Blood Flow Restriction Only Increases Myofibrillar Protein Synthesis with Exercise

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ABSTRACT

NYAKAYIRU, J., C. J. FUCHS, J. TROMMELEN, J. S. J. SMEETS, J. M. SENDEN, A. P. GIJSEN, A. H. ZORENC, L. J. C. VAN LOON, and L. B.VERDIJK. Blood Flow Restriction Only Increases Myofibrillar Protein Synthesis with Exercise. *Med. Sci. Sports Exerc.*, Vol. 51, No. 6, pp. 1137–1145, 2019. **Purpose:** Combining blood flow restriction (BFR) with exercise can stimulate skeletal muscle hypertrophy. Recent observations in an animal model suggest that BFR performed without exercise can also induce anabolic effects. We assessed the effect of BFR performed both with and without low-load resistance-type exercise (LLRE) on *in vivo* myofibrillar protein synthesis rates in young men. **Methods:** Twenty healthy young men (age = 24 ± 1 yr, body mass index = 22.9 ± 0.6 kg·m^{-2}) were randomly assigned to remain in resting condition (REST ± BFR; *n* = 10) or to perform LLRE (LLRE ± BFR at 20% one-repetition maximum; *n* = 10), combined with two 5-min cycles of single leg BFR. Myofibrillar protein synthesis rates were assessed during a 5-h post-BFR period by combining a primed continuous L-[ring-^{13}C$_6$]phenylalanine infusion with the collection of blood samples, and muscle biopsies from the BFR leg and the contralateral control leg. The phosphorylation status of anabolic signaling (mammalian target of rapamycin pathway) and metabolic stress (acetyl-CoA carboxylase)–related proteins, as well as the mRNA expression of genes associated with skeletal muscle mass regulation, was assessed in the collected muscle samples. **Results:** Under resting conditions, no differences in anabolic signaling or myofibrillar protein synthesis rates were observed between REST + BFR and REST (0.044% ± 0.004% vs 0.043% ± 0.004% per hour, respectively; *P* = 0.683). By contrast, LLRE + BFR increased myofibrillar protein synthesis rates by 10% ± 5% compared with LLRE (0.048% ± 0.005% vs 0.043% ± 0.004% per hour, respectively; *P* = 0.042). Furthermore, compared with LLRE, LLRE + BFR showed higher phosphorylation status of acetyl-CoA carboxylase and 4E-BP1 as well as the elevated mRNA expression of MuRF1 (all *P* < 0.05). **Conclusion:** BFR does not increase myofibrillar protein synthesis rates in healthy young men under resting conditions. When combined with LLRE, BFR increases postexercise myofibrillar protein synthesis rates *in vivo* in humans. **Key Words:** SKELETAL MUSCLE, HYPERTROPHY, ANABOLIC SIGNALING, STABLE ISOTOPES, GENE EXPRESSION

Muscle disuse due to reduced physical activity, immobilization, or bed rest has been shown to result in substantial decreases in muscle mass and strength (1,2). These catabolic changes in skeletal muscle tissue have in turn been associated with functional disabilities and an increased risk of developing (chronic) metabolic impairments (3). A recent study from our laboratory, for example, showed that 7 d of bed rest decreases quadriceps muscle cross-sectional area by 3.2% and whole-body insulin sensitivity by 29% in healthy young men (4). Such disuse-induced muscle loss and subsequent metabolic dysfunction underscore the need for effective interventional strategies to counteract these detrimental effects.

High-load resistance-type exercise is a strong anabolic stimulus that can increase skeletal muscle protein synthesis rates (5,6) and augment muscle mass and strength when performed as a training program (7). High-load resistance-type exercise training has also been shown effective in counteracting disuse-induced loss of skeletal muscle mass (8). However, performing such demanding exercise might not be feasible for

certain (clinical) populations (e.g., rehabilitating athletes, elective surgery patients) who are at risk of losing substantial amounts of muscle mass due to disuse (9).

Recent work suggests that combining low-load resistance-type exercise (LLRE) with blood flow restriction (BFR) represents an effective anabolic stimulus (10–15). Although limited, the available literature suggests that combining LLRE with BFR can increase mixed-muscle protein synthesis rates in healthy young and older participants (12,13,15). Furthermore, when applied as a prolonged exercise training intervention, combining LLRE with BFR has also been shown to stimulate skeletal muscle hypertrophy to a similar extent as traditional high-load resistance-type exercise (10,11,16,17).

Interestingly, recent observations in rodents suggest that performing BFR without the addition of LLRE can also induce anabolic effects (18). More specifically, Wistar rats subjected to repeated cycles of BFR under resting conditions showed an acute increase in skeletal muscle p70S6–kinase phosphorylation, a downstream target of the mammalian target of rapamycin (mTOR) pathway associated with protein synthesis (18). In line with this, another study in rodents showed an increase in skeletal muscle fiber size after 6 wk of repetitive cycles of BFR performed without concomitant exercise training (19). These observations suggest that the application of BFR under resting conditions stimulates skeletal muscle hypertrophy, but evidence for this in humans is not yet available. In the current study, we assessed the effects of BFR with and without LLRE on myofibrillar protein synthesis rates *in vivo* in humans.

METHODS

Subjects. Twenty young, healthy male subjects (age = 24 ± 1 yr, weight = 72 ± 2 kg, body mass index = 22.9 ± 0.6 kg·m^{-2}) participated in this randomized controlled study. The participants were recreationally active and exercised no more than 3 d·wk^{-1}, with resistance-type exercise being performed no more than 1 d·wk^{-1}. All subjects were informed about the experimental procedures and possible risks of participation before signing an informed consent. The study was approved by the Medical Ethical Committee of the Maastricht University Medical Centre+, The Netherlands, and was registered at the Nederlandse Trial Register (NTR5914). All procedures were conducted in accordance with the standards stated in the most recent version of the Declaration of Helsinki.

Study design. This study was a randomized controlled trial in which subjects were randomly allocated to the group that performed LLRE or the group that remained in resting conditions (REST). Subsequently, within each group separately, we used a within-subject unilateral-leg design, where one leg was randomly subjected to two 5-min cycles of BFR, while the contralateral leg served as the within-subject non-BFR control leg. Myofibrillar protein synthesis rates were assessed during a 5-h post-BFR period by combining a primed continuous L-[ring-^{13}C$_6$]phenylalanine infusion with the collection of blood samples from a dorsal hand vein catheter, and muscle biopsies from both the blood flow restricted and the control leg in each participant to assess the effect of BFR when combined with LLRE as well as under resting conditions.

