Blunted hypertrophic response in aged skeletal muscle is associated with decreased ribosome biogenesis

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HUMAN AND RODENT STUDIES HAVE reported that skeletal muscle hypertrophy; age; skeletal muscle; microarray; ribosome biogenesis

Blunted hypertrophic response in aged skeletal muscle is associated with decreased ribosome biogenesis. J Appl Physiol 119: 321–327, 2015. First published June 5, 2015; doi:10.1152/japplphysiol.00296.2015.—The ability of skeletal muscle to hypertrophy in response to a growth stimulus is known to be compromised in older individuals. We hypothesized that a change in the expression of protein-encoding genes in response to a hypertrophic stimulus contributes to the blunted hypertrophy observed with aging. To test this hypothesis, we determined gene expression by microarray analysis of plantaris muscle from 5- and 25-mo-old mice subjected to 1, 3, 5, 7, 10, and 14 days of synergist ablation to induce hypertrophy. Overall, 1,607 genes were identified as being differentially expressed across the time course between young and old groups; however, the difference in gene expression was modest, with cluster analysis showing a similar pattern of expression between the two groups. Despite ribosome protein gene expression being higher in the aged group, ribosome biogenesis was significantly blunted in the skeletal muscle of aged mice compared with mice young in response to the hypertrophic stimulus (50% vs. 2.5-fold, respectively). The failure to upregulate pre-47S ribosomal RNA (rRNA) expression in muscle undergoing hypertrophy of old mice indicated that rDNA transcription by RNA polymerase I was impaired. Contrary to our hypothesis, the findings of the study suggest that impaired ribosome biogenesis was a primary factor underlying the blunted hypertrophic response observed in skeletal muscle of old mice rather than dramatic differences in the expression of protein-encoding genes. The diminished increase in total RNA, pre-47S rRNA, and 28S rRNA expression in aged muscle suggest that the primary dysfunction in ribosome biogenesis occurs at the level of rRNA transcription and processing, showing that mTOR activity is absolutely necessary to mount a full hypertrophic response.

In an effort to identify other genes or pathways that might contribute to the age-related difference in skeletal muscle hypertrophy, microarray analyses were performed to identify changes in gene expression between young and old individuals in response to an acute bout of resistance exercise or following a training program (24, 27, 29). Although these studies were able to identify pathways that may have a role in the diminished hypertrophic response of the elderly, the power of these analyses was limited by the small number of time points, typically pre- and postmeasurements. Given the dynamic nature of gene expression during skeletal muscle hypertrophy, it is likely that this design limitation resulted in potentially important changes in gene expression being missed (5). This idea is supported by the finding that acute changes in gene expression are no longer observed following a resistance exercise training program and, therefore, may not truly reflect those biological processes governing hypertrophic growth (29).

The purpose of this study was to perform a comprehensive transcriptome analysis of old skeletal muscle undergoing hypertrophy in an effort to identify differentially expressed genes. Skeletal muscle hypertrophy was induced by synergist ablation with gene expression measured by microarray analysis after 1, 3, 5, 7, 10, and 14 days. To identify age-associated genes that were differentially expressed in response this hypertrophic stimulus, we compared this newly generated data set against our previously published transcriptome analysis in young skeletal muscle subjected to the identical perturbation (5). Despite observing only modest differences in gene expression between the two groups, pathways associated with the regulation of protein synthesis, metabolism, and immune response were highly enriched in old skeletal muscle. Somewhat paradoxical though, we found that ribosome biogenesis was significantly impaired at the level of ribosomal DNA (rDNA) transcription in aged skeletal muscle undergoing hypertrophy. These findings suggest that the blunted hypertrophic response in old skeletal muscle was primarily the result of a failure to sufficiently increase the translation capacity of the muscle more so than changes in gene expression.

METHODS

Animal care and use. All experimental procedures performed in this study were approved by the University of Kentucky Institutional Animal Care and Use Committee. Male C57BL/6J mice, 5 mo of age (The Jackson Laboratory, Bar Harbor, ME) and 25 mo of age

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Young (5 mo of age) and old (25 mo of age) mice were subjected to bilateral synergist ablation surgery to induce hypertrophy of the plantaris muscle as previously described (18). Briefly, following anesthesia with a mixture of 95% oxygen and 5% isoflurane, the soleus and the majority of the gastrocnemius muscles were surgically excised via an incision on the dorsal aspect of the hind limb. Particular attention was made to ensure neural and vascular supply to the plantaris muscle remained intact and undamaged. Animals that served as a control group underwent sham surgery without gastrocnemius and soleus muscle excision. Following recovery from surgery, mice were anesthetized as described above at the designated time point and plantaris muscles were excised, weighed, placed in RNAlater (Ambion, Austin, TX) and stored at 4°C. Plantaris muscle was collected at 1, 3, 5, 7, 10, and 14 days after the surgery (n = 6 per time point) during the same 4-h time period (10:00 A.M. to 2:00 P.M.) after the animals had been fed and were rested, thus ensuring a similar metabolic state between the groups. Plantaris muscles (n = 6) to serve as controls were collected from mice subjected to the sham synergist ablation surgery. Following collection of the plantaris muscles, mice were killed by cervical dislocation under anesthesia.

