Acute molecular responses in untrained and trained muscle subjected to aerobic and resistance exercise training versus resistance training alone

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Abstract

Aim: This study assessed and compared acute muscle molecular responses before and after 5-week training, employing either aerobic (AE) and resistance exercise (RE) or RE only.

Methods: Ten men performed one-legged RE, while the contralateral limb performed AE followed by RE 6 h later (AE + RE). Before (untrained) and after (trained) the intervention, acute bouts of RE were performed with or without preceding AE. Biopsies were obtained from m. vastus lateralis of each leg pre- and 3 h post-RE to determine mRNA levels of VEGF, PGC-1α, MuRF-1, atrogin-1, myostatin and phosphorylation of mTOR, p70S6K, rpS6 and eEF2.

Results: PGC-1α and VEGF expression increased (P < 0.05) after acute RE in the untrained, but not the trained state. These markers showed greater response after AE + RE than RE in either condition. Myostatin was lower after AE + RE than RE, both before and after training. AE + RE showed higher MuRF-1 and atrogin-1 expression than RE in the untrained, not the trained state. Exercise increased (P < 0.05) p70S6K phosphorylation both before and after training, yet this increase tended to be more prominent for AE + RE than RE before training. Phosphorylation of p70S6K was greater in trained muscle. Changes in these markers did not correlate with exercise-induced alterations in strength or muscle size.

Conclusion: Concurrent exercise in untrained skeletal muscle prompts global molecular responses consistent with resulting whole muscle adaptations. Yet, training blunts the more robust anabolic response shown after AE + RE compared with RE. This study challenges the concept that single molecular markers could predict training-induced changes in muscle size or strength.

Keywords concurrent exercise, gene expression, human skeletal muscle, mammalian target of rapamycin, p70S6 kinase.

It is generally held resistance (RE), but not aerobic (AE) exercise augments myofibrillar protein accretion to result in muscle hypertrophy consequent to chronic training (Kraemer et al. 1995, Coffey & Hawley 2007). However, we recently reported that an acute AE + RE insult prompted greater molecular ‘anabolic’
response than RE alone (Lundberg et al. 2012). Intrigued by these acute findings, we subsequently investigated global muscle responses to an identical AE+RE training regimen (Lundberg et al. 2013). It was found that the concurrent exercise approach produced substantially greater increase in muscle size, compared with RE (Lundberg et al. 2013). Thus, and in contrast to the general believe, it appears concurrent AE could potentiate the hypertrophic stimulus to short-term RE training.

Exercise-induced muscle hypertrophy occurs through increased rate of muscle protein synthesis and/or reduced breakdown, enhancing net protein turnover and allowing for protein accretion (Kumar et al. 2009). Acute RE elicits a 2- to 5-fold increase in muscle protein synthesis that may persist for 48 h (Kumar et al. 2009). Somewhat underappreciated, bouts of acute AE also stimulate muscle protein synthesis (Sheffield-Moore et al. 2004, Miller et al. 2005, Harber et al. 2010), albeit partly due to an increased synthetic rate in the sarcoplasmic protein fraction (Wilkinson et al. 2008). Collectively, these reports infer increased contractile activity imposes altered global, rather than mode-specific protein metabolism of untrained muscle.

Skeletal muscle protein synthesis is regulated via molecular signalling routes enhancing mRNA translation. In this regard, the mammalian target of rapamycin (mTOR) pathway is believed to play a major role (Dreyer et al. 2006, Coffey & Hawley 2007), by regulating translation efficiency, including downstream molecules p70S6 kinase (p70S6K), ribosomal protein S6 (rpS6) and eukaryotic elongation factor 2 (eEF2). Activation of these proteins is accompanied by increases in muscle protein synthesis, favouring muscle hypertrophy in response to chronic RE (Mayhew et al. 2009, Dreyer et al. 2010). Interestingly, robust mTOR signalling is evident after both RE and AE (Dreyer et al. 2006, Mascher et al. 2007, Camera et al. 2010), indicating enhanced translational capacity after either exercise mode. However, it is unclear whether this translates into amplified (Lundberg et al. 2012), unaltered (Carrithers et al. 2007) or compromised (Coffey et al. 2009) intracellular signalling responses, when AE and RE are performed concurrently. Notwithstanding, our recent research findings suggest a particular 5-week AE+RE paradigm performed by previously untrained subjects (Lundberg et al. 2013), produced greater muscle hypertrophy than RE alone.

