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Original Article

Reduced Physical Activity Alters the Leucine-Stimulated Translatome in Aged Skeletal Muscle

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Abstract

Periods of inactivity experienced by older adults induce nutrient anabolic resistance creating a cascade of skeletal muscle transcriptional and translational aberrations contributing to muscle dysfunction. The purpose of this study was to identify how inactivity alters leucine-stimulated translation of molecules and pathways within the skeletal muscle of older adults. We performed ribosomal profiling alongside RNA sequencing from skeletal muscle biopsies taken from older adults (n = 8; ~72 years; 6 F/2 M) in response to a leucine bolus before (Active) and after (Reduced Activity) 2 weeks of reduced physical activity. At both visits, muscle biopsies were taken at baseline, 60 minutes (early response), and 180 minutes (late response) after leucine ingestion. Previously identified inactivity-related gene transcription changes (PFKFB3, GADD45A, NMRK2) were heightened by leucine with corresponding changes in translation. In contrast, leucine also stimulated translational efficiency of several transcripts in a manner not explained by corresponding changes in mRNA abundance ("uncoupled translation"). Inactivity eliminated this uncoupled translational response for several transcripts, and reduced the translation of most mRNAs encoding for ribosomal proteins. Ingenuity Pathway Analysis identified discordant circadian translation and transcription as a result of inactivity such as translation changes to PER2 and PER3 despite unchanged transcription. We demonstrate inactivity alters leucine-stimulated "uncoupled translation" of ribosomal proteins and circadian regulators otherwise not detectable by traditional RNA sequencing. Innovative techniques such as ribosomal profiling continues to further our understanding of how physical activity mediates translational regulation, and will set a path toward therapies that can restore optimal protein synthesis on the transcript-specific level to combat negative consequences of inactivity on aging muscle.

Keywords: Aging, Circadian Rhythm, Disuse, Ribosome profiling, Translation

Muscle anabolic sensitivity to branched chain amino acids, such as leucine, plays an important role to promote myofibrillar protein synthesis in muscle, whereas cellular stresses such as aging (1–3), disuse (4–6), and disease (7) can dampen nutrient anabolic sensitivity. Inactivity can induce anabolic resistance to protein, and reduced sensitivity to leucine is thought to be a major contributor to the progressive loss of muscle mass and function with age (ie, sarcopenia) (8,9). Therefore, a better understanding of the implications of inactivity by older adults on leucine-stimulated translation and transcription and related molecular pathways is necessary to develop future targeted treatments to overcome anabolic resistance.

The current understanding of the molecular mechanisms of anabolic sensitivity to leucine is centered on the mTORC1 signaling pathway (10). However, measurement of this pathway using traditional techniques such as immunoblotting poorly explains the clinical outcomes of anabolic resistance with disuse and aging. For example, unilateral leg immobilization for 2 weeks in young participants exhibited a lower myofibrillar protein synthesis response

to amino acid ingestion without corresponding changes in skeletal muscle mTORC1 signaling (5). Additionally, we have shown that short-term bed rest blunted amino acid-induced mTORC1 signaling (ie, S6K1 and S6 phosphorylation) in young and older adults, yet reductions to nutrient-stimulated whole-muscle protein synthesis and loss of lean mass occurred only in the old (11).

Inactivity and age induce a surprisingly large number of transcriptome alterations (12,13) and RNA sequencing (RNA-Seq) has identified most of these transcription changes to be categorized as mitochondrial, metabolic, inflammation, and fibrosis-related (6,14–16). However, transcript levels are an imperfect measure of protein production because mRNA translation is subject to extensive regulation. While translational control mediated by mTORC1 is known to play an important role in mediating gene expression to affect anabolic processes such as synthesis of contractile proteins, and ribosome biogenesis to increase translational capacity, our understanding of the genome-wide translational effects of mTORC1-dependent and -independent pathways lags behind our understanding of transcriptional changes that occur in response to inactivity, aging, and anabolic sensitivity to nutrients. Therefore, dissecting inactivity-induced changes to leucine-stimulated translation of mRNAs requires the resolution and relevance to protein abundance afforded to us through ribosomal profiling (Ribo-Seq).

Traditional RNA-Seq captures total mRNA abundance within a tissue sample, while the emerging technique of Ribo-Seq allows the capture of ribosome-protected fragments measuring translational activity in a transcript-specific manner (17). This technique has been applied recently in mice, showing ribosomal distribution across start and stop codons change with age in the kidney and liver; however, this analysis was not performed in skeletal muscle or in humans (18). Our laboratory has previously used Ribo-Seq in combination with RNA-Seq in mouse muscle to demonstrate that, in response to a leucine gavage, there was a robust "uncoupled" translation response that occurred in the absence of significant alterations to mRNA abundance (transcription), particularly for the translation of transcripts classified within the mTORC1 signaling pathway (19). This prior study also supported that this technique was sensitive to measure leucine-induced translation changes of circadian transcription factors (19). Ribo-Seq has been used to identify the importance of protein translation for circadian coordination in cancer cell lines but not before in human skeletal muscle (20). We hypothesized tandem Ribo-Seq and RNA-Seq would provide new insight to our understanding of transcriptional and translational kinetics in response to leucine ingestion and that inactivity would dampen mTORC1-mediated translation and disrupts circadian coordination.

Method

Participant Characteristics

Older adults (n = 8; ~72 years; 6 F/2 M) were recruited from the Salt Lake City area via local advertisements and Center on Aging registry. To determine eligible older adult volunteers, an initial screening visit occurred which included a medical history, a physical exam, blood tests to determine liver and kidney function (to exclude cardiovascular, liver, and respiratory disease, uncontrolled hypo/hyperthyroidism and hypertension, history of deep vein thrombosis), and an oral glucose tolerance test and HbA1c to exclude those with prediabetes. Participants were on average 164.4 ± 10.4 cm tall, 56 ± 8.1 kg (~body mass index = 23 ± 1.3) in mass, and had normal glucose regulation (fasted glucose: 89.6 ± 4.4; HbA1c: 5.5 ± 0.3%). Participants read and signed the informed consent (IRB_00084354) and the study was reviewed and approved by the University of Utah Institutional Review Board and conformed to the Declaration of Helsinki and Title 45, US code of Federal Regulations, Part 46, "Protection of Human Subjects." This study is found at clinicaltrials.gov (NCT03839628).

