Ribosome biogenesis adaptation in resistance training-induced human skeletal muscle hypertrophy

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Ribosome biogenesis adaptation in resistance training-induced human skeletal muscle hypertrophy. Am J Physiol Endocrinol Metab 309: E72–E83, 2015. First published May 12, 2015; doi:10.1152/ajpendo.00050.2015.—Resistance training (RT) has the capacity to increase skeletal muscle mass, which is due in part to transient increases in the rate of muscle protein synthesis during postexercise recovery. The role of ribosome biogenesis in supporting the increased muscle protein synthetic demands is not known. This study examined the effect of both a single acute bout of resistance exercise (RE) and a chronic RT program on the muscle ribosome biogenesis response. Fourteen healthy young men performed a single bout of RE both before and after 8 wk of chronic RT. Muscle cross-sectional area was increased by 6 ± 4.5% in response to 8 wk of RT. Acute RE-activated induction of the ERK and mTOR pathways were similar before and after RT, as assessed by phosphorylation of ERK, MNK1, p70S6K, and S6 ribosomal protein 1 h postexercise. Phosphorylation of TIF-IA was also similarly elevated following both RE sessions. Cyclin D1 protein levels, which appeared to be regulated at the translational rather than transcriptional level, were acutely increased after RE. UBF was the only protein found to be highly phosphorylated at rest 8 wk of training. Also, muscle levels of the rRNAs, including the precursor 45S and the mature transcripts (28S, 18S, and 5.8S), were increased in response to RT. We propose that ribosome biogenesis is an important yet overlooked event in RE-induced muscle hypertrophy that warrants further investigation.

Ribosomal RNA; cyclin D1; upstream binding protein; transcription initiation factor 1A

RESISTANCE TRAINING (RT) increases both muscle mass and strength. Increased intramuscular protein synthesis in the hours following each resistance exercise (RE) bout appears to be a major mechanism regulating the adaptive mass gain (1). This increased rate of protein synthesis results from a coordinated series of cellular events dictated acutely by an increased translational efficiency (protein synthesized per ribosome) (1, 28). The initiation of ribosomal activity, i.e., mRNA translation, is dependent on the activation of the mammalian target of rapamycin (mTOR) and extracellular signal-regulated kinase (ERK) pathways (8, 39). There are considerable data on the impact of exercise intensity, duration, prior training, and nutritional status on these signaling pathways of translation initiation (1, 6, 28). However, a further, and less well understood, aspect of RT-induced muscle hypertrophy is the potential for greater translational capacity (quantity of ribosome) (19). In fact, ribosomal content dictates the upper limit of protein synthesis of a cell (12), and thus it may be speculated that RE not only activates translational initiation but also increases the total muscle capacity for protein synthesis through de novo ribosome biogenesis.

Ribosome biogenesis is a multistep process compromising ribosomal (r)RNA synthesis, processing, and assembly with ribosomal proteins. The first and key limiting step is the transcription of DNA to the pre-rRNA 45S transcript by the RNA polymerase I (Pol I) (7, 17). The 45S rRNA is subsequently cleaved, with external (ETS) and internal transcribed spacers (ITS) being removed to generate three mature rRNA transcripts: 28S, 18S, and 5.8S (23). These are exported to the nucleus, where along with 5S rRNA (a transcript from Pol III activity) they collectively associate with a number of ribosomal proteins. The assembled mature ribosome is a complex consisting of both rRNA and ribosomal proteins distributed in two subunits: a large 60S subunit (composed of 28S, 5.8S, and 5S rRNAs plus ~49 proteins) and small 40S subunit (composed of 18S rRNA plus ~33 proteins) (21, 41).

Synthesis of 45S rRNA requires the formation and activity of a complex of proteins at the rDNA promoter, including the upstream binding protein (UBF) and selectivity factor (SL1). Activation of this complex is followed by the recruitment of transcription initiation factor 1A (TIF-IA) which interacts with Pol I to drive 45S rRNA synthesis (7, 20). UBF protein content and phosphorylation state are closely correlated with Pol I recruitment and total rDNA transcription rates (31, 37). The phosphorylation of UBF at Ser388 and Ser484 is markedly heightened during cell cycle progression by the cyclins (including cyclin D1) and cyclin-dependent kinases (CDK), which are required for UBF activity (36, 37).

It has been demonstrated that ribosome biogenesis is an important component in rodent models of overload-induced muscle hypertrophy by synergistic ablation (9, 38). However, to date, no study has been performed in a physiologically relevant milieu such as resistance exercise/training in human subjects. Therefore, the purpose of the present study was to examine the mechanisms of muscle ribosome biogenesis in response to a single bout of acute RE as well as chronically following a period of RT in healthy young adults. To achieve this, we aimed to measure the signaling transduction pathways that lead to the activation of UBF and TIF-IA, the key transcriptional regulators of rRNA. Subsequently, we also analyzed the impact of the resulting rDNA transcription, including measurement of the precursor transcript (45S pre-rRNA), pre-
rRNA sequences spanning the intermediary 5′ internal/external transcribed spacer regions (ITS-28S, ETS-18S, and ITS-5.8S), and the final mature rRNA transcripts (18S, 5.8S, and 28S).