Further, it appears acute exercise prompts a more transient and specific cellular response in trained than untrained muscle (Tang et al. 2008, Wilkinson et al. 2008), inferring that chronic training blunts the non-specific response to acute exercise, evident in untrained muscle (Kim et al. 2005, Kumar et al. 2009). Eventually, this would facilitate robust adaptations of specific protein fractions, that is, myofibrillar and mitochondrial, known to be most readily stimulated by RE (Tesch 1988, Kim et al. 2005) and AE (Hawley 2002) training respectively.

While translational signalling events have been associated with the muscle protein synthetic response to RE (Mayhew et al. 2009), a few reports suggest ‘snapshot’ molecular markers may in fact predict muscle adaptive responsiveness to chronic RE training. For example, phosphorylation of p70S6K correlated with the subsequent increase in muscle mass following 6-week electrical stimulation in rats (Baar & Esser 1999), as well as 14-week RE training in men (Terzis et al. 2008). This, however, is not a consistent finding, and a recent study employing three different RE regimens failed to confirm such relationship (Mitchell et al. 2012). At the gene expression level, downregulation of myostatin induced by acute RE correlated with the subsequent gains in 1RM strength and muscle cross-sectional area after training (Raue et al. 2012). Given the ambiguity, more evidence is warranted before single molecular markers can be identified to predict long-term physiological outcomes, with any certainty.

To this background, the primary goal of this study was to explore acute muscle molecular responses before and after 5-week training, employing either AE+RE or RE only. The current study design also allowed for correlating acute molecular changes before training, with rate of muscle hypertrophy and changes in in vivo muscle function elicited by either exercise programme. It was hypothesized that (i) acute gene expression and protein phosphorylation would infer greater ‘anabolic’ response after AE+RE compared with RE in the untrained, but not trained state (ii) molecular responses would display a more ‘mode-specific’ response in trained than untrained muscle; and (iii) p70S6K and myostatin would correlate with changes in muscle size and in vivo muscle function resulting from both AE+RE and RE training.

**Material and methods**

**General design**

Ten young men performed unilateral AE+RE and RE training of either limb over 5 weeks. The schedule comprised 15 AE and 12 RE sessions. Before and after training, an acute RE bout with or without preceding (6 h) AE was completed (Fig. 1). Percutaneous biopsies were obtained from m. vastus lateralis before and 3 h after RE. Tissue samples were subsequently analysed for gene expression and mTOR-related protein phosphorylation. Molecular responses determined in
the untrained and trained condition, respectively, were compared across AE+RE and RE. Equally important, the protocol also allowed for assessing the relationships between molecular responses and training-induced muscle adaptations (Lundberg et al. 2013).

Subjects

Ten male college students (25 ± 4 years, 184 ± 6 cm and 83 ± 13 kg) volunteered for the study. Subjects were healthy and moderately active individuals, engaged in 3–5 h recreational sports (e.g. skiing and team sports) weekly, with limited or infrequent experience of RE. Thus, no subject had participated in structured RE for the past year. Subjects were informed of the purposes and risks involved in the study before giving their informed written consent for participation. The study protocol was approved by the Regional Ethical Review Board in Umeå. Subjects complied with all testing and training protocols prescribed in the study.

Training protocol

Training protocols and equipment have been described in detail elsewhere (Lundberg et al. 2013). In brief, one leg performed concurrent AE+RE, while the opposite limb was subjected to RE only. Legs chosen for AE+RE were randomized in a counterbalanced manner. The 5-week training period comprised 15 AE and 12 RE sessions. To allow for recovery between bouts, RE was performed 6 h after completion of AE on the same day. AE was conducted on a one-legged cycle ergometer emphasizing and isolating the knee extensor muscles (Andersen et al. 1985). An incremental test was performed to assess maximal workload (Wmax) and endurance performance (Lundberg et al. 2013). Resistance was increased by 2.5 N every 2nd min until failure to maintain a cadence of 60 rpm. Wmax was defined as the last successfully completed workload. Subjects cycled 40 min at a load of ~70% of Wmax at 60 rpm. To ensure highly strenuous efforts, ratings of perceived exertion were continuously monitored such that the intensity could be modified during each session. Upon completion, workload was increased by ~20 W and exercise continued until failure to maintain cadence (~2.5 min). RE comprised maximal concentric and eccentric unilateral knee extensions for both legs using a flywheel knee extension (Tesch et al. 2004) ergometer (YoYo® Technology, Stockholm, Sweden). Each leg performed 4 sets of 7 repetitions with 2-min recovery between sets. Visual real-time feedback of power (RE and AE) and cadence (AE) was provided and hence ensured maximal efforts. Changes in muscle size and function evoked by the current exercise regimens have been reported elsewhere (Andersen et al. 1985, Tesch et al. 2004, Lundberg et al. 2013).

