Contraction intensity and feeding affect collagen and myofibrillar protein synthesis rates differently in human skeletal muscle

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Exercise increases the synthesis rate of various skeletal muscle proteins (23, 47, 61, 70, 71, 73, 101), and its regulation is among others thought to be dependent on the mammalian target of rapamycin (mTOR) (30, 76) and the extracellular signal-regulated protein kinase 1/2 (49, 75, 83). Different exercise types, i.e., endurance- vs. resistance-type exercises, have been shown to exert divergent effects on muscle protein turnover and synthesis rates (95, 101). However, except from differences in exercise intensity, decisive differences in contraction type and exercise volume characterize various kinds of exercises; thus, differences in the response may be because of several varying and uncontrolled parameters. Therefore, the isolated effect of contraction intensity cannot be extracted from these studies, nor can it be done from a comparison of the number of studies investigating just one exercise type (9, 17, 27, 36, 70, 78, 80, 89). The present insight of how contraction intensity alone affects the muscle protein synthesis rates is to our knowledge based on few studies. In 2005, Atherton et al. (3) stimulated the extensor digitorum longus muscle in rats at two frequencies, designed to mimic endurance- or resistance-type exercises. Despite unmatched exercise volume, they concluded that only after the resistance-type stimulation the myofibrillar protein synthesis rate was increased with concomitant changes in mTOR-ribosomal protein s6 kinase-binding protein-1 (BP1) and eukaryotic elongation factor (eEF)2 signaling (3). Recently, Kumar et al. (61) investigated how a range of various contraction intensities in young and old humans affected myofibrillar protein synthesis and found that only intensities at ≥60% one-repetition maximum (1RM) in the young subjects induced significant and similar elevations (61). Of concern though in that paper was that the duration of the enhanced protein synthesis rate was markedly shorter than shown elsewhere (70, 78), which, according to the authors, presumably was caused by the lack of exercise volume (61). It therefore might be speculated whether the lack of changes in synthesis rate at the lower intensities may also have suffered from the low exercise volume.

Along with the myofibrillar, contractile proteins the extracellular matrix and thus the collagen proteins make up a crucial structure in the skeletal muscle (57, 77). The importance of the collagen matrix for skeletal muscle function is exemplified by the severe functional abnormalities when pathologies in collagen structures are present (7, 67). 4E-BP1 remained to respond only to the HL contraction intensity. Thus the study design allows us to conclude that the MAPK- and mammalian target of rapamycin-dependent signaling responds to contractile activity, whereas elongation mainly was found to respond to feeding. Furthermore, although functionally linked, the contractile and the supportive matrix structures upregulate their protein synthesis rate quite differently in response to feeding and contractile activity and intensity.

gas chromatography-combustion-isotope ratio mass spectrometry; protein turnover; molecular signaling; exercise; nutrition

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the diversity of the existing data and the lack of direct comparisons of how contractile intensity affects muscle collagen and myofibrillar protein synthesis rate made us design the present study comparing how two distinct contraction intensities affect the synthesis rate of the two protein fractions.

Elevated nutrient availability is known to exert a major impact on muscle protein synthesis rate both at rest (37), as well as during (8) and following (26, 81, 94, 104) exercise. In contrast, the intramuscular collagen proteins have been shown to be insensitive to feeding in the resting state (5), whereas it has not been investigated whether exercise makes the muscle collagen synthesis rate responsive to nutrient intake. It is likely that exercise and elevated nutrient availability interact to affect both the temporal and relative changes in protein synthesis compared with either of the interventions alone (13, 73), but no studies have investigated whether nutrition and exercise intensity interact to change the synthesis rates of either myofibrillar and/or collagen protein fractions.

The aims of the present study were in an intrasubject design to compare how skeletal muscle myofibrillar and collagen proteins change the synthesis rates following two distinct contraction intensities in the overnight fasted state and in the fed state, respectively. Similarly, we investigated the phosphorylation status of selected molecular targets with influence on the translation initiation and elongation regulation in an attempt to link the known molecular pathways to contractile- and feeding-induced actions.

MATERIALS AND METHODS

Through newspapers and web advertisements, we recruited 20 subjects (10 to complete trials in the fasted state and 10 other subjects to complete trials in the fed state). All subjects were apparently healthy and sedentary (more than 6 mo since they may have participated in organized sports or exercise more frequently than once a week) males who fulfilled the following criteria: not on medications, have type 2 diabetes, without knee pain, nonalcoholic, nonsmokers. The subjects were randomly assigned to complete the fasting or the fed trial (see more detailed trial description below). Before enrollment in the study, each subject was individually informed according to the Declaration of Helsinki II and gave written consent to volunteer participation in the protocol, which was approved by the local ethical committee for Frederiksberg and Copenhagen municipalities under journal (KF) 01–024/04.

On a separate day (at least 7 days before study) and after thorough equipment adjustments and significant strength test, familiarization, the subjects had their 1RM determined for each leg on the knee extension equipment (Technogym; Super Executive Line, Gambotto, Italy). After warming up, the 1RM strength was determined in both legs following a protocol applying increasing loads interspersed with sufficient rest periods. Before the trial (1 wk), the subjects were instructed to refrain from alcohol intake. During the last 3 days before the trial, the subjects were instructed to eat sufficiently, drink no caffeine-containing beverages, and refrain from performing strenuous physical tasks or exercise. Restrictions were monitored by activity records and weighed food registration, and these indicated acceptable compliance.