MATERIALS AND METHODS

The study protocol was approved by the Edith Cowan University Human Ethics Committee and was conducted in accordance with the Declaration of Helsinki. An overview of the experimental study design is shown in Fig. 1.

Subjects

Fourteen males (18–36 yr old) volunteered for the study following informed, written consent. For 3 wk prior to study commencement, all participants refrained from intense lifting or strength training.

Pretraining Testing

The pretraining period included two familiarization sessions: 1) an information session where proper nutrition and lifting techniques were discussed and 2) a practical familiarization session. Three days after the familiarization sessions, subjects commenced pretraining testing, which was conducted over 3 separate days. On day 1, total, lean, and fat masses were determined using dual-energy X-ray absorptiometry (DEXA) scanning, and thigh muscle cross-sectional area (CSA) was measured using computed tomography (CT) scanning (details below). On day 2, one-repetition maximum (1-RM) lifting performance was evaluated on the leg press, leg extension, and leg flexion exercises (details below). On day 3, which was completed 4–6 days after the 1-RM testing, muscle biopsy samples were obtained from the right vastus lateralis both before and 1 h after the completion of a single RE session. One hour postexercise was chosen because it is known to promote great response on both ERK and mTOR pathways, and thus we hypothesize that it would also allow us to observe differences in the ribosome biogenesis markers that are dependent on these pathways.

Body Composition

Total, lean, and fat masses of the whole body were measured using a Hologic Discovery QDR-4500 DEXA scanner (Hologic, Bedford, MA). Lean and fat masses (g) were calculated from total and regional analyses of the whole body scan, which provided descriptive data of the subjects and also allowed prebiopsy and posttraining session nutrition to be set (details below). CT scans of the right thigh were taken at 50% of the distance from the lateral condyle of the femur to the greater trochanter (i.e., thigh length) by a qualified radiographer to determine quadriceps muscle CSA using Siemens SOMATOM Dual Source 64 Slice CT system (Siemens, Berlin, Germany). Analysis was performed using ImageJ, with the mean of CSA score of two separate traces being used (33). After 8 wk of training, CT scanning was conducted 6–7 days after the last session for posttraining CSA measurement. The typical error for repeated digitization of 14 scans was 1.13 cm² [90% confidence interval (CI) = 0.86 - 1.68 cm²], and the coefficient of variation was 1.2% (90% CI = 0.9–1.7%).

1-RM Testing

Each subject’s maximum lifting performance was determined on the leg press, leg extension, and leg flexion (leg curl) exercises. After a full warmup, including 5 min of stationary cycling and two practice sets (6 repetitions at loads equal to ~50 and 70% of perceived maximum load), each subject performed single repetitions of an incrementally greater load until a maximum was found. Subjects typically required four to six lifts to achieve maximum. The rest interval for 1-RM attempts was 3 min. These 1-RM scores were used to monitor changes in strength performance with training and to set the training loads.

Acute RE Bout and Muscle Biopsy Procedure

Subjects reported to the laboratory after an overnight fast. They consumed a standardized meal (cereal plus milk) containing 2,220 kJ of energy, 11.6 g of protein, 105 g of carbohydrate, and 5.6 g fat/100 kg lean mass. The same meal was provided for the postraining acute exercise bout and biopsy sampling session. After resting quietly for 1 h, muscle biopsy samples were taken from the right vastus lateralis (untrained rest biopsy) at 50% of the distance from the lateral femoral condyle to the greater trochanter (i.e., at the same thigh location as the CT scan) using the microbiopsy technique. A 13-gauge catheter was inserted into the muscle under local anesthetic (5% lidocaine cream), and then a 14-gauge triggered microbiopsy needle was inserted through the catheter. Three small (10–30 mg) samples were taken, with the catheter remaining inserted for the duration. Subjects remained resting in the supine position for 30 min until warmup for the RE session commenced. The acute RE test session was the same stimulus as that used throughout the RT program described in detail below. Biopsy samples were taken again 1 h after the completion of the RE session (untrained postexercise biopsy). Each sample was immediately frozen in liquid nitrogen and stored at −80°C until further analysis. After 8 wk of training, the muscle biopsy sampling procedure was repeated 3–5 days after the last training session (trained rest biopsy, and 1 h after RE, trained postexercise biopsy). Subjects consumed whey protein isolate/concentrate postexercise (RedBak Whey Protein 50/50 isolate/concentrate; Probiotec) immediately after every 60 min of training, training and testing sessions. The drink contained 38.4 g of protein, 3.8 g of carbohydrate, and 2.1 g fat/100 kg lean mass; thus, postexercise nutrition was identical in training and testing sessions.

