

# Mixed muscle protein synthesis and breakdown after resistance exercise in humans

STUART M. PHILLIPS, KEVIN D. TIPTON, ASLE AARSLAND,  
STEVEN E. WOLF, AND ROBERT R. WOLFE

*Metabolism Unit, Shriners Burns Institute, and Departments of Surgery and Anesthesiology,  
University of Texas Medical Branch, Galveston, Texas 77550*

**Phillips, Stuart M., Kevin D. Tipton, Asle Aarsland, Steven E. Wolf, and Robert R. Wolfe.** Mixed muscle protein synthesis and breakdown after resistance exercise in humans. *Am. J. Physiol.* 273 (*Endocrinol. Metab.* 36): E99–E107, 1997.—Mixed muscle protein fractional synthesis rate (FSR) and fractional breakdown rate (FBR) were examined after an isolated bout of either concentric or eccentric resistance exercise. Subjects were eight untrained volunteers (4 males, 4 females). Mixed muscle protein FSR and FBR were determined using primed constant infusions of [<sup>2</sup>H<sub>5</sub>]phenylalanine and [<sup>15</sup>N]-phenylalanine, respectively. Subjects were studied in the fasted state on four occasions: at rest and 3, 24, and 48 h after a resistance exercise bout. Exercise was eight sets of eight concentric or eccentric repetitions at 80% of each subject's concentric 1 repetition maximum. There was no significant difference between contraction types for either FSR, FBR, or net balance (FSR minus FBR). Exercise resulted in significant increases above rest in muscle FSR at all times: 3 h = 112%, 24 h = 65%, 48 h = 34% ( $P < 0.01$ ). Muscle FBR was also increased by exercise at 3 h (31%;  $P < 0.05$ ) and 24 h (18%;  $P < 0.05$ ) postexercise but returned to resting levels by 48 h. Muscle net balance was significantly increased after exercise at all time points [(in %/h) rest =  $-0.0573 \pm 0.003$  (SE), 3 h =  $-0.0298 \pm 0.003$ , 24 h =  $-0.0413 \pm 0.004$ , and 48 h =  $-0.0440 \pm 0.005$ ], and was significantly different from zero at all time points ( $P < 0.05$ ). There was also a significant correlation between FSR and FBR ( $r = 0.88$ ,  $P < 0.001$ ). We conclude that exercise resulted in an increase in muscle net protein balance that persisted for up to 48 h after the exercise bout and was unrelated to the type of muscle contraction performed.

hypertrophy; muscle damage; fractional synthetic rate; fractional breakdown rate

immediately after the exercise (4). However, the time course of the responses of muscle protein breakdown and, more importantly, muscle protein net balance after resistance exercise has not been examined.

Activities that are comprised of repeated eccentric contractions have been shown to result in disruption of the ultrastructure of skeletal muscle (12, 14, 15). Recently, Gibala et al. (15) showed that, immediately after a single bout of either concentric or eccentric resistance exercise in untrained subjects, there was significant myofibrillar disruption that persisted for 48 h. There was, however, a far greater degree of myofibrillar damage observed after the eccentric phase of the protocol (15). The degree of myofibrillar disruption and myocellular enzyme release (an indicator of myofibrillar disruption) has consistently been shown to be greater after eccentric vs. concentric activities (12, 14, 15). Whereas eccentric contractions result in greater myofibrillar disruption, as seen by electron microscopy, it is not known whether there is an association between the processes of myofibrillar disruption and the breakdown of muscle proteins.

The purpose of this investigation was to examine the time course of muscle protein synthesis (fractional synthesis rate, FSR) and breakdown (fractional breakdown rate, FBR) after an isolated bout of resistance exercise. In addition, we wished to determine whether there was a difference in either FSR or FBR after the performance of either concentric or eccentric exercise.

