Ribosome accumulation during early phase resistance training

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Running title: Ribosome biogenesis in early phase resistance training

Abstract

Increased ribosomal density is a phenomena observed in response to resistance training (RT) supporting elevated. A time course of RT-induced accumulation of ribosomes has not been described. We mapped markers of ribosomal biogenesis (total RNA, ribosomal RNA (rRNA), ribosomal protein S6 (rpS6) and upstream binding factor (UBF) protein content) in response to twelve RT sessions. Additionally we investigated the effect of fluctuations in training volume on these markers and relationship between RNA accumulation and muscle growth measured as muscle thickness (ultra sound). Eighteen participants were allocated to either a training (TRAIN, *n*=11) or control (CTRL, *n*=7) group. In TRAIN, participants performed unilateral knee extension with either constant (CONST, 6 sets) or a variable volume (VAR, 6, 3 and 9 sets in sessions 1-4, 5-8 and 9-12, respectively). Muscle biopsies were sampled from *m vastus lateralis* in TRAIN before and 48 hrs after the first session as well as 48 hrs after session 4, 5, 8, 9 and 12 and after eight days of de-training. Control biopsies were sampled in CTRL at baseline and after 48 hrs and 3-5 weeks. RT led to muscle growth in TRAIN compared to CTRL and concomitant increases in total RNA, rRNA, including precursor rRNA as well as UBF and rpS6 protein through gradual increase throughout the training period, confirmed in comparison to CTRL. Total RNA increased in a curve-linear fashion, most rapidly in response to the first four sessions (8.6%, 95% CI: [5.5, 11.7] per session), followed by a plateau and peak values (49.5% [34.2, 66.5] above baseline) after Session 8. UBF protein levels explained total RNA levels after controlling for time and increases in total RNA levels predicted RT induced muscle hypertrophy. After de-training, total RNA and specific rRNA species decreased without changes in muscle mass indicating reduced concentrations and biosynthesis of ribosomes. These result underlines a determinant role for ribosomal biogenesis in RT-induced muscle hypertrophy and that ribosomal biogenesis is sensitive to training cessation.

Keywords: Resistance training, ribosome biogenesis

Introduction

Skeletal muscle is a critical target for interventions that promotes health across the lifespan (Wolfe, 2006), with resistance training (RT) being the advocated remedy.
Prolonged RT leads to changes in the balance between muscle protein breakdown and synthesis, with one bout of resistance exercise acutely increasing protein synthesis for up to 48 hrs after exercise (Phillips *et al.*, 1997), with subsequent repeated bouts leading to accumulation of muscle protein over time (Phillips, 2014 ; Brook *et al.*, 2015). In recent years, this view has been supplemented by evidence suggesting that chronic RT leads to increased resting synthetic rate of muscle protein (Kim *et al.*, 2005; Wilkinson *et al.*, 2008; Reidy *et al.*, 2017), which has been postulated to be associated with increased translational capacity, i.e. accumulation of ribosomes (Reidy *et al.*, 2017; Figueiredo & McCarthy, 2017). This notion is supported by exercise-induced increases in total RNA, a proxy marker of ribosome abundance, which is closely connected to protein synthesis (West *et al.*, 2016; Millward *et al.*, 1973) and muscle hypertrophy (Figueiredo *et al.*, 2015; Stec *et al.*, 2016; Hammarström *et al.*, 2020). Conversely, inhibition of ribosomal RNA (rRNA) transcription and inhibition of its up-stream transcription factors act to diminish muscle cell growth (Stec *et al.*, 2016 ; Walden *et al.*, 2016; West *et al.*, 2016).

Biosynthesis of novel ribosomes is a complex, highly coordinated and energy demanding process that involves synthesis of both ribosomal proteins and the four mature rRNA transcripts (Warner, 1999; Moss *et al.*, 2007; Walden *et al.*, 2016). Ribosomal accumulation is believed to be determined by the rates of pre-rRNA transcription by RNA polymerase I (Pol I), which in turn is regulated by coordinated assembly of a complex of transcription factors at the rDNA promoter (Moss *et al.*, 2007). Specifically, activation of the of the upstream binding factor (UBF) through phosphorylation is needed to initiate transcription (Tuan *et al.*, 1999; Lin *et al.*, 2007). Such activation is at least partly controlled by the mechanosensitive mTOR pathway, with its inhibition being associated with blocked UBF phosphorylation and subsequent rRNA transcription (Nader *et al.*, 2005; Hannan *et al.*, 2003). Interestingly the availability of UBF *per se* has been shown to be a determinant of rRNA transcription (Hannan *et al.*, 1996) through control of rDNA gene activity (Sanij *et al.*, 2008).