Pretesting. Before being included in the study, each subject first completed a screening session (≥5 d before test day) that consisted of assessing health status through a medical questionnaire and measurements of weight and height. Eligible subjects were then randomized to either the LLRE group ($n = 10$) or the REST group ($n = 10$). Participants randomized to the LLRE group were familiarized with the leg press and leg extension machines (Technogym, Rotterdam, The Netherlands), and their one-repetition maximum (1RM) was estimated using the multiple repetitions testing procedure (20). The 1RM testing was preceded by a short warm-up set of 15 submaximal repetitions, followed by a maximum of 5 sets of exercise at progressively increasing loads until failure. As a result of the unilateral design of the study (one blood flow restricted leg and the other leg as control), the 1RM of each leg was determined separately. The 1RM was used to calculate the 20% 1RM load required for the leg press and leg extension exercise performed during LLRE on test days, similar to previous studies (12,13,16,21).

Standardization of physical activity and diet. All participants were instructed to refrain from any sort of strenuous physical activity in the 48 h before the test day and to avoid consumption of caffeine and alcohol in the 12 and 24 h preceding the test day, respectively. They were also instructed to consume a standardized dinner the day before the test. The standardized meal had the same composition for all subjects (62 ± 2 kJ·kg^{-1} body weight, providing 37 energy% [En%] carbohydrate, 36 En% fat, and 27 En% protein). The standardized dinner was the last meal the subjects consumed before 10:00 PM the day before the test day. Thereafter, subjects remained fasted until the end of the test day, but they were allowed *ad libitum* consumption of water.

Experimental protocol. Subjects reported to the laboratory by car or public transport at 08:00 AM on the test day after an overnight fast. The experimental protocol is depicted in Figure 1. The test day started by the placement of a catheter into an antecubital vein for the stable-isotope amino acid infusion and a second catheter in a dorsal hand vein of the contralateral arm for arterialized blood draws. To allow sampling of arterialized blood, the hand was first placed in a hot box (60°C) for 10 min before drawing blood. After collection of a basal blood sample, the plasma phenylalanine pool was primed with a single dose of L-[ring-^{13}C$_6$]phenylalanine (2.25 μmol·kg^{-1}), after which a continuous L-[ring-^{13}C$_6$]phenylalanine (0.05 μmol·kg^{-1}·min^{-1}) intravenous infusion was initiated, lasting until the end of the test day ($t = -150$ min until $t = 300$ min). Subjects rested in a supine position for another 120 min, whereas the second and the third arterialized blood samples were collected 60 min ($t = -90$ min) and 120 min ($t = -30$ min) into the stable-isotope infusion period, respectively. Subjects then received BFR on one leg for 2 × 5 min. The BFR approach applied (with respect to cuff size and absolute pressure) resembled that of Gundermann et al. (14), who observed a 49% increase in mixed-muscle
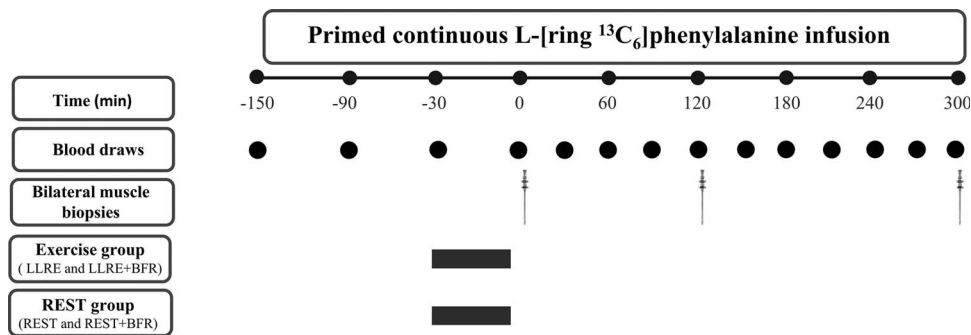
Primed continuous L-[ring $^{13}C_6$]phenylalanine infusion

| Time (min) | -150 | -90 | -30 | 0 | 60 | 120 | 180 | 240 | 300 |

Blood draws

Bilateral muscle biopsies

Exercise group (LLRE and LLRE+BFR)

REST group (REST and REST+BFR)

FIGURE 1—Schematic representation of the experimental protocol. The exercise group performed LLRE (LLRE leg vs LLRE + BFR leg; $n = 10$) and the REST group remained in resting conditions (REST leg vs REST + BFR leg; $n = 10$).

protein synthesis rates after LLRE + BFR. We used a 13-cm wide nylon pressure cuff with a 12-cm pneumatic bag inside (Hokanson SC12, 13 × 85 cm; Hokanson, Bellevue, WA) that was placed on the proximal part of the thigh and connected to a rapid cuff inflator (ID, Maastricht University Medical Centre+, The Netherlands). The other leg served as the within-subject control by not receiving the BFR stimulus. Each of the two cycles of BFR was initiated by inflating the cuff to a pressure of 120 mm Hg for 30 s, followed by 10 s of deflation. This procedure was then repeated three more times in total, while cuff pressure was increased with 20 mm Hg increments (140, 160, and 180 mm Hg), before finally reaching the target pressure of 200 mm Hg, which was maintained for 5 min, as has been done in previous studies (12–14,22). Although a reliable measure of the arterial restriction of blood flow during BFR is still lacking (17), we crudely assessed whether cuff inflation resulted in ischemia/hypoxia in the leg during BFR by placing a pulse oximeter on the big toe of each participant and measuring oxygen saturation. Oxygen saturation was observed to be within normal range (98%–100%) at baseline before cuff inflation in all subjects, but it was reduced to an unmeasurable range when the final pressure of 200 mm Hg was reached (i.e., the oximeter ceased measuring shortly after the saturation reduced to 85%–90%). Oxygen saturation returned to baseline values 30–60 s after pressure was released from the cuff.

Participants allocated to the REST treatment were seated in a semi-upright position (Fowler's position) during the 2 × 5 min cycles of BFR, whereas the LLRE group performed 4 sets of leg press exercise during the first cycle of BFR (first set 30 repetitions, followed by 3 sets of 15 repetitions) and 3 sets of leg extension exercise during the second cycle of BFR (3 sets of 10 repetitions). Between each set, participants had a 30-s resting period during the leg press exercise and a 60-s resting period during the leg extension exercise, while cuff pressure was maintained at 200 mm Hg. The two cycles of BFR were interspaced by 5 min in which the cuff was deflated. For the LLRE group, this period was used to perform the same exercise protocol with the control leg (LLRE at 20% 1RM, without BFR). A metronome was used to assure that participants held the correct cadence of 1.5 s for the concentric and 1.5 s for the eccentric phase of the exercise. This resulted in the last repetition of the leg press exercise being completed after ~5 min,

followed by cuff deflation. For the leg extension exercise, cuff pressure was maintained for another ~1.5 min after the last repetition (at ~3.5 min) to complete the 5-min BFR cycle. Both exercises were performed with a load of 20% 1RM. If a participant failed to complete a set during the 5-min BFR cycle, he would rest until the start of the following set and perform the exercise with a weight that was decreased by 10% of the absolute load.