Acute exercise bouts and muscle biopsies

Acute exercise bouts were performed 5–7 days before (untrained) and 3–4 days after (trained) the 5-week training period (Fig. 1). First, the leg subsequently assigned to AE+RE training performed acute AE as described above. Six h after completion, RE was executed by means of 4 × 7 maximal repetitions for each leg in the knee extension ergometer. Percutaneous muscle biopsies (Bergstrom 1962) were obtained from vastus lateralis immediately before (PRE) and 3 h after (POST) the RE bout. Local anaesthesia was administered to the skin and fascia before incision. Subsequent incisions were made 20 mm proximal to the previous biopsy site. Bergström needles (5 mm) with suction applied were used to obtain ~180 mg tissue samples that were visually inspected, and excess blood, fat and connective tissue were removed, before frozen in liquid nitrogen pre-cooled isopentane, and stored at −80°C until analysed. A standardized dinner (pasta, tomato sauce and juice) was provided the night before the acute exercise bouts consisting of 2.21 g carbohydrates kg⁻¹ body weight (bw), 0.22 g protein kg⁻¹ bw and 0.04 g fat kg⁻¹ bw. Breakfast (1.01 g carbohydrates kg⁻¹ bw, 0.31 g protein kg⁻¹ bw).
bw and 0.24 g fat·kg\(^{-1}\) bw) and lunch (2.02 g carbohydrates·kg\(^{-1}\) bw, 0.62 g protein·kg\(^{-1}\) bw and 0.48 g fat·kg\(^{-1}\) bw) were also standardized on days including biopsies (Ensure Plus\textsuperscript{®}, Abbott Laboratories B.V., Zwolle, Netherlands). Breakfast was provided 1 h prior to AE, and lunch 3 h prior to RE.

RNA isolation, reverse transcription and real-time PCR

Muscle tissue samples (~20 mg wet weight) were homogenized using TRIzol\textsuperscript{®} (Invitrogen Life Technologies, Carlsbad, CA, USA), and total RNA was extracted. Reverse transcription into cDNA was performed on 1 μg of total RNA from each sample following the instructions of a commercial kit in a final volume of reverse transcription into cDNA was performed on

muscle RING-finger protein-1 (MuRF-1; Hs00822397_m1), myostatin (Hs00193363_m1), peroxisome proliferator-activated receptor-γ coactivator-1z (PGC-1z; Hs0101672z_m1) and vascular endothelial growth factor (VEGF; Hs99999070_m1) were derived from the TaqMan\textsuperscript{®} Gene Expression Assays (Applied Biosystems). GAPDH (Hs99999905_m1) and 18S (Hs01375212_g1) were used as reference genes. The expression levels of reference genes were almost identical across time points, and the GAPDH/18S ratio did not change. Hence, the results were related to GAPDH only. All reactions were performed using MicroAmp\textsuperscript{®} Fast Optical 96-well reaction plates (Life Technologies). Amplification mixes (10 μL) contained the diluted (1 : 100) cDNA sample (4.5 μL), 2× TaqMan\textsuperscript{®} Fast Universal PCR Master Mix (5.0 μL) and specific primers (0.5 μL). Thermal cycling protocol employed consisted of 2 min at 50°C and 10 min at 90°C followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Gene expression levels were determined using the 2\(^{-\Delta\Delta C T}\) method, which relates mRNA changes as a ratio to the reference gene.

