SIGNALLING RESPONSES FOLLOWING VARYING SEQUENCING OF STRENGTH AND ENDURANCE TRAINING IN A FED STATE

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Abstract

Purpose: The objective of this study was to compare anabolic signalling responses to differing sequences of concurrent strength and endurance training in a fed state.

Methods: Eighteen resistance-trained males were randomly assigned to the following experimental conditions; i) strength training (ST), ii) strength followed by endurance training (ST-END) or iii) endurance followed by strength training (END-ST). Muscle tissue samples were taken from the *vastus lateralis* before each exercise protocol, upon cessation of exercise, and 1 h-post cessation of strength training. Tissue was analysed for total and phosphorylated (p-) signalling proteins linked to the mTOR and AMPK networks.

Results: Strength training performance was similar between ST, ST-END and END-ST. p-S6k1 was elevated from baseline 1 h post training in ST and ST-END (both $p < 0.05$). p-4E-BP1 was significantly lower than baseline post ST ($p = 0.01$), while 1 h post exercise in the ST-END condition p-4E-BP1 was significantly greater than post exercise ($p = 0.04$). p-ACC was elevated from baseline both post and 1 h post exercise (both $p < 0.05$) in the END-ST condition. AMPK, mTOR, p38, PKB, eEF2 responded similarly to the ST, ST-END and END-ST. Signalling responses to ST, ST-END and END were largely similar. As such it cannot be ascertained which sequence of concurrent strength and endurance training is most favourable in promoting anabolic signalling.

Conclusions: These data indicate that in the case of the present study an acute bout of concurrent training of differing sequences elicited similar responses of the AMPK and mTOR networks.

Key words: Combined exercise, mTOR, AMPK, exercise order, anabolic signalling

Introduction

A number of research studies have reported acute and chronic resistance exercise-induced activation of the PI3k/PKB/mTORC1/S6k1/4E-BP1 growth associated signalling network [1-4]. The repeated activation of the "mTORC1 network", induced by progressively overloaded strength training, can result in increased cross sectional area (CSA) and contractile strength of the trained muscles [5]. It has been hypothesised that the endurance training-induced activation of the energy modulating AMPK signalling network may be antagonistic to the mTORC1 network and any associated strength training adaptations [6-8].

The potential inhibition of intramuscular protein synthesis via endurance training mechanisms that activate AMPK remains a contentious issue in applied physiology. In murine models it is generally accepted that endogenous AMPK mediates a suppressive effect on mTORC1 [5,9,10] activity and consequent muscle growth induced by external loading [5,9,11]. In humans the interactions between the growth-associated and energy modulating pathways are yet to be fully elucidated, as a number of researchers report no inhibitory effect of AMPK on mTORC1 and subsequent signalling [12-15]. Moreover, to date only two published studies have investigated the effects of differing the order of acute concurrent strength and endurance training on molecular and signalling responses associated with protein synthesis [16,17].

Investigations of the molecular responses to acute intra-session sequencing of concurrent training have reported no effect of strength and endurance exercise order (strength followed by endurance or *vice versa*) on the mTORC1 network [16,17]. However, there are notable inconsistencies between studies, as Coffey, Jemiolo, Edge, Garnham, Trappe, Hawley [16] employed repeated high intensity sprints as the endurance stimulus rather than the more commonly used ~30 min of steady state exercise [12-14]. An additional confounding methodological factor is that no study included a condition involving strength training alone. As such, it cannot be accurately determined whether any inhibition of anabolic signalling was caused by the endurance exercise stimulus. Furthermore, in both of these studies the respective exercise protocols were conducted in a fasted state [16,17]. Conducting strength and/or endurance type exercise when fasted is associated with cortisol catabolising protein and phosphorylation of the AMPK network [18]. Furthermore, it has been suggested that low muscle glycogen may impair intracellular signalling pathways responsible for hypertrophy [19]. This may indicate data presented in previous research examining the signalling responses to concurrent training in which participants are fasted may not provide an accurate representation of anabolic signalling. In addition, the real world applications of these studies are lacking, as few athletes perform strength training fasted