General Experimental Design

All medical and experimental procedures were conducted in collaboration with the clinical research core, part of the center for clinical and translational sciences (CCTS) (Figure 1A). Prior to the first experimental day, individuals tracked their habitual physical activity levels for 14 days using a pedometer. On the first experimental day of the study, participants arrived to the clinic fasted, underwent a DEXA scan, and a Leucine Stimulation Test. This was followed by 2 weeks of step reduction (70% reduction from baseline habitual step activity level) in which the participants keep track of their activity levels in order not to exceed their reduced target step goal. On the next day, the participants came in for a second visit to repeat the DEXA scan and Leucine Stimulation Test. A follow-up oral glucose tolerance test was performed the day before the completion of the 2-week step reduction period. Insulin was detected by ELISA (Human Insulin ELISA, Millipore Sigma, Burlington, MA; EZHI-14K) and Metabolic Clearance Rate and Insulin Sensitivity Index were calculated (21).

Leucine Stimulation Test

We administered 2.4 g of leucine mixed in 250 mL of a 5 kcal flavored water solution based on what we have successfully used to observe anabolic resistance after bed rest in older adults (11). Participants arrived to the clinic early at 0700 fasted, and a whole-body DXA scan was performed. Next, a baseline blood sample was collected and then the participant underwent a muscle biopsy procedure from the vastus lateralis (baseline or "pre leucine") using a modified Bergstrom needle biopsy approach with 2% lidocaine and manual suction (22). After ingestion of the leucine bolus, periodic blood sampling was performed, and a second muscle biopsy was taken from the vastus lateralis 60 minutes after

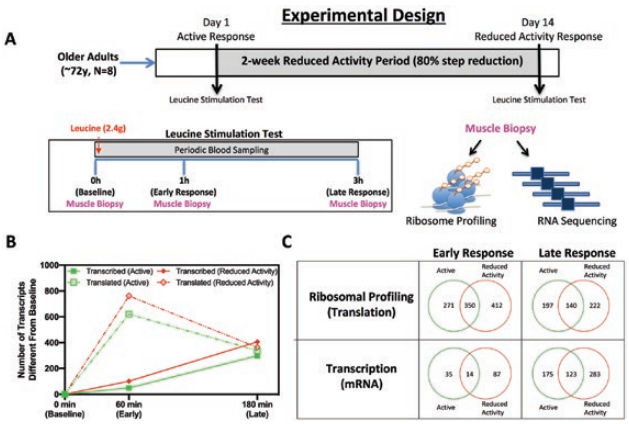

Figure 1. Two weeks of step reduction alters the transcriptional and translational responses to a leucine stimulation test. The experimental design depicts the 2-week step reduction and leucine stimulation tests (A). A graphical depiction of the numbers of transcripts significantly altered for translation and transcription across the various time points respective to the Active or Reduced Activity to leucine (B) as well as greater resolution for directions of transcript changes are presented (C).

leucine (Early Response), followed by a third biopsy at 180 minutes post leucine (Late Response). After 2 weeks of step reduction, the procedure listed above was repeated except biopsies occurred on the opposite leg. The starting thigh for muscle biopsies on the first experimental day was randomized for each participant and balanced with the second visit (left then right or right then left) while repeated muscle biopsies during each visit occurred on alternating thighs (eg, right, left, right). Samples were rapidly frozen in liquid nitrogen and stored at −80°C for ribosomal profiling and RNA-Seq (11,19). Blood samples were placed on ice immediately, spun down at 3000 rpm for 10 minutes, and then stored at −80°C as frozen plasma. Plasma samples were used later to detect leucine concentrations using the EZ-fast amino acid analysis kit (Phenomenex; Cat #KG0-7165).

Tandem Ribosomal Profiling and RNA-Seq

Polysome complexes were isolated, and unprotected mRNA digested with RNase I, and the ribosome-protected mRNA footprints were analyzed by RNA-Seq methods as previously described by our group (19). In summary, ~30 mg frozen muscle was homogenized in the Mini-Beadbeater-8 (Biospec Products) with 3 × 2.3-mm diameter chrome steel balls for 2 × 30 seconds in 1.5 mL lysis buffer (10 mM Tris-Cl, pH 7.5; 300 mM KCl; 10 mM $MgCl_2$; 200 µg/mL cycloheximide; 1 mM DTT; and 1% Triton X-100) at 4°C. 1000 U RNase I (ThermoFisher) was added to each lysate and the mixture was incubated at room temperature for 30 minutes. Insoluble debris was removed by centrifugation for 10 minutes at 4°C. Centrifugation through 50% sucrose at 200 000g for 3.5 hours was carried out to isolate the ribosomes which were then resuspended in Qiazol for RNA isolation (miRNAeasy Mini Kit, Qiagen). For total RNA sequencing (RNA-Seq), ~20 mg of tissue was also homogenized in 1.5 mL Qiazol and RNA was subsequently purified (miRNAeasy Mini Kit). Protein for Western blots was recovered from these Qiazol treated samples using standard protocols available through Qiagen's website (https://www.qiagen.com/us/resources/download.aspx?id=f2e26cce-cb97-44fd-a5ce-714a74a965a0&lang=en). RNA-Seq samples only were then treated with TURBO DNase (ThermoFisher) and purified with RNA Clean and Concentrator 5 Columns (Zymo Research). Ribosomal RNA (rRNA) was then removed from both Ribo-Seq and RNA-Seq samples by using the NEBNext rRNA Depletion Kit (New England Biolabs) as described by the manufacturer with the one exception that the final depleted RNA was purified with an RNA Clean and Concentrator 5 column (Zymo Research).