Protein extraction and western blot

About 30 mg wet muscle of each sample was homogenized using glass homogenizers and RIPA buffer containing 150 mM NaCl, 10 mM Tris-HCl (pH 7.8), 5 mM EDTA, 0.5% NaDeoxycholate, 0.1% SDS, 0.1% Triton X-100, double-distilled water and protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Twenty μL of buffer was used per 1 mg of frozen muscle tissue. After rotating the homogenate for 1 h at 4°C, the samples were centrifuged at 15 000 g during 10 min at 4°C. The supernatant was recovered, and protein concentration subsequently determined using the Bradford technique. Samples containing 40 μg of protein were separated by SDS-polyacrylamide gel (7.5% or 10% acrylamide) electrophoresis together with a protein ladder. The same gel was used to run all the samples for any subject. Upon completion of electrophoresis, gels were equilibrated in transfer buffer for 20 min and then transferred to PVDF membranes (90 min at 90 V). Non-specific binding was blocked by pre-incubation of the membranes in fluorescent blocking buffer (Millipore, Billerica, MA, USA) during 60 min at room temperature (RT). The membranes were then incubated overnight at 4°C with antibodies against phospho-mTOR (Ser2448, 1 : 500), phospho-rpS6 (Ser235/236, 1 : 900), phospho-eEF2 (Thr56, 1 : 1500) and phospho-P70S6K (Thr389, 1 : 1000). The antibodies for mTOR, rpS6 and eEF2 were from Cell Signaling Technology (Beverly, MA, USA), and the antibody against p70S6K from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Membranes were washed (4 × 5 min) in PBST (0.1%) and subsequently incubated with IRDye\textsuperscript{®} secondary antibody (LI-COR Biosciences, Cambridge, UK) for 60 min at RT (concentration 1 : 10 000 or 1 : 20 000). A final wash was then carried out (4 × 5 min in PBST and 2 × 5 min in PBS). An Odyssey\textsuperscript{®} SA Infrared Imaging System (LI-COR Bioscience) was used to scan the membranes, and public domain software ImageJ (National Institutes of Health, Bethesda, MD, USA) was used for quantification of the blots. Target proteins were expressed relative to α-tubulin abundance (1 : 20 000; Sigma-Aldrich, St. Louis, MO, USA) to ensure equal protein loading.

Data analysis

Results are presented as mean ± SD. Gene expression and protein concentration were analysed using repeated-measures ANOVA. First, differences from PRE to POST (time) and AE+RE vs. RE (leg), in the untrained and trained condition, respectively, were assessed by two-way ANOVA. Responses in untrained and trained muscle were compared employing a two-way ANOVA with factors condition (untrained; trained) and leg (AE+RE; RE). Positively skewed variables were log-transformed. When significant interactions or main effects were found, simple effect tests were employed and the false discovery rate procedure used, to compensate for multiple post hoc comparisons (Curran-Everett 2000). Pearson’s product moment correlation was used to examine molecular responses before training (AE+RE; n = 10, RE; n = 10) and combined across legs (n = 20), in relation to...
established end-point outcomes as reported elsewhere (Lundberg et al. 2013). More specifically, the relative changes in selected markers regulating protein turnover (MuRF-1, atrogin-1, myostatin, mTOR, p70S6K, rpS6 and eEF2) on one hand, and whole muscle and fibre size, and strength and power performance, were correlated. The level of significance was set at 5% ($P < 0.05$). All statistical analyses were performed using spss v.20 (SPSS, Chicago, IL, USA).

**Results**

**Functional and muscular adaptations**

Changes in in vivo muscle function and whole muscle and fibre size following AE+RE and RE have been reported in detail elsewhere (Lundberg et al. 2013). In brief, knee extension peak power increased ($P < 0.05$) by 29 and 24% following AE+RE and RE, respectively, with no difference across legs. Likewise, peak torque increased ~30% in both conditions. The increase in m. quadriceps femoris volume, as determined by MRI, was greater ($P < 0.05$) after AE+RE (14%) than RE (8%). Mean fibre cross-sectional area (CSA) increased by 17 ($P < 0.05$) and 9% ($P > 0.05$) after AE+RE and RE training respectively.

**Acute aerobic and resistance exercise**

Power during 40-min one-legged cycling was 35% greater in the trained compared with the untrained state (Table 1). RE peak power was ~30% greater in the trained vs. the untrained state for both AE+RE and RE (Table 1). While the absolute work produced during acute AE and RE was greater after training, perceived exertion was similar on both occasions (Table 1).

