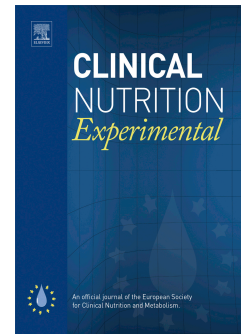


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A 7-day high protein hypocaloric diet promotes cellular metabolic adaptations and attenuates lean mass loss in healthy males.

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Abstract: Mitochondrial quantity and density are associated with increased oxidative metabolism. It has been demonstrated that a hypocaloric high fat/low carbohydrate (HF/LC) diet can up-regulate transcriptional markers of mitochondrial biogenesis; this was yet to be explored *in vivo* subsequent to a high protein/low carbohydrate (HP/LC) diet. Thus the aims of the study were to explore such diets on transcriptional markers of mitochondrial biogenesis, body composition and resting metabolic rate (RMR). Forty-five healthy male participants were randomly assigned one of four intervention diets: eucaloric high protein (PRO-EM), hypocaloric high protein (PRO-ER), eucaloric high carbohydrate (CHO-EM) or hypocaloric high carbohydrate (CHO-ER). The macronutrient ratio of the high protein diet and high carbohydrate diets was 40:30:30% and 10:60:30 % (PRO:CHO:FAT) respectively. Energy intake for the hypocaloric diets were calculated to match resting metabolic rate. Participants visited the laboratory on 3 occasions each separated by 7 days. On each visit body composition, resting metabolic rate and a muscle biopsy from the vastus lateralis was collected. Prior to visit 1 and 2 habitual diet was consumed which was used as a control, between visit 2 and 3 the intervention diet was consumed continuously for 7-days. In the PRO-ER group a significant increase in AMPK, PGC-1 α , SIRT1 and SIRT3 mRNA expression was observed, the increase in AMPK, PGC-1 α was also than the other groups ($p < 0.05$). No change was observed in any of the transcriptional markers in the other 3 groups. Despite ~30 % reduction in calorie intake no difference in lean mass (LM) loss was observed between the PRO-ER and CHO-EM groups. The results from this study demonstrated that a 7-day a high protein hypocaloric diet resulted in increased AMPK, SIRT1 and PGC-1 α mRNA expression at rest, also in a hypocaloric state increased dietary protein attenuated LM mass loss.

Keywords: High protein diet, PGC-1 α , AMPK, hypocaloric, low carbohydrate diet

Introduction

Mitochondria are responsible for energy production via fatty-acid oxidation, Krebs cycle and oxidative phosphorylation. Mitochondria quantity and density has been linked with increased endurance performance [1], reduction in type 2 diabetes and improved insulin sensitivity [2-6]. A seminal paper from Puigserver, Wu [7] first described peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1- α), in the subsequent years PGC-1 α had been identified as key for the regulation and co-activation of mitochondrial biogenesis [8].

Both AMP-activated protein kinase (AMPK) and silent information regulator T1 (SIRT1) mediated deacetylation [9] have been demonstrated to regulate expression of PGC-1 α in human muscle [10]. A substantial body of evidence suggests that SIRT1 expression responds to decreases in nutrient availability [11] and increases in energy expenditure [12]. Similarly AMPK is stimulated by cellular stressors that causes a depletion of adenosine triphosphate (ATP) and elevation of AMP, such as calorie restriction [13], hypoglycaemia [13] and exercise [14]. Subsequently, increases in PGC-1 α mRNA expression can be observed in direct response to such stressors [13]. Acute hypocaloric HF/LC diets (50% fat, 30% carbohydrate and 20% protein) significantly increases AMPK phosphorylation and PGC-1 α deacetylation, this is not observed subsequent a hypocaloric low fat high carbohydrate (LF/HC) diet (20:60:20 %) [15], suggesting increased dietary carbohydrate intake prevents the

activation of the AMPK/SIRT – PGC-1 α axis in skeletal muscle that would otherwise be activated by a low calorie diet.

The preservation of lean mass is important for the maintenance of quality of life [16], it is generally accepted that weight loss strategies which preserve LM are preferential to those that results in skeletal muscle atrophy [17]. During hypocaloric diet-induced weight loss approximately 20 – 30% of mass lost is lean mass (LM) [18], increasing dietary protein is one method which has been demonstrated to attenuate skeletal muscle atrophy in a hypocaloric state [19]. The manipulation of carbohydrate intake as a regulator of weight maintenance / loss is well documented demonstrating improved lipid profile and fat oxidation [20, 21]. However, the majority of literature manipulates dietary fat – protein remains constant. A small number of metabolic perturbation murine studies have manipulated protein intake and it has been shown that high protein intake (35 % protein) increases PGC-1 α expression relative to a control diet (15% protein) [22].

In this field most research focuses on increasing dietary fat to restrict carbohydrate intake. However it is well documented the increased protein intake can attenuate LM loss and may be a preferable choice during weight loss. The impact high protein diets have on transcriptional markers of mitochondrial biogenesis is not fully explored. This study was designed to investigate the impact of increasing protein independently or alongside calorie restriction on metabolic adaptation, body composition and RMR.