RNA isolation. Total RNA was prepared from plantaris muscle using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s directions. RNA samples were treated with TURBO DNase (Ambion) to remove genomic DNA contamination. Total RNA concentration and purity was assessed by measuring the optical density (230, 260, and 280 nm) with a Nanodrop 1000 Spectrophotometer (ThermoFisher Scientific, Wilmington, DE). RNA integrity was assessed using a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA); the average RNA integrity number (RIN) value for all samples was 9.12 ± 0.17 (scale 1-10) indicating high-quality RNA with minimal degradation products.

Microarray analysis. Microarray analysis was performed at the University of Kentucky Microarray Core Facility according to the manufacturer’s protocol (Affymetrix, Santa Clara, CA). Gene expression was measured using the Mouse Gene 1.1 ST chip, which provides coverage of 28,000 protein-coding transcripts and 7,000 noncoding transcripts (degree 2-3) and following 14 days of synergist ablation. These findings are consistent with the general regression model. Any gene with different profiles between the 5- and 25-mo-old groups will show some statistically significant coefficient, and its corresponding regression model will be statistically significant. The P value associated to the F-statistic in the general regression model was used to select significant genes between age groups. Second, once gene models showing significant differences were identified, backward stepwise regression was performed on this set of genes to identify the conditions for which genes shows statistically significant profile changes.

Cluster and pathway analysis. Ingenuity Pathway Analysis (IPA) was used to identify those pathways that were significantly enriched across all differentially expressed genes, restricting it to those pathways associated with whole tissues (analysis conducted in June 2014). To elucidate how these biological pathways were affected by age, genes were categorized on the basis of their response to the synergist ablation (i.e., upregulated or downregulated) and the mean fold change at each time point was then generated for both the upregulated and downregulated genes. Hierarchical clustering was performed on all differentially expressed genes on the basis of similar expression profiles across time.

RT-PCR analysis. Complementary DNA was generated from 250 ng of total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen/Life Technologies, Grand Island, NY) using a combination of random hexamers and oligo(dT) primers. Quantitative PCR for each candidate gene was performed using KAPA SYBR Green qPCR ReadyMix (Sigma-Aldrich, St. Louis, MO) with the following cycle conditions: 95°C for 3 min, 40 cycles at 95°C for 30 s, and at 60°C for 60 s. Primer sequences are available upon request. All transcripts were normalized to the geometric mean of Gapdh and Rn7sk. These genes were selected on the basis of having the lowest variability (0.24% and 0.34%, respectively) across age and treatment as determined by the microarray analysis.

Statistical analysis. The muscle wet weight, total RNA concentration, and quantitative PCR data were analyzed by two-way ANOVA followed by Tukey’s post hoc test with significance set at P ≤ 0.05. Significant gene profiles were determined using polynomial regression (degree = 3) using a false discovery rate-corrected P value of 0.01 to determine statistical significance. For stepwise regression analysis the P value for the regression coefficients was set to α = 0.01. Only genes that had a clear trend and fit the model with an R² value of >0.7 were used for further analysis. A right-tailed Fisher’s exact test was used to determine the top statistically significant canonical pathways from IPA. For this analysis, P < 0.0001 was considered statistically significant.

RESULTS AND DISCUSSION

In response to 14 days of synergist ablation, plantaris muscle weight was significantly increased in both young and old mice; however, the increase in normalized plantaris muscle weight was significantly less (40% vs. 71%, respectively) in old mice than in young mice (Fig. 1). Importantly, there was no difference in the body weight of young and old sham-operated mice and following 14 days of synergist ablation. These findings are
consistent with previous observations in aged animals showing blunted hypertrophic growth following mechanical overload ranging from 7 days to 8 wk (2, 4, 6, 8, 22, 31).

In an effort to identify differentially expressed genes that may contribute to the blunted hypertrophic response observed in old skeletal muscle, we performed a time-course microarray analysis of gene expression following 1, 3, 5, 7, 10, and 14 days of synergist ablation in young and old mice. Regression analysis using the two data sets identified 1,607 genes that were differentially expressed across the time course between the young and old groups (see Supplemental Table 1 online).