Experimental Design

On the day of the study, subjects arrived in the morning at 6:00 A.M. by taxi to the laboratory at The Institute of Sports Medicine, Bispebjerg Hospital, after an overnight fast from 9:00 P.M. the evening before. They rested supine, and catheters were inserted retrogradely in the antecubital vein of both arms. One catheter was used for tracer infusion and one for repeated blood sampling throughout the study. Before tracer priming, a blood sample was drawn for background tracer enrichment determination. Thereafter, a primed (0.8 mg/kg, ~6.1 μmol/kg) continuous (1.0 mg·kg⁻¹·h⁻¹, ~7.6 μmol·kg⁻¹·h⁻¹) infusion of L-[1-¹³C]leucine tracer (sterility and pyrogenity tested, 99% enriched; Cambridge Isotopes Laboratories, Andover, MA) was started, which was maintained until the completion of the study, ~10 h later (Fig. 1). The leucine tracer has previously been shown appropriate for measuring myofibrillar and intramuscular collagen protein fractional synthesis rate (FSR) (5, 70).

The tracer was prepared by dissolving the weighted amount in 60 ml of sterile 0.9% saline, filtered through a 0.2-μm sterile disposable filter (Sartorius, Hannover, Germany) in the final volume of sterile 0.9% saline, and kept at 5°C until infusion. At time points −2:45, −1:45, −0:55, 0:10, 0:25, 1:00, 1:30, 2:00, 3:00, 4:00, and 5:20 [h:min; with time 0 (zero) being immediately after cessation of exercise], blood samples were drawn for the determinations of [¹³C]ketoisocaproic acid (KIC) enrichment, amino acids, and hormonal concentration. At time −2:45, −0:45, 0:30, 3:00, and 5:30 (h:min), muscle biopsies were obtained (Fig. 1). From time point −0:35 to 0:00 the resistance exercise protocol was completed. In the fasted

Fig. 1. Trial design. The 10-h infusion protocol is outlined, and time points for bilateral muscle biopsies are marked with arrows. The exercise protocol (EXER), which is outlined at bottom, shows the repeated shift between the light-load (LL, horizontal bar) and heavy-load (HL, vertical bar) contraction intensities.
trials, the subjects \((n = 10)\) were only allowed to drink water, whereas in the fed trials each subject \((n = 10)\) received a multi-

nutrient supplement \([17\) energy percent \((E\%)\) protein \((\text{soy and milk protein resulting in a high biological value})\), \(52 E\% \text{carbohydrate (hydrolyzed cornstarch)}\), and \(31 E\% \text{fat plus a variety of minerals and vitamins (Komplett Næring; Novartis Healthcare, Copenhagen, Denmark)})\] every half hour containing energy corresponding to \(1/48\) of their estimated daily energy expenditure \((\text{estimated by the Harris-Benedict equation multiplied by } 1.6)\). The first bolus was given just after the tracer priming and infusion initiation as a double dose \((2/48\) of estimated daily energy expenditure). The strategy of feeding frequent small doses was used to maintain a steady and elevated nutrient availability and concomitantly avoiding the nonsteady state of leucine tracer enrichment throughout the trial.

Exercise Protocol

The exercise protocol consisted of unilateral knee extensions. In brief, one leg worked against a light load \((LL; 16\% \text{ of } 1RM)\) while performing 36 repetitions \((1 \text{ repetition every } 5\text{th }s \text{ for } 3\text{ min})\) in each set. The contralateral leg worked against a heavy load \((HL; 70\% \text{ of } 1RM)\) performing eight repetitions \((\approx 25\text{ s})\) in each set. To counterbalance, one-half of the subjects trained their dominant leg using HL and the contralateral leg using LL, while reversed for the other half of the subjects. The exercise consisted of isolated quadriiceps muscle contractions performed in a commercial knee extension device \((\text{Technogym; Super Executive Line})\) using a range of motion of \(100^\circ\) \(\text{full knee extension). The subjects were seated in the training device with an open hip angle, arms folded across their chest, and fastened with rigid straps across their hips and thigh. They alternately exercised each leg (Fig. 1) by shifting between the predefined LL and HL in the device. Importantly, total lifted load was matched within each set between the HL and LL protocols. We added the mean weight of the lower leg and crank arm \((\text{fixed mass})\) to the measured total lifted load \((\text{HL load})\). When calculating the relative intensities at \(16\text{ and }70\%\), the mean weight of the lower leg and crank arm \((\text{fixed mass})\) was adjusted accordingly to match within each set between the HL and LL protocols. The contralateral load was subtracted \((2.3 \times \text{vol} \text{ ice-cold ethanol, and the pellet was left overnight and subsequently washed with } 70\% \text{ ethanol and spun (1,600 } g, 4\text{°C, } 20\text{ min})\) to precipitate the high-salt insoluble collagen proteins. The supernatant was then added (2.3 \times \text{vol} \text{ ice-cold ethanol, and the pellet was left overnight and subsequently washed with } 70\% \text{ ethanol and spun (1,600 } g, 4\text{°C, } 20\text{ min})\) to precipitate myofibrillar proteins. The salt-precipitated total collagen protein pool and the washed myofibrillar pellet were then hydrolyzed in 6 M HCl \((110^\circC \text{ for } 18\text{ h})\). The constituent amino acids in the hydrolysates were purified by cation exchange resin \((\text{Dowex AG-50W; Bio-Rad, Copenhagen, Denmark})\) and diluted in acetonitrile.

Protein-bound amino acid enrichment. A 15- to 25 mg piece of muscle tissue was used for isolating the myofibrillar and collagen proteins using a solubility/centrifugation procedure used elsewhere \((5, 23, 25, 68, 70, 71)\). Briefly, muscle samples were homogenized in 1 ml ice-cold buffer containing 0.15 M NaCl, 5 mM EDTA, 0.1% Triton X-100, and 0.02 M Tris \((\text{pH } 7.4)\) using an automatic homogenizer \((\text{FastPrep 120A-230; Thermo Savant, Holbrook, NY})\). The homogenate was centrifuged \((1,600 \times g, 4\text{°C, } 20\text{ min})\), and the pellet \((\text{myofibrillar and collagen proteins})\) was added 1 ml of 0.7 M KCl to precipitate the high-salt insoluble collagen proteins. The supernatant was then added \((2.3 \times \text{vol} \text{ ice-cold ethanol, and the pellet was left overnight and subsequently washed with } 70\% \text{ ethanol and spun (1,600 } g, 4\text{°C, } 20\text{ min})\) to precipitate myofibrillar proteins. The salt-precipitated total collagen protein pool and the washed myofibrillar pellet were then hydrolyzed in 6 M HCl \((110^\circC \text{ for } 18\text{ h})\). The constituent amino acids in the hydrolysates were purified by cation exchange resin \((\text{Dowex AG-50W; Bio-Rad})\), dried down in a SpeedVac as described above, and finally derivatized as their N-acetyl n-propyl esters using our own optimized protocol for previously applied procedures \((22)\).