RT Program

Subjects were paired for 1-RM knee extension strength and then randomly allocated to one of two training groups: heavy-load group training at 90% of 1-RM (90% 1-RM group) or a moderate-load group training at 70% of 1-RM (70% 1-RM group) for leg press, knee extension, and knee flexion exercises. Each subject was supervised at each session by an experienced strength trainer who was able to motivate the subjects and ensure that they could safely complete each set to absolute failure. The subjects performed as many repetitions as possible in the first set of the first session of testing. The number of
Table 1. RT-PCR primer details

<table>
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<tr>
<th>Target</th>
<th>Catalog No./Primer Sequence</th>
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<tr>
<td>Span 5’-end 5.8S rRNA</td>
<td>PPH82111A</td>
</tr>
<tr>
<td>Internal to 5.8S rRNA</td>
<td>PPH82091A</td>
</tr>
<tr>
<td>Span 5’-end 18S rRNA</td>
<td>PPH82110A</td>
</tr>
<tr>
<td>Internal to 18S rRNA</td>
<td>PPH71602A</td>
</tr>
<tr>
<td>Span 5’-end 28S rRNA</td>
<td>PPH82112A</td>
</tr>
<tr>
<td>Internal to 28S rRNA</td>
<td>PPH82090A</td>
</tr>
<tr>
<td>45S rRNA (5’-ETS)</td>
<td>PPH82089A</td>
</tr>
<tr>
<td>TBP (forward)</td>
<td>TGGTGGTGCTGCCTGCTAGTGG</td>
</tr>
<tr>
<td>TBP (reverse)</td>
<td>CGTGCTGGAACTGCTAAAGGAG</td>
</tr>
<tr>
<td>HPRT (forward)</td>
<td>CGGGGGCTGCTGATGATAGTGG</td>
</tr>
<tr>
<td>HPRT (reverse)</td>
<td>CAGCAAGAGGGCTAGCTAGG</td>
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<td>Cyclin D1 (reverse)</td>
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<td>TIF-IA (forward)</td>
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<tr>
<td>TIF-IA (reverse)</td>
<td>CAGGGGGAAGGCTAGCTGCC</td>
</tr>
<tr>
<td>UBF (forward)</td>
<td>CAGCTCTGGAATCCTGCTCC</td>
</tr>
<tr>
<td>UBF (reverse)</td>
<td>CCACTCTGGAATGCTGTTTC</td>
</tr>
<tr>
<td>POLR1B (forward)</td>
<td>CCTAGCTTGCAGCTGTTTCC</td>
</tr>
<tr>
<td>POLR1B (reverse)</td>
<td>CAGGAAGGAAATGGGAGGTA</td>
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</tbody>
</table>

TBP, TATA box-binding protein; PPIA, cyclophilin A; HPRT, hypoxanthine phosphoribosyltransferase 1; TIF-IA, RRN3 RNA polymerase I transcription initiation factor 1A; UBF, upstream binding transcription factor; POLR1B, polymerase RNA I polypeptide B.

repetitions was recorded, and then loads were adjusted by set during training to maintain the number of repetitions; thus loads were continually increased as the subjects increased their strength. Each subject’s 1-RM load was determined before and after 4 wk of training to allow for adjustment of the repetition range based on the load-repetition relationship changed. Training was performed twice/wk for 8 wk (3 exercises, 4 sets each until failure), with 2 min of rest between sets and 3 min of rest between exercises.

Analysis of Muscle Tissue: Total RNA Extraction and cDNA Synthesis

Total RNA was extracted using the ToTALLY RNA Kit (Ambion, Austin, TX), following manufacturer’s instructions. RNA integrity was determined using the Agilent 2100 BioAnalyzer (Agilent Technologies, Mulgrave, Victoria, Australia). RNA samples were then diluted to an appropriate concentration with nuclease-free water and cDNA synthesis performed using the High Capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA). cDNA was stored at −20°C for subsequent analysis.