## METHODS

### Participants

Subjects were eight (4 male, 4 female) volunteers who were advised of the purposes of the study and associated risks, and all subjects gave written informed consent. The project was approved by the Institutional Review Board and the Clinical Research Centre (CRC) of The University of Texas Medical Branch. The subjects' descriptive characteristics are shown in Table 1. Subjects were moderately active (recreational cycling and running) and did not engage in any forms of resistance training for  $\geq 5$  mo before or during the study. Subjects had their concentric bilateral 1 repetition maximum (RM) determined (Table 1) in the seated position in a Nautilus knee extension machine. A subject's 1 RM was defined as the maximum weight he or she could lift to full extension and hold for a 1-s count. In the seated resting position in this machine, the subject's knee was flexed at  $\sim 100^\circ$ . Full extension required the movement of the subject's knee through this arc to the point at which the subject's knee was fully extended. During the 1 RM testing and the testing protocol no assistance ("spotting") was given, but subjects were verbally encouraged to maintain their effort. After the initial testing session, subjects were assigned in a counterbalanced manner

THE PROCESS OF MUSCLE HYPERTROPHY after resistance exercise is a fundamental adaptation to an increased resistive workload. Muscle growth can only occur, however, if there is net anabolism within the muscle. That is, muscle protein net balance (synthesis minus breakdown) is positive during the period in which hypertrophy occurs. A variety of investigations have shown that in the period after resistance and long-term endurance exercise there is a stimulation of mixed muscle protein synthesis (4, 7, 8) that persists for up to 24 h (33). On the other hand several studies, using indirect measures, have demonstrated that exercise stimulates (10, 21) or does not affect (9, 16) myofibrillar protein breakdown. We have recently confirmed that an isolated bout of high-intensity resistance exercise stimulated muscle protein breakdown over the first 4 h postexercise (4). Nevertheless, because there was a greater relative stimulation of synthesis, the overall effect was an increase in muscle net protein balance

Table 1. *Descriptive characteristics of subjects*

Age, yr	22.6 ± 0.6
Ht, cm	171.0 ± 3.3
Wt, kg	65.3 ± 4.4
1 RM, kg	73.5 ± 6.4
1 RM/Wt	1.43 ± 0.04
Volume, kg	4,365 ± 381

Values are means ± SE ( $n=8$ ). RM, repetition maximum; 1 RM/Wt, repetition maximum per kg body weight; volume, total weight lifted during protocol, which was calculated as the total no. of repetitions completed multiplied by the mass lifted (in kg).

to either an eccentric or a concentric group and were matched for 1 RM (per kg body weight) and gender. All female subjects participating in this study were taking oral contraceptives and were tested in the early (follicular) phase of their menstrual cycle.

### Experimental Protocol

The protocol was designed to examine the time course of mixed muscle protein FSR and mixed muscle protein FBR after an isolated resistance exercise bout. The initial response was examined at ~3 h postexercise and at two subsequent time points (~24 h and ~48 h) postexercise on consecutive days after the initial exercise bout. The effect of contraction type was also examined by looking at the effect of either eccentric or concentric contractions on FSR and FBR. A schematic representation of the infusion protocol is shown in Fig. 1.

Subjects reported to the CRC on the evening before the beginning of testing and did the same for the remaining 4 days of the testing procedure. Subjects were instructed to maintain a meat-free diet before and during the study protocol. During the protocol, subjects consumed one meal at the CRC after the infusion protocol; all other meals were consumed away from the CRC at the subjects' discretion. Subjects were instructed to maintain a consistent dietary pattern throughout the duration of the study. After an overnight fast, at 0500 on the morning of *day 1* (rest) of the protocol, subjects had an 18-gauge catheter inserted into a dorsal hand vein, which was kept patent with a 0.9% saline drip. This hand was also warmed with a heating pad to "arterialize" the blood sample. Another 18-gauge catheter was inserted in a contralateral forearm vein for a primed constant infusion of isotopically labeled amino acids. The catheters were inserted in positions that were not occluded by arm bending. Infusion of [ $^2\text{H}_5$ ]phenylalanine was begun at ~0600 ( $t = 0$ ). The initial (baseline) percutaneous muscle biopsy was taken from the lateral vastus at ~0800 ( $t = 120$ ). The muscle biopsy was taken under suction after subcutaneous adminis-