Resistance exercise is a potent stimuli for rRNA transcription as a single session leads to increases in pre-rRNA (Nader *et al.*, 2014 ; Stec *et al.*, 2015) and repeated bouts lead to accumulation of mature rRNA thus also total RNA and presumably functional ribosomes

(Stec *et al.*, 2015, 2016 ; Bickel *et al.*, 2005; Hammarström *et al.*, 2020; Figueiredo *et al.*, 2015; Brook *et al.*, 2016; Reidy *et al.*, 2017). However, the true time course of ribosomal transcription and accumulation in response to RT remains largely unstudied, with a mere few studies having investigated exercise-induced changes in rRNA over multiple time-points, all of which are either limited to a selected few time-points or a limited time frame. For example, two consecutive bouts of electrically evoked muscle contractions were associated with increased levels of total RNA, with peak values being observed 72 hrs after the second bout (Bickel *et al.*, 2005). Using voluntary contractions, peak values were reported after nine sessions, followed by a slight decrease to after 18 sessions (Brook *et al.*, 2016), resembling data from our lab where five sessions of RT led to marked increase in total RNA levels (per-unit muscle tissue), whereby a numerical lowering occurred to after the last training session of the 12 wk interventions (31 sessions). (Hammarström *et al.*, 2020). Interestingly, during the initial phase of RT, total RNA accumulation seems to be volume-dependent, as three sets per exercise in leg exercises led to augmented total RNA and rRNA levels compared to one set per exercise, coinciding with the differences in muscle hypertrophy seen after 12 weeks of RT. (Hammarström *et al.*, 2020). These data suggest that ribosome accumulation reaches a plateau in the early phase of RT and that increases are sensitive to training volume in constant volume protocols.

Based on these observations we hypothesize that (1) ribosome accumulation occurs during the early phase (3-4 weeks) of RT, within which this accumulation (2) reaches a plateau when RT volume is kept constant, (3) displays fluctuations in response to fluctuating training volume and (4) is partially reversed to one week after cessation of RT. In addition to addressing these hypotheses we aimed to relate RNA accumulation to total UBF levels and muscle growth.

Methods

Study overview

Eighteen volunteers were recruited to the study. Eligible participants were non-smokers between 18 and 35 years of age with a training history of less than one RT session per week during the six months leading up to the study. Exclusion criteria were consumption of dietary supplements or medication with known effects on muscle metabolism, injuries causing impaired strength and/or affecting their ability to perform RT, symptoms or history of disease, and known adverse reactions to local anesthetics. Participants were allocated to either an training group (TRAIN, n = 11) or a non-training control group (CTRL, n = 8; see Table 1 for

participant characteristics; see Figure 1A for overview of the intervention). TRAIN performed a 12 session RT protocol lasting for 3-4 weeks, consisting of 10 repetition maximum (RM) unilateral knee-extension, with the two legs conducting RT with different volume profiles, allowing within-participant comparison of the effects of volume regimes. In TRAIN, one leg conducted RT with constant volume throughout the intervention (CONST, 6 sets per session) and the other leg performed RT with variable volume (VAR, 3 blocks of four sessions with 6, 3 and 9 sets per session, respectively; Figure 1A). CTRL did not partake in RT and were instructed to continue their everyday activities. Muscle biopsies were sampled bilaterally in TRAIN before and 48 hrs after the first session, as well as 48 hrs after the fourth, fifth, eight, ninth and twelfth session, and after a seven days of de-training. Muscle biopsies were obtained from CTRL at three occasions; at baseline and 48 hrs and 3-5 weeks (average (SD) 3.6 (0.7)) after the first sampling event. TRAIN and CTRL performed strength assessments > seven days prior to the first biopsy sampling (TRAIN; CTRL), 72 hrs after the twelfth session (TRAIN) and 24 h after the last biopsy (TRAIN, following de-training; CTRL). Appendicular lean mass (Dual-energy X-ray absorptiometry, DXA) and muscle thickness of *m. vastus lateralis* were assessed prior to the first biopsy (TRAIN and CTRL) as wells as before the second to last (TRAIN) and last (TRAIN and CTRL) biopsy.

RT protocol

Prior to all RT sessions, participants performed a standardized warm-up consisting of 5 min ergometer cycling (rating of perceived exertion (RPE): 12-14), followed by ten repetitions of push-ups, sit-ups and back-extensions. After warm-up, participants performed unilateral knee-extension with the prescribed number of sets. Each set was prescribed with 10 repetitions maximum (RM). When sets were completed with either fewer (8) or more (12) repetitions, the resistance was adjusted accordingly. Inter-set rest periods were 90 sec. Throughout the intervention RT sessions were alternatingly initiated by training the right and left leg, changing every other session, with the contralateral leg being trained in the rest period between sets of the first leg. The second session of each four-session block (session 2, 6 and 10) was performed at a sub-maximal resistance (~90% of the previous session) with the same number of repetitions (10). Within each session, participants also conducted two sets of three upper-body exercises (bench press, lateral pull-down and shoulder press; 10RM). After completion of each session participants were given a standardized drink to aid recovery (0.15 g kg-1 protein, 11.2 g kg-1 carbohydrates and 0.5 g kg-1 fat).