Library Construction

As previously described (19), RNA-Seq samples were heat fragmented (94°C) in 30 mM $MgCl_2$ for 8 minutes. Ribosome profiling and fragmented RNA-Seq samples were treated with T4 Polynucleotide Kinase (New England Biolabs) and ATP to prepare ends for subsequent ligation reactions. Purification of the RNA was carried out with a RNA Clean and Concentrator 5 Column (Zymo Research). NEBNext Small RNA Library Prep Set for Illumina was then used to make libraries and the resulting library was purified with a Monarch PCR and DNA Cleanup kit (New England Biolabs) and then electrophoresed on a 6% polyacrylamide gel for size selection of ribosome-protected fragment and RNA-Seq fragments with inserts between 20 and 40 nucleotides. Gel slices in this size range were excised, the library DNA was eluted overnight in DNA elution buffer (New England Biolabs), and the DNA was then purified with a Monarch PCR and DNA Cleanup Kit (New England Biolabs). Libraries were then subjected to 50-cycle single-end sequencing on an Illumina HiSeq 2000 instrument. Raw sequence data can be obtained from the National Center for Biotechnology Information Gene Expression Omnibus repository entry GSE162730.

Bioinformatic Analyses

As described (19), adapter sequences were trimmed (FASTX-Toolkit hannonlab.cshl.edu/fastx_toolkit/) and contaminating rRNA sequences were removed (alignment to the human 45s rRNA repeat sequence using bowtie (23)). Uniquely mapping sequences were identified by alignments using bowtie to Reference Sequence database (RefSeq) mRNA entries obtained from the University of California, Santa Cruz browser (Hg38 human genome reference assembly) in which all mRNAs derived from the same gene were reduced to a single entry corresponding to the longest isoform. Normalization factors based on the trimmed mean of M-values were determined by using the calcNormFactors function of the Bioconductor package edgeR (24) for ribosome profiling and RNA-Seq. Dispersion estimates were obtained prior to likelihood ratio tests (glmFit and glmLRT functions of edgeR) to determine significance of the $\log_2$ fold change in ribosome-protected fragments or RNA for all transcripts with ≥1 count/million in all samples. Differences were considered significant if the false discovery rate was ≤0.05. Pearson's product-moment correlation coefficients were calculated.

Immunohistochemistry

Muscle was sectioned into 10-µm cross-sections, mounted on slides in −25°C, then left to air-dry overnight. Immunofluorescent staining was used to detect Type I (DSHB, BA.D5 IgG2b, 1:75), Type IIa (DSHB, SC.71 IgG1, 1:75), Type IIx (DSHB, 6H1 IgM Supernatant 1:2) myosin heavy chain over night at 4°C and the membrane (Fisher Scientific, WGA #W849, 1:50) for 10 minutes at room temperature. Secondary antibodies were used for 60 minutes at room temperature (ThermoFisher, Alexafluors 350-#A21140 1:250, 647-#A21240 1:250, 488-#A21042 1:500) and slides were post-fixed in methanol for 5 minutes and slides mounted with Vecta-Shield (Vectorlabs, #H1000). Images of the entire biopsy were taken using a Nikon Widefield Microscope at 20× magnification. Fiber cross-sectional area was determined using the SMASH Matlab plugin (25).

Immunoblotting

Protein concentration was determined by using the Bradford technique, and 30 µg of protein was separated via polyacrylamide gel electrophoresis, transferred onto a polyvinylidene difluoride membrane, and incubated with primary and secondary antibodies. Polyvinylidene difluoride membranes were imaged on a ChemiDoc XRS (Bio-Rad) and quantified with Image lab software (Bio-Rad). To validate that the leucine dose and timing activated mTORC1 signaling, downstream readouts of phosphorylated proteins related to the mTORC1 signaling pathway were measured, and Per2 was also measured. The primary antibodies were purchased from Cell Signaling Technologies (phospho-p70S6K1 Thr389, 1:1000, #9205; total p70S6K1, 1:1000, #9202; phospho-ribosomal protein S6, rpS6, Ser240/244, 1:1000, #2215; and total rpS6, 1:1000, #2217), with the exception of Per2 purchased from Abcam (ab179813). Secondary antibodies (1:2000) were purchased from Santa Cruz Biotechnology (#SC2004). Phosphorylation of these proteins were normalized to total expression of that respective protein, while reporting total protein

expression for p70S6K1 and rpS6 was normalized to Ponceau S staining or GAPDH in the case of Per2. Reported data are fold change from Active baseline.

Statistics

Differences in participant clinical outcomes in response to reduced activity were determined using a paired *t* test (0 minute Post vs 0 minute Pre). To identify statistical differences within our model for immunoblotting and specific transcript changes in the heat map figures, a 2-way ANOVA across the model (Effect of Activity: Active or Reduced Activity; Effect of Leucine: 0, 60, 180 minutes) was implemented ($p \leq .05$ for significance). Only main effects of Activity, or Activity * Leucine interactions are reported in the heat map figures. A simple linear regression was used to determine R^2 coefficients for the relationship of transcription change (RNA-Seq) with translation change (Ribo-Seq). The impact of reduced activity on the translation of mRNAs encoding ribosomal proteins was determined by taking baseline expression for all mRNAs encoding ribosomal proteins (Translation; Ribo-Seq) that changed from leucine stimulation, and taking the fold change of Reduced Activity/Active normalized RPKMs. A 1-sample *t* test was used for this comparison. A similar strategy was used to map the area under the curve of transcripts encoding ribosomal proteins translation across the time course. The areas under the curves for each transcript encoding for a ribosomal protein graphed for each contrast was compared using a paired *t* test. In order to model circadian translation and transcription across the leucine stimulation time course, a nonlinear sine wave with nonzero baseline regression was used. The curves were then compared using an extra sum-of-squares *F* test comparing the 3 parameters of Amplitude, PhaseShift, and Baseline ($p \leq .05$ for significance). The wavelength was constrained to 1440 minutes to emulate the known 24-hour wavelength (circadian expression, validated by CercaDB) of these molecules. These were performed using Prism 8 (GraphPad, San Diego, CA), as were the generation of the Volcano Plots. Ingenuity Pathway Analysis (IPA; Qiagen) was used to determine Canonical Pathways for both Ribo-Seq and RNA-Seq data sets. IPA was also used to determine Upstream Regulators for the RNA-Seq data set. For all IPA comparisons, statistics were informed by the $\log_2$ fold change, with a statistical cutoff of Adj.*p* value ≤ .05.

