Whey protein augments leucinemia and post-exercise p70S6K1 activity compared to a hydrolysed collagen blend when in recovery from training with low carbohydrate availability

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Abstract

We examined the effects of whey versus collagen protein on skeletal muscle cell signalling responses associated with mitochondrial biogenesis and protein synthesis in recovery from an acute training session completed with low carbohydrate (CHO) availability. In a repeated measures design (after adhering to a 36-h exercise-dietary intervention to standardise pre-exercise muscle glycogen), eight males completed a 75-min non-exhaustive cycling protocol and consumed 22 g of a hydrolysed collagen blend (COLLAGEN) or whey (WHEY) protein 45 min prior to exercise, 22 g during exercise and 22 g immediately post-exercise. Exercise decreased ($P<0.05$) muscle glycogen content by comparable levels from pre-to post-exercise in both trials (≈ 300 to 150 $mmol.kg^{-1}$ dw). WHEY protein induced greater increases in plasma BCAAs ($P=0.03$) and leucine ($P=0.02$) than COLLAGEN. Exercise induced ($P<0.05$) similar increases in PGC-1α (5-fold) mRNA at 1.5 h post-exercise between conditions though no affect of exercise ($P>0.05$) was observed for p53, Parkin and Beclin1 mRNA. Exercise suppressed ($P<0.05$) p70S6K1 activity in both conditions immediately post-exercise (≈ 25 $fmol.min^{-1}.mg^{-1}$). Post-exercise feeding increased p70S6K1 activity at 1.5 h post-exercise ($P<0.05$), the magnitude of which was greater ($P <0.05$) in WHEY (180 ± 105 $fmol.min^{-1}.mg^{-1}$) versus COLLAGEN (73 ± 42 $fmol.min^{-1}.mg^{-1}$). We conclude that protein composition does not modulate markers of mitochondrial biogenesis when in recovery from a training session deliberately completed with low CHO availability. In contrast, whey protein augments post-exercise p70S6K activity compared with hydrolysed collagen, as likely mediated via increased leucine availability.

Keywords: autophagy, p70S6K1, CHO restriction, glycogen

Introduction

The role of increased dietary protein intake in facilitating skeletal muscle adaptations associated with endurance training is now gaining acceptance (Moore et al. 2014). Indeed, consuming protein before (Coffey et al. 2011), during (Hulston et al. 2011) and/or after (Rowlands et al. 2015) an acute training session stimulates muscle protein synthesis (MPS). Post-exercise protein feeding has also been shown to modify skeletal muscle transcriptome responses towards those supporting the endurance phenotype (Rowlands et al. 2011). In considering protein-feeding strategies for athletes, it is pertinent to consider the absolute dose (Moore et al. 2009; Witard et al. 2014; Rowlands et al. 2015; MacNaughton et al. 2016), feeding schedule (West et al., 2011; Areta et al. 2013), digestibility (Burke et al. 2012; Phillips, 2016) and source of protein (Tang et al. 2009; Wilkinson et al. 2007). Contemporary guidelines recommend whey protein beverages due to its higher leucine content and rapid aminoacidemia upon ingestion (Thomas et al. 2016), though hydrolysed collagen beverages and gels are now commercially available and marketed to athletic populations. Whilst the use of a gel delivery matrix appears particularly beneficial for endurance athletes given the practical advantages of feeding while in locomotion (Impey et al. 2015), it is noteworthy that collagen based formulations likely have lower leucine content and digestibility compared with whey (Phillips, 2016).

With this in mind, the aim of the present study was to therefore examine the effects of two practically relevant protein-feeding strategies (i.e. whey protein solution versus a hydrolysed collagen blend in a gel format) in modulating skeletal muscle cell signalling responses associated with mitochondrial biogenesis and MPS. Given the increased popularity of training with low carbohydrate (CHO) availability (i.e. the train-low paradigm) in an attempt to enhance mitochondrial related adaptations (Hawley and Morton, 2014; Bartlett et al. 2015; Impey et al. 2016; 2018), we adopted an experimental design whereby male cyclists

completed a non-exhaustive training session in which glycogen remained within an absolute concentration (i.e. pre-and post-exercise concentrations of <350 and >100 mmol.kg dw^{-1}, respectively) considered representative of train-low conditions (Impey et al. 2018).

Methodology

Subjects: After providing informed written consent, eight recreational male cyclists (age: 25 ± 3 years; height: 175 ± 0.1 cm; body mass: 74.4 ± 6.7 kg) who trained between 3 – 10 hours per week took part in this study. Mean VO_{2peak} and peak power output (PPO) was 56.5 ± 3.8 $ml.kg^{-1}.min^{-1}$ and 327 ± 26 W respectively. None of the participants had a history of neurological disease or skeletal muscle abnormality and none were under pharmacological intervention during the study. The study was approved by the Research Ethics Committee of Liverpool John Moores University.