**Gene expression**

PGC-1z. PGC-1z was assessed as a marker for exercise-induced mitochondrial biogenesis. In the untrained state, there was a time x leg interaction ($F = 13.6, P = 0.005$; Fig. 2a). While RE showed a twofold increase from PRE to POST ($P = 0.014$), PGC-1z was unaltered ($P = 0.057$) in AE+RE. Levels were higher in AE+RE at PRE (5.6-fold) and POST (2.1-fold) compared with RE ($P < 0.003$). In the trained state, there was a time x leg interaction ($F = 40.6, P < 0.0005$). Thus, while PGC-1z expression was unchanged from pre to post after RE, there was a 1.6-fold decrease in AE+RE ($P < 0.0005$). Levels were 3.2-fold higher in AE+RE than RE at PRE ($P < 0.0005$). A condition x time interaction was also found ($F = 43.4, P < 0.0005$), due to decreased PGC-1z expression in AE+RE from untrained to trained ($P < 0.0005$), with no change in RE. The data show that PGC-1z is increased in untrained, but not trained muscle after RE, and that any exercise response is more short-lived after training.

VEGF. VEGF is paramount for exercise-induced angiogenesis. In the untrained state, there was a time x leg interaction ($F = 9.1, P = 0.015$; Fig. 2b). VEGF expression increased 1.3-fold ($P = 0.018$) from PRE to POST in RE, but did not change in AE+RE. Levels were higher in AE+RE at PRE (2.7-fold) and POST (2.0-fold) compared with RE ($P < 0.0005$). In the trained state, there was a trend towards a time x leg interaction ($F = 3.6, P = 0.057$) from PRE to POST in AE+RE, with no change in RE. The data show that PGC-1z is increased in untrained, but not trained muscle after RE, and that any exercise response is more short-lived after training.

**Table 1.** Selected performance and metabolic measures during acute exercise bouts in the untrained and trained state

| Aerobic exercise | Untrained | | | Trained | |
|------------------|-----------| | |-----------| |
| Average power (W) | 37 ± 5 AE+RE | 50 ± 11* | | 50 ± 11 | |
| Blood lactate at 40 min (mM) | 3.8 ± 1.1 RE | 5.8 ± 1.8* | | 5.8 ± 1.8 | |
| Blood lactate at completion (mM) | 5.0 ± 1.1 AE+RE | 7.2 ± 2.6* | | 7.2 ± 2.6 | |
| Average HR (bpm) | 122 ± 13 RE | 137 ± 14* | | 137 ± 14 | |
| HR at exercise completion (bpm) | 164 ± 18 RE | 171 ± 17 | | 171 ± 17 | |
| RPE at 40 min (central/local) | 14/17 RE | 15/17 | | 15/17 | |
| RPE at exercise completion (central/local) | 16/19 RE | 17/20 | | 17/20 | |

| Resistance exercise | Untrained | | | Trained | |
|---------------------|-----------| | |-----------| |
| Peak CON power (W) | 347 ± 113 AE+RE | 355 ± 105 | | 451 ± 123* | |
| Peak ECC power (W) | 375 ± 131 RE | 389 ± 122 | | 478 ± 130* | |

AE+RE, leg subjected to aerobic and resistance exercise; RE, leg subjected to resistance exercise only; HR, heart rate; RPE, ratings of perceived exertion; CON, concentric; ECC, eccentric.

Means ± SD.

*Significant differences ($P < 0.05$): untrained vs. trained.
interaction ($F = 4.7$, $P = 0.058$) due to a slight decrease of VEGF in AE+RE with no change in RE. AE+RE showed greater VEGF expression at PRE (2.2-fold) and POST (1.7-fold) than RE (main effect of leg, $P < 0.0005$). A trend towards an interaction condition x leg was found ($F = 4.7$, $P = 0.058$), due to somewhat lower VEGF in AE+RE compared with RE, in the trained state. Thus, the response of VEGF and PGC-1α appeared to occur in parallel.