tration of 1% lidocaine. Baseline and all other muscle samples were blotted dry, frozen in liquid nitrogen, and stored at  $-80^\circ\text{C}$  before analysis. Immediately after the biopsy a primed constant infusion of  $^{15}\text{N}$ -labeled phenylalanine was initiated. Blood samples were drawn before and throughout the infusion protocol at  $t = 0, 120, 180, 210, 240, 260, 280,$  and 300 min (Fig. 1) for determination of the tracer-to-tracee ratio. In addition, serum samples were drawn at  $t = 300$  min for determination of serum creatine kinase activity. After the blood sample at  $t = 240$  min, the  $^{15}\text{N}$ -phenylalanine infusion was terminated for the determination of muscle protein FBR according to the method of Zhang et al. (34). After termination of the  $^{15}\text{N}$ -phenylalanine infusion, muscle biopsies were taken from the same incision made previously ( $t = 120$ ), at  $t = 280$  (40 min after termination of the infusion; Fig. 1) and  $t = 300$  min (60 min after termination of the infusion; Fig. 1). On subsequent days, muscle biopsies were taken from the opposite leg. Moreover, when biopsies were taken from the same leg, the incision was made  $\geq 5$  cm proximal to the initial incision. Care was also taken to ensure that the positioning of the biopsy needle would not sample tissue from a previous biopsy site by ensuring that the biopsy needle was proximal or distal from the incision.

After the last biopsy and blood sample, subjects consumed a meal at the CRC before being discharged. Subjects collected 24-h urine samples beginning at 0500 on *day 1* (rest) for the duration of the study. Urine samples were analyzed for creatinine, urea, and 3-methylhistidine (3-MH) concentration. The same infusion protocol was repeated on *days 1* (rest), 3 (24 h postexercise), and 4 (48 h postexercise), with the exception of the 2nd day (~3 h postexercise), when subjects performed the exercise test between  $t = 0$  and  $t = 120$  min of the infusion of the protocol. The exercise test took place at the University of Texas Medical Branch Alumni Field House. An infusion of [ $^2\text{H}_5$ ]phenylalanine was maintained throughout the exercise protocol, which lasted for ~45 min, by a battery-operated calibrated infusion pump (Travenol, Hooksett, NH). Subjects warmed up by cycling on a stationary cycle ergometer with no load for 10 min. Subjects then performed eight sets of eight repetitions at 80% of their predetermined concentric 1 RM. Each set was followed by 2 min of rest. Subjects who were randomized to the eccentric protocol had the weight lifted for them by the investigators. The subjects then extended their legs to full extension and lowered the weight to a resting position. The opposite was true of the concentric lifting protocol; that is, investigators lowered the weight to a resting position for the subjects while they raised the weight to full extension. All subjects, except for one in the concentric group for whom the weight was lowered to ~73% of his 1 RM after two sets, were able to complete their predetermined workload.

### Isotopes

All isotopes were dissolved in 0.9% saline before infusion. Both [ $^2\text{H}_5$ ]phenylalanine and  $^{15}\text{N}$ -phenylalanine were purchased from Cambridge Isotopes (Andover, MA). All isotopes were infused using a calibrated Harvard syringe pump (Natick, MA), and the exact infusion rate was determined by multiplying the infusate concentration, determined by gas chromatography-mass spectrometry (GC-MS), by the measured infusion rate. The infusion rates of [ $^2\text{H}_5$ ]phenylalanine and  $^{15}\text{N}$ -phenylalanine were  $0.05 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  (priming dose  $2.0 \mu\text{mol}/\text{kg}$ ). All isotopes were filtered through a  $0.2\text{-}\mu\text{m}$  filter before infusion. The infusion protocols were designed so that steady state was achieved in both muscle and plasma pools. This has been confirmed in previous studies (4) and is shown in Figs. 2 and 3.

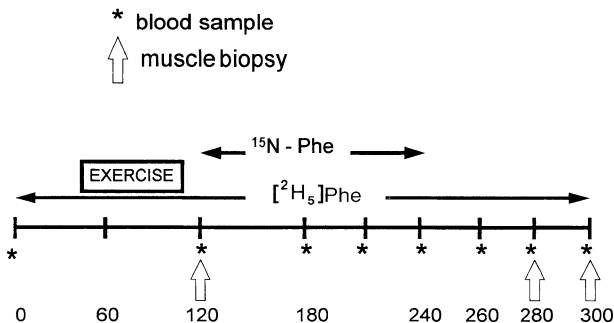


Fig. 1. Schematic representation of infusion protocol on each day. Note that exercise was performed on *day 2* of study only. All values for time are given as time postexercise (~3 h, ~24 h, and ~48 h), except for *day 1*, which was before exercise protocol (Rest).