Muscle strength, body composition and muscle thickness assessments

Muscle strength was assessed as maximal voluntary isokinetic (90° sec-1) and isometric (60° angle, fully extended leg 0°) knee extension torque. After a brief warm-up (5-min cycling, RPE 12-14), participants were seated and secured in the individually adjusted dynamometer. Participants were instructed to gradually increase their effort during three warm-up repetitions (50, 60 and, 70% of subjective maximal effort). After a 30-sec restitution period participants were instructed to perform three repetitions with maximal effort in the concentric phase. Sixty seconds after the isokinetic test the lever automatically moved to a 60° angle and participants were instructed to push against the lever enough to see feedback from the visual feedback system. After an additional 15-sec restitution period, participants were instructed to push against the lever with maximal effort. Within the same assessment session, participants remained seated in the dynamometer for measurement perfomed on both legs. The first measurement was alternated between legs every other session. For statistical treatment of the data, all successful attempts were used. The last strength assessment at baseline was performed at least seven days prior to the first biopsy sampling. At least one of the baseline strength tests was performed on separate day with two sessions allowed to be perform on the same day with a short rest between assessments. Post training assessments were performed 48 hours and eight days after the last session.

For determination of body composition participants were scanned using DXA (Lunar Prodigy densitometer, GE Healthcare, Madison, WI, USA) with the standard scanning mode (13-25 cm). Participants were lying supine within the scanning bed reference lines, with a strap secured around the ankles to ensure a standardized body position in each scan. The scans were conducted with participants in a fasted state between 07.00-10.00 AM, with empty bladder and wearing only under-wear. Prior to each scan, a phantom scan was run to prevent baseline drifting from affecting analyses. The same technician was used at each time point. Analyses was performed using GE enCORE version 17.0 software (GE Healthcare). Region of interest was customized for covering upper thigh, marked with a sqaure from pubic symphysis to lateral part of tuberculum major, and distal to art. genu.

Muscle thickness (MT) was measured using a B-mode ultra sound unit (SmartUS EXT-1M, Telemed, Vilnius, Lithuania). Participants lay supine in a relaxed position for 20 min before assessments, with their feet strapped in a standardized position. A mark was set on the line 60% of the distance between Spinia Iliac Anterior Superior and the lateral femur condyle. MT of *m.vastus lateralis* was measured applying a water-soluble transmission gel (Aquasonic 100

Ultrasound Transmission Gel; Parker Laboratories Inc., Fairfield, NJ, USA), and a 39 mm 12 MHz ultrasound probe was placed perpendicular to the site of interest without pressing the skin. When the quality of the image was satisfactory, evident as distinct upper and lower muscle fascia, three images were captured, where the probe was relocated to the same position between each image. Position of the probe was marked on the skin and subsequently marked on a transparent paper to ensure similar probe placement for both the right and left *m.vastus lateralis* at subsequent assessments. Analyses were done in ImageJ Fiji (Schindelin *et al.*, 2012) with images cropped and coded to ensure blinding of the assessor.

Muscle biopsy sampling

Muscle specimens were sampled bilaterally from *m. vastus laterlis* under local anesthesia (Lidokain 10 mg ml-1, Mylan, Mylan Ireland Limited, Dublin, Ireland) using a disposable needle (12-14 gauge, Universal plus, Medax, Poggio Rusco, Italy), operated with a spring loaded device (Bard Magnum, Bard Norway, Rud, Norway). Two to four passes were made to get sufficient material. One to two aliquots of the samples were quickly dissected free from connective and fat tissue, weighed and frozen in isopentan chilled to -80°C and stored at -80°C until further processing.

RNA and protein extraction

Frozen muscle tissue was homogenized in 1 ml of Trizol (ThermoFisher Scientific, Oslo, Norway) spiked with 0.04 ng of an external, non-mammalian, RNA spike-in (Lambda PolyA External Standard Kit, Takara Bio Europe, Saint-Germain-en-Laye, France). Mechanical disruption of the samples was achieved using Zirconium Oxide Beads (0.5 mm, Next Advance, Inc., New York, USA) and a bead mill (Bullet blender, Next Advance). Chloroform (200 µl) was added prior to centrifugation (12000 g, 15 min at 4°C) to achieve phase separation. Four hundred fifty µl of the upper aqueous phase was transferred to a fresh tube and 500 µl of isopropanol was added to precipitate the RNA. After a 10 min incubation at room temperature, samples were centrifuged (12000 g, 10 min at 4°C), after which a pellet formed. The pellet was washed three times in chilled 75% ethanol with centrifugation between each wash (7500 g, 5 min at 4°C). After the final wash all ethanol was removed and the pellet was eluted in 0.1X Tris-EDTA buffer. RNA concentration and purity was assessed by spectrophotometry. All samples had 260/280 ratios > 1.95.

Protein was extracted from Trizol preparations according to manufacturers instructions and (Kopec *et al.*, 2017) with modifications. The remaining aqueous phase was removed and

DNA was precipitated by the addition of 300 µl of absolute ethanol followed by gentle centrifugation (2000 g, 5 min at room temperature). An aliquot of the phenol-ethanol phase, corresponding to ~1.75 mg of tissue, was transferred to to a fresh tube. After addition of at least two volumes of isopropanol and incubation (10 min at room temperature), samples were centrifuged (7500 g, 10 min 4°C) and a pellet formed. The pellet was washed three times in 95% ethanol with each wash separated by centrifugation (5000g, 5 min at room temperature). After the last wash all liquid was removed and 45 µl of Kopec buffer (Kopec *et al.*, 2017) was added (5% SDS, 10 mM Tris, 140 mM NaCl and 20 mM EDTA, pH 8; containing protease and phosphatase inhibitors). Pellets were incubated at 50°C for three hours after which the majority of samples were dissolved. Any undissolved material was sedimented by centrifugation (10000 g, 10 min at room temperature). Protein concentrations were measured (Pierce Detergent Compatible Bradford Assay, ThermoFisher Scientific). Sample were normalized in 4X Laemmli buffer (Bio-Rad Norway AS, Oslo, Norway), boiled (95°C, 5 min) and stored at -20°C before later use.