Design: In a repeated measures counterbalanced design separated by 7-9 days, subjects completed two non-exhaustive acute exercise trials in conditions of reduced CHO availability with whey (WHEY) or a hydrolysed collagen blend (COLLAGEN) provision before, during and after exercise. At 36-40 h prior to the main experimental trials, all subjects performed a glycogen depletion protocol followed by 36 h of low CHO (3 $g.kg.d^{-1}$) and energy intake (~7.58 ± 0.6 $MJ.day^{-1}$) (as replicated from Impey et al. 2016) in order to standardise pre-exercise muscle glycogen content (see Figure 1). Subjects refrained from CHO intake on the morning of the main experimental trial as well as during exercise, but consumed 1.2 g/kg body mass (BM) of CHO split across two equal 0.6 g/kg doses at 30 min and 60 min post exercise in both trials. Subjects consumed 22 g of whey or collagen protein at 45 minutes prior to exercise, 22 g during exercise and a further 22 g immediately post-exercise. Both trials represented deliberate conditions of reduced CHO and absolute energy availability, but

with high protein availability in the form of whey or collagen throughout. Muscle biopsies were obtained from the vastus lateralis immediately pre-, post- and at 1.5 h post-exercise.

Assessment of peak oxygen uptake: Participants were assessed for peak oxygen consumption (VO_{2peak}) and peak aerobic power (PPO) as determined during an incremental cycle test performed on an electromagnetically braked cycle ergometer as previously described (Impey et al. 2015).

Experimental Protocol:

Day 1 and 2: Participants arrived at the laboratory on the evening (17.00) of day 1. Subjects then performed an intermittent glycogen-depleting cycling protocol lasting ~120 min (as described by Impey et al. 2016). This protocol and all subsequent cycling protocols were conducted on a fully adjustable electromagnetically braked cycle ergometer (Lode Excalibur, Netherlands). The activity pattern and total time to exhaustion (115 ± 5 min; Energy expenditure: 1444 ± 107 kJ) were recorded and repeated exactly during the second experimental condition. Participants then consumed a diet low in carbohydrate (3 $g.kg^{-1}$ BM) but high in protein (2 $g.kg^{-1}$ BM) over the next 36 h to minimise muscle glycogen replenishment to ~300-350 $mmol.kg^{-1}$ dw on the morning of the main experimental trial. During this 36 h period prior to the main experimental trial, total energy intake equated to 7.58 ± 0.6 MJ. Estimated energy expenditure (as calculated from resting metabolic rate using the Harris Benedict equation and PAL level of 1.4 for the sedentary period on Day 2) was 15. 9 ± 1.1 MJ and hence energy balance was – 8.4 ± 0.45 MJ.

Day 3: Subjects reported to the laboratory in a fasted state and an indwelling cannula (Safety Lock 22G, BD Biosciences, West Sussex UK) was inserted into the anticubital vein in the anterior crease of the forearm. Blood samples were collected immediately prior to and every 15 minutes during exercise as well as at 30 minute intervals in the recovery period from

142 exercise. Subjects consumed 22 g of protein from one of two commercially available
143 products consisting of a hydrolysed collagen blend in a gel format (COLLAGEN: Muscle
144 Gel, Muscle Pharm, USA; Ingredients: water, hydrolysed collagen, whey protein isolate,
145 dietary fibre, natural flavours, citric acid, ascorbic acid, malic acid, niacinamide, sodium
146 benzoate, potassium sorbate, sucralose, calcium D pantothenate, pyridoxine HCL, riboflavin)
147 or a whey protein solution (WHEY: Whey Protein, Science in Sport, Nelson, UK;
148 Ingredients: whey protein concentrate, whey protein isolate, fat reduced cocoa powder,
149 natural flavourings, xanthan gum, soy lecithin, sucralose) at 45 minutes prior to beginning
150 exercise. Due to the clear differences in delivery methods of protein sources (i.e. gel versus
151 solutions), neither single nor double blinding of treatments occurred. Fluid intake was
152 matched in both conditions to 500 ml at this time-point. Subjects then rested for 45 minutes
153 prior to commencing exercise. Protein was given 45 min prior to exercise in an attempt to
154 maintain elevated circulatory amino acid availability during the exercise protocol (Impey et
155 al. 2015). Following a 5 min warm up at 150 W, subjects then completed a prescribed cycling
156 protocol consisting of 4 x 30 seconds high intensity intervals at 200% PPO interspersed with
157 2.5 min active recovery at 40% PPO, followed by 45 min steady state cycling at 60% PPO
158 and finally, 3 x 3min intervals at 90% PPO. During the HIT and steady state component,
159 subjects ingested 7.3g of COLLAGEN or WHEY protein every 20 min to provide 22 g of
160 protein per hour. Physiological and perceptual measures were recorded at regular intervals
161 throughout exercise (e.g. heart rate, RPE) and substrate utilisation was assessed during the
162 steady state component of the exercise protocol using online gas analysis (CPX Ultima,
163 Medgraphics, Minnesota, US) according to Jeukendrup and Wallis (2005). Following
164 completion of the training session, subjects consumed an additional 22 g of COLLAGEN or
165 WHEY protein immediately post-exercise as well as 1.2g.kg^{-1} BM carbohydrate in the form
166 of sports drinks (Science in Sport, Nelson, UK) and snacks (Jaffacakes, UK) split as equal

doses of 0.6 $g.kg^{-1}$ BM at 30 and 60 minutes post-exercise. Laboratory conditions remained constant across all experimental trials (19 – 21^{o}C, 40 – 50% humidity).

