals during ER (Table 1). Diets were kept standardised, however, individual preferences (i.e. severe dislike to a certain food) were considered and minor alterations were made to ensure adherence. Water intake was prescribed at 35 $mL \cdot kg^{-1}$ of body mass (2853 (SD 329) mL) and was evenly distributed throughout the day. On ER, in place of breakfast (08:00), subjects consumed a bolus of water equal to the water content of the breakfast provided on EB.

Day 2: Subjects returned to the laboratory the following morning and a 20-gauge cannula was inserted into an antecubital forearm vein. After 30 min seated rest, a fasted blood sample was collected (0 h). Subjects then consumed 75 g glucose dissolved in 250 mL of water, with an additional 50 mL of water used to rinse the beaker to ensure all glucose was consumed. The drink was consumed as quickly as possible and typically within 15 s. Blood samples were collected 0.25, 0.5, 0.75, 1, 1.5 and 2 h after ingestion with subjects remaining seated throughout.

Blood sampling and analysis

Blood samples were drawn in 12 mL volumes, with 5 mL dispensed into pre-chilled tubes containing 1.6 $mg \cdot mL^{-1}$ of potassium EDTA (Sarstedt AG & Co, Nümbrecht, Germany) and stored on ice, and 5 mL dispensed into tubes containing a clotting catalyst (Sarstedt AG & Co, Nümbrecht, Germany) and stored for 15 min at room temperature until completely clotted. Tubes were then centrifuged (1750 *g*; 10 min; 4°C) and plasma/ serum separated. The supernatant was stored at -20°C for later analysis. Two mL of whole blood was mixed with potassium EDTA and used for determination of haemoglobin concentration (via the cyanmethaemoglobin method) and haematocrit (via microcentrifugation) to estimate changes in plasma volume, relative to -24 h[20]. Serum glucose (Horiba Medical, Montpellier, France) and plasma NEFA (Randox Laboratories Ltd, Crumlin, UK) concentrations were determined by enzymatic, colorimetric methods, using a bench-top analyser (Pentra 400, Horiba ABX Diagnostics, Montpellier, France). The intra-assay CV for serum glucose and plasma NEFA were 0.5% and 1.3%, respectively. Plasma GLP-1 (Merck Millipore, Watford, UK), GIP (Merck Millipore, Watford, UK), FGF21 (R&D Systems, Abingdon, UK) and serum insulin (Immunodiagnostic Systems, Boldon, UK) were analysed by enzyme-linked immunosorbent assays. Intra-assay CV for plasma GLP-1, GIP, FGF21 and serum insulin were 7.9%, 6.1%, 3.3% and 4.7%, respectively. Serum glucose, insulin and plasma NEFA concentrations were determined at all sample time points. Plasma GLP-1, GIP and FGF21 concentrations were determined at -24, 0, 0.5, 1, 1.5 and 2 h.

Calculations

The updated homeostatic model of insulin resistance (HOMA2-IR) was used to calculate fasting insulin resistance before and after the dietary intervention using freely available online software (http://www.dtu.ox.ac.uk/homacalculator/). Serum glucose and insulin concentrations from the OGTT were used to assess changes in whole body insulin sensitivity using the Matsuda insulin sensitivity index[22]. Incremental area under the curve (iAUC) was calculated

for glucose and insulin to quantify the glycaemic response during the OGTT (0-2 h)[22]. Total area under the curve (tAUC) was calculated for glucose and insulin, as well as all other variable during the OGTT (0-2 h).

Statistical analysis

Data were analysed using IBM SPSS 23.0 (Somers, NY, USA). Correction of hormone concentrations relative to plasma volume change did not alter the results, so the unadjusted values are presented. Fasted (-24 to 0 h) and postprandial changes (0-2 h) were analysed separately. All data were checked for normality using a Shapiro-Wilk test. Data containing one factor were analysed using a *t*-test or Wilcoxon signed-rank test, as appropriate. Data containing two factors were analysed using a two-way repeated measures ANOVA, followed by *post-hoc* Holm-Bonferroni-adjusted paired *t*-tests or Holm-Bonferroni-adjusted Wilcoxon signed-rank tests, as appropriate. Pearson's *r* was used to explore correlations between variables indicated in text. Data sets were determined to be significantly different when $P<0.05$. Data are presented as mean (SD) unless otherwise stated.