**MuRF-1.** MuRF-1 and atrogin-1 are established markers for targeted protein degradation. There was a time x leg interaction in MuRF-1 expression in the untrained state ($F = 11.8$, $P = 0.007$; Fig. 3a). Thus, while mRNA levels tended to decrease (1.5-fold) from PRE to POST in AE+RE, no change was found in RE. Levels of MuRF-1 were higher (2.2-fold, $P < 0.0005$) in AE+RE than in RE at PRE. In the trained state, there was a time x leg interaction ($F = 33.3$, $P < 0.0005$) due to decreased atrogin-1 levels from PRE to POST in both RE and AE+RE (1.3- to 1.5-fold; Fig. 3b). Additionally, there was a main effect of leg ($P = 0.008$), because values were overall greater in AE+RE than RE. In the trained state, there was a time x leg interaction ($F = 13.1$, $P = 0.006$). Both AE+RE and RE showed decreased ($P < 0.002$) atrogin-1 expression from PRE to POST. The decrease was greater in AE+RE than RE (1.5-fold vs. 1.3-fold). Hence, AE+RE showed lower atrogin-1 expression at POST compared with RE (1.3-fold, $P = 0.006$). There was a condition x leg interaction ($F = 22.5$, $P = 0.001$) resulting from increased atrogin-1 expression in RE after training ($P = 0.04$), with no change in AE+RE. Furthermore, AE+RE showed greater atrogin-1 expression than RE in the untrained condition ($P = 0.002$). The opposite was true in the trained condition, that is, expression levels were lower after AE+RE than RE ($P = 0.028$). Thus, the results suggest that in untrained muscle, but not trained muscle, combined AE+RE produces greater MuRF-1 expression than isolated RE.

**Atrogin-1.** There was no time x leg interaction ($P > 0.05$) in the untrained state. However, there was a main effect of time ($F = 41.8$, $P < 0.0005$) due to decreased atrogin-1 levels from PRE to POST in both RE and AE+RE (1.3- to 1.5-fold; Fig. 3b). Additionally, there was a main effect of leg ($P = 0.008$), because values were overall greater in AE+RE than RE. In the trained state, there was a time x leg interaction ($F = 13.1$, $P = 0.006$). Both AE+RE and RE showed decreased ($P < 0.002$) atrogin-1 expression from PRE to POST. The decrease was greater in AE+RE than RE (1.5-fold vs. 1.3-fold). Hence, AE+RE showed lower atrogin-1 expression at POST compared with RE (1.3-fold, $P = 0.006$). There was a condition x leg interaction ($F = 22.5$, $P = 0.001$) resulting from increased atrogin-1 expression in RE after training ($P = 0.04$), with no change in AE+RE. Furthermore, AE+RE showed greater atrogin-1 expression than RE in the untrained condition ($P = 0.008$). In contrast, there was a trend towards greater atrogin-1 expression after RE
than AE+RE in the trained condition (P = 0.05). Thus, similar to MuRF-1, atrogin-1 showed greater expression after AE+RE in the untrained state.

**Myostatin.** Myostatin is a negative regulator of muscle hypertrophy. In the untrained state, there was a tendency towards a time x leg interaction (P = 0.084) in myostatin mRNA expression due to a slight decrease (1.2-fold) from PRE to POST in RE, with no change in AE+RE (Fig. 3c). Levels of myostatin were higher (P < 0.008) in RE than AE+RE at PRE and POST (3.3- and 2.0-fold respectively). In the trained state, there was a time x leg interaction (F = 18.6, P = 0.002). Myostatin levels decreased in RE from PRE to POST (1.4-fold, P = 0.007), while AE+RE showed no change. Furthermore, AE+RE showed lower myostatin expression than RE at PRE (3.7-fold, P < 0.0005) and POST (1.8-fold, P = 0.001). There was no condition x leg interaction. However, a main effect of leg (P < 0.0005) was found due to the lower myostatin mRNA content in AE+RE than RE. Taken together, exercise-induced downregulation of myostatin was greater after AE+RE than RE.

**Protein phosphorylation**

**mTOR.** Phosphorylation of mTOR was unchanged over time for both legs in the untrained and trained
state, with no difference across legs or conditions (Fig. 4a).

*p70S6K*. In the untrained state, there was a tendency towards a time x leg interaction ($P = 0.078$). Thus, while both AE+RE and RE increased p70S6K phosphorylation from PRE to POST (main effect of time, $P = 0.023$; Fig 4b), AE+RE showed somewhat greater (1.6-fold) increase than RE (1.3-fold). In the trained state, there was no time x leg interaction. However, there was a main effect of time ($P = 0.032$) due to increased levels from PRE to POST in both AE+RE and RE (1.3- and 1.4-fold respectively). There was no condition x leg interaction, yet a main effect of condition ($P = 0.049$). Thus, overall p70S6k phosphorylation levels were higher in the trained compared with the untrained state.