Quantitative polymerase chain reaction (qPCR)

Complementary DNA (cDNA) was synthesized in technical duplicates from 500 ng of total RNA using random hexemer and anchored Oligo-dT primers (Thermo Fisher Scientific) together with Superscript IV (Thermo Fisher Scientific) according to manufacturer's instruction. qPCR reactions were performed with diluted cDNA (2 µl, 1:25 dilution), a SYBR-green based commercial master mix (PowerUp™ SYBR™ Green Master Mix, Thermo Fisher) and, target-specific primers (500 nM) in 10 µl reaction volumes using a real-time detection system (QuantStudio 5 Real-Time PCR System, Thermo Fisher Scientific). Fast cycling was used (1 sec denaturing, 30 sec annealing) after UNG (2 min, 50°C) and polymerase (2 min, 95°C) activation. Melt curves were collected from all reactions to confirm single product amplification. Primers were further evaluated by agarose gel electrophoresis which confirmed primer sizes and non-template control experiments confirming no amplification without template. Primer sequences and their respective average performances are shown in Table 2.

Raw fluorescence data was exported from the QuantStudio software and estimates of quantification cycle (Cq) and amplification efficiency was derived for each reaction using the qpcR package(Ritz & Spiess, 2008).

Immunoblotting

Protein samples (20 μg) were separated on 4-20% Tris-Glycin gels (Criterion TGX Precast Gels, Bio-Rad) at 250 V for 45 min using the recommended running buffer (25 mM Tris, 192 mM Glycin, 0.1% SDS). All samples from the same participant were run on the same gel and all samples were run in at least duplicates. Separated samples were transferred to &#956m PVDF membranes (Immun-Blot, Bio-Rad) using wet transfer (25 mM Tris, 192 Glycin, 10% vol/vol methanol) at a constant voltage of 300 mA for 3 h. Membranes were then stained to confirm transfer and enable total protein quantification using a reversible protein stain (Pierce Reversible Protein Stain, Thermo Fisher Scientific). Primary antibodies were acquired to detect UBF (F-9, sc-13125, Santa-Cruz Biotechnology, Dallas, Texas, USA) and ribosomal protein S6 (54D2, #2317, Cell Signaling Technology, Danvers, MA, USA). After blocking (Tris-buffered saline blocking buffer, 20 mM Tris, 150 mM NaCl, 5% fat-free milk, 0.1% Tween-20), membranes were incubated over-night with primary antibodies diluted in blocking buffer (UBF, 1:200; S6, 1:1000) followed by incubation with a secondary antibody conjugated to horseradish peroxidase (Anti-mouse IgG, #7076, Cell Signaling Technology, 1:10000). Membranes were washed 6 × 5 min after incubation with primary antibodies and 8 × 5 min after incubation with the secondary antibody. All incubation and washing steps were performed at 4°C using an automatic membrane processor (BlotCycler, Precision Biosystems, Mansfield, MA, USA). Chemiluminescent signals from membranes were detected after 5 min incubation in substrate (Super Signal West Femto Maximum Sensitivity Substrate, Thermo Fisher Scientific) using a documentation system. Total protein content was quantified from whole membrane images and defined as the mean gray value of the whole lane. Between-lane gray values were used as background subtracted from protein values. Total protein quantification was done using ImageJ Fiji (Schindelin *et al.*, 2012). Chemiluminescence signals were quantified using Image Studio Lite (LI-COR Biotechnology, Lincoln, NE, USA).

Statistics and data analysis

Descriptive data are presented as mean and standard deviation (SD). The effect of training on muscle strength, muscle thickness, UBF/rpS6 protein, total RNA and gene abundances were assessed using mixed effects regression models. Time and group (TRAIN vs. CTRL) were treated as population (fixed) effects and leg nested within participant included as group level (random) effects. These analyses were performed on data with matching time points between TRAIN and CTRL with the exeption that all post-training data from TRAIN were included (post-training and de-training). Relative interactions between groups were estimated as Δ

TRAIN - Δ CTRL. The effects of different volume conditions and general time-course patterns were assessed using all pairwise observations from the TRAIN group. For protein and total RNA data, segmented regression models were used to estimate changes over sessions in three segments (session 1-4, 4-8 and 8-12; corresponding to blocks of different volume prescription in TRAIN). When no robust effects of volume conditions were detected, group averages are presented. Segmented models were fitted with time and volume condition as population effects and legs nested within participants as group level effects.