Mass Spectrometry

\(\text{[}^{13}\text{C}]\text{KIC enrichment. With the use of the programmed-temperature vaporization (PTV) injection mode, 1 }\mu\text{L \text{derivatized sample was injected and carried by a constant helium flow (}1.8 \text{ ml/min}) in the gas chromatograph (GC, Trace GC 2000 series; Thermo Quest Finnigan, Paris, France) and separated by a capillary column (CP-Sil }8 \text{ CB low bleed }30 \times 0.32 \text{ mm, coating }0.25 \mu\text{m, ChromPack; Varian, Palo Alto, CA})\). In the mass spectrometer \((\text{MS, Automass Multi; Thermo Quest Finnigan})\), electron ionization was applied and the profile mode used to determine the quadrupole-trimethylsilyl derivative fragment ratio \((M+\text{M})\) of the mass-to-charge \((m/z)\) fragment of 232. \(66)\).

Leucine and phenylalanine concentrations. The PTV-inlet injection mode was applied for this analysis as well. The internal tracer standard method \((50)\) was applied to determine the plasma concentrations on the GCMS in the profile mode of leucine and phenylalanine using \(1-[5,5,5-^{2}\text{H}_3]\text{]leucine and }1-[^{2}\text{H}_3]\text{]phenylalanine, respectively, monitoring the tert-butylmethylsilyl derivatives with }m/z\text{ ratios for leucine }\left(M+3/M+M+1=305/302+303\right)\text{ and for phenylalanine }\left(M+8/M=344/336\right)\).

Protein enrichment. Isotopic enrichments of protein-bound leucine were measured by gas chromatography-combustion-isotope ratio mass spectrometry \((\text{Delta Plus XL; Thermo Finnigan, Bremen, Germany})\) against a laboratory standard related to Pee Dee Belemnite limestone. The sample was injected in a PTV inlet and carried by
helium further to the GC capillary column (CP-Sil 19 CB 60 m × 0.25 mm, coating 1.5 μm, ChromPack; Varian).

**FSR calculations.** The FSR was calculated based on the incorporation of tracer ([1-13C]leucine) in tissue proteins, using the standard precursor-product model: FSR = ΔEproduct × Eprecursor × t × atom dilution factor × 100%, where ΔEprecursor is the change in tracer enrichment in two tissue samples taken with a time interval of ∆t in hours during which the precursor pool enrichment was constant and equal to Eprecursor. With the use of human cell culture media, it has been demonstrated that the precursor-product approach validly can be applied both in myocytes and fibroblasts to assess the FSR (63). We used the venous [13C]KIC enrichment being a valid although slightly overestimated surrogate measure of the true leucyl-tRNA precursor pool enrichment in skeletal muscle (53, 62, 65, 66, 97).

**Muscle Tissue Preparation for Western Blotting**

To measure protein phosphorylation status, samples were prepared and analyzed essentially similar to Rose et al. (87). Briefly, 15–20 mg muscle were homogenized using an extraction buffer (0.05 M Tris·HCl, 0.15 M NaCl, 1 mM EDTA, 1 mM EGTA, 0.05 M NaF, 5 mM Na2P2O7, 2 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 0.5% protease inhibitor cocktail, and 1% Nonidet P-40). After centrifugation, the lysate was separated, and, based on BCA protein assay spectrophotometer assessments, each sample was diluted to similar protein concentrations (1.5 g dependent on the target) and resolved in self-cast polyacrylamide gels (SDS-PAGE) and were subsequently transferred to polyvinylidene fluoride membranes (Millipore), which were blocked with either 3% BSA or 2% milk proteins dissolved in 1× TBS and 0.25% Tween 20 (pH 7.4). Samples were thereafter incubated overnight at 4°C with the following primary antibodies: 4E-BP1 (Thr37/46) (no. 9459), AMP-activated protein kinase (AMPK) (Thr172) (no. 2531), acetyl-CoA carboxylase (ACC) (Ser221) (no. 3661), protein kinase B (Akt) (Ser473) (no. 9271), extracellular signal-regulated kinase (ERK)1/2 (Thr202/204) (no. 9101), eEF2 (Thr56) (no. 2331), p38 (Thr180/182) (no. 9211), and Rp-s6k (Thr389) (no. 9430) (Cell Signaling Technology, Danvers, MA). Finally, the membranes were incubated in secondary horseradish peroxidase (HRP) antibody (PO448; Dako, Carpinteria, CA) and visualized by Immobilon Western Chemiluminescent HRP Substrate (Millipore), and images were captured using a Kodak Image Station (2000MM). Band intensities were quantified by imaging software (1D 3.5; Kodak). Preliminary experiments demonstrated that the amounts of protein loaded were within the dynamic range for the conditions used and the results obtained (data not shown). Due to the fact that several studies have shown unchanged absolute amounts of the target molecules acutely following exercise, nutritional interventions, and repeated biopsies (25, 27, 43, 58) and that we had limited muscle lysate-volume, the phosphorylation status is presented as the absolute phosphospecific band intensity in arbitrary units. Representative blots are shown in Fig. 2.