Materials and Methods

92

93 Participants

94

95 Forty-eight healthy males volunteered to participate in the study with forty-five
96 completing (mean \pm SD: age 26.0 ± 5.1 years; body mass 74.9 ± 10.2 kg; height
97 179.5 ± 5.9 cm). One participant was removed due to non-dietary adherence and
98 two did not complete the study. Participants were initially screened against pre-
99 determined criteria to ensure they met the inclusion criteria and were free from any
100 medical condition that would preclude participation in the study. Participants could
101 not be following a restrictive dietary regime (vegetarian, vegan) or suffer from any
102 food allergies/intolerances. Participants could not be participating in physical activity
103 > 2 times per week and could not be consuming or have consumed any dietary or
104 protein supplements in the previous 2 weeks. The experimental procedures and
105 potential risks associated with the study were explained and the participants gave
106 written informed consent prior to participation. None of the participants had a history
107 of any neurological disease or musculoskeletal abnormality. The study was approved
108 by the University of Hertfordshire School of Life and Medical Sciences ethics
109 committee LMS/PG/UH/00196.

110

111 Study Protocol

112

113 In a randomised repeated-measures study design, the participants were assigned to
114 one of four groups: energy matched high protein (PRO-EM, $n = 11$), energy
115 restricted high protein (PRO-ER, $n = 12$), energy matched high carbohydrate (CHO-
116 EM, $n = 12$) or energy restricted high carbohydrate (CHO-ER, $n = 10$) (Table 1). The

participants attended the laboratory on three occasions, each separated by 7 days. Before visit 1 (baseline) and visit 2 (pre) all participants remained on their habitual diet, between visits 2 and visit 3 (post) the participants consumed the randomly assigned intervention diet. All experimental procedures were completed in the same order at the same time of the day. The baseline and pre assessments were used as a control to demonstrate stability of the measures assessed, where no differences were observed between these measures the pre measures were used for analysis.

Table 1 Prescribed macronutrient breakdown for each group.

	Prescribed calorific intake	Carbohydrate (%)	Protein (%)	Fat (%)
PRO-EM	Matched to estimated daily energy requirements	30	40	30
PRO-ER	Restricted to resting metabolic rate	30	40	30
CHO-EM	Matched to estimated daily energy requirements	60	10	30
CHO-ER	Restricted to resting metabolic rate	60	10	30

(% = percentage of total daily calorific intake)

Experimental protocol.

Following an overnight fast the participants arrived at their allocated timeslot (start time range: 07:30 – 09:30 am) each testing visit. The participants were requested to arrive at the laboratory using the same mode of transport. Morning physical activity was not permitted. Prior to the pre and baseline visits the participants were

completed a standardised 4 day food diary. The food diary was visually assessed for accuracy by the investigator and further detail was requested if required. An assessment of body composition (DXA scan) was then undertaken, followed by the measurement of resting metabolic rate. Finally a micro-muscle biopsy was collected (Figure 1).

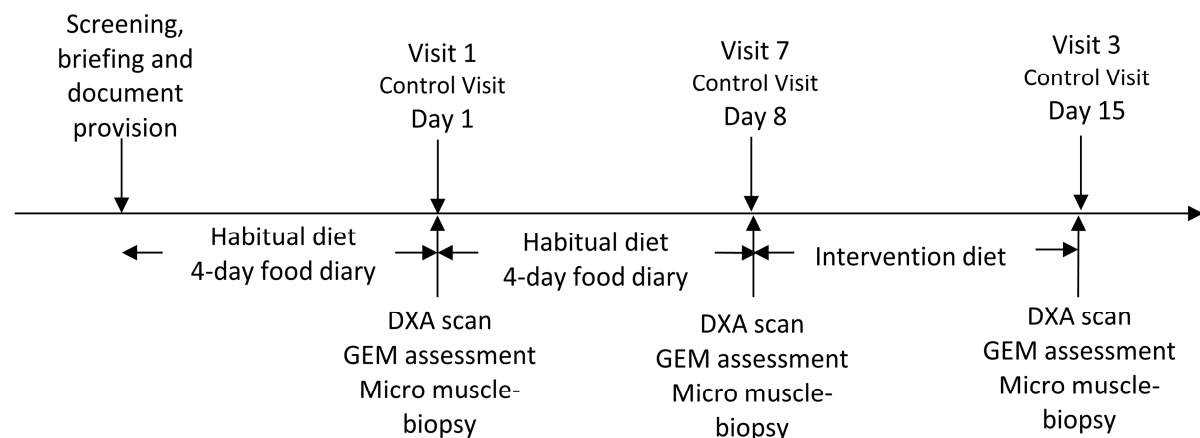


Figure 1. Schematic of experimental protocol.

Measurement of habitual dietary intake

A 4 day food diary using estimated household measures was used to collect habitual dietary intake prior to the pre and baseline visits. In the study briefing the participants were provided with a comprehensive example of the food diary and were instructed on how to complete it. The importance of accuracy and detail were emphasised, as was the importance of maintaining current dietary habits and documenting all food and drink consumed. Participants were requested to record intake on 3 week days and 1 weekend day each week, a total of 8 days dietary intake was collected over the 2 week prior visit 1 and visit 2.

153

154 Body composition assessment: Dual energy x-ray absorptiometry (DXA) scan

155

156 All metal and piercings were removed and fasted body mass (BM) was measured
157 using a digital column scale (seca 704, seca Ltd., Hamburg, Germany). A full body
158 DXA scan (GE Lunar iDXA, GE Healthcare, UK) was performed in accordance with
159 the manufacturer's guidelines for patient positioning and was analysed using
160 enCORE Software, version 14.10 (GE Healthcare, Bucks, UK). The scan measured
161 BM, total LM and total fat mass (FM). All scans were performed by a fully trained
162 operator. In addition to regular machine calibration, a standard quality assurance
163 procedure was performed each testing day.