The molecular responses to concurrent strength and endurance training in humans remain inconclusive, and thus it is difficult to fully elucidate the specific mechanisms regulating adaptations to concurrent training strategies. The purpose of this study was to answer two questions. Firstly, does combining acute bouts of strength and endurance training result in the inhibition of signalling proteins associated with hypertrophy as a result of the activation of the AMPK signalling network? Secondly, does the order in which strength and endurance training are performed influence the responses of the mTORC1 and AMPK signalling networks in a fed state?

Methods

Study population

Eighteen recreationally resistance-trained men (age: 24 ± 3 y; body mass: 80.5 ± 9.9 kg; height: 177.8 ± 7.5 cm; % body fat: 17.5 ± 7.2%; sum of assessed 1 repetition maximums (leg extension and leg press) (1RMs): 375.6 ± 56.3 kg; $\dot{V}O_{2max}$: 50.1 ± 7.2 ml·kg·min^{-1}) volunteered to participate in the study. Prior to all procedures written informed consent was given in accordance with the Declaration of Helsinki. Participants were matched at baseline for age, body mass, body fat %, 1 RM totals and $\dot{V}O_{2max}$ (all $p > 0.05$) and randomly assigned to one experimental condition. Each participant had completed > 2 years of strength training prior to the study. All participants were free from any endocrine or metabolic contraindications and in all cases participants were asked to refrain from nutritional supplementation or pharmacological interventions for 30 days prior to testing.

Design

A balanced, randomised, between-group study design was employed. Participants were randomly assigned to one of three experimental conditions: i) strength training (ST), ii) concurrent training, with strength training first (ST-END) or iii) concurrent training, with endurance training first (END-ST). Participants in the ST group performed strength training alone; the ST-END group performed strength training immediately followed by an endurance training protocol; those participants designated END-ST performed endurance training immediately followed by strength training. In order to assess the effect of each intervention on signalling factors related to morphological adaptation, muscle tissue samples were collected before, 10 min post exercise and 1 h post cessation of the loading protocols. The participants' ability to maintain their designated strength-training load was assessed to determine if endurance exercise prior to strength training resulted in diminished strength performance.

Methodology

Prior to any trials, assessment of $\dot{V}O_{2max}$ and 1RM loads were performed for the purpose of normalising relative training intensity, all assessments were conducted in line with standardised procedures [20,21]. Upon commencing the study, participants attended the laboratory in a fed state. Final nutritional intake was standardised prior to the experimental protocol (Figure 1), it was consumed ≥1 h prior to any loading and consisted of 2 g carbohydrate/kg body mass, 0.5 g protein/kg (milk protein) body mass and 0.15 g fat/kg body mass. Participants were advised to abstain from exercise, alcohol and caffeine for 24 h prior to each visit.

The strength training protocol consisted of seated leg extensions and seated leg press as these exercises have previously been demonstrated to activate the *vastus lateralis* (VL) and have previously been employed in comparable research [16,22]. It was critical that the VL was activated consequent to the strength training protocol as this muscle was used for harvesting tissue. For each exercise within the strength-training bout, 5 sets of 6 repetitions at 80% 1RM were completed. This protocol and intensity of exercise has been shown to be appropriate for eliciting strength and hypertrophic responses in recreationally trained non-athletes [23,24].

Figure 1 about here

In all instances, the endurance exercise protocol involved participants completing 30 min of submaximal cycle ergometry at 70% power at maximal oxygen uptake ($p\dot{V}O_{2max}$). Visual feedback for pedal frequency, power output and elapsed time were provided. All strength

and/or endurance based exercise commenced at the same time of day (0900 h ± 1h) to avoid any diurnal performance or signalling variations [25].