Real-Time PCR

RT-PCR was performed on a LightCycler 480 II using SYBR Green I Master Mix (Roche Applied Science, Penzberg, Germany). Commonly used reference genes hypoxanthine-guanine phosphoribosyltransferase (HPRT), TATA-binding protein (TBP), β2-microglobulin, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA levels were measured (Table 1). Geometrical means of the three most stable and lower-variance [based on the technique described by Mane et al. (18)] mRNAs (HPRT, TBP and PPIA) were calculated and used for normalization of the target mRNAs, by the 2−ΔΔCt method. Target mRNAs were upstream binding protein (UBF), polymerase RNA I polypeptide B (POLR1B), cyclin D1, and transcription initiation factor 1A (TIF-IA; also known as RRN3). Target rRNAs were the mature ribosome species 28S, 18S, 5.8S. Since these primers should amplify both mature RNAs but also pre-rRNAs, primers were designed specifically for pre-rRNAs sequences spanning the 5’ end ITS/ETS regions and a specific primer for the initial region of 5’ end of 45S rRNA, namely 45S, ETS-18S, ITS-5.8S, and ITS-28S (Fig. 2). Primers for rRNAs (Table 1) were designed by Qiagen specifically for this study, using RT2 Profiler PCR Arrays (Qiagen; Venlo, Limburg, The Netherlands). Standard and melting curves were performed for every target to confirm primer efficiency and single product amplification.

Western Blot

Skeletal muscle tissue was homogenized using an OMNI Bead Ruptor Homogenizer (Omni International, Kennesaw, GA) using RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% deoxycholic acid, 1% NP-40, and 1 mM EDTA) supplemented with Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, Waltham, MA). After a centrifugation step, the supernatant had the protein concentration determined with the BCA protein assay kit (Pierce, Rockford, IL). Protein homogenates were mixed with 4X Laemmli’s buffer and boiled. Denatured proteins were separated by SDS-PAGE using 8–18% gel, depending on the protein target. Proteins were electrophoresed to PVDF membranes (Bio-Rad, Hercules, CA) using TransBlot semidry transfer apparatus (Bio-Rad). Gels were cut and transferred together, as described elsewhere (15). This procedure allows different proteins to be quantified in the same run, avoiding multiple stripping and reprobing cycles and saving sample material. Membranes were blocked for 1 h in 5% BSA in TBST solution at room temperature and probed using specific antibodies for pan cyclin D1, pan UBF, p-UBF Ser188, and p-UBF Ser484 (Santa Cruz Biotechnology, Dallas, TX), p-ribosomal protein S6 kinase, 70 kDa, polypeptide 1 (p70S6k) Thr42/44Ser42/44, p-p70S6k Thr42/44, p-ERK1/2 Thr202/Tyr204 and Thr185/Tyr187, p-mitogen-activated protein kinase-interacting kinase 1 (MNKi) Thr197/202, p-S6 ribosomal protein Ser240/244, p-S6 ribosomal protein Ser235/236, p-elf4E Ser209, pan elf4E-4E-binding protein 1 (4E-BP1; Cell Signaling Technologies, Danvers, MA), pan TIF-IA, p-TIF-IA Ser249, and pan GAPDH (Abcam, Cambridge, MA). Primary antibodies were incubated overnight in blocking buffer (1:1,000 dilution, with the exception of GAPDH, which was 1:10,000), followed by the appropriate anti-rabbit- or anti-mouse-linked to horseradish peroxidase secondary antibody (1: 5000) for 1 h at room temperature on the subsequent day. Membranes were exposed using enhanced chemiluminescence reagent (ECL Select kit; GE Healthcare) and bands were detected using ChemiDoc (Bio-Rad). Bands were quantified using ImageJ software (National Institutes of Health, Bethesda, MD). Western blot data were normalized by GAPDH.

Fig. 2. Real-time PCR primers for rRNAs. Pre-rRNA 45S representation; black-filled rectangles represent regions that will generate mature 18S, 5.8S, and 28S, with external transcribed spacer (ETS) and internal transcribed spacer (ITS) in between. Primers used for accessing pre-rRNAs and mature rRNAs were designed against the regions depicted and are shown as lowercase letters: 5’end internal to ETS, specific for 45S; 5’ETS-18S; 5’internal to 18S; 5’ITS-5.8S; 5’internal to 5.8S; 5’ITS-28S; 5’internal to 28S.

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Table 2. Subject baseline anthropometric characteristics

<table>
<thead>
<tr>
<th>Group</th>
<th>Age, yr</th>
<th>Height, cm</th>
<th>Mass, kg</th>
<th>Lean Mass, kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>70% 1-RM</td>
<td>20.5 ± 1.9</td>
<td>181.4 ± 10.3</td>
<td>81.1 ± 22.1</td>
<td>64.6 ± 12.9</td>
</tr>
<tr>
<td>90% 1-RM</td>
<td>22.4 ± 5.2</td>
<td>177.5 ± 9.4</td>
<td>78.8 ± 6.6</td>
<td>61.8 ± 5.0</td>
</tr>
<tr>
<td>All</td>
<td>21.6 ± 4.1</td>
<td>179.2 ± 9.6</td>
<td>79.8 ± 15.1</td>
<td>63.0 ± 8.9</td>
</tr>
<tr>
<td><strong>P value</strong></td>
<td>0.42</td>
<td>0.48</td>
<td>0.78</td>
<td>0.56</td>
</tr>
</tbody>
</table>

Data are means ± SD; 70% 1-RM group, n = 6; 90% 1-RM group, n = 8. 1-RM, 1-repetition maximum.