The last cycle of BFR was followed by a 5-h period in which arterialized blood samples were collected at 30 min intervals ($t = 0–300$ min), and 3 muscle biopsies were collected from both the BFR leg and the control leg ($t = 0, 2$ and 5 h) to determine myofibrillar protein synthesis rates. Biopsies were collected from the middle region of the vastus lateralis muscle using the percutaneous needle biopsy technique under local anesthesia (23). The biopsy samples were collected distal to the area where the BFR cuff was placed, as the area directly underneath the pressure cuff has previously been suggested to show attenuated growth (24). The muscle samples were dissected carefully, freed from any visible adipose tissue and blood, immediately frozen in liquid nitrogen, and stored at −80°C until subsequent analysis. Arterialized blood samples were collected in EDTA-containing tubes and centrifuged at 1000g for 10 min at 4°C. Aliquots of plasma samples were frozen in liquid nitrogen and also stored at −80°C until further analysis.

Plasma and muscle tissue analyses. Plasma phenylalanine concentrations and plasma L-[ring-$^{13}C_6$]phenylalanine enrichments were measured by gas chromatography–mass spectrometry (Agilent 7890A GC/5975C MSD; Agilent Technologies, Santa Clara, CA) as described previously (25). Myofibrillar protein–bound L-[ring-$^{13}C_6$]phenylalanine enrichments were determined by gas chromatography–combustion–isotope ratio mass spectrometry (MAT 253, Thermo-Scientific, Bremen, Germany) analysis as described in our previous work (26). Myofibrillar protein–bound enrichments of the $t = 0$ h biopsy was set to 0 for each individual and subtracted from the $t = 2$ h and $t = 5$ h enrichments, allowing the $t = 2$ h and $t = 5$ h MPE values to represent the increase compared with the $t = 0$ h biopsy.

Western blot analysis was performed on muscle samples homogenized in accordance with previously described procedures (27). The total amount of supernatant that was loaded on gel was based on protein content (50 μg per lane) after a

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BCA protein assay assessment. With the exception of mTOR and acetyl-CoA carboxylase (ACC), protein samples were run on a Criterion Precast TGX 4%–20% gel (Bio-Rad, Hercules, CA). The mTOR and ACC proteins were run on a Criterion Precast XT 3%–8% Tris-acetate gel (Bio-Rad). Specific proteins were detected with the following antibodies: ACC with anti-ACC and anti-phospho-ACC (Ser[79]), mTOR with anti-mTOR and anti-phospho-mTOR (Ser[2448]), S6 protein kinase 1 (p70S6K) with anti-p70S6K and anti-phospho p70S6K (Thr[389]), ribosomal protein S6 (RS6) with anti-RS6 and anti-phospho-RS6 (Ser[235]/Ser[236]), and eukaryotic translation initiation factor 4E-binding protein-1 (4E-BP1) with anti-4E-BP1 and anti-phospho-4E-BP1 (Thr[37/46]) (all from Cell Signaling Technology). Ponceau S staining was used to standardize for the total amount of protein loaded on gel. The additional assessment of total protein within groups and within legs showed no changes over time. Phosphorylation status as a proxy of activation of the signaling proteins was expressed as a ratio, relative to the total amount of each protein.

The determination of skeletal muscle mRNA was performed as previously described (28). Gene expression assays (Applied Biosystems, Foster City, CA) for 18S, mTOR, p70S6K, MuRF1, and MAFbx are listed in Supplemental Digital Content 1 (see Table, Supplemental Digital Content 1, Gene express assays, http://links.lww.com/MSS/B492). Statistical analysis for all mRNA data was performed on the delta Ct values. The mRNA data of the control legs (LLRE and REST) at $t = 0$ h were used as reference and were given the value of 1, and all other values from the control and BFR conditions were expressed as fold changes for figure presentation.

Calculations. In line with previous research (29), myofibrillar protein fractional synthetic rates (FSR) were calculated using the standard precursor–product equation, as follows:

$$FSR = \frac{\Delta E_p}{E_{precursor} \, t} \times 100\%$$

where ΔE_p is the increment in myofibrillar protein–bound L-[ring-$^{13}C_6$]phenylalanine enrichment after an incorporation period, $E_{precursor}$ is the weighted mean plasma L-[ring-$^{13}C_6$] phenylalanine enrichment during that incorporation period, and t is the incorporation period (h). Weighted mean plasma enrichments were calculated by taking the average enrichment between all consecutive time points and correcting for the time between these sampling time points.

Statistical analysis. All data are expressed as mean ± SEM. A sample size of 10 subjects per group was calculated with a power of 80% and an α level of 0.05 to detect a 20% difference in myofibrillar protein synthesis rates between the BFR leg and the control leg within groups (effect size = 1.07). All analyses were performed from the period immediately after the BFR intervention and included the first biopsy ($t = 0$ min) up until the end of the experimental trial ($t = 300$ min). Within-subject differences in myofibrillar protein synthesis rates between BFR and control leg were assessed using paired samples t-tests, for the resting (REST) and exercise (LLRE) groups separately.

Although we did not power for between-group differences in myofibrillar protein synthesis rates, we performed unpaired t-tests as secondary analyses on the data to provide insight into the potential differences between the REST and the LLRE groups. Two-factor repeated-measures ANOVA was performed with time and treatment (BFR vs control) as within-subject factors to assess differences in ACC, key anabolic signaling proteins of the mTOR pathway and mRNA expression. Observed main effects or interactions were further assessed with Bonferroni-corrected *post hoc* testing where appropriate. Mean difference (MD), as well as 95% confidence interval (CI) of the difference, and Cohen's d_z effect size (calculated as MD / SD of difference) are also presented for the within-subject assessments where appropriate. Statistical significance was set at $P < 0.05$. All calculations were performed using SPSS Statistics (version 25; IBM, Armonk, NY).

RESULTS

All subjects managed to complete the 2×5-min BFR cycles without premature cuff deflation. In the LLRE group, the exercise weight was reduced by 10% during the leg press exercise for 2 of the 10 subjects, after failing to complete the third set. No adverse events were reported in any of the subjects.

Plasma concentrations and tracer enrichments. Mean plasma phenylalanine concentrations during the experimental trial were $54.3 \pm 1.5 \, \mu mol \cdot L^{-1}$ in the LLRE group and $55.3 \pm 2.7 \, \mu mol \cdot L^{-1}$ in the REST group, with no differences between groups. Mean plasma enrichments of the infused L-[ring-$^{13}C_6$]phenylalanine during the post-BFR period were 6.88 ± 0.25 and 6.71 ± 0.26 MPE in the LLRE and REST groups, respectively, with no differences between groups. Both phenylalanine concentrations and enrichments were in steady state throughout the experimental period.