Results

Participants decreased their activity by 8651 ± 894 steps per day (from ~10 909, to ~2258; ~80% reduction, $p < .0001$). Participants' lean mass (DXA—Leg Lean Mass; Pre: 12.3 ± 1 kg; Post: 12.3 ± 1 kg) and muscle fiber cross-sectional area was unchanged, but there was a tendency for reduced Type I fiber size (Pre: 3681 μm^2 ± 379; Post: 2944 μm^2 ± 289; paired *t* test, $p = .09$). Glucose tolerance (oral glucose tolerance test 120 minutes; Pre: 112 ± 11 mg/dL; Post: 95 ± 7 mg/dL), Metabolic Clearance Rate (Pre: 9.7 ± 1 mg * kg^{-1} min^{-1}; Post: 9.8 ± 1 mg * kg^{-1} min^{-1}), and Insulin Sensitivity Index (Pre: 6.3 ± 2 mg * kg^{-1} min^{-1} pM^{-1}; Post: 5.3 ± 1 mg * kg^{-1} min^{-1} pM^{-1}) were not altered after 2 weeks of step reduction. Plasma leucine at baseline and in response to leucine ingestion was unchanged by step reduction (leucine area under the curve—Pre: 29 mM * min; Post: 22 mM * min. $p = .09$).

Early Translation Was Followed by Late Transcriptional Changes in Response to Leucine

Tandem Ribo-Seq and RNA-Seq of skeletal muscle biopsy samples taken before, 60 minutes (Early Response), and 180 minutes (Late Response) after leucine (2.4 g) ingestion, repeated before and after 2 weeks of step reduction (Study Design: Figure 1A), demonstrated robust translation (Adj.*p* value ≥ .05) in the Early Response (Active: 271; Common: 250; Reduced Activity: 412) with far fewer transcription changes (Active: 35; Common: 14; Reduced Activity: 87) (Figure 1B and C). The Late Response exhibited fewer transcripts with altered translation compared to the Early time point (Active: 197; Common: 140; Reduced Activity: 222) while the numbers of transcription changes increased (Active: 175; Common: 123; Reduced Activity: 283).

Previously Identified Inactivity-Induced Transcripts Are Further Induced by Leucine

Few changes (translation and transcription) were detected at baseline (fasted) as a result of 2 weeks of step reduction. This is in contrast to our prior RNA-Seq study of 5-day bed rest in which many fasted-state transcription changes were identified (12). This might be expected due to the mild disuse intervention used in this study (compared to bed rest) and in agreement with prior step reduction studies in older adults that also did not demonstrate many transcription changes using microarray analysis of muscle (26). Nonetheless, the muscle anabolic impact of 2 weeks of inactivity was unmasked following leucine stimulation. The 5 most dramatically increased/decreased (by fold change) in translation at each contrast are presented in Figure 2A. Highly altered transcripts appearing in multiple contrasts were included only once to prevent redundancy and allow presentation of more transcripts, and does not mean there was not a significant change to the translation of that transcript in the other contrasts. Inactivity altered the leucine-stimulated translational response for several transcripts (GADD45A, KLF10, KY, IRF7, NMRK2, CAPN6, DUSP6, PFKFB3, and HDCA9), 3 of which were previously identified to exhibit fasted-state transcription changes as a result of bed rest (PFKFB3, GADD45A, NMRK2) (12,13).

Leucine Increased the Translation of mRNAs Encoding Ribosomal Proteins and Circadian Rhythm Transcripts

The Early Response to leucine increased translation of many ribosomal transcripts, and decreased translation of several circadian-interacting factors (NR1D1, KY, PER1, HSPA1A, DBP) some that we have observed previously in mouse muscle after a leucine bolus (19). The relevance of the top translation changes being to ribosomal proteins and circadian regulators are supported by inclusion of mTOR/Translation-related Pathways in the Early Response & the Circadian Pathway in the Late Response for top 10 Ingenuity Canonical Pathways of translation using the Ribo-Seq data (Figure 2B). IPA using RNA-Seq data showed an absence of transcriptional changes to mTOR-related Pathways in both Active and Reduced Activity, while revealing transcriptional activation of the Circadian Rhythm Pathway, but only in the Active response (Figure 2C).

Translation Changes That Are Uncoupled From Changes in mRNA Abundance

The Volcano Plot in Figure 3A depicts Early Translation (Ribo-Seq) contrasted with Early Transcription (RNA-Seq) in response to leucine in the Active and Reduced Activity condition. As described in our previous mouse study (19), the early response to leucine consisted of altered translation of several transcripts in the absence of changes in mRNA abundance (RNA-Seq), such that translational