Statistics

Data are expressed as means ± SE, unless otherwise noted. Quadriceps CSA and percentage changes in quadriceps CSA (%ΔCSA) from pretraining were analyzed by two-way ANOVA with repeated measures [group (intensity) × time]. Baseline anthropometric characteristics were analyzed by Student’s t-tests. Western blot data are presented as fold change against the pretraining rest biopsy sample. The effects of acute RE and chronic RT were assessed using a two-way ANOVA with repeated measures (Sigma Plot version 12.0). Following statistically significant main effects, Student-Newman-Keuls post hoc tests were used to determine the significance of pairwise comparisons between individual acute RE sessions and training. For the scope of the current study, the effect of “resistance exercise” is defined as the acute response of a single session of exercise (i.e., rest vs. post-RE), and the effect of “resistance training” refers to both the chronic response after 8 wk of training (i.e., untrained vs. trained) at rest (untrained rest vs. trained rest) and the response induced by exercise over time (untrained post-RE vs. trained post-RE). When appropriate, correlations between variables were assessed using Pearson’s product moment correlations. Statistical significance was accepted at P ≤ 0.05.

RESULTS

Subject Characteristics

The subject’s baseline anthropometric characteristics are shown in Table 2.

Muscle CSA (%ΔCSA)

Mid-thigh muscle CSA increased significantly following the 8-wk RT program (6.4 ± 2.0 and 7.6 ± 1.0%, 70 and 90% 1-RM groups, respectively, P < 0.05 and P < 0.01), with no difference between groups (P = 0.56) (Fig. 3). Thus, subsequent molecular analysis examined either the pooled group means or each subject’s individual data in cases of correlations between changes in CSA and measurements of ribosome biogenesis.

Effect of RE and training on translation initiation pathways (ERK and mTOR)

p70S6K. Phosphorylation of p70S6K at Thr389 (Fig. 4A) increased post-RE (main effect, P < 0.001) by sixfold in untrained (P < 0.05) and 6.5-fold in trained conditions (P < 0.05). There was also a main effect of RE for p70S6K phosphorylation at Thr421/Ser424 (P < 0.001; Fig. 4B), with increases in response to exercise of 2.3-fold in untrained (trend, P = 0.052) and 2.9-fold in trained conditions (P < 0.05). There was no effect of training for either p70S6K phosphorylation site.

ERK1/2. Phosphorylation of ERK1 (Thr202/Tyr204; Fig. 4C) and ERK2 (Thr185/Tyr187; Fig. 4D) showed a main effect of exercise (both P < 0.001) but no effect of training. ERK1 phosphorylation was increased 3.2-fold post-RE in the untrained condition (P < 0.05) and 2.9-fold in the trained condition (trend, P = 0.064). RE-induced ERK2 phosphorylation was increased 2.2-fold before training (P < 0.01) and 2.1-fold after training (P < 0.05).

S6 ribosomal protein. Phosphorylation of S6rp at Ser235/236 (Fig. 4E) was increased after exercise (main effect, P < 0.01). Before training RE resulted in a 4.5-fold change (P < 0.05), whereas after training a 4.2-fold change was observed (P < 0.05). There was also a main effect for exercise (P < 0.001) for phosphorylation of Ser240/244 (Fig. 4F) with an 8.2-fold elevation pretraining (P < 0.05) and sixfold elevation posttraining (P < 0.05). There was no significant effect of training.

MNK1. Phosphorylation at Thr197/202 (Fig. 4G) was increased markedly in response to RE (main effect, P < 0.001, no effect of training), which was sevenfold both in untrained (P < 0.01) and trained conditions (P < 0.01). elf4E and 4E-BP1. There was no statistical difference acutely or chronically for elf4E phosphorylation at Ser209, and hyperphosphorylation of 4E-BP1 assessed by mobility shift (data not shown).

Effect of RE and Training on Cell Cycle Regulators: Cyclins

Cyclin D1 protein (Fig. 5A) increased 1.9-fold after both bouts of RE (main effect P < 0.01, before and after training, both P < 0.01), and there was no training effect. There was no difference in cyclin D3 protein (Fig. 5B) at any time point. Cyclin D1 mRNA (Fig. 5D) showed a training × exercise interaction (P < 0.001) and a main effect for exercise (P < 0.05). This was due to a small but significant decrease in resting cyclin D1 mRNA following training (P < 0.05) together with an acute increase in mRNA postexercise in the trained condition only (P < 0.05).