### Analyses

**Urine.** Urine collections were pooled, and the total volume for each day was recorded. Analysis of urinary creatinine and urea concentration was performed using commercially available kits (procedures 555 and 640, respectively; Sigma Chemical, St. Louis, MO). The concentration of 3-MH in urine was determined by making the *tert*-butyl dimethylsilyl (*t*-BDMS) derivative of 3-MH, using a GC-MS (Hewlett-Packard 5890/5989B, Palo Alto, CA), and using 3-*l*-methyl-<sup>13</sup>C]MH as an internal standard (2.7 mM). The procedure for preparation of urine samples for urinary 3-MH concentration by isotope dilution was according to the procedures outlined by Rathmacher et al. (23). Briefly, ~500  $\mu$ l of urine (weighed) and ~100  $\mu$ l of internal standard (weighed) were mixed. The sample was then acidified by adding 20  $\mu$ l of 1 N HCl. The urine sample was then passed over an acid-washed cation exchange column (Dowex AG 50W-8X, 100–200 mesh, H<sup>+</sup> form; Bio-Rad Labs, Richmond, CA). The 3-MH and other amino acids were then eluted from the column with two 1.5-ml washes of 2 N ammonium hydroxide. These aliquots were then dried under vacuum using a Speed-Vac rotary drying apparatus (Savant Instruments, Farmingdale, NY). The sample was then incubated with urease (~25 U of Jack bean type IX urease; Sigma Chemical) in a phosphate buffer (30 mM, pH 7.5) for 3 h at 37°C. The sample was again lyophilized to dryness. To the dried sample, 50  $\mu$ l of acetonitrile and 50  $\mu$ l *N*-methyl-*N*-(*t*-butyldimethylsilyl) trifluoro-acetamide (MTBSTFA; Pierce Chemical, Rockford, IL) were added, and the sample was heated at 90°C for 1 h. The sample was then analyzed using GC-MS (Hewlett-Packard 5890, series II; Fullerton, CA) by injecting 1  $\mu$ l and monitoring ions of mass-to-charge ratio (*m/z*) 238 (*m*+0) and 239 (*m*+1) to determine the concentration of 3-MH by use of the internal standard method (3).

**Blood.** Blood samples for determination of amino acid enrichment and concentration were immediately precipitated in preweighed tubes containing 15% sulfosalicylic acid, which contained a weighed amount of internal standard. The internal standard used was [<sup>2</sup>H<sub>8</sub>]phenylalanine (85.5  $\mu$ M), added in a ratio of 100  $\mu$ l/ml of blood. To determine the enrichment of infused phenylalanine and internal standards in whole blood, the *t*-BDMS derivative of phenylalanine was made according to previously described procedures (3). Analysis of *t*-BDMS phenylalanine by GC-MS (Hewlett-Packard 5890, series II) was performed using electron impact ionization and selected ion monitoring of *m/z* 234, 235, 239, and 240, for the *m*+0, *m*+1, *m*+5, and *m*+6 ions, respectively. Appropriate corrections were made for any spectra that overlapped and contributed to the tracer (*t*)-to-tracee (*T*) ratio (*t/T*) (31). Concentration of phenylalanine in both blood and the muscle free pool was calculated as described previously (3) and with the assumption that interstitial water accounts for 13% of the water content in muscle (3).

Serum samples were analyzed for creatine phosphokinase (CPK) activity with a commercially available kit (no. 661; Sigma Chemical, St. Louis, MO). CPK activity is expressed in units (U) per milliliter, where one unit of CPK will phosphorylate one nanomole of creatine per minute at 25°C (Sigma Chemical definition). The intra-assay coefficient of variation (CV) for this assay did not exceed 11% for any given day, and the interassay CV was no greater than 13%.