Muscle protein–bound enrichments and myofibrillar protein synthesis rates. In the group that performed exercise, postexercise myofibrillar protein–bound L-[ring-$^{13}C_6$] phenylalanine enrichments at $t = 2$ h did not differ between the LLRE + BFR and the contralateral control leg (LLRE) (0.0064 ± 0.0009 vs 0.0064 ± 0.0011 MPE, respectively; $P = 0.979$, MD = 0.0000, 95% CI = −0.0018 to 0.0018, $d_z = 0.01$). At $t = 5$ h, myofibrillar protein–bound enrichments tended to be higher in the LLRE + BFR leg (0.0165 ± 0.0014 MPE) when compared with the LLRE leg (0.0151 ± 0.0014 MPE; $P = 0.051$, MD = 0.0014, 95% CI = 0.0000–0.0028, $d_z = 0.71$). In accordance, myofibrillar protein synthesis rates over 0–2 h did not differ between the LLRE + BFR ($0.0463\% \pm 0.0062\%$ per hour) and the LLRE leg ($0.0463\% \pm 0.0078\%$ per hour; $P = 0.997$, MD = 0.0000, 95% CI = −0.0128 to 0.0128, $d_z = 0.001$). Also for the 2- to 5-h period, no significant differences were observed in myofibrillar protein synthesis rates between the LLRE + BFR ($0.0486\% \pm 0.0062\%$ per hour) and the LLRE leg ($0.0411\% \pm 0.0037\%$ per hour; $P = 0.186$, MD = 0.0075, 95% CI = −0.0043 to 0.0193, $d_z = 0.45$). By contrast, $10\% \pm 5\%$ higher myofibrillar protein synthesis rates were
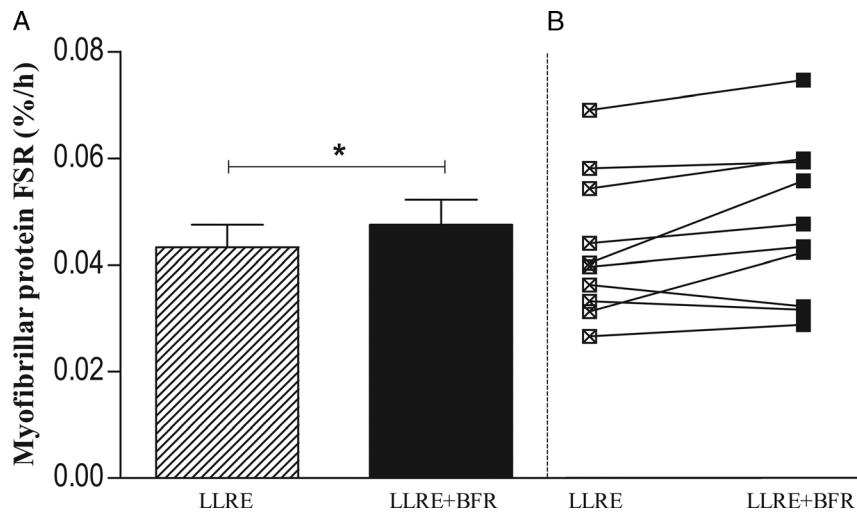
FIGURE 2—Mean ± SEM (A; *n* = 10) and individual (B) myofibrillar protein fractional synthetic rates (FSR) measured over a 0- to 5-h period after LLRE combined with (LLRE + BFR leg) and without (LLRE leg) BFR. Data were analyzed using a paired samples *t*-test (control leg vs BFR leg, within groups). *Indicates a significant difference ($P < 0.05$).

observed over the entire 0–5 h period in the LLRE + BFR leg when compared with the LLRE leg ($P = 0.042$, MD = 0.0043, 95% CI = 0.0002 to 0.0084, $d_z = 0.75$; Fig. 2).

In the group that remained in resting condition, myofibrillar protein–bound L-[ring-$^{13}C_6$]phenylalanine enrichments at $t = 2$ h were $0.0056 ± 0.0005$ MPE in the REST + BFR leg and $0.0063 ± 0.0007$ MPE in the REST leg, with no differences between legs ($P = 0.344$, MD = −0.0007, 95% CI = −0.0022 to 0.0008, $d_z = −0.32$). At $t = 5$ h, myofibrillar protein–bound enrichments were increased to $0.0152 ± 0.0013$ MPE in the REST + BFR leg and $0.0147 ± 0.0012$ MPE in the control leg, with no differences between legs ($P = 0.587$, MD = 0.0006, 95% CI = −0.0017 to 0.0028, $d_z = 0.18$). In accordance with the myofibrillar protein–bound enrichment data, myofibrillar protein synthesis rates measured over 0–2 h did not differ between the REST + BFR ($0.0441\% ± 0.0047\%$ per hour) and the REST leg ($0.0499\% ± 0.0064\%$ per hour; $P = 0.296$, MD = −0.0058, 95% CI = −0.0176 to 0.0060,

$d_z = −0.35$). Likewise, no differences were observed in myofibrillar protein synthesis rates over the 2- to 5-h period between the REST + BFR ($0.0447\% ± 0.0055\%$ per hour) and the REST leg ($0.0392\% ± 0.0037\%$ per hour; $P = 0.233$, MD = 0.0055, 95% CI = −0.0042 to 0.0152, $d_z = 0.41$). In accordance, myofibrillar protein synthesis rates over the entire 0–5 h period were not different between the REST + BFR and the REST leg ($P = 0.683$, MD = 0.00125, 95% CI = −0.0055 to 0.0080, $d_z = 0.13$; Fig. 3).

In addition to the primary, within-group analyses, the secondary between-group analyses (unpaired *t*-test rested vs exercised group) showed no differences in myofibrillar protein synthesis rates between REST + BFR and LLRE + BFR, nor between REST and LLRE ($P \geq 0.213$).

Signaling proteins. In the group that performed exercise, a significant time effect ($P = 0.037$) and treatment effect ($P = 0.046$) was observed for ACC phosphorylation (Fig. 4), indicating an overall higher ACC phosphorylation in
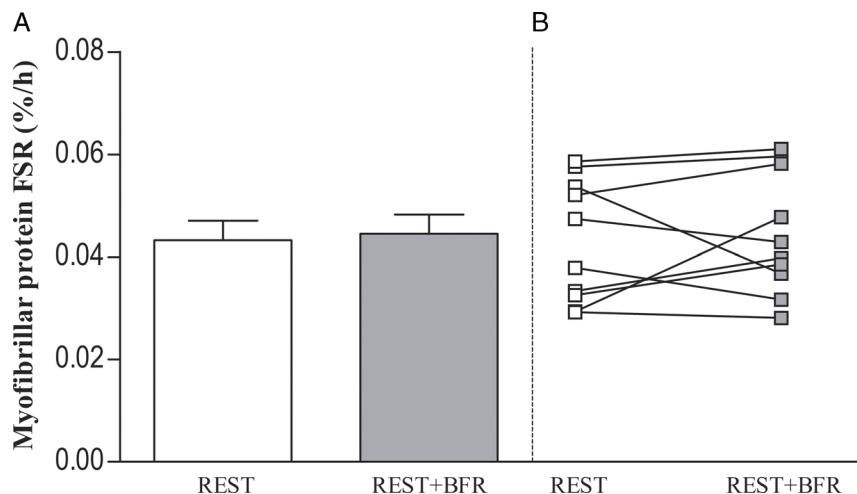