Effect of RE and Training on UBF and TIF-IA

Total and phosphorylated UBF. UBF total protein was markedly increased after exercise (main effect, P < 0.01, although as discussed later, we believe this result is likely an artefact from protein extraction) but was not influenced by resistance training (Fig. 6A). Substantial variation in subject responsiveness to acute exercise was noted for total UBF. Total
UBF protein levels were negatively correlated with UBF phosphorylation at Ser\textsuperscript{484} (r = −0.54, P < 0.001) and Ser\textsuperscript{388} (r = −0.32, P < 0.05; data not shown).

There was no effect of exercise or training on phosphorylation of UBF at Ser\textsuperscript{484} (Fig. 6C). Additionally, there was no effect of acute exercise on the phosphorylation of UBF at Ser\textsuperscript{388} (Fig. 6B). However, UBF phosphorylation at Ser\textsuperscript{388} was chronically increased fivefold in resting muscle following RT (P < 0.05; Fig. 6B).

TIF-IA. Phosphorylation at Ser\textsuperscript{649} of TIF-IA (Fig. 6D) was markedly increased after RE in both conditions (main effect, P < 0.01, 5.6-fold change before, P < 0.01, and 7.6-fold after RT,
Effect of RE and Training on rRNA and Total RNA

Total muscle RNA concentration (μg RNA/mg tissue) tended to be increased post-RT (1.3-fold), although there was considerable subject-to-subject variation, and this data did not reach statistical difference (data not shown). Nevertheless, the extent of each subject’s fold change in muscle total RNA from pretraining to posttraining showed a significant positive correlation with muscle %ΔCSA (r = 0.72, P = 0.01; Fig. 7A).

45S pre-rRNA (primer internal to the 5′-ETS region) expression showed an interaction between RE and training (P < 0.05). Post hoc analysis revealed an increase in 45S expression in resting muscle following RT (>2-fold increase, P < 0.01; Fig. 7B). After RT, but not before RT, there was an acute RE-induced decrease in 45S rRNA expression (P < 0.01; Fig. 7B). Levels of 45S pre-rRNA following exercise were strongly correlated with the extent of TIF-IA phosphorylation at Ser484 (r = 0.66, P < 0.01) but not UBF phosphorylation at Ser388 or Ser484 (r = −0.36 and P = 0.14 and r = 0.23 and P = 0.4, respectively).

There were no significant changes after RE or RT for ETS-18S, ITS-5.8S, or ITS-28S pre-rRNAs (Fig. 7, D, F, and H), although trends were observed for main effects of exercise for ETS-18S (P = 0.08) and ITS-28S (P = 0.055).

There were training effects for all mature rRNAs: 18S (P < 0.01), 5.8S (P < 0.01), and 28S (P < 0.05) (Fig. 7, C, E, and G). Post hoc analysis showed differences between untrained and trained conditions at rest for 18S (4-fold increase, P < 0.01) and 5.8S (~4-fold increase, P < 0.01) and a similar trend for 28S (2-fold increase, P = 0.079). Additionally, there were main effects of exercise to acutely reduce levels for 18S (P < 0.05) and 5.8S (P < 0.05) but not 28S.

Effect of RE and training on mRNA Levels of Pol I-Associated Proteins

Gene expressions of POLR1B and TIF-IA (Fig. 8) were not significantly different at any time point. UBF mRNA showed a significant 73% increase with training (main effect of training, P < 0.01) at rest (P < 0.01). Acutely, there was a small decrease after RE following RT (P < 0.05) but not before RT.

DISCUSSION

The novel finding of present study was that significant muscle hypertrophy induced by 8 wk of RT was associated with increased muscle expression of ribosomal RNA transcripts accompanied by a robust activation of key ribosome biogenesis regulatory factors. Specifically, it was demonstrated that 18S, 5.8S, and 28S rRNAs and the pre-rRNA 45S were increased two- to fourfold in the trained muscle. Acute RE promoted ERK/mTOR pathway activation, increased levels of cyclin D1 protein, and elevated phosphorylation of TIF-IA. Whereas UBF phosphorylation was not increased acutely post-RE, it was markedly elevated on Ser388 in resting muscle posttraining. These data show that key indicators of ribosome biogenesis are responsive to a single bout of RE, whereas repeated training appears to be necessary for the sustained activation of UBF and accretion of muscle rRNA.