164

165 Resting metabolic rate (RMR)

166

167 RMR was measured using indirect calorimetry (coefficient of variation = 1.48 %). All
168 assessments were completed in a temperature controlled environmental chamber
169 (temperature set to 24°C). The participants arrived at the laboratory in the morning
170 following an overnight fast. The participants were requested to expel as little energy
171 as possible prior to arrival. The participant lay in a supine position on a couch and a
172 large towel was placed over their body to maintain comfort. Once comfortable a
173 metabolic hood (Gas Exchange Measurement (GEM), GEMNutrition Ltd, UK) was
174 placed over the participants head. The participant was instructed to relax, breathe
175 normally and not to move or fall asleep. The GEM was set to collect respiratory bins
176 every 60 seconds for 20 minutes, the data analysed was from the final 5 minutes of

collecion. Throughout the test the participant was observed through a window to check for adherence. RMR was calculated using the modified Weir equation [23]:

$$\text{RMR} = 1.44 \times (3.9 \times \dot{V}O_2 + 1.1 \times \dot{V}CO_2)$$

Respiratory exchange ratio was calculated to assess for changes in resting substrate utilisation by dividing the $\dot{V}CO_2$ value ($L \cdot \text{min}^{-1}$) by the $\dot{V}O_2$ value ($L \cdot \text{min}^{-1}$) from each minute bin collected on the GEM.

Micro muscle biopsy

Muscle biopsies were obtained from the midpoint on the lateral aspect of the right vastus lateralis muscle. The biopsy site was cleaned using Betadine (Pardue Products, USA) and samples were obtained under local anaesthesia, 2 ml of 1 % v/v without adrenaline Lidocaine Hydrochloride (Hameln Pharmaceuticals: cat. no PL01502) was injected into the subcutaneous fat of the selected biopsy site. Once the anaesthetic had taken effect (~5 minutes), a 14 gauge co-axial was inserted ~2 cm into the muscle, a disposable core biopsy instrument (TSK Stericut Biopsy Needle 14 Gauge, TSK Laboratories, Japan) was then inserted through the co-axial and activated. A single biopsy pass was used collecting approximately 10 - 20 mg of muscle tissue. The biopsy instrument was immediately removed from the leg and within 10 seconds the muscle tissue was removed from the biopsy instrument using a sterile scalpel and flash frozen in liquid nitrogen. The muscle sample was placed in an RNase free plastic vial and stored at -80 OC under the HTA license number 12202 until analysis.

Dietary prescription

For the two energy restricted (ER) groups the participants were provided an intervention diet with an energy intake that met RMR. RMR was calculated from the GEM result attained from Visit 1. For the energy matched (EM) groups, energy intake was calculated according the World Health Organisation [24] lifestyle dependent physical activity multiplier:

$$\text{EI} = \text{PAF} \times \text{RMR}$$

EI = Energy intake, PAF = physical activity factor, RMR = resting metabolic rate

RMR was attained from the GEM result on visit 1, and PAF was selected following a series of lifestyle questions concerning daily activity levels, type of work and exercise activity (Table 2).

Table 2. Lifestyle dependent physical activity multiplier used to calculate energy intake [24].

Activity level	Calculation
Low	1.53 x RMR
Moderate	1.77 x RMR
Active	2.25 x RMR

A total of 34 (17 high carbohydrate, 17 high protein) different diet menus were created starting at 1,075 kcal, increasing by 150 kcal for each different diet up to 3,325 kcal. Participants were assigned the diet closest to their calculated

interventional energy intake. The greatest difference between estimated intervention diet and prescribed diet was 75 kcal per day.

All intervention meals were created by Soulmatefood® (Waterfoot, Lancashire, UK) and directly delivered to the participant's door (Table 3). Each participant received two deliveries; the first delivery contained three day's food, the second four day's food. Each day consisted of 5 pre packaged/cooked meals (breakfast, morning snack, lunch, afternoon snack and dinner). Included with the delivery was a daily menu with consumption instructions. The participants were required to refrain from consuming any additional food and just drink water or fluids free from caffeine or energy.

Table 3. Prescribed daily calorie intake and macronutrient breakdown for each group throughout the 7-day intervention.

Group	Dietary intake (kcal•day ⁻¹)	CHO (•day ⁻¹)		PRO (•day ⁻¹)		FAT (•day ⁻¹)	
		g	g•kg ⁻¹	g	g•kg ⁻¹	g	g•kg ⁻¹
PRO-EM	2828 ± 331	217 ± 26	3.0 ± 0.5	289 ± 34	4.0 ± 0.7	96 ± 11	1.3 ± 0.2
PRO-ER	1876 ± 116	143 ± 9	1.9 ± 0.3	191 ± 12	2.5 ± 0.4	64 ± 4	0.8 ± 0.1
CHO-EM	2881 ± 213	444 ± 33	6.1 ± 0.6	75 ± 6	1.0 ± 0.1	97 ± 7	1.3 ± 0.1
CHO-ER	1735 ± 246	267 ± 37	3.7 ± 0.7	45 ± 6	0.6 ± 0.1	59 ± 8	0.8 ± 0.2

Quantitative PCR (qPCR)

242 *RNA Isolation:* Human muscle biopsies were homogenised in 700 µL MagNA Pure
243 LC RNA Isolation Tissue Lysis buffer (Roche, Mannheim, Germany) in Roche
244 MagNA Lyser Green bead tubes at 6500 rpm for 30 seconds. After homogenisation,
245 the tubes were centrifuged for 10 min at 13,000g before 350 µL of each homogenate
246 was used for RNA extraction. Total RNA was extracted using the MagNA Pure 96
247 Cellular RNA Large Volume Kit on a MagNA Pure 96 (Roche, Mannheim, Germany),
248 in an elution volume of 50 µL according to the manufacturer's protocol. RNA was
249 stored at -80 °C. RNA concentrations were determined (A260) using a NanoQuant
250 plate on a Tecan Infinite 200PRO. RNA quality (RNA integrity number equivalent,
251 RINe) was assessed using RNA ScreenTape on a 2200 TapeStation (Agilent, Santa
252 Clara, USA).