**Muscle.** Muscle biopsy tissue samples were analyzed for protein-bound and free intracellular enrichment, as well as intracellular concentration, as described previously (3, 4). Briefly, each sample was weighed and muscle protein was precipitated with 800  $\mu$ l of 14% perchloric acid (PCA). An

internal standard (2  $\mu$ l/mg tissue) containing 3.3  $\mu$ M L-[ring-<sup>13</sup>C<sub>6</sub>]phenylalanine was added to the precipitate. The tissue was then homogenized and centrifuged. The supernatant was collected, and this procedure was repeated twice more with additional 500- $\mu$ l washes of 14% PCA. The remaining pellet of muscle tissue was washed in distilled deionized H<sub>2</sub>O, washed three times in absolute ethanol, and then placed in a 50°C oven to dry overnight. The dried pellet was placed in 6N HCl and hydrolyzed for 24 h at 110°C. The protein hydrolysate was then deionized using ion exchange columns as described for blood analysis (3, 4). Briefly, an aliquot of the acid hydrolysate (~4 mg wet weight of muscle) was passed over an acid-washed cation exchange column (Dowex AG 50W-8X, 100–200 mesh, H<sup>+</sup> form; Bio-Rad Labs). The pooled PCA washes (~1.3 ml) were prepared in the same manner as the protein-bound acid hydrolysates for determination of the intracellular phenylalanine enrichment. Amino acids were eluted from the column with 3 ml of 3M NH<sub>4</sub>OH, and the resulting eluate was collected and dried under vacuum with a Speed-Vac rotary drying apparatus (Savant Instruments, Farmingdale, NY). To make the heptafluorobutyric (HFB) derivative of phenylalanine, 500  $\mu$ l of 3.5 N HBr propanol reagent were added to the dry residue, which was vortexed and heated at 110°C for 1 h. The sample was then dried down under a stream of dry N<sub>2</sub> gas, and 100  $\mu$ l of HFB anhydride was added to the dry residue. The sample was then heated at 60°C for 20 min and was then dried down under N<sub>2</sub>. Ethyl acetate (100  $\mu$ l) was added to resuspend the HFB-phenylalanine derivative for injection into the GC-MS. All phenylalanine enrichments were determined using chemical impact ionization with methane gas and selected ion monitoring at a variety of ions, depending on what was to be determined. Protein bound [<sup>2</sup>H<sub>5</sub>]phenylalanine enrichment was determined by monitoring *m/z* 407 and 409, which are the *m*+3 and *m*+5 enrichments, respectively, where *m*+0 is the lowest molecular weight of the ion. The ratio of *m*+5 to *m*+3 (*m*+5/*m*+3) was used, since it is much more sensitive than the traditional *m*+5/*m*+0 (used for plasma samples). Enrichment from the protein-bound samples was determined using a linear standard curve of known *m*+5/*m*+3 ratios and corrected back to the absolute change in *m*+5 enrichment over the incorporation period. Precursor enrichment for calculation of FSR was determined from intracellular [<sup>2</sup>H<sub>5</sub>]phenylalanine enrichment by monitoring the *m*+5/*m*+0 (mass 409 and 404 amu) enrichments of the HFB-phenylalanine. For calculation of FBR, the decay in intracellular enrichment of the infused <sup>15</sup>N-phenylalanine was measured by monitoring the ions of *m/z* 404 and 405 and was calculated according to the calculations and assumptions outlined by Zhang et al. (34). Muscle free phenylalanine concentration was determined using a [ring-<sup>13</sup>C<sub>6</sub>]phenylalanine internal standard (3.3  $\mu$ M) and monitoring ion of *m/z* 410 and 404 and assuming that interstitial water accounts for 13% of the water content in muscle (3).

### Calculations

FSR of muscle protein was calculated from the determination of the rate of tracer incorporation into muscle protein and with use of the muscle intracellular free phenylalanine enrichment as the precursor, according to the equation

$$\text{FSR (\%/h)} = \{[Et_1 - Et_0]/[E_p \cdot (t_1 - t_0)]\} \cdot 100 \quad (1)$$

where  $Et_0$  is the enrichment in the protein-bound phenylalanine tracer from the first biopsy at  $t = 120$  min,  $Et_1$  is the enrichment of the protein-bound phenylalanine tracer from the second or third biopsy at  $t = 280$  and  $t = 300$  min,  $(t_1 - t_0)$  is the incorporation time (~3 h); and  $E_p$  is the mean intracellular