Results

Body mass change

Body mass was not different between trials at -24 h (P=0.311) but was lower at 0 h during ER (P<0.05). Body mass decreased between -24 h and 0 h during both trials (P<0.0001), but to a greater extent during ER (EB: 0.43 (SD 0.31) kg; ER: 1.26 (SD 0.43) kg; P<0.0001).

Fasting metabolic measures

Values for fasting variables collected before (-24 h) and after (0 h) the dietary intervention are presented in Table 2. There were trial (P<0.05) and interaction (P<0.001) effects, but no time effect (P=0.099) for serum glucose concentrations. Glucose concentrations were lower at 0 h during ER compared to EB (P<0.01). Between -24 h and 0 h, serum glucose concentrations decreased during ER (P<0.0001), but did not change during EB (P=0.578). There were time (P<0.01) and interaction (P<0.05) effects, but no trial effect (P=0.079) for serum insulin concentrations. Insulin concentrations were lower at 0 h during ER compared to EB (P<0.05). Between -24 h and 0 h, serum insulin concentrations decreased during ER (P<0.01), but did not change during EB (P=0.178). There were time (P<0.01), trial (P<0.05) and interaction (P<0.05) effects for HOMA2-IR, which was lower at 0 h during ER compared to EB (P<0.05) and decreased between -24 h and 0 h during ER (P<0.01), but did not change during EB (P=0.303; Figure 1).

There were time (P<0.0001), trial (P<0.05) and interaction (P<0.0001) effects for plasma NEFA concentrations. NEFA concentrations were greater at 0 h during ER compared to EB (P<0.0001). Between -24 h and 0 h, plasma NEFA concentrations increased during ER (P<0.0001), but did not change during EB (P=0.166). There were no time (P=0.545), trial (P=0.227) or interaction (P=0.628) effects for plasma GLP-1 concentrations. There was a time

effect (P<0.01), but no trial (P=0.088) or interaction (P=0.096) effects for plasma GIP concentrations. GIP concentrations decreased between -24 h and 0 h during ER (P<0.05) and tended to decrease during EB (P=0.055). There was a time effect (P<0.0001), but no trial (P=0.776) or interaction (P=0.098) effects for FGF21 concentrations. Plasma FGF21 concentrations decreased between -24 h and 0 h during ER (P<0.0001) and EB (P<0.01).

Postprandial metabolic responses

Glucose, insulin and NEFA

There were time (P<0.0001), trial (P<0.01) and interaction (P<0.0001) effects for serum glucose concentrations, with lower concentrations at 0 h and greater concentrations between 0.75-1 h (P<0.05; Figure 2A) during ER compared to EB. Serum glucose iAUC (EB: 96 (SD 74) mmol·L^{-1}·2 h^{-1}; ER: (171 (SD 102) mmol·L^{-1}·2 h^{-1}; P<0.001; Figure 2B) and tAUC (EB: 692 (SD 101) mmol·L^{-1}·2 h^{-1}; ER: (757 (SD 107) mmol·L^{-1}·2 h^{-1}; P<0.001; Figure 2B), were greater during ER than EB and there was a trend for greater peak glucose concentrations during ER (EB: 7.93 (SD 1.52) mmol·L^{-1}; ER: 8.44 (SD 1.46) mmol·L^{-1}; P=0.073). Glucose time-to-peak was delayed during ER compared to EB.

There was no trial effect (P=0.920), but there were time (P<0.0001) and interaction (P<0.001) effects for serum insulin concentrations, with greater insulin concentrations at 2 h during ER compared to EB (P<0.05; Figure 2C). Serum insulin iAUC was greater during ER than EB (EB: 23335 (SD 10964) pmol·L^{-1}·2 h^{-1}; ER: 26094 (SD 10807) pmol·L^{-1}·2 h^{-1}; P<0.05; Figure 2D), but tAUC was not different between trials (EB: 31678 (SD 11598) pmol·L^{-1}·2 h^{-1}; ER: (32685 (SD 11987) pmol·L^{-1}·2 h^{-1}; P=0.487; Figure 2D). There were no differences between

trials for peak serum insulin concentrations (EB: 452 (SD 168) pmol·L^{-1}; ER: 433 (SD 163) pmol·L^{-1}; P=0.564) but time-to-peak was delayed during ER compared to EB.