253
254 *Reverse Transcription:* As a result of the large sample number and the automated
255 process, RNA concentration was not taken into account for each individual reaction.
256 14 µL of each RNA sample was reverse transcribed in a total volume of 20 µL using
257 the High capacity cDNA reverse transcription kit (without RNase inhibitor, Applied
258 Biosystems, Thermo Fisher, Loughborough, UK). The average RNA concentration
259 across the 142 samples was 32.4 ng•µL⁻¹ equating to an average of 453ng
260 RNA/reverse transcription reaction. Reactions were performed in 96 well PCR plates
261 on a PTC-225 Peltier thermal cycler (MJ Research, Quebec, Canada) using the
262 following profile; 25°C for 10 minutes, 37°C for 60 minutes, 85°C for 5 minutes, 4°C
263 hold. Minus RT control reactions were set up for 14 samples, in which the RNA
264 component was replaced with nuclease free water (Ambion, Fisher Scientific,
265 Loughborough, UK).

266

qPCR: For quantitative real-time PCR (qPCR), Human TaqMan® gene expression assays were purchased as a 20X assay ready stock from Life Technologies (Carlsbad, USA) (primers 18 mM and probes 5 mM) (Table 4). 1 µL cDNA was added to each qPCR reaction mixture which also contained gene expression assay mix (primers 900 nM final, probe 250 nM final), LightCycler 480 Probes Master and nuclease free water to give a 5 µL total reaction volume. Reactions were prepared in white multiwell 384 plates (Roche, Mannheim, German) using a Mosquito HV (TTP Labtech, Melbourn, UK). The plates were sealed using optical seals (Roche, Mannheim, Germany) and centrifuged at 290g for 2 min before being run on a LightCycler® 480 instrument (Roche, Mannheim, Germany) with the following thermal cycling parameters: initial de-naturation step 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 10 s and primer annealing/extension at 60°C for 30 s. A cooling step at 40°C for 30 s was the final stage of the run.

Table 4. Taqman Assays on Demand information (Applied Biosystems)

Gene name	Gene abbreviation	Assay on Demand number
Silent information regulator-T1 (SIRT1)	SIRT1	Hs01009005_m1
Silent information regulator-T3 (SIRT3)	SIRT3	Hs00953477_m1
AMP-activated protein kinase 1 (AMPK)	PRKAA1	Hs01562315_m1
Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α)	PPARGC1	Hs01016719_m1
Peroxisome proliferator-activated receptor delta (PPARδ)	PPARδ	Hs00987011_m1

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	GAPDH	Hs99999905_m1
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The crossing point (Cp) value for each sample was calculated using the second derivative maximum method applied directly by the Roche software to the real-time amplification curves. This value represents the cycle at which the increase of fluorescence is highest and where the logarithmic phase of a PCR begins.

Amplification Efficiency of qPCR assays: The efficiency of each of the Taqman® assays on demand was confirmed by performing standard curves in a 384 well qPCR assay on the LightCycler® 480 under the same conditions as already described. Six point standard curves with 1 in 10 serial dilutions were prepared in nuclease free water for human plasmid DNA for each of the genes being tested alongside Human genomic DNA with a top concentration of 3e5 copies/μL and 1e5 copies/μL respectively. qPCR reactions were performed in triplicate and the amplification efficiency calculated for each Taqman® assay on the basis of the equation $E = (10^{-1/\text{slope} - 1}) \times 100$ with the logarithm of the template concentration on the x axis and the average Cp plotted on the y axis.

Statistical analysis

The RMR and body composition data were analysed using a 2-way repeated measures ANOVA with dietary intake (between) and time point (within) as the main variables. Significant interaction effects were analysed with the use of LSD post hoc test to determine the location of the pairwise differences within (time) and/or between

(dietary intake). The muscle biopsy qPCR analysis raw Cp values were normalised to the house keeper gene (GAPDH) using an analysis of covariance method, before relative fold change over baseline (visit 1) were calculated using $2^{-\Delta\Delta CT}$. Visit one and visit two were used as control. Statistical analysis of the qPCR fold change data was assessed using a paired sample t-test to determine the difference between the control trials, where no difference was observed a paired sample test was run to assess pre-post intervention relative fold change (time) difference. Pre – post intervention group difference was assessed using a one way ANOVA. Statistical significance was set at $p < 0.05$. Statistical analysis was conducted using the statistical package for the Social Science software program (SPSS; version 22, IBM, Armonk, NY). All data are presented mean \pm SEM unless specified.

Results

Habitual dietary intake

No time or group interactions were observed for habitual total calorie intake or macronutrient breakdown (Table 5). The mean daily calorific intake across all groups was $2,340 \pm 473 \text{ kcal}\cdot\text{day}^{-1}$ (range $2,192 \pm 574$ to $2423 \pm 330 \text{ kcal}\cdot\text{day}^{-1}$).