Muscle strength, muscle thickness, protein and total RNA data was modeled after log transformation. Gene abundance data were fitted with number of sessions as a categorical variable in comparisons of volume conditions, and Cq values converted to counts as suggested by Matz et al. (2013). A Poisson-lognormal model was used to fit these count, using data from all genes and including group level effects for each technical duplicate, controlling for technical errors during sample preparation. An offset consisting of a normalization factor proportional to the amount of muscle used to prepare cDNA was used to model gene abundance per tissue weight. The external reference gene was used to calculate the normalization factor (External reference counts × muscle weight (mg) in each Trizol preparation). The offset was specified as a predictor with the coefficient fixed to 1.

A linear model was used to estimate the increase per session and average total RNA for every leg in the TR group. These estimates were then used to estimate the effect of training-induced increase per session and average total RNA abundance on muscle hypertrophy. For each leg, session was used as the independent variable centered on Session 6 and log transformed RNA per tissue weight as the dependent variable. Mean-centering of the independent variable was done to obtain an estimate of the average RNA concentration per leg. This also assured that the slope and intercept did not correlate, something that could lead to colinearity issues in subsequent modeling. A mixed effects model was subsequently fitted with differences in muscle thickness pre- to post-training as the dependent variable and estimated percentage per session increases in total RNA, the mean total RNA scaled as standard deviations from the mean and sex as independent variables. Leg nested within participant was used as group levels effects. The robustness of this model was assessed by leave-one-out analysis on the level of individual data points in the relationship between total RNA and sessions and on the level of participants (see Results).

All models were fitted using a Bayesian framework using either the brms (Bürkner, 2017) or MCMCglmm (Hadfield, 2010) package written for R (R Core Team, 2020). Inference about effects of interest was done based on point estimates and their 95% credible intervals (CI). Credible intervals not containing null effects were interpreted as robust. Models were fitted with default priors which also makes CI analogous to confidence intervals but with the interpretation being that the CI contains the true population value with the specified certainty (95%), given the data. Fitting performance was assessed by confirming convergence of at least four different chains of MCMC samples (graphically assessed and confirmed with $\hat{R} \approx 1$). Model performance was assessed from comparing simulated data from each model to observed data graphically (posterior predictive checks).

Results

In TRAIN, all participants successfully completed their prescribed RT on both legs, with the two volume conditions resulting in diverging volume profiles (load × repetitions) over the course of the study (Figure 1B). Exercise intensities (resistance at 10RM) increased similarly in both conditions from the first to the second (30%, 95% CI: [21, 41]) and third (47% [35, 61]) training block, with each block consisting of four training sessions. Concomitantly, in TRAIN, isokinetic strength and thickness of *m. vastus lateralis* increased from baseline to after Session 12 compared to CTRL (isokinetic strength ~ 9.2%-point difference; muscle thickness ~ 3.6%-point difference, Figure 1C and D), a difference that was sustained to after eight days of de-training (~ 6.7%-point and ~ 3.5%-point difference in change in isokinetic strength and muscle thickness, respectively; Figure 1C and D). Isometric strength showed the same general pattern to after Session 12 (~ 3.5%-point difference), though with considerably larger degrees of uncertainty, as indicated by wider 95% CI normalization compared to CTRL after de-training (~ 1.9%-point; Figure 1C). No differences were observed between volume conditions for either strength or muscle thickness.

For both rpS6 and UBF, protein levels increased linearly throughout the training intervention, with rpS6 showing estimated increments per session corresponding to 4.2% [1.1, 7.3] during block 1 (session 1-4), 2.6% [-0.4, 5.6] during block 2 (session 5-8) and 4.6% [1.2, 8.1] during block 3 (session 9-12), and UBF showing increments corresponding to 7.3% [2.1, 12.7], 4.6% [-0.4, 9.9] and 6.1% [0.3, 12.2]. This general pattern was confirmed when comparing TRAIN to CTRL where UBF and rpS6 protein levels were higher in TRAIN compared to CTRL after Session 12 and elevated after eight days of rest (Figure 2A and B), however with no

differences being observed after the first training session (48 hrs). Increases did not differ between volume-conditions but for UBF, there was a tendency towards lower levels in VAR after Session 12 (-19.7% [-42.8, 11.6]). After de-training, UBF-levels tended to decrease in CONST (-22.5% [-44.4, 7.0]) while levels in VAR remained at elevated level compare to after Session 12 (8.0% [-21.4, 49.6]; interaction effect: 34.4% [-14.8, 113.1]). For rpS6, de-training affect protein levels, which remained similar between volume conditions period and remained similar between volume conditions. At the mRNA level, UBF showed robust increase from before to 48 hrs after the first session in TRAIN compared to CTRL (Figure 2D), while rpS6 showed no robust differences between TRAIN and CTRL at any time point. No differences were observed between volume conditions for either transcripts (Figure 2D and E).