There were time (P<0.0001), trial (P<0.01) and interaction (P<0.0001) effects for plasma NEFA concentrations, with greater plasma NEFA concentrations between 0-0.5 h during ER compared to EB (P<0.01; Figure 3A). Plasma NEFA tAUC was 45% greater during ER compared to EB (EB: 22.06 (SD 9.00) mmol·L^{-1}·2 h^{-1}; ER: 32.09 (SD 9.44) mmol·L^{-1}·2 h^{-1}; P<0.01; Figure 3B).

Serum glucose iAUC and pre-OGTT (0 h) plasma NEFA concentrations tended to be positively correlated (r=0.472; P=0.089), but serum glucose iAUC did not correlate with NEFA tAUC (r=-0.049; P=0.868). Serum glucose tAUC did not correlate with either plasma NEFA tAUC (r=0.112; P=0.703) nor pre-OGTT plasma NEFA concentrations (r=0.326; P=0.255).

Matsuda Index

The Matsuda Index of insulin sensitivity was not different between trials (EB: 7.50 (SD 4.75); ER: 7.93 (SD 5.06) P=0.603).

GLP-1 and GIP responses

There was a time effect (P<0.05), but no trial (P=0.219) or interaction (P=0.055) effects for plasma GLP-1 concentrations. GLP-1 tAUC was not different between trials (EB: 3207 (SD 1321) pmol·L^{-1}·2 h^{-1}; ER: 4123 (SD 3203) pmol·L^{-1}·2 h^{-1}; P=0.155; Figure 4B).

There was a time effect (P<0.0001), but no trial (P=0.473) or interaction (P=0.150) effects for plasma GIP concentrations. GIP tAUC was not different between trials (EB: 23874 (SD 10283) pmol·L^{-1}·2 h^{-1}; ER: 24287 (SD 10143) pmol·L^{-1}·2 h^{-1}; P=0.698; Figure 4D).

FGF-21 response

There was a time effect (P<0.01), but no trial (P=0.513) or interaction (P=0.763) effects for plasma FGF-21 concentrations. FGF-21 tAUC was not different between trials (EB: 8000 (SD 4038) pg·mL^{-1}·2 h^{-1}; ER: 7553 (SD 5171) pg·mL^{-1}·2 h^{-1}; P=0.511; Figure 5).

Discussion

The aim of this study was to determine the acute effects of 24 h severe energy restriction on indices of insulin sensitivity. The results demonstrate that postprandial glycaemic control is impaired, despite a reduction in HOMA2-IR after 24 h severe energy restriction. These findings may have implications for the efficacy of intermittent energy restriction diets, particularly for weight maintenance, where weight loss related improvements in insulin sensitivity might not be anticipated.

Undergoing short periods of severe energy restriction (consuming ~25% of EER) is a requisite component of an IER diet, and has been shown to be an effective method of reducing daily energy intake in lean[9,11] and overweight/ obese[10,14] populations. Three to twelve weeks of IER has been demonstrated to cause significant weight and fat mass losses, comparable to that achieved with moderate daily energy restriction of similar duration[8]. Importantly, several studies have reported improvements in fasting insulin sensitivity indexes after 4-6 months of IER[6,23]. In the current study, HOMA2-IR decreased 23% after 24 h of severe energy restriction (ER) compared to an adequate energy intake control trial (EB). However, in response to an oral glucose challenge, serum glucose tAUC ~was 9% greater (iAUC was ~78% greater) and serum insulin iAUC was ~12% greater, during ER compared to EB. In addition, peak serum glucose concentration was 6% greater and serum glucose remained elevated for longer, during ER. This data suggests that glycaemic control was impaired after a single 24 h period of severe energy restriction in a group of young, lean men.