**Statistics**

The fasted FSR and phosphorylation status results following LL and HL contractions are compared by a two-way ANOVA with repeated measures. The feeding effect on FSR and molecular signaling was evaluated separately within each contraction intensity by comparing resting and individual postexercise values in the fasted and fed states using a two-way ANOVA. When significant main effects appeared, post hoc Holm-Sidak tests were completed to compare across interventions. Only selected post hoc comparisons were made; values within each of the two contraction intensities were compared with rest, and between the two contraction intensities the two values were compared only at each time point. The KIC enrichment results in the three time periods obtained during fasting and feeding and the leucine and phenylalanine concentrations were tested by a two-way ANOVA with repeated measures applying a Holm-Sidak post hoc test when significant effect appeared. Finally, the insulin areas under the concentration curve (AUC) during the fasted and fed trials were calculated for each subject. Each fasted and fed state individual AUC was normalized to the fasted state mean AUC to determine a degree of change in insulin availability. The degree of change data were compared by an unpaired t-test. A P level <0.05 was considered significant, whereas, when 0.10 > P > 0.05, a tendency is discussed. All analyses were completed in SigmaStat 3.5 (Systat Software, San Jose, CA).

**RESULTS**

**Subject Characteristics**

The two groups of sedentary, young, healthy males who were included were similar in age: 25.6 ± 1.4 yr, body mass index: 23.6 ± 0.8 and 24.4 ± 1.7 kg/m², and IRM strength: 77.4 ± 5.1 and 74.3 ± 2.4 kg, fasted and fed groups, respectively (all P > 0.10).

**Table**

<table>
<thead>
<tr>
<th>Target and phosphorylation site</th>
<th>Fasted state</th>
<th>Fed state</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Light load</td>
<td>Heavy load</td>
</tr>
<tr>
<td>Biopsy times [hr:min]</td>
<td>0:30</td>
<td>5:30</td>
</tr>
<tr>
<td>Rp-s6k-Thr389</td>
<td>REST</td>
<td>REST</td>
</tr>
<tr>
<td>4E-BP1-Thr37/46</td>
<td>REST</td>
<td>REST</td>
</tr>
<tr>
<td>Akt-Thr473</td>
<td>REST</td>
<td>REST</td>
</tr>
<tr>
<td>eEF2-Thr56</td>
<td>REST</td>
<td>REST</td>
</tr>
<tr>
<td>ERK1/2-Thr202/204</td>
<td>REST</td>
<td>REST</td>
</tr>
<tr>
<td>p38-Thr180/182</td>
<td>REST</td>
<td>REST</td>
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<tr>
<td>AMPK-Thr172</td>
<td>REST</td>
<td>REST</td>
</tr>
<tr>
<td>ACC-Ser221</td>
<td>REST</td>
<td>REST</td>
</tr>
</tbody>
</table>

Fig. 2. Examples of representative Western blots for all signaling proteins measured, in all conditions, in this study. All bands for a particular antibody were obtained from a single blot.
Table 1. Plasma $^{13}$C/KIC atom percent excess enrichment

<table>
<thead>
<tr>
<th>FSR Period</th>
<th>Rest</th>
<th>Early</th>
<th>Late</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fasted state</td>
<td>4.24±0.16</td>
<td>5.00±0.13</td>
<td>5.17±0.13</td>
</tr>
<tr>
<td>Fed state</td>
<td>3.84±0.12</td>
<td>4.24±0.13</td>
<td>4.17±0.10</td>
</tr>
</tbody>
</table>

Values are means ± SE. Units are %. FSR, fractional synthesis rate; KIC, $^{13}$C-ketoisocarpoic acid. Significant effects of time and feeding were apparent from a two-way ANOVA.

Precursor Enrichment

Table 1 shows the $^{13}$C/KIC enrichments within each time period in which an FSR value is calculated, i.e., at rest and at early (0.5–3 h) and late (3–5.5 h) exercise recovery. Within each of the time periods (rest, early, and late), we applied the mean enrichment of $^{13}$C/KIC to the equation of FSR. We observed a slight increase in plasma $^{13}$C/KIC enrichment ($P < 0.05$) throughout both the fasted and fed trials and saw, as expected, a lesser enrichment ($P < 0.05$) during fed trials compared with the fasted trials, presumably because of absorption of unlabeled leucine in the bloodstream originating from the ingested proteins.

Leucine, Phenylalanine, and Insulin Concentration

Circulating leucine and phenylalanine concentrations are shown in Table 2. For phenylalanine, an interaction between feeding and time was found ($P < 0.05$), revealing a constant concentration throughout the fasted trials and an elevated concentration in the fed state. Also, a significant effect of feeding and time on the leucine concentrations was found ($P < 0.05$). Insulin concentrations at pretrial and through out the trials are shown in Table 3. In the fasted trials, insulin concentrations did not change from the overnight fasted, pretrial level. The basal level in the fed group was not different from the fasted group ($P > 0.05$), but the intermittent feeding increased insulin concentrations 2.1 ± 0.3-fold above resting and fasting levels ($P < 0.05$).

Intramuscular Total Collagen Protein Synthesis Rate

There was a clear effect of prior exercise on skeletal muscle collagen FSR whether studied in the fasted or fed state ($P < 0.05$, Fig. 3). These changes in collagen FSR were unaffected by contractile intensity ($P > 0.10$). Feeding did not increase resting nor postexercise collagen protein synthesis rate and nor was the postexercise temporal response different compared with fasting [fasted state: 1.7 ± 0.3 to 2.1 ± 0.2-fold from the resting level at 0.08 ± 0.01%/h; fed state: 1.5 ± 0.3 to 2.4 ± 0.5-fold from the resting level at 0.06 ± 0.01%/h (Fig. 3)].