FIGURE 3—Mean ± SEM (panel A; *n* = 10) and individual (panel B) myofibrillar protein fractional synthetic rates (FSR) measured over a 0- to 5-h period during resting conditions with (REST + BFR leg) and without (REST leg) BFR. Data were analyzed with paired samples *t*-test (control vs BFR leg, within groups). No significant differences were observed between the treatment legs.
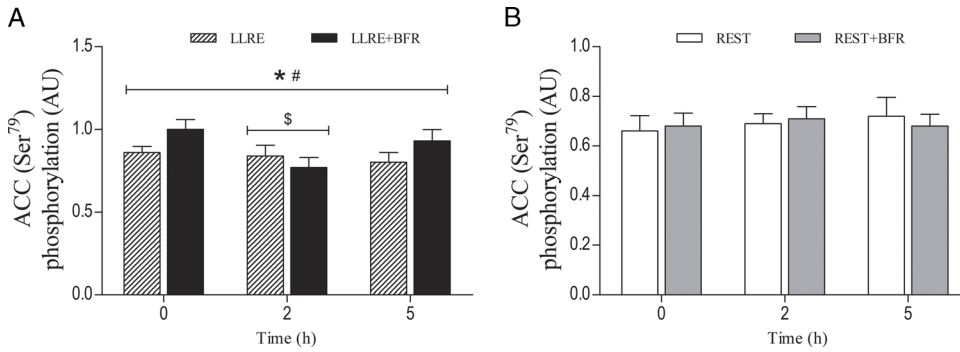
FIGURE 4—Mean ± SEM skeletal muscle phosphorylation status of ACC, after LLRE (A) with (LLRE + BFR; $n = 10$) and without (LLRE; $n = 10$) BFR. Mean ± SEM skeletal muscle phosphorylation status of ACC, during resting conditions (B) with (REST + BFR) and without (REST) BFR. Data were analyzed with a two-way repeated-measures ANOVA (time–treatment leg) within groups. Differences were only observed within the exercise group. *Significant treatment effect ($P = 0.046$). #Significant time effect ($P = 0.037$). $Post hoc testing for the time effect showed lower ACC phosphorylation at the 2-h vs the 0-h time point (i.e., for both legs combined; $P = 0.024$).

LLRE + BFR versus LLRE and an overall lower ACC phosphorylation at 2 h versus 0 h ($P = 0.024$). No differences between legs and/or changes over time were observed for mTOR, p70S6K, or RS6 (Fig. 5A–C). For 4E-BP1 phosphorylation, a significant time–treatment interaction was observed ($P = 0.009$), with higher 4E-BP1 phosphorylation in the LLRE + BFR versus LLRE leg at $t = 2$ h ($P = 0.038$, MD = 0.081, 95% CI = 0.0058–0.1564, $d_z = 0.77$; Fig. 5D). In the group that remained in resting condition, no differences between legs or changes over time were observed for any of the proteins measured (Fig. 5E–H).

mRNA expression. In the group that performed exercise, a time–treatment interaction was observed for MuRF1 ($P = 0.002$), with *post hoc* analysis showing higher mRNA expression for LLRE + BFR when compared with LLRE at $t = 2$ h ($P = 0.001$, MD = 0.857, 95% CI = 0.4799–1.2341, $d_z = 1.626$; Fig. 6A). For the LLRE + BFR leg, greater MuRF1 mRNA expression was also observed at $t = 2$ h when compared with the other time points ($P < 0.01$). Furthermore, MuRF1 mRNA expression was lower at $t = 5$ h when compared with the other time points for both the LLRE + BFR

and the LLRE leg ($P < 0.01$). No differences between legs or changes over time were observed for MAFbx, mTOR, or p70S6K mRNA expression in the LLRE group.

In the group that remained in resting condition, mTOR showed a time–treatment interaction ($P = 0.019$), with higher mRNA expression observed for REST + BFR versus REST at $t = 5$ h ($P = 0.004$, MD = 0.147, 95% CI = 0.0610–0.2330, $d_z = 1.22$; Fig. 6G). Within the REST leg, mTOR mRNA expression at $t = 5$ h was observed to be lower than $t = 2$ h ($P = 0.027$). No further differences were found in mRNA data between the REST + BFR and the REST leg (Fig. 6).

DISCUSSION

The aim of the current study was to assess the effect of BFR with and without LLRE on myofibrillar protein synthesis rates *in vivo* in healthy young men. Combining LLRE with BFR resulted in higher myofibrillar protein synthesis rates than LLRE alone, whereas BFR applied during resting conditions did not change myofibrillar protein synthesis rates in healthy young men.
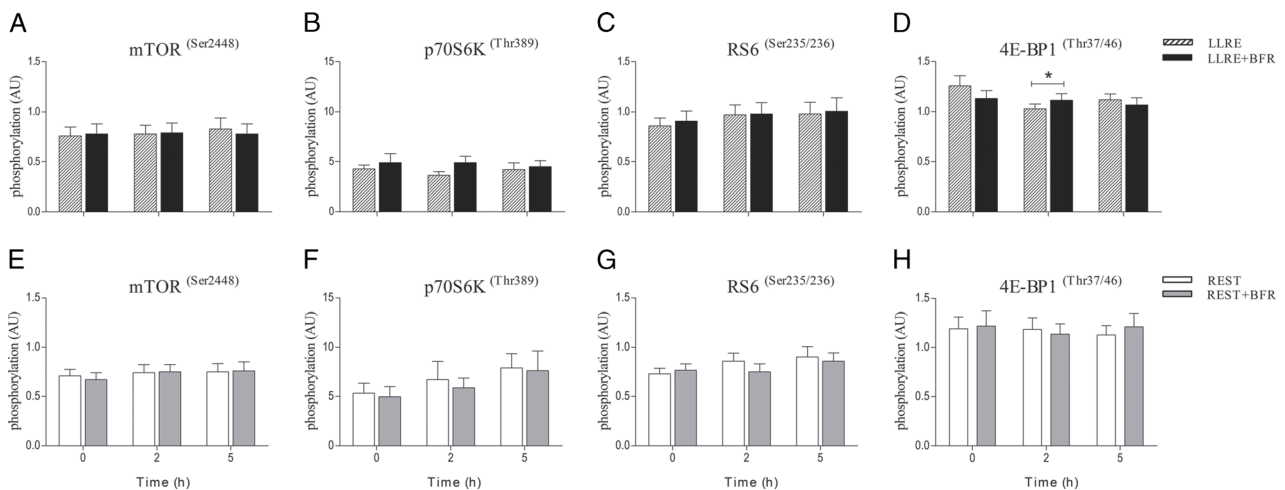