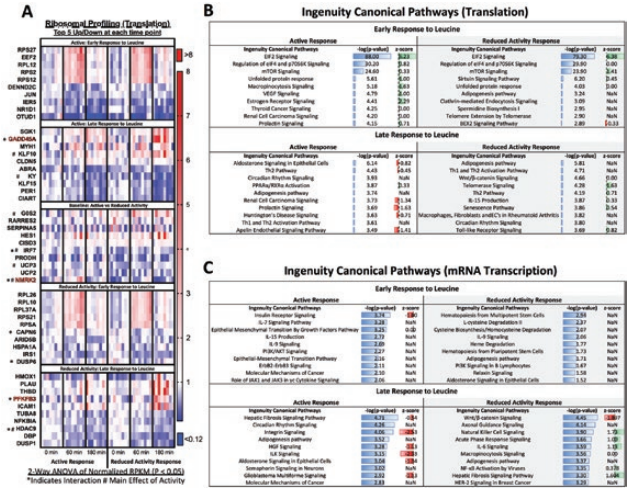

Figure 2. Leucine alters transcription and translation of Circadian Rhythm coordinators and reinforces the expression of inactivity-induced transcripts. The top 5 increased and top 5 decreased transcripts, by translation (ribosome-protected fragment [RPF]) fold change, are presented for each contrast. Duplicates were skipped. 2-way ANOVA (Activity * Leucine) was used on RPKM of translation values to describe inactivity main effects or Leucine * Activity interactions. Identified in previous reduced activity studies, GADD45A, NMRK2, and PFKFB3 are highlighted in red font (A). Ingenuity Canonical Pathway groupings were determined for the Active and Reduced Activity Early and Late Responses for translation (RPF) (B) and transcription (RNA-Seq) (C). $N = 8$.

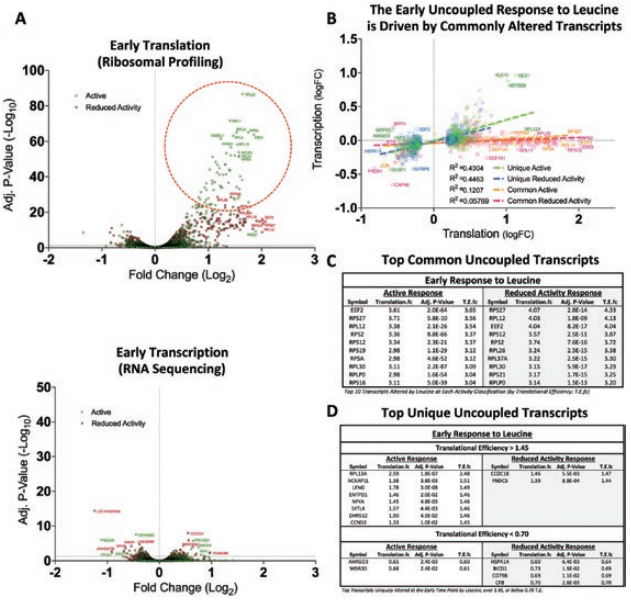

Figure 3. Inactivity affects leucine selectivity for altering the translational efficiency of proteins involved in muscle health. Volcano plots of Translation (Ribo-Seq) and Transcription (Ribo-Seq) in the Early condition are compared (A). These were correlated with groupings reflective of those that had significant differences both for the Active and Reduced Activity leucine stimulation tests, as well as those uniquely altered within those activity status contrasts (B). The top 10 transcripts with the largest translational efficiency changes in the early leucine stimulation period for both the Active and Reduced Activity conditions are presented (C) and transcripts with large changes in translation (TE ≥ 1.45) unique to either Active or Reduced Activity conditions are presented (D).

control is "uncoupled" from transcriptional control. The strongest example of this occurred for mTORC1-related pathways. In this example (Figure 2B vs C), "EIF2 Signaling," "Regulation of eIF4 and p70S6K Signaling," and "mTOR Signaling" built from Translation (Ribo-Seq) are completely absent when the pathways are created using Transcription (RNA-Seq). Shown in the red circle in Figure 3A, inactivity reduced leucine-stimulated TE of these select transcripts.

Correlation of translation (Ribo-Seq; logFC) with transcription (RNA-Seq; logFC) confirmed that the Early Response to leucine triggered changes in translation of select transcripts that are truly uncoupled from transcriptional changes (Supplementary Figure 1C). The Active and Reduced Activity coupled responses were highly correlated (Active: $R^2 = 0.95$; Reduced Activity: $R^2 = 0.94$) representing translation changes driven by changes in mRNA abundance (transcription), in dramatic contrast with the uncoupled changes where translation was not correlated with transcription. This phenomenon appeared to be nearly absent in the Late Response to leucine, demonstrated in Supplementary Figure 1.

Inactivity Changes Leucine-Stimulated Translation of mRNAs Involved in Muscle Health

Figure 3B further dissects the phenomenon of uncoupled translation and demonstrates transcripts with the greatest changes in translational efficiency exhibited significant changes (Adj.p value ≤ .05) in response to leucine under both the Active and Reduced Activity conditions. The top 10 are presented by translational efficiency respective to the Active and Reduced Activity contrasts (Figure 3C). The majority of these transcripts with the highest TE changes (>1logfc = 2-fold) in the Early Response are not dramatically altered by Inactivity beyond what is described in Figure 4. However, several transcripts relevant to muscle health were stimulated by leucine for a TE > 1.45 that were uniquely increased in the Active condition and no longer altered after Reduced Activity (RPL13A, NCKAP1L, LFNG, NFYA, and CCND2). Translation of FNDC5 increased uniquely under the Reduced Activity condition, with corresponding decreases in the translation of HSPA1A, BICD1 (27) (Figure 3D).

Inactivity Reduces Translation of mRNAs Encoding Ribosomal Proteins at Baseline and in Response to Leucine

Immunoblotting for phosphorylated p70S6k^{Thr389} ($p = .0051$) and phosphorylated rpS6$^{Ser240/244}$ ($p < .0001$) (downstream of mTORC1) were increased as a result of leucine at the Early Response, with rpS6 activation persisting into the Late Response for both Active and Reduced Activity with no interaction. Similarly, total p70S6K ($p = .0012$) and rpS6 ($p = .001$) increased with leucine for both of these groups (Figure 4A). The transcripts driving the IPA Translational Pathways (Figure 2B: "EIF2 Signaling," "Regulation of eIF4 and p70S6K Signaling," and "mTOR Signaling") for both the Active Early and Reduced Activity Early Responses to leucine are presented as a volcano plot in Figure 4B. The transcripts with the greatest translation increases in the Active Response were also significant in the Reduced Activity Response; however, the cumulative translation of 72 ribosomal transcripts (increased as a result of leucine ingestion) was reduced by Inactivity at baseline (fasted state) (1-sample t test: $p < .0001$), and in response to leucine (AUC—paired t test: $p < .0001$) (Figure 4C).