Muscle biopsies were taken from the VL at baseline, 10 min post and 1 h post cessation of the loading protocols. All muscle tissue was extracted via the puncture biopsy technique from the VL. Prior to incision, local anaesthetic (Bupivacaine Hydrochloride, 0.5% Marcaine) was injected into biopsy site. Initially 1 ml was injected, and if a visible raise in the subcutaneous volume did not appear the needle was slightly retracted and an additional 1 ml injected. The needle was then removed and reinserted into the injection location at 45° and a further 1 ml injected. Following a ≥ 3 min period, an incision was made longitudinally to the line of the VL to cut through subcutaneous tissue and fascia. Following insertion the biopsy needle (Bard Biopsy Systems, Tempe, AZ, USA) was then inserted perpendicular to skin surface. When the tip of the needle passed the muscle facia the angle of the needle was flattened to ~45°, fired, and then immediately withdrawn. The extracted tissue was immediately removed, cleaned with saline, weighed and snap frozen in liquid nitrogen before storage at -80°c. This process was repeated until sufficient tissue (40 – 60 μg) was obtained for biochemical analysis.

Processed muscle tissue was analysed for total and phosphorylated signalling proteins associated with the mTOR and AMPK signalling networks. The analysed signalling proteins within the mTOR network included; 4E binding protein 1 (4E-BP1), mammalian target of rapamycin (mTOR), protein kinase B (PKB) and 70-kDa S6 protein kinase (S6k1). The analysed signalling proteins of the AMPK network included; acetyl-CoA carboxylase (ACC), AMPK, eukaryotic elongation factor 2 (eEF2) and tuberous sclerosis complex 2 (TSC2), the stress activated protein mitogen-activated protein kinase (p38) was also analysed. Muscle tissue (10–15 ųg) was scissor minced in lysis buffer on ice (50 mM Tris, pH 7.5, 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM NaVO4, 50 mM NaF, 0.50% protease inhibitor cocktail). Samples were then shaken for 1 h (800 rpm) at 4°C before centrifugation for 60 min at 12000 g. The supernatant was subsequently removed from the pellet to a clean tube and used to determine protein concentration via a DC Protein Assay (Bio-Rad Laboratories, Hertfordshire, UK). Equal amounts of protein were first boiled in Laemmli sample buffer (250 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue, 5% β-mercaptoethanol). Subsequently 10-30 μg protein from each sample was separated on precast Criterion (Bio-Rad Laboratories) SDS polyacrylamide gels (4–20% gradient gels) for ~75 min at 150 V. Proteins were transferred to a Protran nitrocellulose membrane (Whatman, Dassel, Germany) at 30 V for 4 h. Membranes were then blocked in 5% BSA-Tris-buffered saline with 0.1% Tween-20 (TBST) and then incubated overnight at 4°C with the appropriate primary antibody. The antibodies were used at the following dilutions: rabbit monoclonal GAPDH 1:5,000, rabbit polyclonal 1:1,000 4E-BP1$^{ser37/41}$, ACC^{ser79}, AMPK^{Thr172}, eEF2^{Thr56}, mTOR^{ser2448}, p38$^{Thr80/Thy182}$, PKB^{ser437}, S6k1^{Thr389} and TSC2^{ser939} (14C10; Cell Signaling Technology, Danvers, MA).