FIGURE 5—Mean ± SEM skeletal muscle phosphorylation status of selected anabolic signaling proteins, after LLRE (A–D; $n = 10$) and during resting conditions (E–H; $n = 10$). Data were analyzed with a two-way repeated-measures ANOVA (time–treatment leg) within groups. Differences were only observed within the exercise group. 4E-BP1: time–treatment leg interaction, $P = 0.009$. *Significant difference when compared with LLRE at the same time-point ($P = 0.038$).
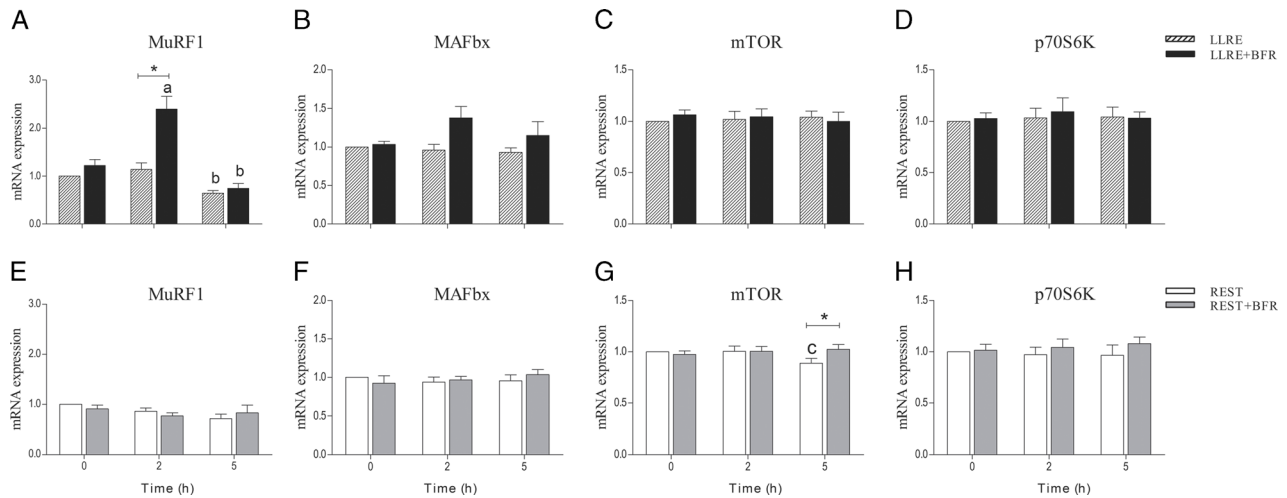
FIGURE 6—Mean ± SEM skeletal muscle mRNA expression of selected genes, after LLRE (A–D; $n = 10$) and during resting conditions (E–H; $n = 9$). Data were analyzed with a two-way repeated-measures ANOVA (time–treatment leg) within groups. Exercise group: MuRF1 time–treatment leg interaction, $P = 0.002$. *Significant within-group difference between treatment legs ($P < 0.05$). [a]Significantly different from corresponding treatment leg at $t = 0$ and $t = 5$ h ($P < 0.01$). [b]Significantly different from corresponding treatment leg at $t = 0$ and $t = 2$ h ($P < 0.01$). Resting condition group: mTOR time–treatment leg interaction, $P = 0.013$. [c]Significantly different from corresponding treatment leg at $t = 2$ h ($P = 0.027$).

Although high-load resistance-type exercise has been shown to be a strong stimulus for skeletal muscle hypertrophy (7), injured athletes or rehabilitating patients might be limited in their ability to perform high-load exercise. An alternative approach suggested to promote skeletal muscle anabolism without the need of heavy weight resistance is the application of BFR with, or even without LLRE (16,18,19). As there are only limited data available of the acute anabolic response to BFR under both conditions, the current study assessed the effects of BFR on myofibrillar protein synthesis rates in healthy young men. Using a within-subject unilateral design, the effect of BFR was assessed during LLRE (LLRE + BFR vs LLRE), as well as during resting conditions (REST + BFR vs REST).

We observed that myofibrillar protein synthesis rates over the 0- to 5-h period were higher with LLRE + BFR when compared with performing an identical bout of LLRE without BFR (Fig. 2). These observations are in line with the limited but consistent findings by others (12–15). For example, the first study in this area by Fujita et al. (13), showed increased mixed-muscle protein synthesis rates measured over 3 h after a single cycle of BFR combined with 20% 1RM LLRE (13). Using a similar BFR protocol (200 mm Hg cuff pressure and multiple exercise sets within a BFR cycle, albeit with a wider cuff), the current study confirms and extends on those findings by showing that combining BFR with LLRE increases myofibrillar protein synthesis rates. As such, the current study for the first time shows that LLRE + BFR induces an anabolic effect on the contractile protein pool of skeletal muscle tissue.

Although the exact mechanism behind the anabolic effect of LLRE + BFR is still unclear, the occurrence of metabolic stress has been proposed to play a role (30). Increased metabolic stress during LLRE + BFR is believed to result in greater skeletal muscle activation by speeding up type I muscle fiber fatigue and stimulating early recruitment of type II muscle fibers (30,31). As metabolic stress has been shown to increase AMP-activated protein kinase (AMPK) activity (32), the

current study included measures of intramuscular ACC phosphorylation, which is a downstream target of AMPK. The phosphorylation of ACC has been shown to strongly correlate with changes in AMPK activity (33) and was therefore used as a proxy of metabolic stress. In line with several studies that found greater metabolic stress by measuring systemic plasma lactate concentrations (12,13,31), we observed a higher phosphorylation of ACC after LLRE + BFR when compared with LLRE (Fig. 4A). Although caution is warranted given the small differences observed, our findings suggest greater intramuscular metabolic stress when combining LLRE with BFR.

Because the stimulation of mTOR and its downstream effectors has been shown to correlate with increased muscle protein synthesis rates (34), we also assessed whether the phosphorylation of several key anabolic signaling proteins (i.e., mTOR, p70S6K, RS6, and 4E-BP1) differed between LLRE + BFR and LLRE. Performing LLRE + BFR resulted in a small but significantly greater phosphorylation of 4E-BP1 at $t = 2$ h when compared with LLRE (7% ± 9%; Fig. 5D). Notably though, differences of approximately the same magnitude but in opposite direction were observed for 4E-BP1 phosphorylation at $t = 0$ h and $t = 5$ h, although these did not reach statistical significance. Other anabolic signaling proteins also showed no differences between LLRE + BFR and LLRE (Fig. 5A–C). This general lack of difference may however be related to the timing of biopsies, as previous studies did observe higher p70S6K phosphorylation after a similar LLRE + BFR protocol (12,13). Thus, although from previous work it appears that the anabolic effect of LLRE + BFR may at least partly be mediated by the mTOR signaling pathway, the current findings do not support a major role.

In view of the fast, albeit transient, increase in gene expression generally observed after exercise, we also determined whether LLRE + BFR versus LLRE showed differences in transcriptional activation during the postexercise period.

