Human Muscle Gene Expression following Resistance Exercise and Blood Flow Restriction

MICAH J. DRUMMOND, SATOSHI FUJITA, ABE TAKASHI, HANS C. DREYER, ELENA VOLPI, and BLAKE B. RASMUSSEN

Departments of Physical Therapy and Internal Medicine and Division of Rehabilitation Sciences, University of Texas Medical Branch, Galveston, TX; and Department of Human and Engineered Environmental Studies, Graduate School of Frontier Sciences, University of Tokyo, Chiba, JAPAN

ABSTRACT


Introduction: Blood flow restriction in combination with low-intensity resistance exercise (REFR) increases skeletal muscle size to a similar extent as compared with traditional high-intensity resistance exercise training. However, there are limited data describing the molecular adaptations that occur after REFR. Purpose: To determine whether hypoxia inducible factor-1 alpha (HIF-1α) and REDD1 mRNA are expressed differently in REFR compared with low-intensity resistance exercise with no blood flow restriction (CONTROL). Secondly, to determine whether low-intensity resistance exercise is able to induce changes in mRNA expression of several anabolic and catabolic genes as typically seen with high-intensity resistance exercise. Methods: Six subjects were studied at baseline and 3 h after a bout of leg resistance exercise (20% 1RM) in REFR and CONTROL subjects. Each subject participated in both groups, with 3 wk separating each visit. Muscle biopsy samples were analyzed for mRNA expression, using qRT-PCR. Results: Our primary finding was that there were no differences between CONTROL and REFR for any of the selected genes at 3 h after exercise (P > 0.05). However, low-intensity resistance exercise increased HIF-1α, p21, MyoD, and muscle RING finger 1 (MuRF1) mRNA expression and decreased REDD1 and myostatin mRNA expression in both groups (P < 0.05). Conclusion: Low-intensity resistance exercise can alter skeletal muscle mRNA expression of several genes associated with muscle growth and remodeling, such as REDD1, HIF-1α, MyoD, MuRF1, and myostatin. Further, the results from REFR and CONTROL were similar, indicating that the changes in early postexercise gene expression were attributable to the low-intensity resistance exercise bout, and not blood flow restriction. Key Words: mRNA, HIF-1α, REDD1, mTOR, ISCHEMIA–REPERFUSION

Recent work has shown that low-intensity resistance exercise with blood flow restriction (REFR) causes muscle hypertrophy (1,2,26,30–32), which can be measured after only 2 wk of REFR training (2). In addition, when walking exercise (3 × wk⁻¹ at a distance of 1 km·d⁻¹) is combined with blood flow restriction, muscle hypertrophy is also stimulated (1). Thus, it seems that low-intensity muscle contractions, when performed under conditions of restricted blood flow, have the potential to initiate the necessary cellular signals promoting muscle protein synthesis and cell growth. The stimulation of muscle growth with very-low-intensity resistance exercise (20–50% of one-repetition maximum (1RM)) and a shortened duration of training (~2 wk) is of significant interest for understanding the mechanisms regulating human muscle hypertrophy, because traditional resistance exercise recommendations for muscle hypertrophy have generally been accepted as > 70% 1RM, 3 × wk⁻¹, for at least 5–12 wk (18). In an attempt to begin identifying the mechanisms behind REFR-induced muscle hypertrophy, we have recently shown that muscle protein synthesis and ribosomal S6 kinase 1 (S6K1) phosphorylation are increased after a single bout of REFR (12). This suggested that mRNA translation was increased after REFR; however, changes in mRNA expression of muscle hypertrophy regulators may also be involved.

A potential mechanism for how REFR promotes muscle hypertrophy may be the subsequent reperfusion on release of the pressure cuff after the acute muscle hypoxia during the exercise bout. This may promote cell survival and cell-growth adaptations within muscle, such as the activation of the mammalian target of rapamycin (mTOR), a signaling pathway linked to muscle hypertrophy (23). Previous

Address for correspondence: Blake B. Rasmussen, Ph.D., University of Texas Medical Branch, Department of Physical Therapy, Division of Rehabilitation Sciences, 301 University Blvd., Galveston, TX; E-mail: bbras@utmb.edu.