Table 5. Average daily habitual calorific intake and macronutrient breakdown for each group prior to intervention diet. (mean \pm SD and percentage (%) of total energy intake).

Mean daily	CHO	PRO	FAT
calorie intake			

	(kcal•day ⁻¹)	g•day ⁻¹	%	g•day ⁻¹	%	g•day ⁻¹	%
PRO-EM	2192 ± 173	232 ± 22	42.4	95 ± 6	17.2	86 ± 6	35.2
PRO-ER	2391 ± 133	267 ± 16	44.7	100 ± 8	16.7	93 ± 5	35.0
CHO-EM	2355 ± 152	274 ± 23	46.6	103 ± 9	17.5	89 ± 8	33.8
CHO-ER	2407 ± 107	307 ± 16	51.7	97 ± 7	16.0	84 ± 5	31.4

A total of 8 days dietary intake was collected over the 2 week prior visit 1 and visit 2; participants were requested to record intake on 3 week days and 1 weekend day each week using the estimated household measures method. (CHO = carbohydrate, PRO = protein, FAT = fat). % = percentage of dietary intake

Gene expression

No significant difference was observed between TP1 and TP2 in any of the genes, thus it can be assumed that any post intervention changes observed were due to the intervention rather than the internal variability of the measure.

No significant group interactions were observed in any of the genes, however it is important to note trends towards group interactions were observed within both PGC-1 α ($f = 2.654$, $p = 0.063$) and AMPK ($f = 2.771$, $p = 0.056$) expression. Within the PRO-ER group significant pre-post intervention time point difference was observed in PGC-1 α (fold increase = 1.27, $p = 0.0402$), AMPK (fold increase = 2.09, $p = 0.027$), SIRT1 (fold increase = 1.50, $p = 0.026$) and SIRT3 (fold increase = 1.19, $p = 0.010$) mRNA expression). No time point gene expression changes were observed in any other dietary group (Figure 2 A-E), or in the expression of PPAR mRNA.

A significant change in LM was observed post intervention as were significant group difference. Post intervention 3 or the groups lost LM ($p < 0.05$) (Figure 3A), the CHO-

ER losing the greatest amount (-1.26 ± 0.14 kg), the PRO-ER losing -0.82 ± 0.3 kg and CHO-EM losing -0.53 ± 0.19 kg respectively. LM was maintained in the PRO-EM group (-0.21 ± 0.17 kg). The CHO-ER group lost significantly more LM than both the PRO-EM and CHO-EM groups ($p < 0.05$), no difference in LM was observed between the PRO-ER and CHO-EM groups.

The PRO-ER and CHO-ER groups lost greater ($p < 0.05$) BM than both CHO-EM and PRO-EM (Figure 3B). No difference was observed between groups matched for energy intake. All groups exhibited a significant loss in FM ($p < 0.01$) post 7-days dietary intervention (Figure 1B). The PRO-ER lost the greatest amount of FM (-0.99 ± 0.11 kg) and the CHO-EM group lost the least (-0.50 ± 0.14 kg). The difference between these two groups was the only significant group difference observed ($p < 0.05$),