Although we did not observe changes in the mRNA expression of genes associated with muscle protein synthesis (p70S6K and mTOR), LLRE + BFR induced a greater expression of the muscle-specific ubiquitin ligase MuRF1 when compared with LLRE (Fig. 6A). Elevated MuRF1 mRNA expression has frequently been observed early into the recovery period after high-load resistance-type exercise (35,36), as well as after acute and chronic LLRE + BFR (24). This may be associated with increased protein breakdown as an inherent part of the postexercise muscle remodeling process (35). In addition, there are some suggestions that MuRF1 is also involved in the regulation of energy metabolism, especially under conditions of metabolic stress (37). The latter would be in line with the greater ACC phosphorylation observed in the present study (Fig. 4A), and it suggests certain homeostatic perturbations with LLRE + BFR, perhaps similar to what has been observed with high-load resistance-type exercise (35,36). Collectively, the current findings indicate that combining LLRE with BFR stimulates skeletal muscle remodeling to a greater extent than a comparable bout of LLRE, by increasing anabolic protein signaling as well as promoting protein turnover-related gene expression and, more importantly, by increasing myofibrillar protein synthesis rates.

Based on rodent data suggesting that BFR performed at rest might also stimulate skeletal muscle anabolism (18,19), we also determined whether BFR could increase myofibrillar protein synthesis rates in the absence of concomitant exercise (REST + BFR). In contrast to our hypothesis, we observed no differences in myofibrillar protein synthesis rates (Fig. 3) or the phosphorylation of anabolic signaling proteins (Fig. 5) in the REST + BFR leg when compared with REST. We also observed no differences in metabolic stress between REST + BFR and REST as assessed by ACC phosphorylation (Fig. 4B). Although animal data presented by Nakajima et al. (18) also observed no changes in metabolic stress after repeated bouts of REST + BFR versus REST (quantified as AMPK phosphorylation), they did observe greater p70S6K and ribosomal S6 phosphorylation in skeletal muscle tissue. It could be argued that between-species differences may explain the discrepancy between that study and the current study. Yet, using a longitudinal design in humans, Takarada and colleagues (22) showed that the daily application of BFR without exercise was effective in attenuating skeletal muscle mass loss in subjects undergoing 14 d of non-weight-bearing leg immobilization. As the decrease in skeletal muscle mass during disuse has been associated with substantial reductions in muscle protein synthesis rates (38), it could be speculated that the atrophy-attenuating effects of REST + BFR would primarily affect muscle protein synthesis rates. However, the fact that we did not observe an effect of REST + BFR on myofibrillar protein synthesis rates (Fig. 3) may suggest REST + BFR to only be effective in stimulating skeletal muscle protein synthesis during disuse, rather than further increasing muscle protein synthesis rates in habitually active individuals. Alternatively, it could be speculated that the number of BFR cycles, which were purposely kept similar between REST + BFR and LLRE + BFR in the current study (two cycles of 5 min), may have also played a role. Previous studies assessing the atrophy-attenuating effects of REST + BFR instead performed five repetitive 5-min cycles of REST + BFR within a single session. However, as the assessment of a dose–response relationship between the number of REST + BFR cycles and skeletal muscle anabolism is currently lacking, it is unclear whether performing more than two cycles of REST + BFR might have shown greater effects on anabolic signaling or myofibrillar protein synthesis rates.

Clearly, future research will need to provide further insight into whether REST + BFR can be effective in preserving basal and/or postprandial muscle protein synthesis rates and, as such, maintaining skeletal muscle mass during disuse. Furthermore, the contribution of factors such as the number of repetitive BFR cycles within each session, but also the number of sessions performed daily, as well as the (individualized) pressure required to stimulate skeletal muscle anabolism will need to be further elaborated on to optimize recommendations for BFR performed at rest, as well as when combined with exercise. With respect to individualized pressure, it is important to note that the extent of BFR depends on the specific combination of factors such as cuff width and the pressure applied. How these impact the muscle protein synthetic response to BFR remains to be established, as these factors have until now only been associated with differences in the cardiovascular and perceptual response (39,40).

Over the past years, a growing number of studies reported increases in muscle mass when BFR is combined with LLRE (10,11,16). The present study is the first to show that this is attributed, at least partly, to an increase in myofibrillar protein synthesis rates. Applying LLRE + BFR may therefore be useful to maintain skeletal muscle mass and stimulate muscle hypertrophy in a clinical setting, when individuals are unable to perform exercise at a high resistive load (e.g., for injured athletes or as pre- and postoperative training programs).

In conclusion, BFR performed at rest does not increase myofibrillar protein synthesis rates *in vivo* in humans. When combined with LLRE, BFR further increases postexercise myofibrillar protein synthesis rates.

REFERENCES

1. Clark BC. In vivo alterations in skeletal muscle form and function after disuse atrophy. *Med Sci Sports Exerc*. 2009;41(10):1869–75.

2. Dirks ML, Backx EM, Wall BT, Verdijk LB, van Loon LJ. May bed rest cause greater muscle loss than limb immobilization? *Acta Physiol (Oxf)*. 2016;218(1):10–2.

3. LeBlanc AD, Schneider VS, Evans HJ, Pientok C, Rowe R, Spector E. Regional changes in muscle mass following 17 weeks of bed rest. *J Appl Physiol (1985)*. 1992;73(5):2172–8.

4. Dirks ML, Wall BT, van de Valk B, et al. One week of bed rest leads to substantial muscle atrophy and induces whole-body insulin resistance in the absence of skeletal muscle lipid accumulation. *Diabetes*. 2016;65(10):2862–75.

5. Miller BF, Olesen JL, Hansen M, et al. Coordinated collagen and muscle protein synthesis in human patella tendon and quadriceps muscle after exercise. *J Physiol*. 2005;567(Pt 3):1021–33.

6. Phillips BE, Hill DS, Atherton PJ. Regulation of muscle protein synthesis in humans. *Curr Opin Clin Nutr Metab Care*. 2012;15(1):58–63.

7. Damas F, Phillips SM, Libardi CA, et al. Resistance training-induced changes in integrated myofibrillar protein synthesis are related to hypertrophy only after attenuation of muscle damage. *J Physiol*. 2016; 594(18):5209–22.

8. Ferrando AA, Tipton KD, Bamman MM, Wolfe RR. Resistance exercise maintains skeletal muscle protein synthesis during bed rest. *J Appl Physiol (1985)*. 1997;82(3):807–10.

9. Backx EMP, Hangelbroek R, Snijders T, et al. Creatine loading does not preserve muscle mass or strength during leg immobilization in healthy, young males: a randomized controlled trial. *Sports Med*. 2017;47(8):1661–71.

10. Martin-Hernandez J, Marin PJ, Menendez H, Ferrero C, Loenneke JP, Herrero AJ. Muscular adaptations after two different volumes of blood flow-restricted training. *Scand J Med Sci Sports*. 2013;23(2): e114–20.

11. Yasuda T, Loenneke JP, Thiebaud RS, Abe T. Effects of blood flow restricted low-intensity concentric or eccentric training on muscle size and strength. *PLoS One*. 2012;7(12):e52843.