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reports have indicated that hypoxia-inducible factor-1 alpha (HIF-1α) and REDD1 (regulated in development and DNA damage responses) are upregulated after hypoxia (4,13,14,19,27). An increased expression of these proteins has been shown to downregulate the mammalian target of rapamycin (mTOR) signaling pathway (4,7,19,33). However, in our previous publication, mTOR signaling to S6K1 was enhanced after REFR (12). This may have been attributable to muscle hyperemia–reperfusion (after exercise when the pressure cuff was released) inhibition of HIF-1α and REDD1. Therefore, it is unknown whether HIF-1α and REDD1 have a regulatory role during the early postexercise recovery period (3 h) after REFR.

Another potential mechanism for acute changes in muscle hypertrophy after REFR may be the upregulation of hypertrophy-associated genes in skeletal muscle during postexercise recovery. Many reports have identified that several mRNA increase/decrease transiently within 24 h after a single bout of resistance exercise in human skeletal muscle (5,6,15,17,21,24,36,37). However, there is no information available identifying changes in mRNA expression after REFR. Our previous report focused on mechanisms supporting translation initiation (12) while neglecting other hypertrophy signals such as changes in mRNA expression of genes associated with satellite cell control (p21, cyclin D1, insulin-like growth factor-1 receptor (IGF-1R), mechano growth factor (MGF), MyoD, myogenin), cell size (myostatin) and protein turnover (muscle atrophy F-box (MAFbx), muscle RING finger 1 (MuRF1), mTOR, S6K1).

Therefore, we hypothesized that selected mRNA associated with muscle growth and remodeling would be altered to a greater extent in the early postexercise recovery period after REFR compared with low-intensity resistance exercise without blood flow restriction (CONTROL). The aims of the study were 1) to determine whether HIF-1α and REDD1 mRNA are expressed differently in REFR compared with CONTROL, and 2) to determine whether low-intensity resistance exercise is able to induce changes in mRNA expression of several anabolic and catabolic genes as typically seen with high-intensity resistance exercise.

METHODS

Subjects. We studied six young male subjects in a crossover design on two separate occasions, 3 wk apart. All subjects were healthy and physically active but were not currently engaged in an exercise training program. All subjects gave informed written consent before participating in the study, which was approved by the institutional review board of the University of Texas Medical Branch. Screening of subjects was performed, with clinical history, physical exam, and laboratory tests, including complete blood count with differential, liver and kidney function tests, coagulation profile, fasting blood glucose and oral glucose tolerance test, hepatitis B and C screening, HIV test, TSH, lipid profile, urinalysis, drug screening, and ECG. The subjects’ characteristics are published in an article by Fujita et al. (12). The subjects were initially randomized to REFR and CONTROL groups.

1RM testing. On two separate occasions (>5 d apart) and more than 5 d before conducting the study, each subject was tested for muscle strength by measuring their 1RM on a leg extension machine (Cybex-VR2, Medway, MA) located within the general clinical research center’s (GCRC) exercise laboratory. Briefly, 1RM consisted of a single 10-repetition warm-up at 22.5 kg. At this time, the exercise supervisor determined a suitable weight for the subject to lift one time. Subsequently, the subject rested for 3 min, the weight was increased/decreased, and the subject attempted again to lift the assigned weight. This process continued until the subject could no longer lift the weight. The previous completed lift was considered their 1RM. About 5 d later, the subjects repeated this protocol, and the higher of the two 1RM values obtained was used to determine the exercise weight (20%) for the resistance exercise portion of this study.