Following overnight incubation, the membranes underwent 3 × 5 min washes in TBST. The membrane was then incubated for 1 h at room temperature with horseradish peroxidase-linked anti-rabbit IgG (1:10,000; Abcam, Cambridge, UK) or anti-mouse (1:10,000; Abcam, Cambridge, UK), diluted in 5% BSA-TBST. The membrane was then cleared of the antibody using TBST. Antibody binding was detected using enhanced chemiluminescence (GE Healthcare Biosciences, Pittsburgh, PA). Molecular weight was estimated using molecular weight Kaleidoscope Prestained Standards (Bio-Rad Laboratories). To improve antibody performance, reduce nonspecific bands and the variability of quantifying different membranes the following procedure was performed: prior to transfer, the gels were cut at the molecular

weight markers which corresponded to the molecular weight (KDa) of the proteins analysed in the respective runs. All of the gel segments for the entire data set were transferred onto a single membrane for each protein. This allowed clearer visualization of the time course response of the proteins. Following imaging and band quantification of phosphorylation of the analysed proteins membranes were stripped for 30 min at 50°C in stripping buffer (65 mM Tris HCl, 2% SDS vol/vol, 0.8% mercaptoethanol vol/vol) and reblocked, followed by an overnight incubation in the corresponding total primary antibody. All imaging and band quantification were carried out using a bioimaging Gel Doc system (Bio-Rad Laboratories). Quantified phosphorylated 4E-BP1$^{ser37/41}$, ACC^{ser79}, AMPK^{Thr172}, eEF2^{Thr56}, mTOR^{ser2448}, p38$^{Thr80/Thy182}$, PKB^{ser437}, S6k1^{Thr389} and TSC2^{ser939} were divided by the total corresponding protein.

Statistical Analysis

Performance data presented as mean ± standard deviation and molecular data are presented as mean ± standard error. Molecular and signalling data were transformed to arbitrary units and normalised to individual baseline data [12,16]. Prior to analysis dependant variables were verified as meeting required assumptions of parametric statistics and changes in molecular variables were analysed using mixed model repeated measures ANOVA tests. ANOVA analysed differences between 3 conditions (ST, ST–END and END–ST) and 3 time points (pre, post and 1 h post exercise cessation). Participant's ability to maintain their individual required training intensity were analysed using one way ANOVA tests. ANOVA analysed differences between 3 conditions ST, ST–END and END–ST). The alpha level of 0.05 was set prior to data analysis. Assumptions of sphericity were assessed using Mauchly's test of sphericity, if the assumption of sphericity was violated Greenhouse Gessier correction was employed. If significant effects between conditions or over time were observed *post-hoc* differences were analysed with the use of Bonferroni correction. Statistical power of the study was calculated post-hoc using G*Power statistical software (v3.1.3, Düsseldorf, Germany) using the effect size, group mean, SD and sample size of the primary outcome measures, in this case being the signalling proteins of the mTOR network. Power was calculated as between 0.8 and 1 indicating sufficient statistical power [26].

Results

Participants' ability to maintain strength training load was not affected by experimental condition ($F_{(2, 15)} = 0.491$, $p = 0.621$; Figure 2).

Figure 2 about here

No time x group interaction was observed for p-4E-BP1 ($F_{(4, 28)} = 0.405$, $p = 0.804$). p-4E-BP1 changed significantly over time ($F_{(2, 28)} = 4.943$, $p = 0.015$). p-4E-BP1 was significantly lower than baseline values post exercise in participants following in ST (29.6 ± 13.6%) ($p = 0.01$). 1 h post exercise in ST-END p-4E-BP1 was 41.1 ± 7.2% greater than post exercise ($p = 0.04$) although no difference was observed between pre and post exercise ($P = 0.10$). No differences were observed in END-ST (both $p > 0.05$).

No time x group interactions were observed for p-S6k1 ($F_{(4, 28)} = 0.638$, $p = 0.64$). A time effect was observed for p-S6k1 ($F_{(2, 28)} = 11.733$, $p < 0.001$). Both ST and ST-END elicited increases from baseline (18.5 ± 58.7% and 57.9 ± 93.9% respectively) in p-S6k1 1 h post exercise (113.4 ± 119.3% and 145.6 ± 191.4%; both $p < 0.05$). No such increase from baseline was observed

1 h post exercise in END-ST ($p = 0.19$). END-ST did however elicit significant increases in p-S6k1 from post to 1 h post exercise ($p = 0.04$).