Myofibrillar Protein Synthesis Rate

Fasting myofibrillar protein FSR was influenced by the contraction intensity of a prior exercise bout (interaction: $P < 0.05$, Fig. 4A). Myofibrillar FSR was 0.08 ± 0.01%/h at rest, and LL contractions were not sufficient to enhance the myofibrillar FSR level significantly above that level (early: 0.11 ± 0.01 and late: 0.09 ± 0.02%/h; not significant). In contrast, HL contractions resulted in a delayed improvement (late: 0.14 ± 0.02%/h, 2.0 ± 0.4-fold, $P < 0.05$). Oral feeding elevated myofibrillar protein FSR at rest 2.3 ± 0.3-fold up to 0.18 ± 0.03%/h ($P < 0.05$; Fig. 4B), and this elevated level was maintained at all postexercise time points irrespective of prior contraction intensity. When food was provided, LL contractions kept the myofibrillar protein FSR elevated above fasting conditions at the late time point ($P < 0.05$, Fig. 4B).

Molecular signaling. Representative blots are shown in Fig. 2 for all targets, in all conditions. For more details, see MATERIALS AND METHODS.

Rp-s6k and 4E-BP1

In the fasted state, the phosphorylation of Rp-s6k-Thr389 revealed a tendency toward an interaction between time and contraction intensity ($P = 0.06$), which was due to a delayed rise in the phosphorylation level at 3 and 5.5 h following HL (Fig. 5A). This was matched with a similar interaction ($P < 0.01$), i.e., a delayed increase, in 4E-BP1-Thr37/46 following HL (Fig. 5B). Feeding increased Rp-s6k phosphorylation at rest and LL contractions on top elevated the phosphorylation

Table 2. Plasma phenylalanine and leucine concentrations pretrial and in subsequent blood samples drawn throughout the trial

<table>
<thead>
<tr>
<th>Phenylalanine, µM</th>
<th>Pretrial</th>
<th>−2:45</th>
<th>−0:55</th>
<th>0:25</th>
<th>2:50</th>
<th>5:20</th>
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</thead>
<tbody>
<tr>
<td>Fasted state</td>
<td>115±11</td>
<td>138±27</td>
<td>116±18</td>
<td>116±15</td>
<td>105±14</td>
<td>102±12</td>
</tr>
<tr>
<td>Fed state</td>
<td>126±16</td>
<td>194±26</td>
<td>170±20</td>
<td>171±26</td>
<td>161±23</td>
<td>165±20</td>
</tr>
<tr>
<td>Leucine, µM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasted state</td>
<td>193±7</td>
<td>188±10</td>
<td>184±27</td>
<td>165±26</td>
<td>174±25</td>
<td>194±4</td>
</tr>
<tr>
<td>Fed state</td>
<td>202±9</td>
<td>222±16</td>
<td>197±28</td>
<td>199±10</td>
<td>195±12</td>
<td>220±21</td>
</tr>
</tbody>
</table>

Values are mean ± SE. Pretrial, before tracer priming and infusion and before subjects were given the first nutritional drink in the fed trials. Subsequent blood samples were drawn throughout the trial and are presented as h:min, with 0:00 corresponding to immediately after completion of exercise. A significant interaction effect was observed for phenylalanine concentrations, whereas for leucine a time as well as a feeding effect was apparent.
Fig. 3. Intramuscular total collagen protein fractional synthesis rate (FSR) in the fasted state (A) and the fed state (B). +P < 0.05 compared with resting value. A: effect of exercise was found for the fasted state intramuscular total collagen protein FSR (P < 0.01). B: in the fed state, the exercise effect remained (P < 0.05). The total collagen protein FSR was not affected by feeding; thus, no differences appeared from the corresponding fasted state levels (P > 0.05). Data are means ± SE.

Fig. 4. Myofibrillar protein FSR in the fasted state (A) and the fed state (B). A: interaction appeared for the fasted state myofibrillar protein FSR (P < 0.05) revealing a divergent response following LL and HL resistance exercises. B: statistical even myofibrillar protein FSR throughout all conditions was seen in the fed state. The level was elevated from fasted values at rest (P < 0.05), and following light (P < 0.05) and heavy loading (P < 0.10) in the late recovery period, whereas at the early recovery neither LL (P = 0.33) nor HL (P = 0.17) reached statistical significance. Data are means ± SE. *P = 0.10 and **P < 0.05, LL vs. HL responses at the same time point. +P < 0.05 compared with resting value. ^P < 0.10 and §P < 0.05, fasted vs. fed state values.

immediately after exercise (feeding, P < 0.01; Fig. 6A). When HL was added to feeding, Rp-s6k phosphorylation rose markedly in the whole postexercise period (feeding, P < 0.001; exercise, P < 0.05; Fig. 6B). Feeding did not affect 4E-BP1-Thr37/46 phosphorylation neither at rest nor when exercise was completed.

**Akt and eEF2**

Akt-Ser473 phosphorylation was elevated in response to exercise (exercise, P < 0.001; Fig. 5C), and contraction intensity tended to have a positive effect (contraction intensity, P = 0.06; Fig. 5C). In contrast, we could not find any effect of feeding on Akt-Ser473 phosphorylation (feeding, P > 0.10; Fig. 6, E and F). Only exercise remained to exert an effect (P < 0.001). eEF2-Thr56 phosphorylation was unaffected by exercise at the time points assessed in the present study (Fig. 5D), whereas significant overall effects of feeding were apparent (feeding, P < 0.05 and P < 0.01 following LL and HL contractions, respectively; Fig. 6, G and H).

**ERK1/2 and p38**

Both for ERK1/2 and p38 the phosphorylation revealed a marked dependency of exercise and contraction intensity (exercise, P < 0.01; contraction intensity, P < 0.05; Fig. 5, E and F), being more phosphorylated following HL than LL contractions. Feeding did not affect the mitogen-activated protein kinase (MAPk) phosphorylation markedly (Fig. 7, A and B, and Fig. 7, C and D), although an interaction (P < 0.05) appeared for ERK1/2 following HL contractions because of a faster drop at 3 h.