These results could be explained by a simple alteration in substrate availability. A short period of severe energy restriction may deplete hepatic glycogen stores and reduce endogenous glucose production[24]. Consequently, circulating glucose and insulin are also reduced[25]. As HOMA2-IR is a product of fasting glucose and insulin concentrations, these acute metabolic changes that occur with severe energy restriction limit the validity of HOMA2-IR to assess insulin sensitivity in this context. The reduction observed in this and similar studies may reflect a reduced requirement for insulin secretion, rather than an improvement in insulin sensitivity *per se.* Similarly, despite increases in fed-state serum glucose and insulin concentrations during the OGTT, the composite Masuda Index of insulin sensitivity was unaffected by ER. This may be due to the incorporation of fasting glucose and insulin concentrations in the calculation of the index[26,27].

When exogenous glucose availability is low, insulin concentrations are also low, stimulating lipolysis to mobilise triglycerides for oxidation[28]. As evidenced in the current study, this leads to an increase in plasma NEFA concentrations, and previous studies, utilising a very similar energy restriction protocol, have also reported an increase in fat oxidation and a concomitant decrease in carbohydrate oxidation in both the fasted and postprandial state[10,11,14]. A consequence of increased fat oxidation is the accumulation of acetyl-CoA, NADH and citrate, which can inhibit both upstream (via inhibition of phosphofructo-kinase) and downstream (via inhibition of GLUT4 translocation and pyruvate dehydrogenase) glycolysis[29]. Elevated plasma NEFA concentrations have also been postulated to cause mitochondrial overload, resulting in incomplete fatty acid oxidation and the accumulation of toxic fatty acid intermediates, such as diacylglycerol and ceramide which may impair insulin signalling[30]. However, impairments in skeletal muscle insulin signalling are not a prerequisite for reduced muscle glucose uptake, and rapid impairments in the ability to process exogenous (ingested or infused) glucose might be explained by reduced glycolytic flux/ oxidative disposal. For

example, Lundsgaard *et al.*[31] reported that 3 days of overfeeding with carbohydrate, increased leg glucose uptake during a hyperinsulinemic-euglycemic clamp, whereas 3 days of high-fat overfeeding reduced glucose uptake despite normal insulin signalling. It was suggested that greater TCA influx from beta-oxidation-derived acetyl-CoA might explain the reduced glucose uptake in the absence of changes in insulin signalling. Evidence for this was provided by the observations that high fat diet adherence led to a significant decrease in total PDH-E1α protein content (the enzyme responsible for catalysing the conversion of pyruvate to acetyl-CoA) as well as increased Ser^{300} phosphorylation (i.e., reduced PDH activity) and increased glucose-6-phosphate accumulation[31]. Hence, in the context of the current study, elevated NEFA (a surrogate for increased lipolysis and greater dependency upon fat oxidation) likely decreased glucose uptake/ oxidation by a similar mechanism.

Several findings from the current study are analogous to a similar study which investigated the effects of 24 h severe energy restriction in overweight and obese subjects[14]. Postprandial insulin iAUC was greater after severe energy restriction in the current study, a finding that differs from Antoni et al.[14], but average time-to-peak insulin concentration appeared to be delayed after severe energy restriction in both studies, suggesting an impaired early-phase insulin response. Early-phase insulin has been shown to more potently lower blood glucose concentrations compared to late-phase insulin[32]. This might therefore explain the greater peak glucose concentrations observed after severe energy restriction in the current study and Antoni *et al.*[14]. Together, these findings demonstrate that 24 h severe energy restriction impairs glycaemic control in both lean (current study) and overweight/ obese[14] subjects, with both studies indicating that early-phase insulin response may be a casual factor.