Muscle biopsies: Muscle biopsies were obtained from separate incision sites (2 – 3 cm apart) from the lateral portion of the vastus lateralis muscle. Biopsies were obtained using a Bard Monopty Disposable Core Biopsy Instrument (12 guage x 10 cm length, Bard Biopsy Systems, Tempe, AZ, USA). Samples were obtained under local anaesthesia (0.5% marcaine) and immediately frozen in liquid nitrogen and stored at – 80^{o}C for later analysis.

Blood analysis: Blood samples were collected in vacutainers containing K_2 EDTA, lithium heparin or serum separation tubes, and stored on ice or at room temperature until centrifugation at 1500 g for 15 min at 4℃. Serum and plasma were aliquoted and stored at -80℃ until analysis. Plasma glucose, lactate, non-esterifed fatty acids (NEFA), glycerol, β-hydroxybutyrate (β-OHB), insulin and amino acids were analysed as previously described (Impey et al. 2016).

RNA extraction and analysis and Reverse transcriptase quantitative Real-Time Polymerase Chain Reaction (rt-qRT-PCR): Muscle samples (~ 20 mg) were immersed and homogenized in 1ml TRIzol (Thermo Fisher Scientific, UK). RNA was extracted according to the manufacturer's instructions. RNA concentration and purity were assessed by UV spectroscopy at ODs of 260 and 280 nm using a Nanodrop 3000 (Fisher, Rosklide, Denmark). 70 ng RNA was used for each PCR reaction. Primer were purchased from Sigma (Suffolk, UK) and forward (F) and reverse (R) sequences were as follows: PGC-1 (F: TGCATGAGTGTGTGCTCTGT; R: CAGCACACTCGATGTCACTC), p53 (F: ACCTATGGAAACTACTTCCTGAAA; R: CTGGCATTCTGGGAGCTTCA), Parkin (F: TCCCAGTGGAGGTCGATTCT; R: GGAACCCCCTGTCGCTTAG), Beclin1 (F: ATCTCGAGAAGGTCCAGGCT; R: TCTGGGCATAACGCATCTGG). rt-qRT-PCR

191 amplifications were performed using QuantiFast™ SYBR® Green RT-PCR one step kit on a
192 Rotogene 3000Q (Qiagen, Crawley, UK) supported by rotogene software (Hercules, CA,
193 USA). Detailed procedures are described by Impey et al. (2016).

194 **Muscle glycogen concentration:** Muscle glycogen concentration was determined from 10-
195 20 mg muscle tissue according to the acid hydrolysis method described previously (Impey et
196 al. 2016). Glucose concentrations were quantified using a commercially available kit (GLUC-
197 HK, Randox Laboratories, Antrim, UK).

198 **[γ^{-32}P] ATP Kinase Assay:** Twenty mg muscle tissue was used for the measurement of
199 p70S6K1 and PKB (Akt) activity as previously described (McGlory et al. 2014).

200 **Statistics:** Statistical analyses were performed using Statistical Package for the Social
201 Scientist (SPSS version 21). Changes in physiological and molecular responses between
202 conditions (i.e. muscle glycogen, circulatory metabolites, amino acids, mRNA and kinase
203 activity) were analysed using two way repeated measures General Linear Model, where the
204 within factors were time and condition. Where a significant main effect was observed,
205 pairwise comparisons were analysed according to Bonferoni post hoc tests in order to locate
206 specific differences. A P value < 0.05 was deemed significant and all data in text, figures and
207 tables are presented as mean ± SD.

208

209 **Results**

210 ***Physiological and metabolic responses to exercise***

211 Exercise intensity and substrate metabolism during the steady state component of the exercise
212 protocol is displayed in Table 1. No significant differences (P>0.05) were observed between
213 trials for any parameter. Exercise reduced (P<0.001) muscle glycogen stores to comparable

214 levels (150 mmol.kg^{-1} dw) with no difference (P=0.485) between conditions (Table 2).
215 Plasma NEFA, glycerol and β-OHB increased during exercise (P<0.001) though plasma
216 glucose did not display any change (P = 0.112) (Figure 2 A, B, C and D, respectively).
217 Changes in plasma NEFA availability across the whole sampling period were suppressed in
218 WHEY compared with the COLLAGEN trial (P=0.046) whereas no differences were
219 observed between trials for glycerol (P=0.080), β-OHB (P = 0.070) or glucose (P=0.963).
220 Despite differences in NEFA availability during exercise, no differences were observed in
221 either CHO (P=0.640) or lipid oxidation (P=0.750) during the steady state component of the
222 exercise protocols (Table 1, respectively).

223 ***Markers of mitochondrial adaptations***

224 The magnitude of the exercise-induced increase (P = 0.001) in PGC-1α mRNA expression at
225 90 min post-exercise was not different (P = 0.731) between trials (Figure 3A). Neither
226 exercise (P = 0.354) nor experimental condition (P = 0.472) affected p53 mRNA expression
227 (Figure 3B). As markers of mitophagy, Parkin mRNA displayed no effect of exercise (P =
228 0.417) or experimental condition (P = 0.301), whereas Beclin 1 displayed a trend towards an
229 effect of exercise (P = 0.058) but no effect of condition (P = 0.968).