**AMPk and ACC**

The phosphorylation status of these targets only exists from the fasted state. AMPk-Thr172 was only increased by exercise, whereas contraction intensity did not have any impact (exercise, P < 0.001; Fig. 8A), although the post hoc tests revealed that HL contractions induced a higher phosphorylation status at 3 h (P < 0.05) and tended at 5.5 h (P < 0.10). The phosphorylation status of the direct read out of AMPk activity, ACC-Ser21, revealed a significant interaction between exercise and contraction intensity (interaction, P < 0.05; Fig. 8B) that appeared as a marked elevation immediately following HL contractions only.

**DISCUSSION**

The present paper investigated how contraction intensity and feeding separately and in combination affected the FSR of the contractile and intramuscular collagen protein fractions and related molecular signaling in human skeletal muscle. An important finding was that, in the fasted state, the postexercise collagen protein FSR rose irrespective of the contractile intensity of the prior exercise. This response was quite different from the response of the myofibrillar proteins, where the synthesis rate increased significantly following heavy contractions only. Furthermore, we found that, during intermittent feeding, the intramuscular total collagen protein FSR was not responsive to improved availability of nutrients after contractile work of any intensity, which also was very distinct from the pronounced myofibrillar protein FSR responsiveness to feeding. We showed that, in the fasted state, the mTOR complex 1-dependent Rp-s6k-4E-BP1 pathway and the MAPk was phosphorylated dependent on the contraction intensity of the previous exercise, whereas, when feeding was provided, Rp-s6k and eEF2 phosphorylation levels were markedly affected in a way that would enhance protein synthesis rate.

**Intramuscular Collagen Protein Synthesis Rate**

We showed here that exercise irrespective of contraction intensity enhanced the muscle collagen protein FSR by twofold (Fig. 3A). Although the stimulating effect of...
exercise on muscle collagen FSR has previously been shown (5, 23, 47, 68, 70, 71), only the effect of contraction type (lengthening vs. shortening contractions) but not intensity has been investigated earlier. Contraction types induced slightly divergent improvements in the FSR of the mature human muscle collagen protein in the first hours after exercise (23, 71). In contrast, studies of fibroblasts have revealed that collagen protein synthesis is affected in an “either/or” fashion independent of the intensity of the mechanical stimuli. Findings in rat Achilles tendons exposed to eccentric or concentric contractions via stimulation of the sciatic nerve during passive movement of the lower leg revealed an even gene regulation of collagen mRNA suggesting an insensitivity toward differences between tensile stress (51). This is supported by the gene expression of signaling molecules in fibroblasts following mechanical stress (19, 20).

We could not assess any effect of feeding on resting muscle collagen FSR (Fig. 3B), which was in accordance with one previous report administering amino acids (5). In addition, we investigated whether exercise made the collagen synthesis rate sensitive to elevated nutrient availability, which it did not (Fig. 3B). To our knowledge, this is the first study to investigate the interplay between exercise and nutrients on collagen FSR, and the finding supports the view that intramuscular collagen synthesis rate is basically insensitive to nutrient availability and suggests, as was the case for contractile intensity, that the connective tissue collagen network is differently regulated than the contractile structure in response to nutrients.

In relation to the muscle collagen synthesis measurement, it is relevant to address that we measured the synthesis rate of the total muscle collagen pool similar to methodologies used for other collagenous tissues (5, 25, 46, 47, 69, 70). For reasons that are unknown to us, the pool of matured and cross-linked collagen proteins has been isolated for determination of the muscle collagen protein FSR (5, 23, 47, 68, 70, 71), despite the fact that the turnover rate is dependent on the maturation stage (4, 6). The present data suggest that muscle collagen is synthesized at least as fast as collagen in tendon, ligament, and bone (5, 46, 47, 69, 70).

Myofibrillar Protein Synthesis Rate

Exercise contraction intensity is decisive for the FSR response of myofibrillar proteins. LL contractions were not sufficient to induce any significant changes in myofibrillar protein FSR (Fig. 4A), which was in accordance with two other reports measuring myofibrillar protein FSR following LL resistance exercise (36, 61) or 45 min cycling exercise at 75% \( \dot{V}O_2 \text{peak} \) (101). Although earlier studies reported an elevated effect of walking at 40% \( \dot{V}O_2 \text{max} \) on muscle protein turnover using protein unspecific measures (18, 89), it is more likely to be because of an increase in the turnover of, e.g., the mitochondrial proteins (101). As expected, heavy load contraction intensity induced a robust twofold improvement in myofibrillar protein FSR (Fig. 4A), which is in agreement with previous findings (71, 73, 101). However, we found that the improvement was delayed and thus absent during the immediate 3-h period after exercise (Fig. 4A). Some previous data have reported elevations in postexercise mixed muscle FSR (27, 78, 79) and myofibrillar protein FSR measurements (71, 73, 101) within the early time period. Other studies, however, lend support to the fact that elevations in muscle synthesis following

![Fig. 5. Phosphorylation status in the fasting condition at rest and 0:30, 3:00, and 5:30 (h:min) after end of exercise following LL and HL exercise. A: Rp-s6k-Thr389. B: 4E-binding protein 1 (BP1)-Thr37/46. C: protein kinase B (Akt)-Ser473. D: eukaryotic elongation factor (eEF) 2-Thr56. E: extracellular signal-regulated kinase (ERK)1/2-Thr202/204. F: p38-Thr180/182. P < 0.05, LL vs. HL responses at the same time point # and vs. resting value +. Rest is the pooled mean of two biopsies that demonstrated even phosphorylation status. Light gray bar, values following LL contractions; dark gray bars, following HL contractions. 0:30, 3:00, and 5:30 refer to the time point h:min after end of exercise. Data are means ± SE.](https://ajpendo.physiology.org/doi/10.1152/ajpendo.00570.2010)
strenuous exercise are delayed in the recovery (81, 88, 96), which is a phenomenon also shown in rodents (42). Recently, Kumar et al. (61) reported an improvement following the heavy loading resistance exercises similar to our protocol already in the second hour after exercise. However, when compared with the present study, there were marked differences in total exercise volume of this study (61); therefore, it can be speculated whether exercise volume may be a determinant for not only the duration of the FSR response following exercise but also when it peaks. Despite some diversity in the results about exactly when in the exercise recovery period the turnover rate is elevated, no doubt exists that heavy resistance exercise enhances the synthesis rate markedly of the contractile proteins (60). Although matched for total lifted load, LL contractions did not reveal any significant change in myofibrillar FSR. Therefore, we suggest that contractile intensity determines the