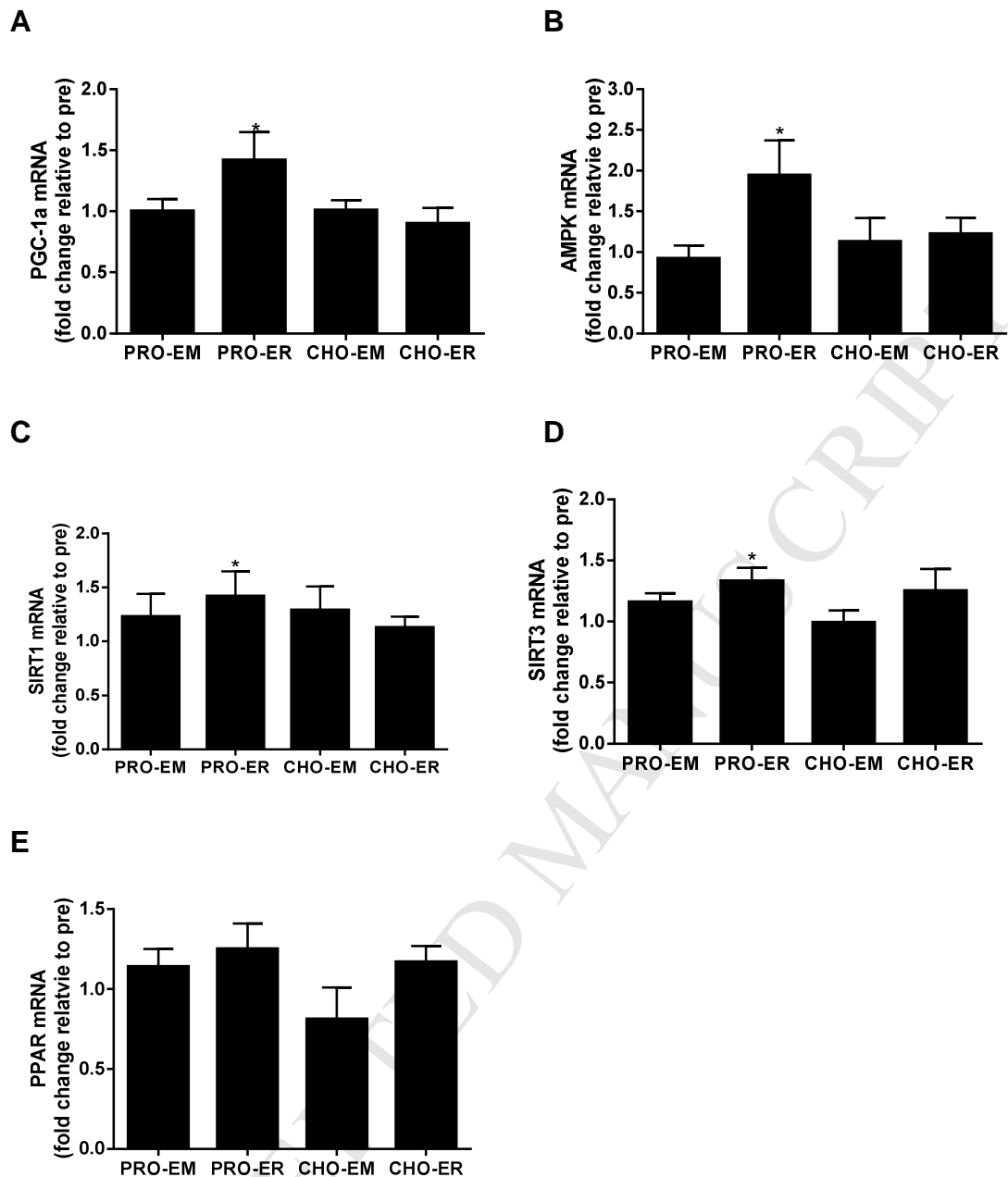


Figure 2. Effect of feeding 7-days diet intervention on mRNA expression at rest.

Effect of 7-days high protein energy matched (PRO-EM), high protein energy restricted (PRO-ER), high carbohydrate energy matched (CHO-EM) or high carbohydrate energy restricted (CHO-ER) diet in healthy lean individuals on PGC-1 α (A), AMPK (B), SIRT1 (C), SIRT3 (D) and PPAR (E) mRNA expression. Muscle samples were obtained following an overnight fast prior to the intervention and repeated again following 7-days dietary intervention (Post). Values are expressed as fold change (pre to post) after normalization to the reference gene (GAPDH) and are reported as the mean \pm SE. For abbreviations of genes see Table 4. *p < 0.05 versus Pre intervention.

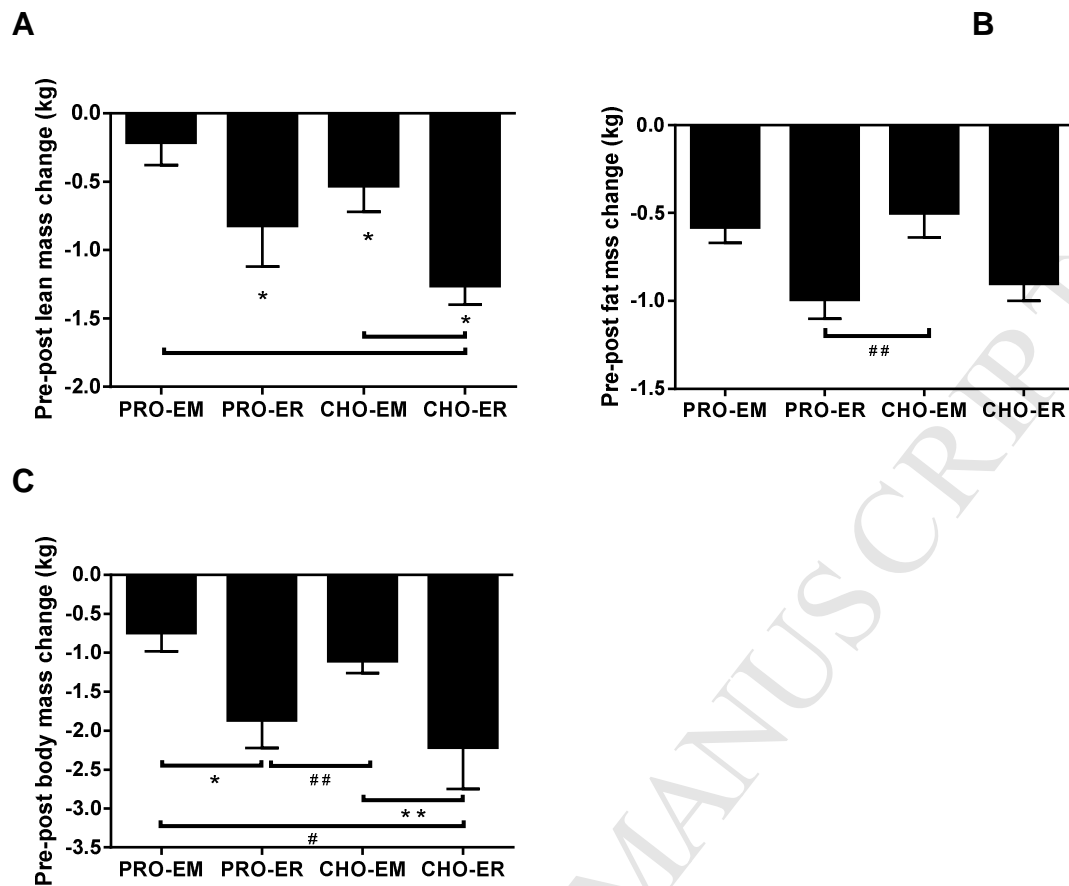


Figure 3. Effect of either 7-days continuous high protein energy matched (PRO-EM), high protein energy restricted (PRO-ER), high carbohydrate energy matched (CHO-EM) or high carbohydrate energy restricted (CHO-ER) diet on lean mass (kg) (A), fat mass (kg) (B) and body mass (kg) (C) at rest in lean healthy individuals. The data presented as mean \pm SEM; * denotes significant difference between PRO-EM and PRO-ER, ** denotes significant difference between CHO-EM and CHO-ER, # denotes significant difference between PRO-EM and CHO-ER and ## denotes significant difference between PRO-ER and CHO-EM. ($p < 0.05$).