*rpS6*. There was no time x leg interaction in the untrained state (Fig. 4c), but a trend towards a main effect of time ($P = 0.09$), due to increased rpS6 phosphorylation from PRE to POST in both AE+RE and RE (1.1- 1.2-fold). There were no differences over time or across legs in the trained state, and no condition x leg interaction found.

*eEF2*. Phosphorylation of eEF2 was unchanged over time, with no differences across legs or conditions (Fig. 4d).

Representative Western blots for all proteins are shown in Fig. 5. For any antibody, samples from each subject were run on the same gel.

**Correlations**

There were no significant correlations between changes in molecular markers (MuRF-1, atrogin-1, myostatin, mTOR, p70S6K, rp66 and eEF2) and endpoint adaptations (muscle volume, fibre size and strength or power; Lundberg et al. 2013). There was a tendency towards a significant correlation ($R = 0.41, P = 0.076$) between increases in p70S6K phosphorylation and whole muscle volume, when combining AE+RE and RE data.
Discussion

The main object of this study was to assess molecular responses of untrained and trained skeletal muscle, to an acute RE insult with or without preceding AE. We report several novel findings, such that performing AE 6 h prior to RE augmented p70S6K signalling and MuRF-1/atrogin-1 expression in untrained muscle compared with RE alone. Likewise, acute molecular responses were more ‘refined’ in the trained than untrained state, as indicated by the failure of RE to elicit increased transcriptional activity of genes involved in endurance-type adaptations. It is also worth noting that amid molecular adaptations at large coincided with the global muscle changes shown after 5-week training, no single molecular marker could predict training-induced changes in muscle size, strength or power.

Phosphorylation of p70S6K tended to be greater after AE+RE than RE. Yet, there was no distinct sign of superior mTOR signalling after concurrent exercise in the untrained state. Consequently, molecular changes governing translation efficiency cannot fully explain the more substantial muscle hypertrophy noted after 5-week AE+RE training (Lundberg et al. 2013). This, however, does not preclude p70S6K as a key mediator for enhanced protein synthesis. In fact, training augmented the p70S6K response, supporting previous work in lower mammals (Karagounis et al. 2010) that showed summated anabolic and attenuated catabolic signalling following repeated bouts of RE. Thus, cumulative training may boost the p70S6K response, perhaps allowing for more rapid translation initiation after acute exercise. This concerts with the shorter duration, yet more pronounced initial increase, in muscle protein synthesis after acute RE apparent in the trained state (Tang et al. 2008).

Gene expression of PGC-1α and VEGF was assessed to reflect mitochondrial biogenesis and angiogenesis respectively (Gustafsson et al. 1999, Lira et al. 2010). These ‘endurance markers’ responded to a novel RE bout in the untrained, but not trained, state. Moreover, exercise-induced expression of these genes was more short-lived in the trained state, to concord with the acute attenuation of PGC-1α and VEGF with training (Richardson et al. 2000, Coffey et al. 2006, Perry et al. 2010). The finding that PGC-1α and VEGF responded to unaccustomed contractile activity in the untrained state, yet remained unchallenged by RE in the trained state, commensurates with the increased citrate synthase activity evident following AE+RE, but not the RE, regimen (Lundberg et al. 2013). The current results infer that increased global PGC-1α expression after RE does not necessarily enhance muscle oxidative capacity or mitochondrial biogenesis after cumulative training. In view of the recent findings of isoform-specific PGC-1α expression (Norrbon et al. 2011, Ruas et al. 2012), the greater induction of this gene after AE+RE than RE may provide a potential mechanism for the exaggerated muscle hypertrophy reported after AE+RE that warrants exploration in the future.

MuRF-1 and atrogin-1 showed greater expression after acute AE+RE than RE in the untrained state. Given that both genes are involved in proteasome-dependent protein breakdown (Coffey & Hawley 2007), an event necessary for exercise-induced tissue remodelling (Leger et al. 2006, Cunha et al. 2012) and muscle growth (Hwee et al. 2013), it is likely the exacerbated response in the untrained state enabled for more marked muscle hypertrophy following AE+RE. Interestingly, MuRF-1 was lower in AE+RE, and atrogin-1 was similar across legs, in the trained state. Collectively, the responsiveness of MuRF-1 and atrogin-1 to the different exercise challenges imposed here, supports the hypothesis that AE+RE, and more so than RE, produces a greater stimulus for tissue protein turnover in the untrained than trained state. Further and concerted the exercise responses of other selected genes, MuRF-1 and atrogin-1 were attenuated after chronic training. This notion supports the dogma of fast progression in functional and muscular adaptations during the initial training phase.