230

231 ***Plasma amino acids, serum insulin and p70S6K1 related signalling***

232 Plasma leucine, BCAAs and EAAs all displayed a significant main effect of time (P=0.043,
233 0.028 and 0.021, respectively) during the sampling period (Figure 4 A, B, C respectively).
234 Pairwise comparisons demonstrated that leucine and BCAAs were significantly different
235 from pre-exercise after 30 and 45 minutes of exercise and that BCAAs were also different
236 from pre-exercise values after 30, 60 and 90 minutes of recovery. Such main effects of time

appear to be predominantly due to those changes occurring in the WHEY trial given that no differences are apparent in the COLLAGEN trial. Additionally, leucine (P=0.02) and BCAA concentrations (P=0.03) also demonstrated a main effect for condition such that WHEY was greater than COLLAGEN whereas differences in EAA between trials only approached statistical significance (P=0.060). When expressed as AUC data, only plasma leucine (P=0.025) was different between trials whereas AUC for BCAA (P=0.135) and EAA (P=0.062) were not different (data not shown).

In accordance with post-exercise CHO intake, insulin increased from pre- and post-exercise values (P = 0.034) though the magnitude of change was not different between trials (P = 0.159) (Figure 4D). As such, no difference (P=0.187) was apparent between trials for insulin AUC data (data now shown)

PKB activity was elevated at 90 min post-exercise (P = 0.003) compared with pre-exercise values, irrespective of nutritional condition (P=0.370) (Figure 4E). Exercise suppressed (P=0.015) p70S6K activity to comparable levels immediately post-exercise (≈ 25 fmol.min^{-1}.mg^{-1}). However, post-exercise feeding increased p70S6K activity at 1.5 h post-exercise (P=0.004), the magnitude of which was greater (P=0.046) in WHEY (180 ± 105 fmol.min^{-1}.mg^{-1}) versus COLLAGEN (73 ± 42 fmol.min^{-1}.mg^{-1}) (Figure 4F).

Discussion

We examined the effects of whey versus collagen protein on skeletal muscle cell signalling responses associated with mitochondrial biogenesis and protein synthesis in recovery from an acute training session completed with low CHO availability. We deliberately studied two forms of protein feeding that we consider have practical relevance for endurance athletes i.e.

a whey protein solution versus a hydrolysed collagen blend administered in a gel format. We also adopted an acute training session intended to mimic situations in which endurance athletes deliberately train with low endogenous and exogenous CHO availability in an attempt to promote oxidative training adaptations (Impey et al. 2018). Whilst we observed no effects of protein composition on acute adaptations associated with mitochondrial biogenesis, whey protein induced greater leucinemia and post-exercise activity of p70S6K activity than collagen.

In accordance with the well-documented differences in amino acid composition between whey and collagen (Castellanos et al. 2006), we observed marked differences in the extent of leucinemia induced by the two protein feeding strategies. In this regard, leucine was elevated to a greater extent with the whey protein solution when compared with the hydrolysed collagen gel format. In agreement with previous reports from our laboratory (Taylor et al. 2013) and others (Breen et al. 2011), we observed that amino acid availability does not apparently modulate acute markers of mitochondrial adaptations. In contrast, we observed whey protein induced greater increases in post-exercise p70S6K activity. The effects of post-exercise whey protein consumption on activation of the mTOR-p70S6K pathway is well documented (Phillips, 2016) and hence, the greater effect of whey compared with collagen on activation of p70S6K is likely related to the increased leucine availability (Moberg et al. 2014; Apro et al. 2015a). Nonetheless, we acknowledge that direct assessment of muscle protein synthesis using stable isotope or deuterium methods would have provided greater insight to the functional relevance of the nutritional strategies used here. In addition, future studies could also assess if such divergent signalling responses are still apparent if the collagen formulation was fortified with additional leucine content to match that of the whey solution.

Although we readily acknowledge that the total leucine delivery in the WHEY trial may appear excessive in terms of that required to facilitate protein synthesis as well as likely resulting in elevated leucine oxidation (as suggested by the fall in leucine and BCAA after 30 and 45 minutes of exercise), we deliberately chose this dosing strategy for a number of practical reasons. Firstly, given that exercising in CHO restricted states augments leucine oxidation (Lemon and Mullin, 1980; Wagenmakers et al. 1991; Howarth et al. 2009), it was our deliberate aim to administer higher exogenous leucine so as to deliver both substrate to promote muscle protein synthesis (Breen et al. 2011; Pasiakos et al. 2011; Churchward-Venne et al. 2013) but yet, also compensate for the higher levels of endogenous leucine oxidation (Lemon and Mullin, 1980; Howarth et al. 2009). Second, unpublished observations by the corresponding author on elite professional cyclists indicated that this is the type of protein feeding strategy actually adopted during morning training rides that are deliberately undertaken in the absence of CHO intake before and during exercise. As such, our aim was to replicate these "real world" strategies and determine the effects of such high protein availability on substrate metabolism and post-exercise signalling responses. Finally, given that many elite cyclists are potentially in daily energy deficits (Vogt et al. 2005) with low energy availability (Loucks et al. 2011), and also that 3 g/kg body mass of daily protein has been recommended to maintain lean mass during energy restriction (Stokes et al. 2018), we therefore considered this feeding strategy to be in accordance with daily protein intakes for both quantity and frequency (Areta et al. 2013). For example, over the 3.5 h data collection period (i.e. 9 am to 1230 pm), the present subjects (ranging from 70-80 kg) consumed 66 g protein and hence for the daily target to be achieved (i.e. approximately 210-240 g), our approach is therefore in accordance with a feeding strategy where subsequent 30-40 g doses could be consumed at 3 h intervals (e.g. 1, 3, 6 and 9 pm if required).