A single session of RT (Session 1) led to robust increases in pre-rRNA 47S ETS and 45S ETS abundance per unit tissue weight, measured as changes from baseline to 48 hrs after exercise within TRAIN (Figure 3B), as well as compared to CTRL (Figure 3C). After Session 1, pre-rRNA 47S ETS and 45S ETS levels remained at similar levels at all measured time-points in TRAIN (Figure 3D), confirmed in comparison to CTRL after Session 12 (Figure 3C). Other rRNA transcripts showed increases in response to training with slightly different temporal patterns with exception of rRNA 5S which did not change and rRNA 5.8 which tended to follow other mature transcript spliced from pre-rRNA 45S ETS, but without statistical robustness (Figure 3C and D). After eight days of rest, 18S and 28S remained at elevated levels compared to CTRL (Figure 3B). This general pattern of rRNA expression was reflected by total RNA expression per unit tissue weight, which increased robustly and steadily in TRAIN throughout the initial part of the intervention (Figure 3E and G), leading to robust increase compared to CTRL after Session 12 (Figure 3F), followed by decreased levels after de-training (-19.2%, [-29.1, -8.1]). For both rRNA expression and total RNA levels, the training-associated increases in abundances occurred predominately during the first four sessions, evident as 8.6% [5.5, 11.7] increase per session, followed by sustained levels from sessions four to eight (1.9% [-0.9, 4.7] increase per session) and from sessions eight to twelve 0.0% [-3.1, 3.2], corresponding to 38.9% [23.9, 55.4], 49.5% [34.2, 66.5] and 49.5% [32.5, 68.6] increases from baseline to 48 hrs after session four, eight and twelve, respectively. In TRAIN, the two volume conditions led to similar changes for most variables (Figure 3D and G), with 45S ETS abundance only showing differential expression, evident as robustly higher

levels in VAR compared to CONST after the 12th session (Figure 3D), coinciding with the increased training volume towards the end of the intervention for this condition.

In TRAIN, total RNA levels were robustly predicted by UBF levels (after controlling for time), with 6.3% [1.8, 11.0] increases in total RNA per unit tissue weight coninciding with one unit increase in UBF levels (corresponding to one standard deviation; Table 3). In contrast, no evidence was found for a relationship between total RNA and rpS6 protein levels (Table 3).

In TRAIN, there was a robust positive relationship between rates of increase in total RNA in response to training and muscle growth measured as increases in *m. vastus lateralis* thickness (Table 4, Figure 4A), with changes in total RNA over the course of the training intervention being estimated in each leg using a regression model containing number of sessions as the independent variable. Conversely, there was a tendency towards a negative relationship between average total RNA levels at Session 6 and changes in muscle thickness (Table 4, Figure 4B), with the average total RNA levels estimated as the predicted value at Session 6 (estimated as the intercept-term) from the model used to estimate the rate of total RNA increase per session.

To assess the robustness of the model for predicting muscle growth, individual relationships between sessions and total RNA levels (Figure 4C) were recalculated after removal of single data points from each participant. The model predicting muscle growth was refitted using new estimates of changes in total RNA abundances and increases thereof per session. Each refitted model resulted in slightly different estimates (displayed as means and 95% CI in Figure 4D). No single data point influenced the r4esults in any meaningful way. Next we assessed the robustness by iteratively removing one participant from the data set, similarly this showed that estimates of the effect of total RNA increase on muscle growth was robust but the effect of average total RNA estimates were more variable (Participant 8 and 3 in Figure 4D).

Discussion

Here, we confirm that resistance training leads to increased abundance of markers of ribosome density, measured as total RNA, ribosomal RNA subspecies and rpS6 protein in previously untrained individuals compared to a non-training control group. These markers accumulated progressively during the initial part of the intervention before it leveled out, establishing a plausible time course for changes in ribosomal concentration in response to RT

that plateaus after ~ 8 sessions. This increase in total RNA was interconnected with increases in UBF protein abundance, suggesting UBF levels *per se* to play a role in regulation of rRNA transcription regulation in response to RT. Total RNA increases was not affected by weekly fluctuations in training volume, however, eight days of de-training led to lowered levels of total RNA and rRNA content, suggesting that training cessation halts ribosome biogenesis. Finally, individual rates of increases in total RNA abundance predicted the magnitude of muscle growth, confirming the likely link between ribosomal biogenesis and muscle mass accretion (West *et al.*, 2016; Millward *et al.*, 1973) and muscle hypertrophy (Figueiredo *et al.*, 2015; Stec *et al.*, 2016; Hammarström *et al.*, 2020).