Experimental design. Details to the infusion protocol and tracer methodology previously used can be found in the study by Fujita et al. (12). Each subject was admitted to the GCRC of the University of Texas Medical Branch the day before the exercise study. The subjects were then fed a standard dinner, and a snack was given at 2200 h. The subjects were studied after an overnight fast under basal conditions, and they refrained from exercise for 24 h before study participation. The next morning, a baseline muscle biopsy was obtained from the lateral portion of the vastus lateralis of the leg, with the biopsy site between 15 and 25 cm from the midpatella. The biopsy was performed using a 5-mm Bergström biopsy needle, under sterile procedure and local anesthesia (1% lidocaine). Muscle tissue was immediately blotted and frozen in liquid nitrogen and stored at −80°C until analysis.

REFR. After the baseline biopsy, a lower-extremity pressure cuff (Kaatsu-Master Mini, Sato Sports Plaza, Tokyo, Japan) was placed around the most proximal portion of each leg. While the subject was seated on a chair, the cuff was pressurized to 120 mm Hg for 30 s, and then the pressure was released for 10 s. This was done four more times while the pressure in the cuff was increased by 20 mm Hg with each subsequent reinflation until 200 mm Hg was reached. With the pressure maintained at 200 mm Hg, the subjects then performed a set of 30 repetitions of bilateral knee extension exercise at 20% 1RM, followed by a 30-s rest period. The subjects performed three more sets of 15 repetitions separated by 30-s rest intervals, for a total of four sets and 75 repetitions. The cuff pressure was maintained at 200 mm Hg during the entire 4–5 min of exercise, and the total exercise time was approximately 4–5 min. Immediately after the fourth set, the pressure cuff was released, and the subject rested in bed for 3 h. At this time, a postexercise biopsy was sampled from the same incision
Three weeks after the first visit, subjects initially assigned to the REFR group repeated the protocol without blood flow restriction (CONTROL), whereas subjects initially assigned to the CONTROL group then completed the blood flow–restricted exercise protocol (REFR). The subjects in the CONTROL group performed the identical exercise protocol as that of the REFR group, except that the cuff was not inflated and no pressure was applied to the legs. Therefore, all six subjects performed both trials in a randomized, crossover design.

RNA extraction and cDNA synthesis. Total RNA was isolated by homogenizing 30–40 mg of tissue with a homogenizing dispenser (T10 Basic Ultra Turrax, IKA, Wilmington, NC) in a solution containing 1.0 mL of TRI REAGENT and 4 μL of polycarrier (Molecular Research Center, Inc, Cincinnati, OH). The RNA was separated into an aqueous phase using 0.2 mL of chloroform, and it was precipitated from the aqueous phase using 0.50 mL of isopropanol. Extracted RNA was washed with 1 mL of 75% ethanol, dried, and then suspended in a known amount (1.5 μg/mL) of nuclease-free water.

RNA was quantified spectrophotometrically (Bio-Rad, Hercules, CA) at a wavelength of 260 nm. RNA concentration was calculated on the basis of total RNA yield. RNA quality was assessed by RNA agarose gel electrophoresis followed by visualization of the 18S and 28S ribosomal RNA bands under ultraviolet light. RNA was DNase treated, using a commercially available kit (DNA-free, Ambion, Austin, TX). One microgram of total RNA was reverse transcribed into cDNA, according to the manufacturers’ directions (iScript, BioRad, Hercules, CA).

Briefly, a 20-μL reaction mixture was constructed, consisting of 1 μg of total RNA, 4 μL of 5× iScript Reaction Mix, 1 μL of iScript Reverse Transcriptase, and a known amount of nuclease-free water. RNA was reverse transcribed into cDNA using a thermocycler (iQ5 Real-Time PCR cycler, BioRad, Hercules, CA) and the following temperature/time protocol: 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min. All isolated RNA and cDNA samples were stored at −80°C until further analysis.