When considered in combination with our recent data (Impey et al. 2016; Hammond et al. 2016), the present study also adds to our understanding of the regulation of p70S6K activity both during and after exercise. Indeed, whereas other researchers have reported that acute endurance exercise does not suppress post-exercise p70S6K phosphorylation (Coffey et al. 2006) or activity (Apro et al. 2015b), we have consistently observed an exercise-induced suppression in p70S6K activity. We suggest that such differences between studies may be due to the magnitude of energy deficit associated with the CHO restriction and glycogen taxing exercise protocols used both here and previously (Impey et al. 2016). Indeed, whilst it is difficult to directly compare the total energy expenditure between this study and the data of Apro et al. (2015b), the exercise intervention studied here elicited considerably lower muscle glycogen concentrations (i.e. ~150 $mmol.kg^{-1}dw$ vs 350 $mmol.kg^{-1}dw$). The potential effects of low muscle glycogen availability on post-exercise signalling (albeit in response to resistance exercise) was also evidenced by Camera et al. (2012) who observed that low muscle glycogen availability (i.e. 150-200 $mmol.kg^{-1}dw$) reduced mTOR phosphorylation compared with higher glycogen concentration (i.e. 350-400 $mmol.kg^{-1}dw$). Nonetheless, these workers also observed the apparent disconnect between snapshots of cell signalling and functional outcomes given that glycogen concentrations did not affect myofibrillar protein synthesis.

In relation to the re-activation of p70S6K activity in the recovery period from exercise, it is noteworthy that we previously observed that the sustained presence of reduced CHO (and energy availability) and/or high post-exercise fat availability also suppresses the re-activation of p70S6K1, even when leucine enriched whey protein was consumed in the post-exercise period (Impey et al. 2016). Based on these studies, we therefore suggested that the apparent suppression of p70S6K1 activity may be due to 1) reduced insulin and PKB signalling or, 2) a direct effect of increased fat availability (Kimball et al. 2015) and/or reduced glycogen

mediating suppression of mTORC1 complex via energetic stress related mechanisms. The present data lend support for the latter mechanism for several reasons. First, we observed that the whey-induced increase in p70S6K1 activity when compared with collagen feeding was independent of post-exercise insulin and PKB activity. Second, at the termination of exercise (i.e. the 75 min time point that corresponds low muscle glycogen availability and energy deficit) the absolute circulating NEFA concentrations observed in our collagen trial (i.e. approximately 1.5 $mmol.L^{-1}$) was similar to that achieved with both CHO restriction (Impey et al. 2016) and post-exercise high fat feeding protocols (Hammond et al. 2016). The apparent suppression of NEFA in the WHEY trial may be due to the higher insulin responses associated with feeding whey protein before and during exercise (Impey et al. 2015; Taylor et al. 2013), thereby causing a reduction in lipolysis that manifests itself as reduced circulating NEFA availability during the exercise period. Nonetheless, we acknowledge that the current assessments of insulin concentration were limited to pre-and post-exercise time-points per se. We also acknowledge the limitations associated with making inferences on muscle free fatty acid (FFA) uptake on snapshot assessments of circulating NEFA per se. Nonetheless, given recent data demonstrating that acute increases in fat availability (as achieved via lipid infusion protocols) impairs MPS in human skeletal muscle despite similar circulating insulin and leucine concentrations (Stephens et al. 2015), it remains possible that subtle alterations in FFA availability (as caused by "acute" dietary manipulations) can have associated implications on mTOR related signalling. When considered with previous studies (Impey et al. 2016; Hammond et al. 2016), the present data suggest that in those exercise conditions in which muscle glycogen is near depletion, the beneficial effects of whey protein (i.e. leucine mediated activation of mTOR) are especially apparent when co-ingested with post-exercise CHO feeding. Whilst there may be benefits of commencing training with reduced endogenous and exogenous CHO availability, we suggest the post-exercise meal should

contain a combination of both protein and CHO, the latter to provide the necessary substrate, energy and metabolic environment to stimulate cell signalling processes.

In summary, we demonstrate that when in recovery from an acute training session undertaken with low CHO and energy availability, whey protein induces greater leucinemia and post-exercise p70S6K activity compared with a hydrolysed collagen blend. Data suggest that hydrolysed collagen blends are a sub-optimal protein source in relation to the goal of stimulating those signalling pathways that regulate muscle protein synthesis. Future studies are now required to directly assess the acute effects of whey versus collagen protein feeding on muscle protein synthesis as well as to examine the long-term effects of such feeding strategies on training-induced skeletal muscle adaptations and performance outcomes.