From the correlation analysis performed here, it is quite evident that relationships between acute molecular markers and muscle adaptations that manifest after chronic training, if present at all, are weak. For example, and purported as the ‘proxy-marker’ of hypertrophy (West et al. 2010), p70S6K did not correlate with the training-induced increase in muscle size. Similarly, p70S6K phosphorylation, determined 1 h post-RE, failed to correlate with the subsequent gains in whole muscle or fibre size after 10-week RE training (Mitchell et al. 2012). Collectively, these findings (current (Mitchell et al. 2012)) contrast the notion of a relationship between p70S6K phosphorylation and training-induced gains in muscle mass in rodents (Baar & Esser 1999) and humans (Terzis et al. 2008). Imperatively, discrepancies in study results could be explained by multiple factors inherent in the study design, for example, time of biopsy sampling, subject physical conditioning level and the particular exercise insult employed. In this context, it should be recalled that Baar and Esser (Baar & Esser 1999) analysed p70S6K phosphorylation in four different rat muscles 6 h after electrical stimulation (twice weekly for 6 weeks) and plotted the percentage change in phosphorylation against the subsequent increase in wet muscle mass. As this design allowed for analysis of responsiveness between muscles, not the relationship...

between acute changes and the resulting chronic responses across animals, it remains open if the magnitude of acute p70S6K phosphorylation could serve as a valid predictor of responsiveness to chronic RE or AE training.

At the transcriptional level, acute exercise downregulates myostatin (Louis et al. 2007, Raue et al. 2012), and such effect appears to correlate with increases in muscle strength and cross-sectional area following 12-week RE training (Raue et al. 2012). In the current study, the more substantial suppression of myostatin after AE+RE than RE was paralleled by whole muscle and fibre size differences across legs (Lundberg et al. 2013). Hence, our group data support the premise that net protein turnover was enhanced in response to the AE+RE regimen employed. Yet, and at odds with findings reported by Raue and associates (Raue et al. 2012), there was no correlation between changes in myostatin and whole muscle or fibre size. Inevitably and given that our RE programme produced marked global muscle adaptations, the current results cast doubt to the hypothesis that acute changes in myostatin mRNA could serve in predicting muscle hypertrophy at the individual level.

It should be acknowledged that total work executed at any time was markedly greater in the leg subjected to AE+RE. Thus, while it cannot be ruled out that any difference in response across legs was due to the total amount of contractile activity, rather than specific exercise mode, this would be an inherent drawback of any experimental design exploring the effects of concurrent training. The limitation in obtaining single post-exercise biopsies should also be appreciated. We chose the 3 h post-RE time point to accommodate for changes in both protein phosphorylation and gene expression. Thus, genes regulating protein turnover typically show responsiveness to acute RE within this time frame (Louis et al. 2007), accompanied by elevated phosphorylation of selected proteins (Wilkinson et al. 2008, Delidicque et al. 2010, Vissing et al. 2013). Yet, it cannot be ruled out that the particular time point chosen to study selected markers could have impacted our results. Also, the molecular response to acute exercise appears to be influenced, and interpretations could be scrutinized by genetic variability (Timmons 2011), nutritional intake (Karlsson et al. 2004, Dreyer et al. 2008) and extent of cross-talk, feedback regulation, redundancy and transiency of activation (Coffey & Hawley 2007). To understand muscle heterogenetic adaptability (Timmons 2011), future studies should look beyond ‘master regulators’ of specific muscle adaptations and explore, for example, global maps of transcriptional regulation (Keller et al. 2011, Raue et al. 2012) and microRNA expression (Davidsen et al. 2011).

Conclusions

This study shows 5-week AE+RE and RE training alters the response of key genes and signalling proteins after acute exercise in concert with the resulting chronic muscle adaptations. The acute gene expression response was typically more short-lived in the trained state. It also appears 5-week training blunts the overall inference of greater acute ‘anabolic’ response shown after AE+RE in untrained muscle. This would infer that RE acts synergistically with RE only during short-term training, as it relates to initiating muscle hypertrophy. Finally, the current study provides no evidence to support the idea that selected skeletal muscle molecular markers could predict diverse inter-individual adaptive responses to chronic RE or AE+RE training.

Conflict of Interest

The authors report no conflict of interest.

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