Figure 3 about here

No time x group interactions were observed for p-ACC ($F_{(4, 28)} = 1.884$, $p = 0.141$), however phosphorylation changed over time ($F_{(2, 28)} = 5.751$, $p = 0.008$; Figure 3). END-ST resulted in significant elevations from baseline both post (91.2 ± 22.4%) and 1 h post exercise (51.5 ± 33.9%; both $p < 0.05$).

Figure 4 about here

Figure 5 about here

Neither time x group interactions ($F_{(4, 28)} = 0.873$, $p = 0.492$), nor effects of time ($F_{(2, 28)} = 2.494$, $p = 0.101$) were reported for p-mTOR, p-PKB, p-AMPK, p-eEF2, p-p38, p-TSC2 (Figures 3 and 4).

Discussion

The aim of this study was to examine whether manipulating the order of acute loadings of strength and endurance training influenced the responses of the mTOR and AMPK signalling networks in a fed state.

The responses of the mTOR and AMPK networks to ST, ST-END and END-ST were similar. As such, the findings of this study indicate that when strength and endurance training are performed in close proximity (following a feeding strategy) the order of the respective exercise modalities does not influence the anabolic nor energy modulating signalling responses. Similar to the present study, previous research has also reported elevations in p-S6k1 following both strength training in isolation and concurrent training [15-17,27,28]. The similar increases in p-S6k1 following strength training and strength training closely followed by endurance training are consistent with recent comparable research [14]. Additionally, Coffey *et al.* [16] also reported up-regulation of S6k1 activity following strength training, however no such increases were observed when strength training was performed following a bout of high intensity interval training. It was suggested that strength training performed after repeated sprints was undertaken in the presence of greater metabolic acidosis (confirmed by greater elevated blood lactate concentrations) when compared with the initial exercise bout, which contributed to the attenuated increase in S6k1. This hypothesis is supported by research demonstrating associations between metabolic acidosis and protein degradation in both rodents and humans [29,30].

Unlike much other research investigating signalling proteins in response to concurrent strength and endurance training [15-17], the present study was conducted following a standardised feeding strategy. This was designed to replicate conditions in which strength training would typically be conducted (i.e. real life practical application). Furthermore, low glycogen content has been shown to blunt signalling responses consequent to strength training [31]. It should be noted that one study has observed rates of myofibrillar protein synthesis to be similar when resistance training was conducted in the presence of high and low nutrient availability [32], although this study involved resistance training in isolation. As previously stated, contrasting findings to the present study have been reported by Lundberg *et al.* [27], who observed p-S6k1 to be greater

when strength training was conducted following endurance exercise than strength training alone. Not only did the authors allocate a 6 h interval between endurance and strength training, but also provided participants with a meal (containing; 2.02 g CHO·kg^{-1} bw, 0.62 g protein·kg^{-1} bw and 0.49 fat·kg^{-1} bw) and a commercially available energy drink following endurance exercise/prior to the strength training protocol. This resulted in glycogen levels being similar between trials involving endurance prior to strength training and strength training in isolation, and may account for the augmenting effect of prior cycling exercise on the anabolic responses to strength training. These findings indicate that if strength training is to be performed subsequent to endurance training on the same day, or within the same session, a feeding strategy between exercise bouts may prevent "unfavourable" signalling responses for strength training related adaptation. In addition, Lundberg *et al.* [27] allowed 6 h recovery between strength and endurance training. These data elude to residual fatigue due to close proximity of strength and endurance training resulting in differing anabolic signalling associated with concurrent training. This may explain why previous studies involving short time periods between strength and endurance training [16,17] observed attuenated anabolic signalling. Additionally, research has demonstrated that the inhibition of strength development within a concurrent regimen may be avoided if sufficient recovery periods (6 – 8 h) are allowed between strength and endurance training [33]. These findings have implications for periodized programming, specifically if hypertrophy is the priority of a particular macro/micro cycle. In this scenario endurance and strength type training should be isolated from each other to ensure that adequate recovery time is allowed to facilitate "optimal" anabolic responses.