We next used cluster analysis to determine how age affected the temporal pattern of expression of differentially expressed genes. We found that 12 clusters provided a sufficient number of discrete clusters with enough genes in each cluster to allow a biologically meaningful pathway analysis (Fig. 2). Cluster analysis showed that the pattern of expression of differentially expressed genes was remarkably similar between young and old groups, with the one exception being cluster 12 (Fig. 2). This cluster contained genes that were expressed at a much lower level in old muscle across the time course and showed a different response to synergist ablation compared with young muscle. Within this cluster, about one-third of the genes were from either the major urinary protein family (Mup1-4, 7, 11, 19, and 20) or the serine (or cysteine) peptidase inhibitor family (Serpina1a, b, c, and e) (see Supplement Table 1).

Although few studies have examined the effect of the Mup genes on skeletal muscle function, increasing levels of circulating Mup1 have been reported to enhance whole-body energy expenditure that was associated with the activation of Akt signaling and mitochondrial biogenesis in skeletal muscle (13). Thus elevated expression of these genes in young skeletal muscle suggests a possible role in regulating the metabolic adaptations that occur with synergist ablation; however, it was when total RNA concentration peaked in the young group. After which expression remained higher in the old group (Fig. 3A). The ribosomal protein genes showing the greatest age-dependent differences were Rpl24, Rps19, Rpl10a, Rpl13 and, in particular, Rpl11 (Fig. 3B and Supplement Table 1). RT-PCR confirmed that Rpl11 expression was significantly increased by 4.6-fold in the old mice at day 10 but was unchanged from baseline at this time point in the young group (Fig. 3C). These results are consistent with findings from a recent human study that found increases in lean mass were negatively correlated with expression of 30 ribosomal protein genes (9).

Given the higher expression of ribosomal protein genes in old skeletal muscle in response to synergist ablation, we were curious to see what effect this might have on ribosome biogenesis. We and others have reported that synergist ablation-induced muscle hypertrophy is associated with a significant increase in ribosome biogenesis (12, 19, 32). To determine whether ribosome biogenesis was altered in response to synergist ablation in old mice, we measured total RNA per unit of muscle mass at each time point; given that 85% of total RNA is ribosomal RNA (rRNA), total RNA per unit of tissue is considered a measurement of ribosome content (34). There was no difference in the ribosome concentration of plantaris muscle of young and old animals that underwent sham operation animals because total RNA per unit of muscle was the same between these two groups (Fig. 4). In response to synergist ablation, total RNA concentration of plantaris muscle of young mice significantly increased from day 5 through day 14, with an approximately 2.5-fold peak expression occurring at days 7 and 10 (Fig. 4). In contrast, total RNA concentration of the plantaris muscle of old mice showed a modest ~50% increase at days 10 and 14 that was significantly less compared with young mice (Fig. 4).

The lower ribosome content of plantaris muscle from old animals in response to synergist ablation might be caused by a failure to fully activate rDNA transcription in response to synergist ablation. To determine whether rDNA transcription was altered in plantaris muscle of old animals, we measured pre-47S rRNA abundance in sham-operated animals and after 3, 7, and 10 days of synergist ablation in both young and old mice. We chose to focus on these time points because day 3 was the last time point before total RNA concentration diverged between the two groups and days 7 and 10 because it was when total RNA concentration peaked in the young group. There was no difference in pre-47S rRNA expression between young and old sham-operated groups suggesting that rDNA transcription at baseline was not affected by age (Fig. 5A). Following 3 days of synergist ablation, there was a significant increase in pre-47S rRNA expression in both young and old

Fig. 1. Aged skeletal muscle demonstrates blunted hypertrophic growth following mechanical overload. Normalized (to body weight) plantaris muscle weight from 25-mo-old mice showed impaired hypertrophy compared with young mice following 14 days of synergist ablation (SA-14). *Significant age-effect; †significant increase relative to sham (P < 0.05).
groups, though the increase in the young group was significantly greater than in the old (3-fold vs. 1.7-fold, respectively). Of note, the increased expression of pre-47S rRNA observed at day 3 in both groups preceded the increase in ribosome content as assessed by total RNA concentration (compare Fig. 4 and 5A). Although the current study does not provide a mechanism to explain the delay between 47S pre-rRNA expression and total RNA accumulation, Nagatomo et al. (21) reported that an acute increase in 60S ribosome formation preceded an increase in total RNA at day 5 in response to cardiac pressure overload. The authors proposed that this delay in total RNA accumulation was a reflection of ribosome half-life of 10–12 days. Given that this was observed in the heart, it is reasonable to speculate that a similar process may be occurring in skeletal muscle in response to mechanical overload induced by synergist ablation and might be affected by age. At day 7, pre-47S rRNA expression in the young group remained significantly elevated by 3-fold, whereas pre-47S rRNA expression returned to baseline level in the old group (Fig. 5A).