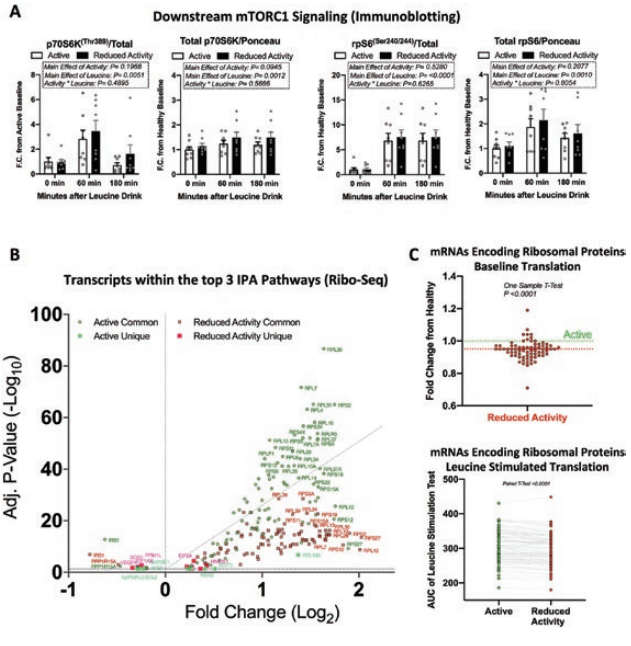

Figure 4. Inactivity reduces the translation of mRNAs encoding for ribosomal proteins. Immunoblotting for total and phosphorylated p70S6k$^{(Thr389)}$ and rpS6$^{(Ser240/244)}$ mTOR signaling targets are presented (A). A volcano plot demonstrating the translational responses of transcripts from the top 3 most activated pathways from Figure 2B are presented (B). Transcripts encoding ribosomal proteins were selected from this list and on a transcript-specific basis, their fold change at baseline was compared between the Active and Reduced Activity responses as well as comparing the Active or Reduced Activity areas under the curves in for translation in response to leucine (C).

Physical Inactivity Altered Circadian Coordination in Response to Leucine

To thoroughly examine the circadian-related findings mentioned previously, we used the IPA Upstream Regulator tool (Figure 5A). We first used an IPA filter to select all significant translation changes (Ribo-Seq) at the Early response to leucine that were only classified as "*transcription regulator*" synonymous with transcription factor. We then uploaded the RNA-Seq results at the Late time point to IPA and used the Upstream Regulator prediction software, again filtering for "*transcription regulator*." There were several instances where the RNA-Seq data predicted a significant change in transcription factor activity for a transcript that Ribo-Seq measured to have a translation change at the early time point. We interpret this result as the translation of a particular transcription factor at 60 minutes post leucine, then caused a measurable transcription response 180 minutes post leucine. This occurred for 11 transcripts in the Active and 17 transcripts in the Reduced Activity Response to leucine. Indicated in bold font are unique transcription factors with measured translation changes Early with corresponding predicted transcriptional activity Late (*Active*—Increased: NFYA, DDIT3, PER2, SP1; Decreased: ZBTB20, CREBBP and *Reduced Activity*—Increased: KLF11, MYOD1, SIRT1, HDAC6, GPS2; Decreased: NCOR1, LDB1, TSC22D3, KLF6, NFKBIA). Interestingly, several transcripts involved in circadian regulation were included on this list (Common: BHLHE41, CEBPB; Unique Active: NFYA, PER2, CREBBP; Unique Reduced Activity: MYOD1) supporting the relevance of observed Circadian Rhythm Signaling pathway differences mentioned previously.

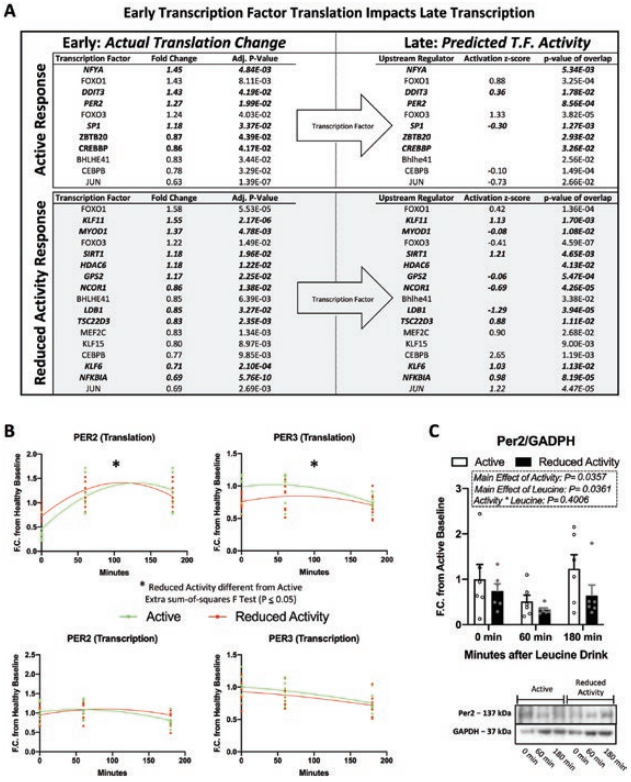

Figure 5. Physical inactivity alters Circadian coordination by leucine. Significantly altered transcription changes (RNA-Seq) in the Late Response to leucine was imported into IPA software and run to predict Upstream Regulator Activity. This list was compared to actual translation changes (ribosome-protected fragment [RPF]) measured in the Early Response to leucine (A). PER2 and PER3 modeled using a sine wave regression with a constrained wavelength of 24 hours demonstrate significantly different translation curves as a result of inactivity using a 3-parameter (Amplitude, Phase Shift, and Baseline) extra sum-of-squares *F* test (B). Immunoblotting for Per2 (subset; $N = 6$) (C).