Fig. 6. Fasted (open bars) and fed (hatched bars) state phosphorylation status at rest and 0:30, 3:00, and 5:30 (h:min) after end of exercise following LL and HL exercise. Rp-s6k-Thr389 (A and B), 4E-BP1-Thr37/46 (C and D), Akt-Ser473 (E and F), eEF2-Thr56 (G and H) phosphorylation status. *P < 0.10 and #P < 0.05, fasted vs. fed state responses at the same time point. +P < 0.05 vs. resting value in the same nutritive condition. Rest is the pooled mean of two biopsies that demonstrated even phosphorylation status in the fasted and fed states, respectively. Light gray bars, values following LL contractions; dark gray bars, values following HL contractions. Data are means ± SE.

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relative change in synthesis rate of the myofibrillar proteins, whereas the volume may be decisive for when the increase appears and how prolonged it is.

We verified the potency of feeding to improve the resting synthesis rates of myofibrillar protein (10, 24, 44) and mixed muscle protein (37, 38, 74). Also following exercise, nutrients and especially amino acids enhance muscle protein turnover markedly (93, 105), and, when applied in long-term training studies, the nutritional supplementation and timing are important determinants for the accretion of muscle mass (48, 54, 102). In the present setup, LL contractions did not improve the myofibrillar FSR above the nutrient-induced level achieved at rest. However, according to earlier studies in the resting state, the feeding-induced stimulation of myofibrillar FSR is abolished after a few hours (11, 73). We showed that LL contractions prolonged the feeding-induced elevation of myofibrillar protein FSR for up to 5.5 h after exercise (Fig. 4). This ability of LL contractions may be the mechanism leading to our earlier reported muscle hypertrophy following 12 wk of LL resistance training (55). Adding HL contractions on top of the repetitive feeding did not reveal any further increase in myofibrillar FSR compared with the feeding effect in the resting condition (Fig. 4B). However, comparing the fed and fasted state postexercise FSR levels, it becomes apparent that the effect of heavy loading was delayed in the fed conditions as well. As seen sometimes during fasting conditions and reported once except from this study when fed continuously (23) and also following a single bolus intake dependent on its timing (38, 96), the postexercise increase in muscle protein synthesis can be delayed. Thus controversy exists as to why an immediate intake of protein and nutrient supplementation is of major importance for the overall protein accretion in response to training (1, 33, 54). It may be speculated that an early intake initiates processes that, despite their immediate inhibition, extend the period where the exercise is anabolic. Thus these data suggest that the contraction-induced and nutrition-induced mechanisms responsible for regulating muscle protein synthesis rates diverge and act independent of one another.

Contraction-Induced Molecular Signaling

Maximal contractile efforts have been shown to be a prerequisite for obtaining marked phosphorylation of the Rp-s6k-Thr\(^{389}\) in the fasted state (31), although some submaximal interventions have succeeded in finding smaller changes (27, 61, 64). Thus the trend toward an increased phosphorylation of

![Image](http://ajpendo.physiology.org/)

Fig. 7. Image of the fasted (open bars) and fed (hatched bars) state phosphorylation status at rest and 0:30, 3:00, and 5:30 (h:min) after end of exercise following LL and HL exercise. ERK 1/2-Thr\(^{202/204}\) (A and B) and p38-Thr\(^{180/182}\) (C and D) phosphorylation status. \(\ast P < 0.05\), fasted vs. fed state responses at the same time point. \(+ P < 0.05\) vs. resting value in the same nutritive condition. Rest is the pooled mean of two biopsies that demonstrated even phosphorylation status in the fasted and fed states, respectively. Light gray bars, values following LL contractions; dark gray bars, values following HL contractions. Data are means \(\pm\) SE.

![Image](http://ajpendo.physiology.org/)

Fig. 8. Phosphorylation status in the fasting condition at rest and 0:30, 3:00, and 5:30 (h:min) after the end of exercise following LL and HL exercise. AMP-activated protein kinase (AMPk)-Thr\(^{172}\) and acetyl-CoA carboxylase (ACC)-Ser\(^{221}\) phosphorylation status in fasting conditions. \(\ast P < 0.05\), LL vs. HL responses at the same time point. \(+ P < 0.10\) and \(\ast\# P < 0.05\) vs. resting value. Rest is the pooled mean of two biopsies that demonstrated even phosphorylation status. Light gray bars, values following LL contractions; dark gray bars, values following HL contractions. Data are means \(\pm\) SE.
Rp-s6k-Thr\textsuperscript{389} following heavy loading in the fasting state (Fig. 5A) is in line with the literature, and we believe that it should be interpreted as a physiologically relevant finding. This is supported by the corresponding delayed phosphorylation of one of its targets, the 4E-BP1-Thr\textsuperscript{37/46} (Fig. 5B). This finding is in line with the recent findings by Kumar and coworkers (61), who applied a similar resistance exercise protocol. Known to be a direct read out of the rapamycin-sensitive mTOR complex 1, which recently was demonstrated to be inhibited by rapamycin along with the postexercise increase in muscle protein synthesis (30), the present crossover design verified that the Rp-s6k-4E-BP1 pathway is likely to be part of the contraction intensity sensitive regulator of muscle myofibrillar protein synthesis (16, 29, 30).