366

367

368 A reduction in BM was observed post intervention in all groups ($p < 0.01$, Figure 3C).

369 The PRO-EM group lost the least BM (-0.74 ± 0.24 kg) whereas the CHO-ER lost the
 370 greatest (-2.21 ± 0.17 kg). Significant post intervention group differences were

371 observed ($p < 0.01$), this was not seen in grouped matched for energy intake,

372 however both ER groups lost significantly more BM than EM groups.

No time or group interaction effects were observed in RMR (Table 6). Mean RMR across all groups pre was 1910 ± 180 kcal vs. 1894 ± 184 post intervention.

Table 6. Resting metabolic rate (RMR) ($\text{kcal} \cdot \text{day}^{-1}$) pre and post 7-day dietary intervention (Mean \pm SEM).

	RMR PRE ($\text{kcal} \cdot \text{day}^{-1}$)	RMR POST ($\text{kcal} \cdot \text{day}^{-1}$)
PRO-EM	1916 ± 73	1910 ± 70
PRO-ER	1904 ± 37	1950 ± 54
CHO-EM	1972 ± 38	1946 ± 44
CHO-ER	1848 ± 69	1768 ± 52

Discussion

This is the first acute dietary intervention study in healthy sedentary males to demonstrate that increased dietary protein intake in a hypocaloric state positively increased mRNA expression of PGC-1 α and AMPK.

In a myoblast cell line it has previously been demonstrated that both calorie and carbohydrate restriction independently increase transcriptional markers of mitochondrial biogenesis. Following 36- hours incubation in a low glucose solution (5 mM) a > 2.5 fold increase in PGC-1 α transcriptional activity was observed relative to

a high glucose solution (25 mM), additionally the same authors also demonstrated that following a 20-hour fast, relative PGC-1 α mRNA levels were increased > 2 fold, suggesting positive activity *in vitro* of energy and glucose restriction independently [25]. However, *in vivo* results from the current study demonstrated no change in mRNA expression in the PRO-EM (carbohydrate restriction) or CHO-ER (energy restriction) groups, suggesting that acute carbohydrate restriction and energy restriction independently do not exert sufficient metabolic stress to elicit metabolic changes at an mRNA level. Furthermore the results from this study showed that subsequent to a continuous, hypocaloric, high protein diet (40% protein (~ 2.5 g•kg⁻¹•day⁻¹) and ~33 % energy deficit) a small, but significant, increase in resting mRNA expression of AMPK, SIRT1, SIRT3 and PGC-1 α in the vastus lateralis muscle was observed. Furthermore, although non-significant, a trend towards a group interaction of AMPK ($p = 0.056$) and PGC-1 α ($p = 0.063$) gene expression was observed. Due to two lost samples in the CHO-ER group at TP3 this resulted in unbalanced groups. When statistics were re-run with phantom values for these two missing points the groups interactions were apparent with the POR-ER group eliciting a significantly greater response in post intervention gene expression relative to all other groups. This would also suggest that the study may have been slightly under powered. These results imply that, in contrast to independent restriction, the combination of energy and carbohydrate restriction provides sufficient metabolic stress to cause skeletal muscle adaptation to cope with the energy demands.

These results are corroborated by similar studies investigating the impact of high fat feedings on mitochondrial biogenesis [15, 25]. It was shown that five days on a hypocaloric HF/LC diet positively increased phosphorylation of AMPK and

deacetylation of PGC-1 α , where as the hypocaloric LF/HC diet negatively reduced the phosphorylation of AMPK and deacetylation of PGC-1 α [15]. These results, along with the findings from the current study suggest that high carbohydrate intake prevents the activation of the AMPK-SIRT1 axis that is otherwise observed in a hypocaloric state. Furthermore, and importantly the method of carbohydrate restriction does not seem important, increasing both dietary fat and/or protein result in similar up-regulation of metabolic markers of mitochondrial biogenesis.

It is considered that weight loss strategies that result in maintenance of LM and promote greater FM loss are preferential [4]. It has previously been demonstrated that ~70 % of weight lost, induced by a hypocaloric diet, is attributed to FM and 30 % from LM [26]. Furthermore it has been reported in obese women that increased protein intake (1.6 g•kg⁻¹•day⁻¹ vs. 0.8 g•kg⁻¹•day⁻¹) increased FM loss and improved maintenance of lean mass [27]. The results from this study corroborate this in a healthy male population. No between group difference was observed in energy-matched conditions for BM, FM or LM. The CHO-ER group lost significantly more LM than the CHO-EM group, interestingly however no difference in LM loss was observed between the PRO-ER and CHO-EM groups. The PRO-EM group had a higher protein intake relative to the CHO-EM group (2.5 g•kg⁻¹•day⁻¹ vs. 1.0 g•kg⁻¹•day⁻¹). Thus, although the calorie intake of the PRO-ER group was ~33 % less than that of the CHO-EM group (total deficit of ~7,000 kcal over the 7-day intervention) no change in LM was observed between groups, further highlighting the key role for protein in LM maintenance. The mechanism of this is still not fully defined. The dietary protein intake of the CHO-EM group was greater than the current reference nutrient intake in the UK for adults of 0.75 g•kg⁻¹•day⁻¹ [28], however it has been

demonstrated the proteolysis of skeletal muscle is suppressed when consuming $\geq 1.5 \text{ g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ of protein [29]. The PRO-ER group consumed $2.5 \text{ g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ of protein, significantly above this threshold.