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Figure 1. Schematic representation of the experimental design. On the evening of day 1, subjects completed a glycogen depleting protocol followed by consumption of 22 g of whey protein. Throughout the entirety of day 2, subjects consumed a low CHO and low energy dietary protocol that was matched for both protein and fat intake. During the main experimental trial on day 3, subjects ingested 22 g of collagen (COLLAGEN) or whey (WHEY) protein before, during and after completion of an acute train-low exercise protocol. In addition to protein, subjects also consumed CHO (0.6 $g.kg^{-1}$ BM) at 30 min and 1 h post-exercise. Muscle biopsies were obtained immediately pre-exercise, post-exercise and 1.5 h post-exercise. This experimental protocol represents an amalgamation of train-low paradigms as subjects effectively performed sleep low on the evening of day 1, consumed a low CHO diet on day 2 and finally, completed an acute training session on the morning of day 3 with CHO restricted before and during exercise.

532 **Figure 2**. Plasma (A) NEFA, (B) glycerol, (C) βOHB and (D) Glucose during and in
533 recovery from exercise. Shaded area represents exercise duration. * P<0.05 significant
534 difference from pre-exercise (i.e. time-point 0), ^ P<0.05 significant main effect of condition.

535

536 **Figure 3**. mRNA expression of (A) PGC-1α, (B) p53, (C) Parkin and (D) Beclin1. * P<0.05
537 significant difference from pre-exercise.

538

539 **Figure 4**. Plasma (A) leucine, (B) total BCAA, (C) total EAA and (D) insulin. Kinase
540 activity of (E) PKB and (F) p70S6K. Shaded area represents exercise duration. *P<0.05
541 significant difference from pre-exercise, **P<0.05 significant difference from post-exercise,
542 ^P<0.05 significant main effect of condition.

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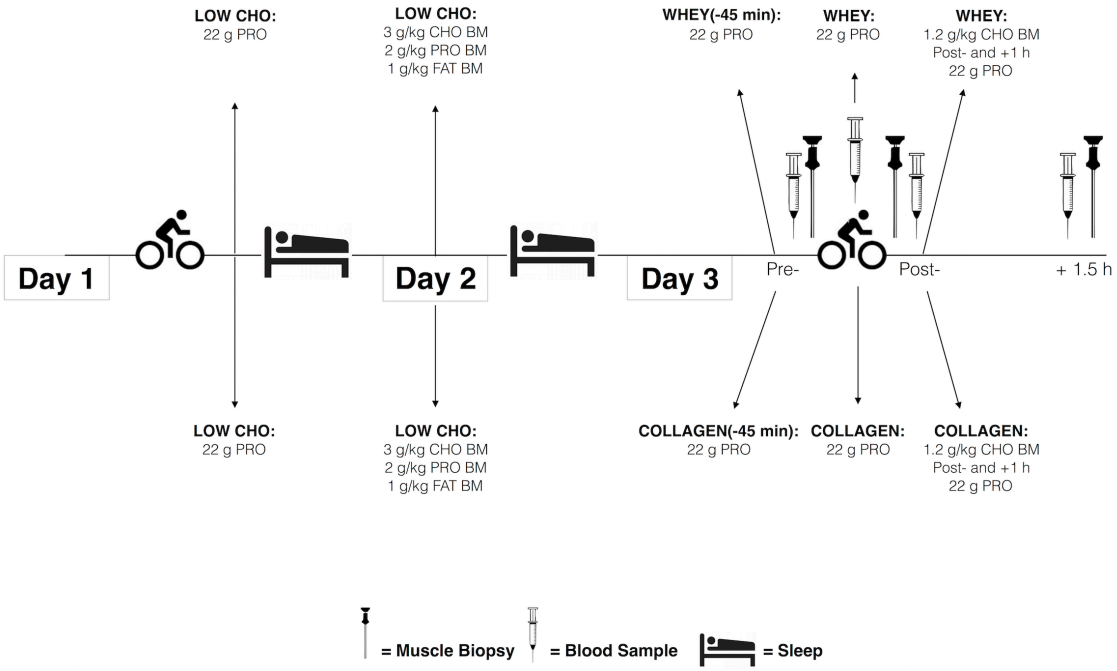
553 **Figure 1**