Using the same model of hypertrophy as we did, von Walden et al. (32) also reported an increase in pre-47S expression after 3 days of synergist ablation which, in contrast to our
findings, returned to baseline by day 7. In both studies, however, pre-47S expression paralleled the change in total RNA, further lending support to the idea that the rate of rDNA transcription dictates ribosome biogenesis. These results suggest that polymerase I (Pol I) activity is compromised in aged animals during hypertrophic growth, which may underlie an impaired ability in increase ribosome biogenesis, potentially limiting growth.

To determine whether processing of pre-47S rRNA was altered in the muscle of old animals undergoing hypertrophy, we measured the 28S rRNA expression. There was no difference in 28S rRNA expression in young or old sham-operated groups, indicating that pre-47S processing was unaffected by age in resting skeletal muscle (Fig. 5B). In good agreement with pre-47S rRNA expression, 28S rRNA expression was significantly increased in response to synergist ablation at days 3, 7, and 10 in the young group; in contrast, 28S rRNA expression remained unchanged in the old group until day 10 (Fig. 5B). These findings suggest that rDNA transcription and processing had become uncoupled in the muscle of old animals during hypertrophic growth.

On the basis of our findings, we present a novel notion that dysfunctional ribosome biogenesis is one the primary factor inhibiting muscle hypertrophy in aged animals. We suggest that in young animals, the expression levels of the core components of mature ribosomes promotes a cellular environment that is permissive for maximizing ribosome biogenesis during muscle hypertrophy, which does not occur in aged animals. The attenuated increase in total RNA, pre-47S, and 28S content in muscle from old mice suggests that the primary dysfunction is occurring at the level of rDNA transcription and/or processing. Because the synergist ablation model of hypertrophy induces such a robust hypertrophic growth, it would be of interest to determine whether ribosome biogenesis would be altered in a more physiological model that induced a more modest level of hypertrophy with age.

Results from the current study suggest that although age-dependent differences exist across in the transcriptome profile between the skeletal muscle of young and old animals during hypertrophy, the differences appear to be modest. Moreover, the response of individual genes to the mechanical overload resulting from synergist ablation appear to be conserved across age groups, with little evidence to suggest that disparities in transcript levels or misexpression are driving the impaired hypertrophic growth in aged animals. Furthermore, it is important to factor in acute gene expression changes vs. chronic changes, because we observed that most of the changes occur early in the time course. Recently, Nader and colleagues (20) challenged the idea that using acute changes in gene expression to better understand the mechanisms regulating muscle hypertrophy are of limited value because most of these changes are not observed with chronic overload. Thus the findings from this study provide support for the hypothesis that attenuated translational capacity is one of the primary factors governing the growth of skeletal muscle with age. To this end, many studies have focused on examining the translational efficacy of muscle and how this process is affected by age, but the role of age on translational capacity has been largely unexplored. Moreover, studies indicate that even acute differences in protein synthesis, via increased translational efficacy, are not sufficient to predict the degree of muscle hypertrophy (17). These findings further argue for an important role for ribosome biogenesis in regulating chronic changes in muscle size. By utilizing multiple time points during hypertrophic growth, our data indicate that one key determinant of translational capacity (i.e., ribosome biogenesis) is severely impaired in aged skeletal muscle, and this appears to be at the level of rDNA transcription.

**Conclusion.** Currently, the regulation of rDNA transcription and ribosome biogenesis in skeletal muscle adaptation is poorly understood, with the majority of studies focusing on assessing the activation of downstream components of the mTORC1 pathway as a readout of ribosome biogenesis.