Next, we contrasted all significant leucine-stimulated translation changes with all known circadian transcripts using CercaDB (28) (Early—Common: 13, Active: 9, Reduced Activity: 14; Late—Common: 18, Active: 12, Reduced Activity: 14) (Supplementary Figure 2). CercaDB is a collaborative database where various tissues sampled at intervals in the day are used to validate or discover circadian factors by modeling sine and cosine wave regressions across a 24-hour wavelength. Using the Leucine stimulation time course measured in our study, we modeled core circadian regulators (CLOCK, ARNTL, CRY1, CRY2, PER1, PER2, PER3) using sine wave regression with a constrained wavelength of 24 hours (Supplementary Figure 2). Using an extra sum-of-squares *F* test for the 3 parameters of amplitude, phase shift, and baseline, we identified a significant difference in the translation curve for PER2 and PER3 in the absence of changes in transcription (Figure 5B). As a follow-up, we performed immunoblotting for Per2 and demonstrated a reduction in Per2 protein content 60 minutes after leucine ingestion and an overall reduction in Per2 content due to inactivity (Main Effect of Leucine, $p = .0361$; Main Effect of Activity, $p = .0357$) (Figure 5C).

To capture transcripts under circadian regulation (validated on CercaDB) that were most dramatically altered by inactivity, 64 transcripts were identified to have significantly different

translation or transcription at any time point (*t* test; $p \leq .05$), and further selected for presentation in Supplementary Figure 3 by those which demonstrated a significant main effect of Activity, or Activity * Leucine interaction when a 2-way ANOVA was run across the entire time course. Finally, we used our previously published data set to contrast fasted-state transcriptional changes in response to 2-week step reduction versus 5-day bed rest. The prior study similarly enrolled active healthy older adults but also contained young controls. The methods and participant characteristics can be found in the previous publication (12). Not reported in our previous publication, this more potent disuse paradigm (5-day bed rest) resulted in fasted-state transcriptional changes to core circadian regulators CLOCK, ARNTL, NR1D2, and BHLHE40 only in old and not in young participants (Supplementary Figure 2). This change was not apparent after 2 weeks of step reduction by the older adults within the current study.

Discussion

The purpose of this study was to uncover unique mechanisms and pathways by which a short-term period of physical inactivity alters leucine-stimulated mRNA translation (protein synthesis) in otherwise healthy and physically active aging muscle. In this study, we used RNA-Seq and Ribo-Seq in tandem across a single sample to scan for changes in ribosomal distribution across mRNAs of interest and compared the translation of mRNAs encoding for ribosomal proteins between Active and Reduced Activity conditions. We also determined that leucine-stimulated translation of mRNAs encoding for transcription factors at an early time point was associated with transcription changes at a later time point, and identified uncoupled transcripts susceptible to inactivity; the study of which is only possible using this unique experimental design and innovative methodological approach. Our main findings were that (i) baseline and leucine-stimulated translation of mRNAs encoding for ribosomal proteins was decreased after 2 weeks of reduced physical activity and that (ii) inactivity deregulated circadian rhythm coordination, specifically shifting PER2 and PER3 translation in the absence of transcription changes.

The potent and rapid increase in translation in the Early (60 minutes post) period following leucine ingestion, with very few changes in transcription until the Late (180 minutes post) period is a simple yet interesting observation considering the general thinking that transcription changes precede changes in translation (29,30). Experimentally, however, it has been demonstrated by us and others (31,32) that translation is affected by a stimulus rapidly (seconds to hours), while transcriptional changes occur more slowly (hours to days) (30). While, of course, in order for mRNA to be translated, it must exist in the mRNA pool. In this study, we find 2 distinct mechanisms of translational regulation: one which appears driven by acute changes to the mRNA pool in response to leucine, and another whereby translation changes are promoted posttranscriptionally to increase translational efficiency of select transcripts as noted previously in mouse muscle (19). The data presented in Figure 5 in which we matched predicted transcription factor activity (RNA-Seq) in the Late response with corresponding measured transcription factor synthesis (Ribo-Seq) in the Early response provided evidence that immediate perturbations to translation could explain one mechanism by which inactivity leads to eventual changes in gene expression (transcription).

The top translation changes induced by leucine and affected by physical inactivity in aged muscle revealed 3 previously identified inactivity-related molecules (PFKFB3, GADD45A, and NMRK2) (12–14,33) where translation mirrored corresponding transcription changes. We and others have previously identified these with traditional RNA-Seq (12,13) but we further confirm that these unique molecules are also robustly altered at the translation level in response to physical inactivity. PFKFB3 is a glycolytic enzyme increased by inactivity, but also may serve as a target for myopathy (34), GADD45A is an inflammation-related gene that induces muscle atrophy (35), and NMRK2 is decreased by inactivity, increased by resistance exercise (13), and is important for NAD+ bioavailability (36). In contrast to our prior bed rest study in which many fasted-state transcription changes were identified (12), the mild disuse intervention used in this study induced few baseline differences in transcription (and few in translation) which is in agreement with a prior step reduction study in older adults (26). However, the impact of inactivity on muscle in this study was uncovered with a leucine challenge resulting in a host of changes to the transcription of molecules either by 60 or 180 minutes post ingestion, and, for the first time reported, we demonstrate corresponding changes in translation. These results indicate that feeding (protein ingestion) or other stress cues (eg, exercise) may be required to fully unmask how inactivity affects the transcription and translation of cell growth and function.