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564 **Figure 2**

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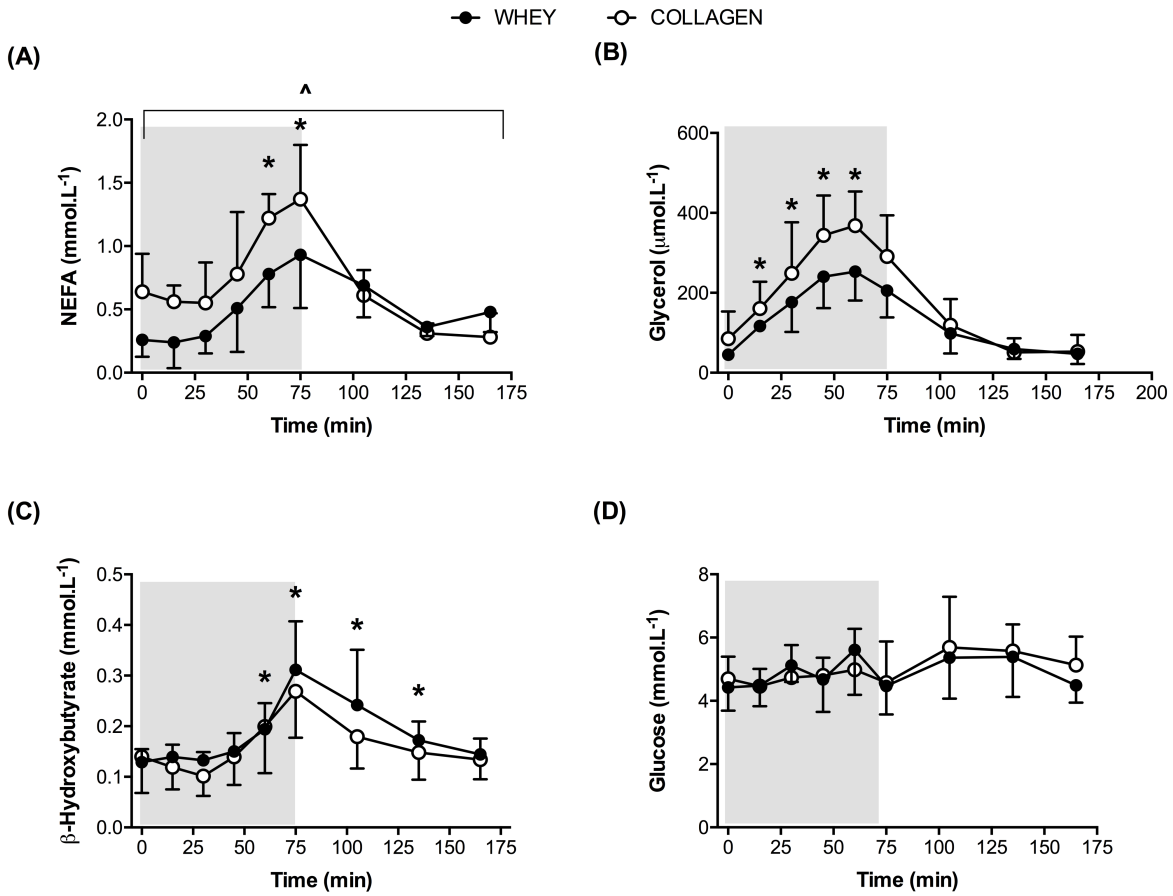

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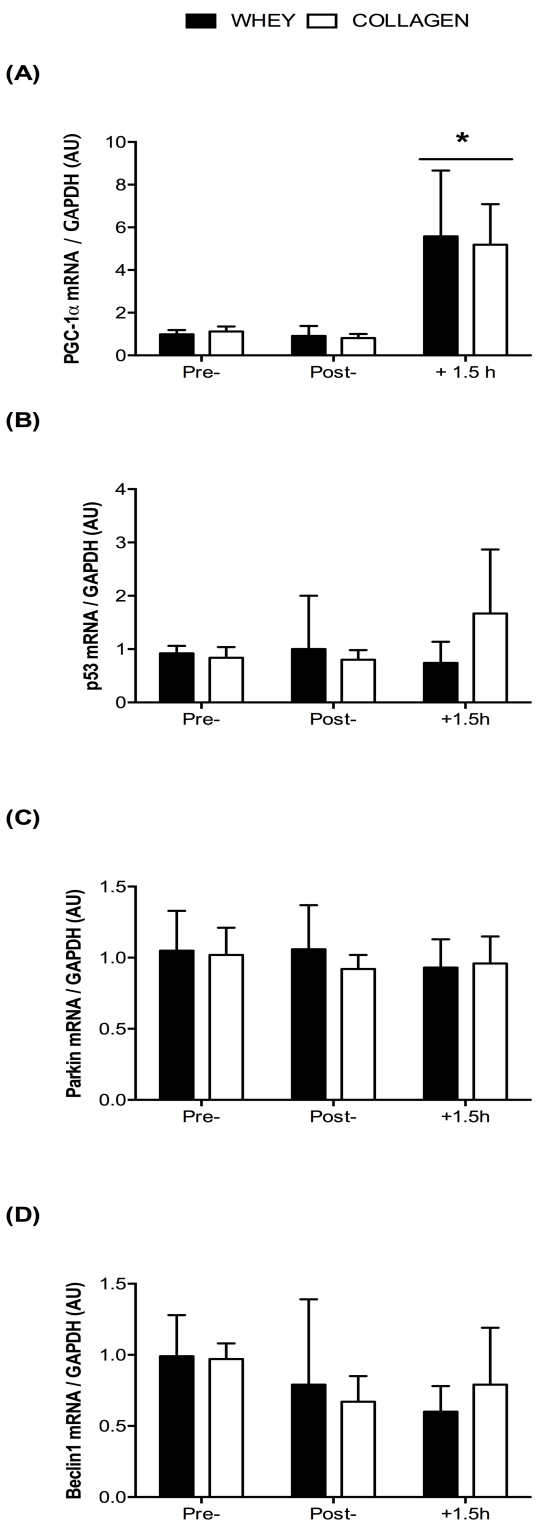
573 **Figure 3**