With regards to weight loss ratios, the results demonstrate that in a hypocaloric diet dietary protein intake can positively impact LM and FM loss. Following a continuous hypocaloric diet ($\sim 33\%$ energy deficit) and independent of macronutrient content both groups lost a significant amount of LM. Approximately 44% of total BM loss in the PRO-ER group was attributed to LM loss compared to 56% in the CHO-ER group. This was coupled with a greater percentage of FM loss in the PRO-ER group (56% vs. 44% respectively). As such, increased dietary protein intake ($2.5 \text{ g}\cdot\text{kg}^{-1}$ vs. $0.6 \text{ g}\cdot\text{kg}^{-1}$) attenuated LM loss by $\sim 12\%$ relative to a hypocaloric diet low in protein. A recent meta-analysis [30] investigating the role of dietary protein intake and long term body composition changes (12 ± 9 weeks) concluded higher protein diets led to greater BM loss (-0.79 kg), FM loss (-0.87 kg) and preserved more LM ($+0.43 \text{ kg}$) relative to lower protein diets. Although over a shorter period, the body composition results from the hypocaloric groups observed in this study followed the same trends with -0.35 kg BM, -0.09 kg FM and $+0.44 \text{ kg}$ LM body composition changes observed. It has been demonstrated that a positive curvilinear ($r^2 = 0.92$) relationship exists between LM and RMR [31], and RMR is known to decline during prolonged (> 9 months) periods of energy restriction [32]. The thermic effect of macronutrients should also be considered, protein has a greater thermic effect compared with the equivalent energy intake of fat or carbohydrate [33]. However the acute nature of the intervention was not long enough to elicit change in RMR and no time point difference or group interaction was observed in RMR, thus it may be assumed that

the duration of the study was not long enough to elicit changes in RMR, as such metabolic rate is not impacted by such a short dietary intervention.

A marked reduction in RER post intervention was observed in both ER groups; however caution should be taken when drawing strong conclusions from this data. Pre intervention both ER groups had a higher RER (PRO-ER = 0.89, CHO-ER = 0.85, PRO-EM = 0.83, CHO-EM = 0.83) and post intervention all groups had an RER of 0.79. Thus a greater relative change was observed pre-post intervention in the ER groups, but no group interaction was apparent. In mice and subsequent to 8 weeks on a hypocaloric diet a significant reduction in RER was observed compared to relative to energy matched diets [34]. Similar to the results from this study, RER was reduced to the same level in the hypocaloric group's independent of dietary protein intake (5% vs. 33% vs. 60%). Solon-Biet, Mitchell [34] also reported a significant reduction in RER in the eucaloric high protein group relative to the eucaloric high protein diet, however this was not to the amplitude of the any of the hypocaloric diets. This suggests that in an eucaloric state restricted carbohydrate (increased protein intake) results in a reduction in RER, however calorie restriction has a greater metabolic impact, independent on macronutrient ratio.

Interestingly this study raises further considerations for dietary prescription studies and methods used to measure energy intake. The mean calorie intake calculated from 2 x 4 day food diaries for each participant in the PRO-EM and CHO-EM groups were $2,192 \pm 574 \text{ kcal} \cdot \text{day}^{-1}$ and $2,355 \pm 527 \text{ kcal} \cdot \text{day}^{-1}$, respectively. However, mean calculated energy intake from RMR X Activity Factor (AF) were $2,828 \pm 331 \text{ kcal} \cdot \text{day}^{-1}$ and $2,881 \pm 213 \text{ kcal} \cdot \text{day}^{-1}$, a difference of 636 and 526 $\text{kcal} \cdot \text{day}^{-1}$,

respectively. Despite meticulous effort to match the dietary intake of the PRO-EM and CHO-EM to energy expenditure the results demonstrated a small reduction in BM in both group (0.74 kg and 1.1 kg respectively), as such dietary prescription was slightly underestimated. Despite this, habitual diet, as recorded using estimated household measures, under predicted energy intake calculated using $RMR \times PA$ by ~20%, a similar percentage to previous findings in athletes [35] (~28 %) and sedentary participants [36] (~20%).

1.1 Conclusions

Mitochondrial quantity and density have been linked with increased endurance performance [1] and a reduction in type 2 diabetes [2-6]. Also, it is suggested that dietary strategies that maintain LM are preferential. This is the first study to demonstrate the impact of increased protein intake in a hypocaloric diet on metabolic adaptation. The results from this study demonstrated that a 7-day a high protein hypocaloric diet resulted in increased AMPK, SIRT1, SIRT3 and PGC-1 α mRNA expression at rest, all of which are upstream markers of mitochondrial biogenesis. Additionally increased protein intake helped to maintain LM mass in a hypocaloric state. Thus, it may be concluded that a high protein, hypocaloric diet is preferential to a high carbohydrate, hypocaloric diet with respect to cellular markers of fat metabolism and also the maintenance of lean mass. However future research is needed to understand the long-term impact of a high protein hypocaloric diet on markers of mitochondrial biogenesis and body composition.

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