Signaling transduction via the ERK and mTOR pathways was greatly upregulated 1 h following an acute bout of RE in both the untrained (i.e., pre-RT) and trained (post-RT) conditions. Phosphorylation of p70S6K at Thr389 (mTOR dependent) and Thr421/Ser424 (predominantly ERK pathway depen-
dent) (13), as well as S6rp at Ser240/244 (p70S6K dependent) and Ser235/236 (both p70S6K and p90 ribosomal S6 kinase, RSK, dependent) (30), was greatly increased following RE. Phosphorylation of ERK1/2 at Thr202/Tyr204 and Thr185/Tyr187 and its downstream target MNK1 at Thr197/202 was also greatly increased. However, none of these kinase responses were altered after 8 wk of training. Our results do not corroborate the view that chronic training causes an attenuation of acute exercise-induced signal transduction pathways related to muscle hypertrophy, as hypothesized previously (5, 25, 40). At least within the time frame of 8 wk of training, both acute exercise bouts resulted in a similar activation of these kinases despite significant training-induced gains in muscle mass. It remains to be determined whether the activation of these kinases is diminished with ongoing training or when a plateau in training intensity and exercise adaptation occurs.

Signal transduction via the mTOR and ERK pathways enhances translational initiation and elongation of a series of specific mRNAs. Although it is known that ERK and mTOR are activated in the early hours of postexercise recovery, less is

**Fig. 6.** Effect of RE and training on total and phosphorylation of upstream binding protein (UBF) and transcription initiation factor 1A (TIF-IA). A: UBF content before and after training and exercise; B: phosphorylation of UBF at Ser388; C: at Ser484; D: phosphorylation of TIF-IA at Ser649; E: TIF-IA content before and after training and exercise. F: representative Western blot figures for UBF and TIF-IA phosphorylation residues and total protein levels and GAPDH are shown. Western blot data were normalized to GAPDH. Values are means ± SE. *P ≤ 0.05; # P ≤ 0.05 vs. the same exercise state (rest or post) of the opposite training condition (untrained or trained). Main effects and interactions are presented in the text.
known of the specific proteins that are translated due to this effect. In the present study, an increase in muscle levels of cyclin D1 protein after both acute RE bouts were observed without concurrent increases in cyclin D1 mRNA or cyclin D3 protein. Consistently, cyclin D1 protein expression has been shown to be regulated primarily at the translational rather than transcriptional level (11, 22, 29). Although it has been hypothesized that the downstream mTOR effector 4E-BP1 may be involved in the translation of cyclin D1, it was shown recently that mTOR does not specifically regulate its expression (34). Alternatively, eIF4E was shown to be responsible for the specific translation of cyclin D1 mRNA (29), and the phosphorylation of eIF4E at Ser209 by MNK1 appears to be required for this effect (2, 35). We were not able to detect an increase in eIF4E phosphorylation at Ser209 after RE, but heightened phosphorylation of the two upstream kinases MNK1 and ERK was observed. It is possible that our single 1-h postexercise biopsy time point may have missed an increase in eIF4E phosphorylation, since cyclin D1 protein was already increased by this time. We suggest that cyclin D1 is a novel molecular target that is specifically upregulated in human muscle after RE, possibly driven by the ERK/MNK pathway.
The role of cyclin D1 in the regulation of the cell cycle/proliferation is known; however, cyclin D1 also plays a key role in mature cells by promoting ribosome biogenesis. Cyclin D1 forms a complex with CDK4 and promotes its catalytic activity (16), which promotes subsequent phosphorylation and activation of UBF (37). Active UBF binds to the rDNA promoter, leading to increased rDNA transcription. In the current study, phosphorylation of UBF appeared to be reduced or almost undetectable at 1 h post-RE in some subjects. UBF has several high-mobility group box domains that confer UBF as having a strong binding affinity for rDNA (27, 42). However, once UBF is dephosphorylated, it will release rDNA and potentially redistribute from the nucleolus to the nucleus or even cytoplasm (26). Interestingly, those subjects who had a blunted phosphorylation of UBF after exercise also displayed a robust increase in total UBF levels. We hypothesize that this effect may be due to the protein extraction protocol employed in the current study. Detachment of dephosphorylated UBF protein from rDNA would enrich the protein-soluble fraction during tissue extraction. It has been reported that even nucleolus-specific protein extraction protocols recover only 50% of nucleolar proteins (4). It is likely that the protein extraction protocol employed in this study failed to recover the entire nucleolar fraction and that this accounted for the increased UBF levels after RE. Convincingly, those subjects who had a blunted phosphorylation of UBF at 1 h post-RE, but not chronically. Although rRNA synthesis relies greatly upon UBF activation, it may in some specific cases be increased by TIF-IA activation by the ERK pathway, which may still cause a proportional increase in UBF level, but these may be below the limit of detection of the Western blot technique employed. Third, one cannot rule out the possibility that the synergistic ablation model simply increases muscle mass by different mechanisms compared with the more physiological stimulus of human RT.