Total RNA seems to be a valid proxy marker of ribosomal density, as most of the RNA is assumed to be ribosomal RNA (Young, 1970), which in turn is a valid marker of translational capacity (Millward *et al.*, 1973). Several studies have shown that total RNA content is altered by RT (Haun *et al.*, 2019; Haddad *et al.*, 2005; Stec *et al.*, 2015, 2016 ; Bickel *et al.*, 2005; Hammarström *et al.*, 2020; Figueiredo *et al.*, 2015; Brook *et al.*, 2016; Reidy *et al.*, 2017), as was also the case in the present data set. However, the time course of total RNA/rRNA changes in response to RT has so far remained speculative, with no study investigating responses to prolonged interventions with multiple sampling time points. In the present data, RT led to a clear session-to-session increase in total RNA per unit tissue weight in response to the first four session, whereupon the changes gradually leveled out before peaking after the 8th session, with the peak increase from baseline being ~ 50%, defining an accumulation phase. This corroborates well with previous suggestions of peak values being reached within four to nine sessions in young males and females (Brook *et al.*, 2016; Hammarström *et al.*, 2020), and may be essential for preparing muscle fibers for subsequent growth (Brook *et al.*, 2016; Figueiredo *et al.*, 2015; Hammarström *et al.*, 2020). After the 8th session, no meaningful increase or decrease were observed for total RNA/rRNA content within the training period, suggesting a plateau phase with attenuated net synthesis of novel ribosomes. Within this last part of the intervention, synthesis of novel rRNA still seemed to be elevated per weight unit muscle tissue compared to baseline, as suggested by sustained elevation of pre-rRNA transcripts, coinciding with peak values of UBF protein levels. This may indicate that during the plateau phase, the ribosomal concentration is balanced by muscle growth (Figueiredo & McCarthy, 2019).

The observed rates of RNA accumulation over the entirety of the intervention were found to be a determinant of changes in muscle thickness (after controlling for average total RNA

levels). Individuals with higher rates of accumulation showed larger accretion of muscle mass. This supports the notion that ribosomal biogenesis is an important determinant of RT-induced muscle hypertrophy, with previous studies showing that increases in total RNA are positively correlated with increases in muscle mass (Figueiredo *et al.*, 2015; Reidy *et al.*, 2017; Mobley *et al.*, 2018), differs between individuals displaying low vs. high levels of muscle hypertrophy in response to RT (Stec *et al.*, 2016) an contribute to explain RT volume-dependent changes in muscle mass and strength (Hammarström *et al.*, 2020). In addition, supression of ribosomal biogenesis in *in vitro* models leads to halted muscle cellular growth in some (Nader *et al.*, 2005; West *et al.*, 2016 ; Stec *et al.*, 2016) but not all studies (Crossland *et al.*, 2017). Conversely, individual variation in fixed amounts of total RNA was not found to determine muscle mass accretion, and higher levels of total RNA was instead associated with a tendency towards lowered muscle growth. Overall, the rate of increases in ribosomal density thus seems to be a better predictor of individual RT-induced changes in muscle mass than absolute ribosomal density, suggesting that net increases in ribosomal biogenesis may be a core determinant of RT responsiveness. Interestingly, the interaction between rRNA synthesis rate and muscle mass accretion (but not between ribosomal content and muscle mass accretion) may shed light on observed differences in muscular responses to RT between young and old individuals. Whereas aged muscle display higher levels of total RNA at rest (Stec *et al.*, 2015) they show reduced changes in total RNA levels in response to RT (Brook *et al.*, 2016), potentially explaining their alleged poorer overall hypertrophic responses (Brook *et al.*, 2016). Whether these cellular characteristics are related to e.g. differences in fiber type distributions (Habets *et al.*, 1999) remains to be determined. Together, these results and perspectives emphasizes on the potentially crucial role of RT-induced ribosomal synthesis for adaptations to training, making ribosomal responses to RT an interesting biomarker in relation to manipulation of training loads for specific populations.

In the present study, training induced increases in rRNA and total RNA coincided with increases in rpS6. Changes in total RNA levels and rpS6 in response to de-training did however not correspond as rpS6 protein levels remained elevated after the de-training period. Training induced increases in rpS6 seen in the present study are in agreement to what has previously been reported in young men (Brook *et al.*, 2015), but not in elderly men and women where a decrease was observed in response to training despite increases in total RNA and rRNA (Stec *et al.*, 2016). Although increases were seen in both rpS6 and and total RNA, rpS6 did not explain variations in total RNA when number of sessions were controlled for.

Together with a disconnect after the de-training period, this suggest that regulations of rpS6 expression and ribosomal RNA transcription displays different temporal characteristics in response to RT. Additionally, ribosomal proteins may have extra-ribosomal functions affecting their expression (Warner & McIntosh, 2009).

UBF levels robustly explained total RNA levels over the entire course of the intervention. These analyses were done with the number of sessions accounted for, allowing unbiased estimates. Unrealistically strong relationships could have been otherwise expected as both the dependent variable (total RNA) and the covariate (UBF levels) varies with the number of sessions. From a mechanistic perspective, UBF is an important transcription factor for rDNA transcription as it, in its active state recruits a secondary transcription factor (SL1) to the rDNA promoter and enables transcription by RNA Pol I (Lin *et al.*, 2007). Activation of UBF is controlled by the mechanosensitive mTOR pathway, and rapamycin, a specific mTOR inhibitor, blocks UBF from recruiting SL1 and subsequent rRNA transcription (Nader *et al.*, 2005; Hannan *et al.*, 2003). Evidence from human exercise studies confirms training-induced activation of UBF through phosphorylation (Figueiredo *et al.*, 2016, 2015). In addition to exercise-induced activation of UBF, mechanical loading also leads to increased levels of total UBF (Figueiredo *et al.*, 2016, 2015). Increases in UBF was determined to be rapamycin insensitive after synergist ablation in mice (Goodman *et al.*, 2011) pointing to an effect observed in cell models where c-Myc induces UBF mRNA transcription (Poortinga *et al.*, 2004). Interestingly the availability of UBF *per se* has been shown to regulate rRNA transcription (Hannan *et al.*, 1996) through control of rDNA gene activity (Sanij *et al.*, 2008). Together with our observations, this underlines the importance of UBF as a regulator of RT-induced ribosomal biogenesis.