This response is similar to the 'second meal effect', which describes an improved glycaemic response to a meal after consumption of glucose at a prior eating occasion[33]. It is thought that the impairment in early-phase insulin response observed with the 'second meal effect' is

mediated by prolonged exposure of the pancreatic islet cells to elevated NEFA concentrations, shown *in vitro* to inhibit insulin secretion[34]. Whilst this cannot be determined in the present study, plasma NEFA concentrations were greater prior to the OGTT during ER, indicating that plasma NEFA concentrations were also likely greater during ER in previous 24 h. This would suggest pancreatic islet cells were exposed to prolonged elevated plasma NEFA concentrations during ER, possibly leading to impaired early-phase response to the glucose load. This is partially supported by a tendency for a positive correlation between pre-OGTT plasma NEFA concentrations and serum glucose iAUC, and an apparent delay in time-to-peak insulin concentration during ER.

It is interesting to note that, despite several studies demonstrating an impairment in glycaemic control after severe energy restriction at rest, a recent study found that restricting carbohydrate intake after evening exercise improved post-prandial glycaemic control the following morning, compared to when carbohydrate was consumed in a quantity equal to that expended during exercise (90 min running at 70% VO2max)[35]. This is quite different to the present and previous studies, which have restricted total energy intake during periods of minimal physical activity. Under such conditions, energy restriction will have little influence on muscle glycogen content (the primary site of insulin-mediated glucose disposal). It also demonstrates that the so-called [acute] insulin sensitising effect of exercise centres on creating a 'sink' for glucose disposal. Further investigation is certainly necessary in this field as both exercise and dietary restriction are important components of successful weight management strategies[36].

There are several biological mechanisms involved in the regulation of energy homeostasis. GLP-1 and GIP are incretin hormones secreted rapidly from the intestine in response to food ingestion[37]. These hormones respond prior to nutrient absorption, and stimulate the secretion of insulin from the pancreas to assist with the disposal of glucose from the blood[37]. In the current study, whilst GIP was elevated after consumption of the glucose solution in both trials,

severe energy restriction did not appear to differentially affect circulating incretin hormone concentrations, compared to an energy balance control trial. Plasma GLP-1 and GIP concentrations were similarly unaffected by short-term (seven days) high-fat (65% of energy) overfeeding (~150% EER), despite subjects in this study also exhibiting impaired postprandial glycaemic control[38]. It should be noted that total GLP-1 and GIP were assessed in the current study and Parry *et al.*[38], as oppose to the biologically active ($GLP\text{-}1_{7\text{-}36}$; $GIP_{1\text{-}42}$) form. However, assessing total GLP-1/ GIP is considered appropriate for estimating the secretion of active GLP-1/ GIP from the intestine[39]. None-the-less, these studies suggest incretin hormones are resistant to short-term fluctuation in energy balance and are unlikely to be involved in acute impairments in glycaemic regulation in these settings.

FGF21 is a novel hepatokine secreted in response to fasting and feeding cycles[39], which positively correlates with obesity, type-2 diabetes, insulin resistance and impaired glucose tolerance in humans[40,41]. FGF21 is thought to be involved in coordinating the adaptive response to energy restriction via several mechanisms, such as encouraging ketosis, lowering blood glucose, increasing insulin sensitivity, and potentially modulating appetite regulation via the Agouti-related peptide and neuropeptide Y pathways[42]. It should be noted that most studies that have found a physiological effect of energy restriction on FGF21 have been rodent studies, with FGF21 concentrations shown to increase rapidly (within 6 h) after the onset of fasting[43]. In contrast, human studies have observed no change in fasting or postprandial (OGTT) plasma FGF21 concentration after 16 h fasting[44], and one study found that it may take 7-10 days of fasting to elicit an increase in FGF21 in humans[45]. In line with this, the current study found no effect of 24 h severe energy restriction on fasting or postprandial plasma FGF21 concentrations. This strengthens evidence that nutritional regulation of FGF21 differs between rodents and humans[44].

Whilst the exact mechanism of metabolic dysregulation may be elusive at present, results from several acute studies now indicate that a short period of severe energy restriction leads to a subsequent period of impaired glycaemic control[9-11,14]. The clinical significance of these findings cannot be extrapolated from these acute studies, but oscillating postprandial glucose concentrations are thought to directly contribute to the development of cardiovascular disease[46], and a delay in the postprandial glucose curve is associated with impairments in β-cell function and insulin secretion[47]. Whether these acute impairments in glycaemic control are improved or exacerbated with multiple restriction and refeeding cycles is not fully known. The only available data on long-term IER is from a rodent study, which found that 32 weeks of intermittent fasting and refeeding promoted redox imbalance, oxidative modification of insulin receptors and a progressive decline in glucose tolerance, despite an initial improvement in glucose tolerance after 4 weeks[48]. These data suggest that irregular feeding patterns leading to increased exposure to elevated blood glucose concentrations may have the potential to impair insulin-mediated glucose uptake.