12. Fry CS, Glynn EL, Drummond MJ, et al. Blood flow restriction exercise stimulates mTORC1 signaling and muscle protein synthesis in older men. *J Appl Physiol (1985)*. 2010;108(5):1199–209.

13. Fujita S, Abe T, Drummond MJ, et al. Blood flow restriction during low-intensity resistance exercise increases S6K1 phosphorylation and muscle protein synthesis. *J Appl Physiol (1985)*. 2007;103(3):903–10.

14. Gundermann DM, Fry CS, Dickinson JM, et al. Reactive hyperemia is not responsible for stimulating muscle protein synthesis following blood flow restriction exercise. *J Appl Physiol (1985)*. 2012;112(9): 1520–8.

15. Gundermann DM, Walker DK, Reidy PT, et al. Activation of mTORC1 signaling and protein synthesis in human muscle following blood flow restriction exercise is inhibited by rapamycin. *Am J Physiol Endocrinol Metab*. 2014;306(10):E1198–204.

16. Lixandrao ME, Ugrinowitsch C, Laurentino G, et al. Effects of exercise intensity and occlusion pressure after 12 weeks of resistance training with blood-flow restriction. *Eur J Appl Physiol*. 2015;115(12): 2471–80.

17. Jessee MB, Buckner SL, Mouser JG, et al. Muscle adaptations to high-load training and very low-load training with and without blood flow restriction. *Front. Physiol*. 2018;9:1448.

18. Nakajima T, Yasuda T, Koide S, et al. Repetitive restriction of muscle blood flow enhances mTOR signaling pathways in a rat model. *Heart Vessels*. 2016;31(10):1685–95.

19. Sudo M, Ando S, Kano Y. Repeated blood flow restriction induces muscle fiber hypertrophy. *Muscle Nerve*. 2017;55(2):274–6.

20. Mayhew JL, Prinster JL, Ware JS, Zimmer DL, Arabas JR, Bemben MG. Muscular endurance repetitions to predict bench press strength in men of different training levels. *J Sports Med Phys Fitness*. 1995;35(2):108–13.

21. Cook CJ, Kilduff LP, Beaven CM. Improving strength and power in trained athletes with 3 weeks of occlusion training. *Int J Sports Physiol Perform*. 2014;9(1):166–72.

22. Takarada Y, Takazawa H, Ishii N. Applications of vascular occlusion diminish disuse atrophy of knee extensor muscles. *Med Sci Sports Exerc*. 2000;32(12):2035–9.

23. Bergstrom J. Percutaneous needle biopsy of skeletal muscle in physiological and clinical research. *Scand J Clin Lab Invest*. 1975;35(7): 609–16.

24. Ellefsen S, Hammarstrom D, Strand TA, et al. Blood flow-restricted strength training displays high functional and biological efficacy in women: a within-subject comparison with high-load strength training. *Am J Physiol Regul Integr Comp Physiol*. 2015;309(7):R767–79.

25. Gorissen SH, Burd NA, Hamer HM, Gijsen AP, Groen BB, van Loon LJ. Carbohydrate coingestion delays dietary protein digestion and absorption but does not modulate postprandial muscle protein accretion. *J Clin Endocrinol Metab*. 2014;99(6):2250–8.

26. Kramer IF, Verdijk LB, Hamer HM, et al. Impact of the macronutrient composition of a nutritional supplement on muscle protein synthesis rates in older men: a randomized, double blind, controlled trial. *J Clin Endocrinol Metab*. 2015;100(11):4124–32.

27. Trommelen J, Holwerda AM, Kouw IW, et al. Resistance exercise augments postprandial overnight muscle protein synthesis rates. *Med. Sci. Sports Exerc*. 2016;48(12):2517–25.

28. Holloway TM, Snijders T, Van Kranenburg J, Van Loon LJC, Verdijk LB. Temporal response of angiogenesis and hypertrophy to resistance training in young men. *Med Sci Sports Exerc*. 2018;50(1): 36–45.

29. Burd NA, Cermak NM, Kouw IW, Gorissen SH, Gijsen AP, van Loon LJ. The use of doubly labeled milk protein to measure postprandial muscle protein synthesis rates in vivo in humans. *J Appl Physiol (1985)*. 2014;117(11):1363–70.

30. Suga T, Okita K, Takada S, et al. Effect of multiple set on intramuscular metabolic stress during low-intensity resistance exercise with blood flow restriction. *Eur J Appl Physiol*. 2012;112(11):3915–20.

31. Loenneke JP, Wilson JM, Balapur A, Thrower AD, Barnes JT, Pujol TJ. Time under tension decreased with blood flow-restricted exercise. *Clin Physiol Funct Imaging*. 2012;32(4):268–73.

32. Ponticos M, Lu QL, Morgan JE, Hardie DG, Partridge TA, Carling D. Dual regulation of the AMP-activated protein kinase provides a novel mechanism for the control of creatine kinase in skeletal muscle. *EMBO J*. 1998;17(6):1688–99.

33. Park SH, Gammon SR, Knippers JD, Paulsen SR, Rubink DS, Winder WW. Phosphorylation-activity relationships of AMPK and acetyl-CoA carboxylase in muscle. *J Appl Physiol (1985)*. 2002; 92(6):2475–82.

34. Kumar V, Selby A, Rankin D, et al. Age-related differences in the dose–response relationship of muscle protein synthesis to resistance exercise in young and old men. *J Physiol*. 2009;587(Pt 1):211–7.

35. Louis E, Raue U, Yang Y, Jemiolo B, Trappe S. Time course of proteolytic, cytokine, and myostatin gene expression after acute exercise in human skeletal muscle. *J Appl Physiol (1985)*. 2007; 103(5):1744–51.

36. Glynn EL, Fry CS, Drummond MJ, et al. Muscle protein breakdown has a minor role in the protein anabolic response to essential amino acid and carbohydrate intake following resistance exercise. *Am J Physiol Regul Integr Comp Physiol*. 2010;299(2):R533–40.

37. Koyama S, Hata S, Witt CC, et al. Muscle RING-finger protein-1 (MuRF1) as a connector of muscle energy metabolism and protein synthesis. *J Mol Biol*. 2008;376(5):1224–36.

38. de Boer MD, Selby A, Atherton P, et al. The temporal responses of protein synthesis, gene expression and cell signalling in human quadriceps muscle and patellar tendon to disuse. *J Physiol*. 2007;585(Pt 1): 241–51.

39. Jessee MB, Buckner SL, Dankel SJ, Counts BR, Abe T, Loenneke JP. The influence of cuff width, sex, and race on arterial occlusion: implications for blood flow restriction research. *Sports Med*. 2016; 46(6):913–21.

40. Rossow LM, Fahs CA, Loenneke JP, et al. Cardiovascular and perceptual responses to blood-flow-restricted resistance exercise with differing restrictive cuffs. *Clin Physiol Funct Imaging*. 2012;32(5): 331–7.