The importance of ribosome biogenesis for skeletal muscle growth (37,38), and that anabolic resistance with age is related to reduced ribosomes (39,40), has been widely studied and demonstrated to be under control of the mTORC1 signaling pathway (41). Standard methods of assessing mTORC1 signaling (ie, immunoblotting) are often inconsistent to capture mTORC1-mediated molecular events. For example, resistance exercise was shown to increase ribosomes despite a decrease in mTORC1 and ribosome biogenesis-related signaling (42). Measuring the translation of mRNAs encoding for ribosomal proteins using Ribo-Seq in our study provided enhanced resolution for detecting inactivity-induced deficits to ribosome production, complimenting previous observations that disuse leads to decreased ribosome biogenesis (43). The appearance of mTORC1-related signaling pathways in the top Canonical Pathways informed by translation (Ribo-Seq) but not RNA-Seq demonstrated this occurred on the translational level only, as previously described (19). Though inactivity reduced translation of mRNAs encoding for ribosomal proteins, these transcripts were among the largest leucine-stimulated TE changes and were increased under both the Active and Reduced Activity conditions. Several aging studies have shown impaired sensitivity of mTORC1 to nutrients concomitant with higher baseline mTORC1 signaling when compared with young controls (44). The robust mTORC1 response observed in both the Active and Reduced Activity conditions on the surface suggest the older adult participants in this study maintained nutrient sensitivity after reduced activity, at least by traditional protein proxies (immunoblotting), but as detected by the current innovative methods, this was not entirely so. Moreover, we do not suspect that baseline muscle mTORC1 signaling in these older participants were higher than young individuals, but a young comparator group would be needed to confirm. Together, these results confirm the importance of nutrient stimulation for ribosome biogenesis and how short-term inactivity, even in habitually active aged adults, disrupts this response.

Muscle circadian rhythms are reinforced by physical activity and feeding (45), and this has importance for the regulation of protein synthesis and prevention of muscle atrophy with age (19,27,46–48). In flies, circadian clock disruption prior to death, specifically increased expression of CLOCK and altered regulation by PER, was associated with impaired locomotor activity (49). In a mouse model

of denervation, an increase in CLOCK mRNA expression preceded the onset of muscle atrophy (50), while genetic manipulation of CLOCK or ARNTL reduced muscle force output and caused disorganized sarcomere architecture (51). Using ribosomal profiling combined with RNA-Seq, we showed that a short-term period of reduced activity disrupted leucine-stimulated circadian coordination in the skeletal muscle of older adults. Under closer scrutiny we found that PER2 and PER3 (transcriptional repressors of CLOCK/ARNTL activity and thus suppressors of their own transcription) exhibited translation rhythm alterations (as determined by sine wave regression) in the absence of transcription. While leucine stimulated an increase in PER2 translation (Ribo-Seq) 60 minutes after ingestion, counterintuitively, Per2 protein expression (immunoblotting) was decreased at this time point, most likely due to increased Per protein turnover. We suspect that the increased translation of PER2 from 60 to 180 minutes might explain the rebound in Per2 protein abundance at 180 minutes post.

In this study, we did not observe changes in lean mass, fiber size, or insulin sensitivity after 2 week of step reduction, nor transcription changes to the core clock regulators, CLOCK and ARNTL. This is in contrast with our previous 5-day bed rest study in which lean mass was reduced and became highly insulin-resistant and this only occurred in old but not in young participants. Interestingly, in that prior study, the muscle morphological and metabolic changes were concurrent with increased muscle transcription of the core circadian transcription factors, CLOCK and ARNTL (and only in old participants), as well as an increase in NR1D2; a negative repressor of ARNTL (previously found to be increased in muscle after 3 months of bed rest (52)). Though the mild 2-week reduced activity intervention did not alter muscle mass and metabolism, it is tempting to speculate that if inactivity persisted longer (or was more robust), the observed translation changes to circadian regulators (eg, PER2 and PER3) may alter transcription changes in core regulators and thus influence muscle size and insulin sensitivity. This hypothesis is partly supported by the decreased protein abundance of Per2 in this current study as a result of reduced activity, as less Per2 theoretically leads to less repression of CLOCK and ARNTL transcription (27). Though there remain several unanswered questions, it is clear from the data presented that aging and activity levels affect the circadian rhythm.

This study was not without limitations. First, a longer time course and the addition of a non-leucine-stimulated time control group and a young comparator might provide new insight by which inactivity affects circadian rhythms in aged skeletal muscle. Second, the participants in this study were highly active and deemed fairly healthy compared with older adult volunteers within other bed rest and step reduction studies (6,15,53–55). The daily steps of studied participants averaged ~11 000 steps/day and had an average body mass index of 23 kg/m^2; thus, it is unclear if healthy aging might be more resistant to inactivity-induced muscle maladaptation.

In summary, the strategic clinical design (early and late leucine stimulation, pre and post short-term inactivity) and unique methodology used in this study identified a translational and transcriptional signature describing the onset of nutrient-induced anabolic resistance. Together, we report that transcriptional and translational changes induced by leucine and further modified by a short-term period of inactivity include decreased translation of mRNAs encoding for ribosomal proteins and alteration of circadian regulators which may precede adaptations to muscle size and metabolic function.

Supplementary Material

Supplementary data are available at *The Journals of Gerontology, Series A: Biological Sciences and Medical Sciences* online.

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Conflict of Interest

None declared.

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Author Contributions

M.T.H. and M.J.D. conceived the study and designed the experiments; Z.S.M., A.I.M., J.J.P., N.M.H., D.K.F., J.J.K. performed the experiments; Z.S.M., N.M.H., L.M.B., M.T.H., M.J.D. analyzed the data; Z.S.M., M.T.H., M.J.D. wrote the manuscript; Z.S.M., A.I.M., J.J.P., N.M.H., D.K.F., J.J.K., L.M.B., M.T.H., M.J.D. edited and approved the final draft of the manuscript.

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