Future studies should investigate the long-term effects of an IER diet on glycaemic control in humans, including the dynamic assessment of glucose uptake and oxidation, as alterations may not be evident in the fasted state[16]. A recent study compared the effects of achieving ~5% weight loss via IER (consuming ~25% EER on two consecutive days, with a self-selected adequate-energy diet on the remaining five days of the week) or CER (consuming 2510 kJ below EER for seven days of the week), in a group of overweight/ obese subjects. Fasted variables showed no difference between the dieting methods, however postprandial insulin sensitivity markers revealed a significant reduction in C-peptide after IER whilst C-peptide was unaltered after CER[50]. C-peptide is secreted in equimolar amounts to insulin, but undergoes minimal extraction at the liver so may be a more robust measure of insulin secretion than circulating insulin concentrations[51]. This change in C-peptide did not appear to influence

postprandial glycaemic control, and comparable reductions in postprandial insulin concentrations were observed with both diets. However, this finding does indicate differences in mechanisms of action between IER and CER, potentially suggesting IER may improve insulin sensitivity to a greater extent than CER after semi-chronic (~2 months) adherence. This warrants further investigation, as does identifying the effects of long-term IER in the absence of weight loss. This will be crucial for determining whether IER can be used as an effective weight maintenance strategy, with this being an important target for reducing rates of obesity related comorbidities in the future[3].

In conclusion, this study has demonstrated that 24 h severe energy restriction leads to impaired postprandial glycaemic control, which cannot be detected in the fasted state. These findings have implications for IER diets and demonstrate the need for future studies to identify the accumulative impact of repeated episodes of short-term severe energy restriction on glycaemic control in lean individuals.

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LJJ and CJH, with assistance from DJS, JAK and JAS. All authors read and approved the manuscript.

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500 **Tables**

501 **Table 1.** Energy and macronutrient intake at each meal (meal time in brackets) during day 1

	Energy balance (EB)		**Energy restriction (ER)**	
Breakfast (08:00)				
	Mean	SD	Mean	SD
Protein (g)	14	1	0	0
Carbohydrate (g)	89	7	0	0
Fat (g)	9	1	0	0
Fibre (g)	1	0	0	0
Energy (kJ)	2097	163	0	0
Foods	Cereal, semi-skimmed milk, orange juice		Water	
Lunch (12:00)				
	Mean	SD	Mean	SD
Protein (g)	46	3	36	3
Carbohydrate (g)	72	6	7	2
Fat (g)	29	3	3	1
Fibre (g)	5	0	2	0
Energy (kJ)	3124	243	874	68
Foods	White bread, mayonnaise, chicken, lettuce, tomato, red pepper, balsamic vinegar, chocolate-chip cookies		Chicken, lettuce, tomato, red pepper, balsamic vinegar	
Snack (16:00)				
	Mean	SD	Mean	SD
Protein (g)	5	0	0	0
Carbohydrate (g)	31	2	0	0
Fat (g)	11	1	0	0
Fibre (g)	1	0	0	0
Energy (kJ)	1040	80	0	0
Foods	Yoghurt, cereal bar		NA	
Dinner (19:30)				
	Mean	SD	Mean	SD
Protein (g)	45	3	33	2
Carbohydrate (g)	138	11	55	4
Fat (g)	28	2	7	1
Fibre (g)	5	0	3	0
Energy (kJ)	4180	326	1748	136
Foods	Pasta, Bolognese sauce, chicken, olive oil, chocolate-chip cookies		Pasta, Bolognese sauce, chicken, olive oil	

Total

	Mean	SD	Mean	SD
Protein (g)	110	7	69	4
Carbohydrate (g)	329	25	62	6
Fat (g)	78	8	10	1
Fibre (g)	12	1	4	0
Energy (kJ)	10441	812	2622	204

Table 2. Blood variables after 24 h of an energy balance (100% EER; EB) or severely energy restricted diet (25% EER; ER).