PCR primers. Primer pairs were customized using Beacon Designer 2.0 software (Premier Biosoft Int., Palo Alto, CA). Custom-designed primers are located in Table 1. MyoD primer pairs have been used previously (25). All primers were checked for specificity to our genes of interest by conducting a Blast analysis and minimizing primer selection across secondary structures. Primer efficiencies were optimized, and the PCR product was verified by melt analysis and a single DNA product, as identified by a DNA agarose gel.

Semiquantitative real-time PCR. Determination of relative mRNA expression was performed by real-time RT-PCR, using the iQ5 Multicolor Real-Time PCR cycler (Bio-Rad, Hercules, CA). cDNA was diluted (1:8) and analyzed using SYBR Green fluorescence (iQ SYBR Green Supermix, BioRad, Hercules, CA). The reaction vessel contained 12.5 μL of iQ SYBR Green Supermix, 0.5–0.7 μL of forward and reverse primers, 2.0 μL of cDNA, and a known amount of sterile water. The total volume of the reaction tube was 25 μL. All samples were run in duplicate. An initial cycle for 5 min at 95°C was used to denature the cDNA. This was followed with 40 PCR cycles consisting of denaturation at 95°C for 20 s, and primer annealing and extension at 55°C for 30 s. Relative fold changes in mRNA within groups, and baseline

<table>
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<tr>
<th>Name</th>
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<th>Product Size (bp)</th>
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differences between groups, were determined as described by Livak and Schmittgen (20) after normalizing to GAPDH.

**Statistical analysis.** All values are expressed as means ± SEM. Comparisons were performed using a one-way analysis of variance with repeated measures, to determine main effects for group (REFR, CONTROL) and time (baseline, post). *Post hoc* testing was performed using independent paired *t*-tests when appropriate. Significance was set at *P* < 0.05.

**RESULTS**

There were no differences in our selected hypoxia-related genes between groups at baseline (*P* > 0.05) or after exercise (*P* > 0.05); however, HIF-1α mRNA was significantly increased (Fig. 1A; *P* < 0.05), and REDD1 mRNA was significantly reduced (Fig. 1B; *P* < 0.05), at 3 h after low-intensity resistance exercise in both groups.

There were no differences in our selected genes associated with satellite cell function between groups at baseline (*P* > 0.05) or after exercise (*P* > 0.05); however, p21 (Fig. 2A; *P* < 0.05) increased in both groups. Cyclin D1 was unchanged 3 h after an acute bout of low-intensity resistance exercise (Fig. 2B; *P* > 0.05). Further, the mRNA expression of IGF-1R (Fig. 3A) and MGF (Fig. 3B) had not changed in either group at 3 h after the low-intensity resistance exercise bout (*P* > 0.05), whereas MyoD mRNA expression had increased in both groups (Fig. 4A; *P* < 0.05). Further, myogenin mRNA expression (Fig. 4B; *P* > 0.05) was unchanged at 3 h after the bout of low-intensity resistance exercise.

There were no differences in our selected gene involved in the regulation of cell size (myostatin) between groups at
baseline \((P > 0.05)\) or after exercise \((P > 0.05)\); however, myostatin mRNA was significantly reduced in both groups at 3 h after a bout of low-intensity resistance exercise (Fig. 5; \(P < 0.05\)).

There were no differences in our selected genes involved in muscle protein turnover between groups at baseline \((P > 0.05)\) or after exercise \((P > 0.05)\). For the genes involved in the regulation of mRNA translation and muscle protein synthesis (mTOR and S6K1), we found no changes in mRNA expression in either group at 3 h after the bout of low-intensity resistance exercise (Fig. 6; \(P > 0.05\)). Finally, in our selected genes associated with the regulation of muscle protein breakdown, we found an increase in the mRNA expression of MuRF1 (Fig. 7A; \(P < 0.05\)) and no change in MAFbx (Fig. 7B; \(P > 0.05\)) for both groups at 3 h after an acute bout of low-intensity resistance exercise.
DISCUSSION

The primary finding from our study was that human skeletal muscle gene expression associated with muscle hypertrophy and remodeling is not different when blood flow restriction is performed during a single bout of low-intensity resistance exercise. However, we did detect that HIF-1α, REDD1, and other anabolic and catabolic skeletal muscle mRNA were altered after a bout of low-intensity resistance exercise (20% 1RM). The finding that human skeletal muscle REDD1 decreased after resistance exercise is novel and suggests that REDD1 may play a regulatory role in controlling human muscle protein synthesis.