Unlike the chronic effect on UBF phosphorylation, TIF-IA was greatly phosphorylated at the ERK-RSK dependent site (Ser649) after acute RE, but not chronically. Although rRNA synthesis relies greatly upon UBF activation, it may in some specific cases be increased by TIF-IA activation by the ERK pathway despite a lack of a change in UBF phosphorylation status (45). Along with cyclin D1 protein, TIF-IA may be an earlier activator of ribosome biogenesis after RE that may promote rRNA synthesis at a later time point.

Synthesis of rRNA has been found to be an early event in rodent models of skeletal muscle hypertrophy (9, 38). However, whether rRNA synthesis plays a similar role in resistance training-induced muscle hypertrophy in humans remains unclear. In the current study, relative levels of mature rRNAs and the pre-rRNA 45S (primer designed against the 5'-ETS region not spanning any mature rRNA) were markedly increased in resting muscle following 8 wk of resistance training. Since the pre-rRNA 45S was similarly increased, we asked whether the mature rRNA data showed higher levels due to the pre-rRNAs, since primers designed for mature rRNAs will also amplify the pre-rRNA version. To differentiate pre-rRNA from mature
rRNA, we also analyzed the 28S, 18S, and 5.8S segments that were still bound to the ITS (ITS-28S and ITS-5.8S) and ETS (ETS-18S) within the pre-rRNA 45S strand. Unlike the primers designed for the internal region of the mature rRNA alone, none of the pre-rRNA fragments (ITS-28S, ETS-ITS, and ITS-5.8S) were increased after training. The training-induced increase in pre-rRNA 45S, but not ETS-18S, ITS-5.8S, or ITS-28S, may suggest that pre-rRNA is rapidly processed. Overall, our data show that resistance training induces long-term increases in muscle ribosome machinery. A major implication of this finding is that rRNAs, which are commonly used as reference genes in the literature, clearly cannot be used as reference genes in longitudinal studies, especially when there are gains and/or losses of muscle mass.

Despite there being chronic increases in mature rRNA abundances posttraining in the current study, we did not find an acute increase in 45S pre-rRNA levels in response to RE. For long-term increases in rRNA, rDNA transcription should be initiated at some point following acute RE. We hypothesize that rDNA transcription may be upregulated at a later time point during postexercise recovery. In line with this hypothesis, Nader et al. (24) recently reported an increase in 45S rRNA 4 h post-RE, 3 hours later than our biopsy sampling time point. Convincingly, although the levels of 45S pre-rRNA after exercise were decreased in the present study, they were positively correlated with the levels of TIF-IA phosphorylation (but not UBF phosphorylation). This may indicate that the transcription of rDNA induced by resistance exercise is mediated by the ERK-RSK-TIF-IA pathway independently of UBF.

Total RNA per milligram of muscle tissue appeared to be slightly increased following chronic resistance training, but this change showed a high interindividual variability, and the overall change did not reach statistical significance. However, perhaps more importantly, the extent of change in a subject’s total muscle RNA concentration was found to be positively correlated with the extent of changes in muscle CSA (Fig. 7A). Total RNA is known to be a proxy measurement of ribosomal RNA content, since >80% of total RNA is ribosomal RNA (43). Therefore, the degree to which subjects were capable of muscle mass gains appears to be related to their extent of increase in muscle ribosome density.

In conclusion, the current data indicate that ribosome biogenesis is a relevant event potentially driving muscle adaptation to resistance training. We propose a testable model (Fig. 9) where dephosphorylated UBF is rapidly removed from rDNA promoter in the nucleolus during or shortly after contraction. The muscle cell requires energy during contraction, which will shut down high-energy consumption activities such as protein synthesis and ribosome biogenesis. However, at the same time, RE sends antagonistic cues for latter adaptation, where cyclin D1 protein expression may be its earliest triggering regulator, possibly by the ERK-MNK1-eIF4E pathway, and TIF-IA is activated independently of UBF. Moreover, the increase in ribosome density may explain the degree of muscle hypertrophy; i.e., reduced accretion of rRNA may limit muscle growth. Some studies have found that RT increases basal muscle protein synthesis (14, 40), which appears not to be mediated by the signaling pathways that increase protein translation effi-

![Fig. 9. Theoretical model for the early and delayed effects of RE in human skeletal muscle. RE promotes TIF-IA phosphorylation, mTOR, and ERK signaling that specifically increases translation of cyclin D1. Increased cyclin D1 may promote activation of UBF protein, increasing rDNA transcription and sustaining muscle growth chronically. Depicted are the nucleus and nucleolus (darker region) in the center, where rDNA is concentrated. mTORC1, mTOR complex 1; eIF4E, eukaryotic initiation factor 4E; 4E-BP, eIF4E-binding protein; P, phosphorylation.](http://ajpendo.physiology.org/)

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Efficiency. We show evidence that the increase in protein synthesis at rest might be mediated by increased ribosome biogenesis in response to resistance training.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS


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