	EB				ER				Interaction
	-24 h		0 h		-24 h		0 h		effect
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Glucose ($mmol \cdot L^{-1}$)	5.4	0.4	5.5	0.6	5.3	0.3	5.0 † *	0.4	0.002
Insulin ($pmol \cdot L^{-1}$)	76	32	70	30	76	34	55 † *	20	0.029
HOMA2-IR	2.68	1.23	2.49	1.36	2.63	1.26	1.79 † *	0.77	0.022
NEFA ($mmol \cdot L^{-1}$)	0.37	0.12	0.43	0.19	0.32	0.16	0.69 † *	0.22	0.001
GLP-1 ($pmol \cdot L^{-1}$)	27	14	27	11	30	20	32	14	0.628
GIP ($pmol \cdot L^{-1}$)	59	26	50	31	77	47	48 *	22	0.096
FGF21 ($pg \cdot mL^{-1}$)	102	63	71 *	39	118	85	65 *	47	0.098

HOMA2-IR, homeostatic model of insulin resistance; NEFA, non-esterified fatty acids; GLP-1, glucagon-like peptide-1; GIP, glucose-dependant insulinotropic peptide. † indicates values were significantly different to EB ($P<0.05$); * indicates values are significantly different to -24 h during the corresponding trial ($P<0.05$).

Figure Legends

Figure 1. Bar chart (A) represents mean homeostatic model of insulin resistance (HOMA2-IR) values calculated from overnight fasting serum glucose and insulin concentrations before (-24 h) and after (0 h) consumption of a 24 h energy balanced (EB; ■) or energy restricted (ER; □) diet. Data are mean with vertical error bars representing standard deviation. Line graph (B) shows individual HOMA2-IR values at 0 h during EB (■) and ER (○). † indicates values were significantly different to EB at 0 h; # indicates values were significantly different to -24 h during ER (P<0.05).

Figure 2. Serum glucose (A) and insulin (C) concentrations during a 2 h oral glucose tolerance test (OGTT) conducted after consumption of a 24 h energy balanced (EB; ■) or energy restricted (ER; ○) diet. Bar charts represent serum glucose (B) and insulin (D) incremental (iAUC) and total (tAUC) area under the curve during the OGTT (0-2 h) for EB (■) and ER (□). Data are means with vertical error representing standard deviation. † indicates iAUC values were significantly different to EB; # idicates tAUC values were significantly different to EB (P<0.05).

Figure 3. Plasma non-esterified fatty acid (NEFA) (A) concentrations during a 2 h oral glucose tolerance test (OGTT) conducted after consumption of a 24 h energy balanced (EB; ■) or energy restricted (ER; ○) diet. Bar chart represents plasma NEFA (B) total area under the curve during the OGTT (0-2 h) for EB (■) and ER (□). Data are means with vertical error representing standard deviation. † indicates values were significantly different to EB (P<0.05).

Figure 4. Plasma glucagon-like peptide-1 (GLP-1) (A) and glucose-dependant insulinotropic peptide (GIP) (C) concentrations during a 2 h oral glucose tolerance test (OGTT) conducted after consumption of a 24 h energy balanced (EB; ■) or energy restricted (ER; ○) diet. Bar charts represent plasma GLP-1 (B) and GIP (D) total area under the curve during the OGTT

(0-2 h) for EB (■) and ER (□). Data are means with vertical error representing standard deviation.

Figure 5. Plasma fibroblast growth factor-21 (FGF21) (A) concentrations during a 2 h oral glucose tolerance test (OGTT) conducted after consumption of a 24 h energy balanced (EB; ■) or energy restricted (ER; ○) diet. Bar chart represent plasma FGF21 (B) total area under the curve during the OGTT (0-2 h) for EB (■) and ER (□). Data are means with vertical error representing standard deviation.