The interaction between low-intensity resistance exercise and blood flow restriction does produce muscle hypertrophy similar to that seen with high-intensity resistance exercise (1,2,26,30–32). The underlying mechanisms for this are still rather unclear. A possible mechanism may be that REFR caused brief ischemia to the muscle during exercise, which was followed by significant hyperemia during reperfusion when the cuff was released. Although ischemia/reperfusion was not directly measured in our study, lactate levels were elevated, suggesting at least partial ischemia and/or hypoxia during REFR (12). Because hypoxia has been shown to activate the transcription factor HIF-1α, we chose to identify the response of HIF-1α and its downstream target, REDD1, during the 3-h recovery period of REFR, when the reduced blood flow and hypoxic conditions during exercise have been reversed. HIF-1α and REDD1 mRNA were not different between our groups; rather, HIF-1α was elevated, and REDD1 decreased as a result of low-intensity resistance exercise alone (Fig. 1A, B). Our data are consistent with other studies that have reported increased HIF-1α mRNA and protein expression in human skeletal muscle after acute bouts of endurance exercise (3,22) and muscle contraction in rats (28). On the other hand, there have been no reports of REDD1 expression in human skeletal muscle after exercise. The relative significance of this finding is perplexing, because HIF-1α mRNA expression did not correlate with a corresponding elevation in REDD1. Because the intensity of our resistance exercise protocol more closely resembled that of muscular endurance protocols, an upregulation of HIF-1α may signify recruitment of metabolic genes (rather than an interaction with REDD1) that promote oxygen delivery. Indeed, HIF-1α can directly activate the transcription of several metabolic genes such as VEGF, EPO, and GLUT4 (3,28,35). Thus, it would be interesting to determine whether HIF-1α is upregulated during high-intensity resistance exercise (e.g., 70% 1RM) in which aerobic adaptations are less likely to occur. Therefore, additional research is needed to determine HIF-1α involvement after chronic low- and high-intensity resistance exercise.

Similarly, REDD1 is induced after cellular stress (7,27,33). Recent reports have determined a connection between REDD1 and the mTOR pathway, revealing its role as a repressor of mTOR phosphorylation and protein synthesis (4,7,11,19,33). Therefore, we were interested in determining whether REDD1 mRNA expression was different in REFR, because we have previously shown that mTOR signaling to S6K1 after exercise was greater in REFR as compared with CONTROL (12). We report that there were no differences between CONTROL and REFR. However, we show that REDD1 mRNA expression was significantly reduced in both groups (Fig. 1B), indicating an exercise-independent response to REDD1. We believe that this is the first study in human skeletal muscle to show that REDD1 mRNA is altered by exercise. However, it is likely that other factors regulate REDD1 (because HIF-1α mRNA expression did not correlate with REDD1 expression). Further, our finding of reduced REDD1 mRNA expression may prove to be important because a reduction in REDD1 would apparently relieve inhibition on mTOR promoting a stimulation of mTOR signaling, mRNA translation, and muscle cell growth. Although changes in mTOR or S6K1 mRNA were not detected in either of our groups (Fig. 6A and B), it may be that REDD1 plays a permissive role by relieving mTOR inhibition and, thereby, opening the door to activation from other anabolic regulators. Future work is needed to define the roles of REDD1 or REDD2 in human skeletal muscle.