The MAPk are activated by cellular stress in the form of exercise (2, 49, 52, 83, 98–100, 106). The ERK has been proposed to be sensitive to exercise intensity (83, 100), whereas the literature suggests a certain stress threshold for p38 activation (103), an observation that is supported by a lesser p38 response to accommodated exercise type/intensity (21, 107). We showed that both ERK and p38 were only significantly phosphorylated above basal conditions following the HL contractions (Fig. 5, E and F). In line with the lack of ERK phosphorylation following rapamycin treatment, which abolished the exercise-induced increase in muscle protein synthesis rate (30), these data together strongly point toward the fact that ERK and p38 are involved in the regulation of contraction-induced elevations in myofibrillar protein synthesis. In addition, it should be stated that, since the present exercise intensity protocols were equalized for total lifted load, the observed differences in MAPk phosphorylation suggest that contraction intensity may be a stronger stimulus for cellular stress than completed work.

Although the peak AMPk phosphorylation was numerically even at 30 min after light and heavy loading, it remained slightly elevated over the whole postexercise period following heavy loading only. Additionally, as a read out of AMPk activity, the ACC phosphorylation status supports the fact that AMPk activity may have been higher immediately following the HL than following the LL intensity (Fig. 8). Strenuous resistive exercise types are known to exert a metabolic stress in myocytes that induces an elevated AMPk phosphorylation (101) and activity (27). In addition, the present findings are in accordance with recent data from Rose et al. (85) who found that enhanced exercise intensity and contractile strain induced a higher AMPk phosphorylation. Thus, when completed as repeated sets within the same exercise bout, high-intensity contractions seem to be stronger inducers of AMPk activity than contractions performed with lower intensities. This relation coincides with AMPk as a candidate to repress translation-enhancing signaling and thus protein synthesis (27, 82, 92).

Feeding and Exercise Signaling

We found a marked effect of feeding on Rp-s6k-Thr\textsuperscript{389} phosphorylation and a maintained elevated phosphorylation early after exercise, which is in accordance with previous reports (26, 28, 35, 37), although exceptions exist (72). Here we show for the first time that feeding interacts with contractile intensity to activate the Rp-s6k. HL contraction intensity maintained the elevated phosphorylation until 5.5 h after exercise, whereas, after LL contraction intensity, the Rp-s6k-Thr\textsuperscript{389} phosphorylation had returned to the basal level already at 3 h after exercise (Fig. 6, A and B). Our data revealed a similar phosphorylation status of 4E-BP1-Thr\textsuperscript{37/46} in the fed state as was found in the fasted state, which is in contrast to earlier reports (26, 41). In those studies, however, the feeding was provided as one big bolus in contrast to ours, which was a smaller but repeated bolus feeding. Despite 4E-BP1 being shown to be phosphorylated equally at three different insulin levels (45), it is likely that the provision of amino acids may affect the 4E-BP1 signaling (32). Similarly, we could not detect any elevated phosphorylation by feeding (Fig. 6, E and F), although Akt-Ser\textsuperscript{473} by any means is known to be highly sensitive to feeding (35, 56, 90). Recently, it was demonstrated that a threshold of insulin/amino acid availability has to be obtained for Akt to be phosphorylated (44, 45), which may not have been obtained by the intermittent feeding regimen (Tables 2 and 3). Therefore, we ascribe the small-bolus feeding protocol applied in the present study to be the main reason for the absence of enhanced Akt and 4E-BP1 phosphorylation. In addition, we found 4E-BP1 phosphorylation in the fed state to follow the same temporal pattern as seen in the fasted state, which implies a maintained contraction-dependent responsiveness despite insufficient nutrient availability.

Although we did not assess any significant effect of exercise on eEF2 phosphorylation, it may be likely, especially after the heavy loading protocol (Fig. 5D), that eEF2 may have been more phosphorylated immediately after cessation of exercise (84, 86), thereby braking the protein synthetic processes during exercise as shown by others (27). Alternatively, we saw a diminished phosphorylation of eEF2 in the fed trials compared with the fasted trials (Fig. 6, G and H). It is now known that hyperinsulinemia (34) and single-bolus feeding (37) have been shown to decrease skeletal muscle eEF2-Thr\textsuperscript{36} phosphorylation. Again, the lack of significant post hoc tests at single time points between fed and fasted eEF2 phosphorylation levels may just underline the insufficiency of the repeated small-bolus feeding regime as mentioned for the Akt and 4E-BP1 targets above. The present data supported by other results suggest an interaction between feeding and exercise on eEF2 phosphorylation, although this needs to be investigated directly.

With relevance mainly for clinical settings during rehabilitation and immobilization/bed rest periods, the present study has two major outcomes: first, even light contractile activity with adequate nutritional intake seems to be adequate to maintain muscle mass, which is supported by our earlier long-term training study (55); and second, muscle connective tissue collagen protein synthesis rate is elevated markedly by light contractile intensities, and, accordingly we showed earlier that patella tendon cross-sectional area improved significantly following the light loading knee extensor protocol (59). These findings suggest a robust effect of tolerable low-intensity contraction intensities on the recovery of collagen structures, making such recommendable at an early time point following, e.g., surgery and as conservative rehabilitation to improve regeneration of connective tissue.

In conclusion, the regulation of the protein turnover of contractile and supportive connective tissue structures varies markedly despite their interaction as a functional entity. The present data imply that fibroblasts in skeletal muscle mainly improve turnover of the connective tissue network by gross...
mechanical stress in an “either-or” manner providing a new set point after exercise somewhat independent of its intensity and insensitive to nutrient availability. In contrast, changes in myofibrillar protein synthesis rate are dependent on the contractile intensity of the exercises, revealing only an improvement following heavy loads. In accordance, only when HL contractions are conducted, the MAPK and mTOR complex 1-dependent pathways are activated. Although ERK1/2 and p38 respond to exercise intensity, the Rp-s6k-4E-BP1 pathway reflects the actual changes in myofibrillar protein synthesis. Feeding and exercise interacted to elevate myofibrillar protein synthesis rate via eEF2 and Rp-s6k activity that was adjusted slightly by contractile intensity, whereas 4E-BP1 remained to be solely dependent on contractile intensity.

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