### Table 1. Most enriched biological pathways among differentially expressed genes

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<thead>
<tr>
<th>Ingenuity Canonical Pathways</th>
<th>P</th>
<th>Ratio</th>
<th>Genes in Pathway</th>
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<tbody>
<tr>
<td>EIF2 signaling</td>
<td>6.69E-09</td>
<td>2.04E-01</td>
<td>RPL11, RPL24, EIF2B4, Rpl36a, HRAS, EIF2B2, Pppl1 ml, RPL18A, RPL27a, RPS13, MAPK3, PAPI, RPL9, RPS3, RPS5, RPL8, RPL31, RPL13, AKT2, RPS19, EIF3I, RPL7L1, RPL12, RPL23, EIF3E, RPLP0, RPS4Y1, RPS26, PIK3R6, RPS15, RPL10, RPS27A, RPL5, EIF3L, RPL13A, RPSA</td>
</tr>
<tr>
<td>Mitochondrial dysfunction</td>
<td>4.35E-07</td>
<td>1.89E-01</td>
<td>NDUF4A, SDHB, NDUF9A, ATP5H, Cox6c, UQCR11, AC02, TRAK1, NCSTN, NDUF4A1, PHDHA1, SOD2, ATP5J2, NDUF52, NDUF6, ATP5J1, ATP5J, NDUF4F1, CYCS, GLRX2, SURF1, COX7A1, APP, FSI1, PRDX3, NDUF2, NDUF4A1, UQCR10, NDUF6, NDUF8, SDHD, NDUF82</td>
</tr>
<tr>
<td>CTLA4 signaling in cytotoxic T-lymphocytes</td>
<td>3.53E-06</td>
<td>2.27E-01</td>
<td>AP2M1, AKT2, CD3E, HLA-A, AP1M1, CLTC, CLTB, TRG, CD8B, PPP2R5A, AP2S1, AP1S1, CD3G, LCK, PPP2R1A, API1G2, PTEN11, LAT, PIK3R6, PTEN22</td>
</tr>
<tr>
<td>Mechanistic target of rapamycin (mTOR)</td>
<td>3.82E-06</td>
<td>1.72E-01</td>
<td>ULK1, PLD2, PRKAB1, HRAS, RHOD, RPS13, MAPK3, TSC2, RPS6KB2, PRKAA2, FIGF, RPS3, RPS5, AKT2, PLD3, STK11, RPS19, EIF3M, EIF3E, PPP2R5A, PLD4, RPS4Y1, PPP2R1A, RPS26, PIK3R6, RPS15, PRR5, RPS27A, RPS6KA1, FNBP1, EIF3I, RPSA</td>
</tr>
<tr>
<td>Oxidative phosphorylation</td>
<td>7.91E-06</td>
<td>2.04E-01</td>
<td>NDUF4A, ATP5J, SDHB, ATP5H, NDUF49, Cox6c, UQCR11, CYCS, SURF1, COX7A1, NDUF4A1, NDUF2, NDUF4A1, NDUF6, UQCR10, NDUF87, ATP5J2, NDUF86, NDUF82, SDHD, ATP5F1, NDUF82</td>
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We propose that the regulation of ribosome biogenesis, in particular, the regulation of rDNA transcription, may provide additional insights into the regulation of protein synthesis during skeletal muscle hypertrophy and its importance with aging.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

K.A.E. and J.J.M. conception and design of research; J.D.L. and J.H.E. performed experiments; T.J.K., J.D.L., J.H.E., and T.C. analyzed data; T.J.K. interpreted results of experiments; T.C. prepared figures; T.J.K. drafted man-

Fig. 3. Increased expression of ribosomal proteins during hypertrophy with old age. A: geometric mean expression of all differentially expressed ribosomal protein genes following synergist ablation. B: Rpl11 increases earlier during mechanical overload in both young and old animals; however, the aged animals maintain higher expression levels at the later time points following synergist ablation. C: RT-PCR confirmed a significant increase in Rpl11 expression in old animals following 10 days of synergist ablation (SA-10), which did not occur in young animals. *Significant age-effect; †significant increase relative to sham (P < 0.05).

Fig. 4. Increased ribosome content during hypertrophy is blunted in aged animals. Total RNA content, of which >85% is ribosomal RNA, increased in response to synergist ablation (SA), indicating increased ribosome biogenesis. Conversely, skeletal muscle of old animals demonstrated a compromised ability to increase total RNA content in response to synergist ablation. Old animals demonstrated a delayed increase in RNA content, not occurring until at SA10 and SA14. Furthermore, at SA5 through SA14, RNA content was significantly lower in the old animals relative to the young animals. *Significant age-effect at that time-point; †significant increase from sham (P < 0.05).

Fig. 5. Increased pre-47S and 28S rRNA expression during muscle hypertrophy are attenuated in old animals. A: pre-47S rRNA expression, a readout of polymerase I activity, increased in response to synergist ablation in young animals after 3 and 7 days of synergist ablation with old animals only showing a modest increase at day 3. Furthermore, pre-47S expression was significantly higher in young animals compared with old animals at both days 3 and 7 of synergist ablation. B: 28S expression was significantly attenuated at days 3 and 7 in old animals. *Significant age-effect at that time-point; †significant increase from sham (P < 0.05).
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