An unexpected, yet novel finding from this data is that several mRNA associated with skeletal muscle hypertrophy and atrophy were altered after a single bout of resistance exercise at 20% 1RM. Markers of satellite cell activity (p21; Fig. 2A and MyoD; Fig. 4A) were significantly elevated 3 h after exposure to low-intensity resistance exercise (P < 0.05), whereas others were unchanged (P > 0.05; cyclin D1; Fig. 2B, IGF-1R; Fig. 3A, MGF; Fig. 3B, myogenin, Fig. 4B). Further, our data suggest a potential increase in muscle protein turnover, as indicated by a significant increase in MuRF1 mRNA (Fig. 7A), and a relieved inhibition on cell growth, as shown by a significant reduction in myostatin mRNA (Fig. 5). Previous reports have demonstrated similar changes in these mRNA after high loads of resistance exercise (6,21,24,25,36), but our data are the first to show that these genes are altered after an acute bout of low-intensity resistance exercise. Therefore, our findings may suggest a level of remodeling (albeit potentially small compared with high-intensity resistance exercise) that has occurred to reinforce and strengthen the fiber in preparation for future loads. It is also likely that the changes in mRNA expression after low-intensity resistance exercise are temporary (i.e., a few hours), whereas high-intensity resistance exercise produces a more sustained upregulation of hypertrophy-associated genes (i.e., up to 24 h or more), as has been previously shown (6,16,21,25,36).

Our data also support the work of others showing that during the early recovery period (after a bout of resistance exercise in humans), it is primarily enhanced mRNA translation that is responsible for the increase in muscle protein synthesis (10,34). For example, we have shown that
REFR (but not low-intensity resistance exercise alone) increases muscle protein synthesis and S6K1 phosphorylation at 3 h after exercise (12); however, no differential effect of REFR was detected on our 13 selected muscle genes in this study. It is likely that a differential response may have been detected at later time points, such as 24 h after exercise, because REFR training clearly induces muscle hypertrophy over time (1,2,12,26,30–32). As pointed out previously (9), mRNA translation seems to be playing a key role in the early stages after exercise, with transcription of muscle genes becoming involved to a greater extent over time with training.

Low intensity/high repetition is characteristic of muscular endurance and may not be optimal for muscular hypertrophy. This is supported by a study by Takarada et al. (32), which reports that 8 wk of low-intensity resistance exercise at approximately 20% 1RM did not increase skeletal muscle cross-sectional area. This has also been demonstrated by others (8), but, in addition, these authors identify an increase in the percentage of type IIAB fibers after 8 wk of high repetitions. It is quite possible that skeletal muscle hypertrophy at a low exercise intensity may take longer than 8 wk and/or may be a more suitable exercise paradigm for populations other than healthy young adults. For instance, Taffe et al. (29) report that in older subjects performing exercise at an intensity of 40% 1RM for 52 wk, type I fiber significantly increased, whereas type II fibers demonstrated a trend for an increase in size. In support of our data, these studies indicate that remodeling of human skeletal muscle can occur with repeated bouts of exercise at a low exercise intensity of 20% 1RM. Nevertheless, it is evident that muscle hypertrophy will be less in comparison with traditional high-intensity resistance exercise training.

In summary, we demonstrate that changes at the mRNA level of various anabolic, catabolic or hypoxia genes are not different after REFR as compared with CONTROL. Further, we present novel data showing that a single bout of low-intensity resistance exercise can alter mRNA expression during early postexercise recovery in human skeletal muscle for genes associated with muscle growth and protein turnover, such as REDD1 (j), HIF-1α (j), p21 (j), MyoD (j), MuRF1 (j), and myostatin (j). We conclude that acute blood flow restriction in combination with resistance exercise does not influence the selected mRNA differently, compared with when resistance exercise is performed with no blood flow restriction. Future studies are needed to evaluate gene expression at later stages of postexercise recovery after REFR and in response to REFR training.

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