Strength training performance in the present study was similar between conditions, as the preceding bout of endurance training had no effect on participant's ability to maintain the required training intensity. This is contrary to previous research that has indicated strength-training quality and quantity is decremented when performed after endurance training [33-37]. This may indicate the endurance training protocol employed in the present study was of insufficient volume and intensity to induce any noteworthy fatigue and impair subsequent strength training performance. A similar protocol was employed by Coffey *et al.* [17] (30 min cycling at a power output that elicited ~70% of $\dot{V}O_{2peak}$). Whilst these authors did not report strength-training performance it was observed that p-AMPK did not significantly increase from baseline at any point of the experimental protocol (strength then endurance training or *vice versa*). These data may suggest that those seeking to further investigate the molecular adaptations to strength and endurance training should employ an endurance protocol that does not consist of 30 min cycling at ~70% $\dot{V}O_{2peak}$/p$\dot{V}O_{2max}$, as limited phosphorylation of the relevant analysed signalling protocols occur following this protocol. Perhaps high intensity interval exercise should be considered as an alternative due to its potency to upregulate relevant signalling cascades [16,17,38].

The present study is not without limitations. Firstly, signalling responses were only assessed immediately post and 1 h post exercise, as such it is possible that differing signalling responses would had been observed if muscle tissue collection took place at ≥3 h post exercise, as others have observed difference signalling responses at this time point [17]. It may also be reasonable to suggest that the (although small) protein bolus provided to the trail may have influenced the signalling responses to the exercise loadings. Additionally, fractional protein synthesis rate was not directly measured but merely anabolic signalling as a proxy of protein synthesis. As such future research should perhaps employ a similar design to that of the present study but extend the post exercise period in which signalling was assessed. Furthermore a direct assessment of total protein synthesis would provide a more robust representation of the influence of concurrent training sequencing on anabolic responses.

Conclusions

This study investigated the molecular responses to acute diverse contractile activity in a fed state. Data indicate that ST, ST-END and END-ST elicited similar responses of both the mTOR and AMPK networks. As such, data presented in the study indicate that when performed in close proximity, the order of strength and endurance training is inconsequential to anabolic and energy modulating signalling when performed in a fed state.

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Competing interests

The authors have no real or perceived conflict of interest in respect of this manuscript.

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Figures and Tables

Figure 1. Schematic representation of experimental time line. ST (*n = 6*), ST-END (*n = 6*, END-ST (*n = 6*).

Figure 2. Mean training load achieved in the ST (*n = 6*), ST – END (*n = 6*) and END–ST (*n = 6*) conditions. ST, strength training alone; ST–END, strength training followed by endurance training; END–ST, endurance followed by strength training. Dashed line indicates required training intensity.

Figure 3. Mean responses of the mTOR signalling network in ST (*n = 6*), ST–END (*n = 6*) and END–ST (*n = 6*) conditions. ST, strength training alone; ST–END, strength training followed by endurance training; END–ST, endurance followed by strength training. (**A**) p-4E-BP1, (**B**) p-PKB, (**C**) p-mTOR and (**D**) p-S6k1. * Significantly greater than pre ($p < 0.05$). ** Significantly greater than post ($p < 0.05$). † Significantly lower than pre ($p < 0.05$).

Figure 4. Mean responses of the AMPK signalling network in the ST (*n = 6*), ST–END (*n = 6*) and END–ST (*n = 6*) conditions. ST, strength training alone; ST–END, strength training followed by endurance training; END–ST, endurance followed by strength training. (**A**) p-ACC, (**B**) p-AMPK, (**C**) p-eEF2 and (**D**) p-p38. * Significantly greater than pre ($p < 0.05$).

Figure 5. Representative images of proteins analysed.