After eight days of de-training, total RNA and rRNA levels per weight unit muscle tissue returned toward baseline levels, though without concomitant reversal of muscle thickness, which remained at elevate levels. This was likely caused by attenuated rRNA transcription, a notion that was supported by reversal of pre-rRNA abundances and possibly by lowered UBF protein levels, though this was not confirmed as statistically robust. This supports the idea that ribosomal biogenesis is a cellular activity on demand, possibly relating to its relative expense (Warner, 1999) also in muscle tissue. Based on this notion, and the fact that RT volume is known to be a potent modulator of molecular mechanisms determining protein synthesis and ribosomal biogenesis including induction of c-Myc expression, mTOR activation (Hammarström *et al.*, 2020; Ahtiainen *et al.*, 2015; Burd *et al.*, 2010), subsequent total RNA

increases (Hammarström *et al.*, 2020) and post exercise protein synthesis (Burd *et al.*, 2010) and subsequent training outcomes (Schoenfeld *et al.*, 2016; Hammarström *et al.*, 2020), we hypothesized that fluctuations in training volume would be reflected in markers of ribosomal biogenesis. When comparing VAR to CONST in the present study we found only one part of the pre-rRNA, 45S ETS, to be differentially expressed and only so after Session 12 in favor of VAR together with a tendency towards rescued UBF levels after de-training in response to increased volume in the VAR but not CONST protocol. These observations do not give support to a clear effect of fluctuations in training volume on total RNA levels or rRNA expression within a relatively short and training-intensive intervention, though it should be noted that the time point with increased 45S ETS expression was preceded by a period of increased training volume, suggesting a potential interaction between time and volume. Indeed, both training protocols utilized in the present study increased muscle strength and induced muscle hypertrophy to a similar degree. From a general perspective, albeit volume is an important determinant of increases in muscle strength and mass (Schoenfeld *et al.*, 2016; Ralston *et al.*, 2017), differences in organization of training loads is likely of minor importance when training volumes are equated over time (Grgic *et al.*, 2017). It is important to note that RT in the current study was performed with the same volume in the first four sessions, something that could have been more than enough to maximize rRNA transcription in previously untrained individuals. This is supported by the observation that pre-rRNA increased rapidly initially in both protocols with minimal changes in response to subsequent sessions, regardless of exercise volume. The CONST protocol in the present study corresponded to volumes used in the moderate volume condition in a previous study from our lab (three sets in two exercises activating knee extensor muscles) (Hammarström *et al.*, 2020). There, higher levels of total RNA were observed after four sessions in the moderate compared to a low volume protocol (Hammarström *et al.*, 2020). Interestingly, using a progressive volume protocol in well-trained participants, increases in total RNA have been reported throughout six weeks of training (Haun *et al.*, 2019). Altough this observation was done in well-trained participants performing a high volume protocol without a control condition with constant volume, compared to constant volume protocols [Brook *et al.* (2016); Hammarström *et al.* (2020); and the present study], progressive volume may thus increase ribosomal abundance to a higher degree and provide a measure to avoid the plateau phase seen in the present study.

In conclusion, RT-induced ribosome accumulation reached peak values in the initial phase of RT (8 sessions) and was interconnected with increases in UBF protein levels. The rate of total RNA accumulation predicted RT-induced muscle hypertrophy. Fluctuations in training volume did not transfer to fluctuations in ribosomal biogenesis, but training cessation led to decreased ribosomal content.

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Figure legends

Figure 1. Study design (A) observed training loads in response to Constant and Variable volume protocols (B) and training outcomes (muscle strength, C; muscle thickness D).

Figure 2. Protein (A and B) and mRNA abundances (D and E) of rpS6 and UBF. Black points and error bars represents statistically robust results (a 95% CI not containing 0). C shows western-blots and total protein stains from a representative participant. mRNA data is normalized per total RNA.

Figure 3. Total RNA and ribosomal RNA subspecies in response to trainin. Total RNA increased compared to non-training controls to post-training (12 sessions) and tended to normalize after de-training (A). There were no robust differences between different volume conditions in the training group (A). B shows primer locations targeting different ribosomal RNA subspecies. Subsets of ribosomal RNA showed robust increases compared to control 48 h after the first session and other after the training period (C). Error bars shows 95% CI. Asterisk in C indicates robust differences between volume conditions (a 95% CI of pairwise differences not containing 0).

Figure 4. Predictions of muscle thickness increase based on total RNA increases (A) and total RNA abundance (B; see Table 4). Values are averaged over values from men and women. Individual plots of estimates total RNA increases over time is shown in C together with results from leave-one-out analysis (D). Leave-one-out analysis shows the effect of removing a single participant (black point and error-bars) and individual values from the total RNA per time estimates where green points represents bounds of the 95% CI and yellow points represents mean estimates.