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577 **Figure 4**

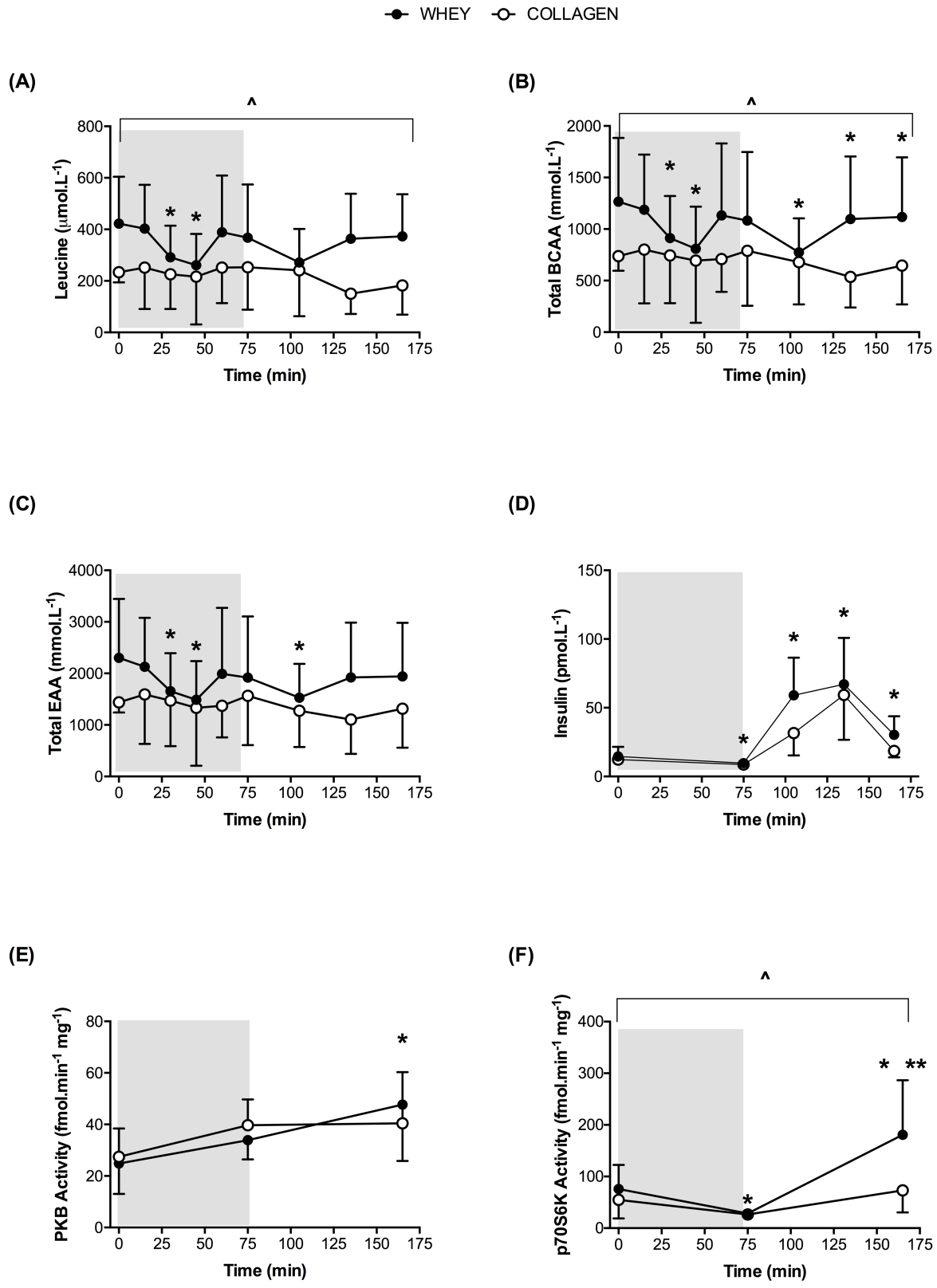

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Table 1 – Exercise intensity and substrate metabolism during the steady state component of the exercise protocol.

	Time (min)		
	15	30	45
VO_2 (% VO_{2max})			
WHEY	66 ± 1	67 ± 2	68 ± 2
COLLAGEN	67 ± 2	67 ± 1	68 ± 1
Heart Rate ($b.min^{-1}$)			
WHEY	165 ± 12	167 ± 12	166 ± 12
COLLAGEN	167 ± 6	168 ± 8	168 ± 8
RER (AU)			
WHEY	0.86 ± 0.05	0.86 ± 0.05	0.86 ± 0.06
COLLAGEN	0.86 ± 0.05	0.87 ± 0.03	0.86 ± 0.03
CHO Oxidation ($g.min^{-1}$)			
WHEY	1.9 ± 0.8	1.9 ± 0.8	1.9 ± 0.9
COLLAGEN	2.1 ± 0.6	2.1 ± 0.6	2.1 ± 0.6
Lipid Oxidation ($g.min^{-1}$)			
WHEY	0.7 ± 0.4	0.7 ± 0.3	0.7 ± 0.4
COLLAGEN	0.6 ± 0.2	0.6 ± 0.2	0.7 ± 0.2

Table 2 – Muscle glycogen concentration before and after exercise. * denotes significant different from pre-exercise, P<0.05.

	Time (min)		
	Pre-	Post-	+ 90 min
Glycogen (mmol.kg^{-1} dw)			
WHEY	339 ± 66	158 ± 80 *	183 ± 35 *
COLLAGEN	356 ± 44	141 ± 25 *	173 ± 23 *