Table 2. *Urinary indexes of protein metabolism*

	Day 1 (Rest)	Day 2 (3 h)	Day 3 (24 h)	Day 4 (48 h)
Urea, mol/day	0.46 ± 0.04	0.51 ± 0.03	0.50 ± 0.03	0.49 ± 0.05
Creatinine, mmol/day	12.7 ± 1.6	13.0 ± 2.0	12.3 ± 1.3	13.5 ± 2.0
3-MH, $\mu\text{mol}/\text{day}$	173 ± 11	180 ± 13	189 ± 14	205 ± 15
3-MH/creatinine, $\mu\text{mol} \cdot \text{mmol}^{-1} \cdot \text{day}^{-1}$	14.4 ± 1.0	15.6 ± 1.9	15.9 ± 1.0	17.1 ± 2.2

Values are means ± SE;  $n = 8$  subjects. 3-MH, 3-methylhistidine. Day 1 (rest), day 2 (3 h), 3 h postexercise; day 3 (24 h), 24 h postexercise; day 4 (48 h), 48 h postexercise.

lar ( $t = 120, 280,$  and  $300$ ) [ $^2\text{H}_5$ ]phenylalanine enrichment during the time period for determination of protein incorporation.

Phenylalanine was chosen as the tracer because it is not oxidized in muscle or synthesized in the body. Thus appearance of phenylalanine results entirely from protein breakdown. Whole body phenylalanine appearance was calculated according to the equation

$$R_a = F/(E_p) \quad (2)$$

where  $R_a$  is rate of appearance ( $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ),  $F$  is infusion rate ( $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ), and  $E_p$  is whole blood [ $^2\text{H}_5$ ]phenylalanine enrichment ( $t/T$ ).

By use of the tracee release method described previously (34), the intracellular dilution of both plasma and muscle amino acid enrichment after the cessation of infusion can be measured and fit to equations to measure muscle FBR. This was calculated in the current protocol by using the decay in enrichment of  $^{15}\text{N}$ -phenylalanine and the equations outlined previously (34). In calculating FBR in this manner, we have assumed that our arterializing blood samples were representative of arterial enrichments (6). We have also assumed that the arterial blood is the only source of tracer entering the muscle intracellular free pool, such that there is no tracer recycling (34), which during such a short infusion is a valid assumption. Because we did not take a biopsy immediately before stopping the  $^{15}\text{N}$ -phenylalanine infusion, we calculated the intramuscular  $^{15}\text{N}$ -phenylalanine at this time point (240 min) by using the ratio of the mean intracellular [ $^2\text{H}_5$ ]phenylalanine (calculated as the mean of 120, 280 and 300 min) to arterial [ $^2\text{H}_5$ ]phenylalanine enrichment and multiplying by the arterial  $^{15}\text{N}$ -phenylalanine enrichment (see Figs. 2 and 3). The validity of this approach has been tested in animals (X.-J. Zhang, unpublished observations). We will not discuss the mathematical assumptions or derivations of the equations necessary to calculate FBR, since this has been previously presented (34). Given the assumptions just presented, however, we were able to calculate a muscle net balance by using FSR from Eq. 1 and FBR, as

$$\text{net balance (\%/h)} = \text{FSR (\%/h)} - \text{FBR (\%/h)} \quad (3)$$

### Statistics

Data were analyzed using a two-way repeated-measures analysis of variance (ANOVA), with time (days) and group (eccentric or concentric) as the within and between factors, respectively. Statistical analysis revealed that there were no between-group differences in the measures of FSR, FBR, net balance, 3-MH excretion, or any relevant variables. Sample size analysis revealed that at least 22 subjects per group would have to be studied to detect a difference between groups. Hence, the data presented here are shown as one group, with only the time effects analyzed and with use of a one-way repeated-measures ANOVA. Wherever significant differences were found, a Tukey post hoc test was used to locate the pairwise difference. Correlations were performed

using a Pearson product correlation and analyzed according to the appropriate degrees of freedom.  $AP$  value of  $<0.05$  was considered significant. Data are expressed as means ± SE.

## RESULTS

### Urine

There was no difference in the excretion of urinary urea throughout the study (Table 2). Urinary excretion of creatinine was also unaffected during the protocol (Table 2). The absolute excretion of 3-MH ( $\mu\text{mol}/\text{day}$ ) was unchanged throughout the protocol, although the time effect ( $P = 0.084$ ) did show a trend toward an increase (Table 2). The ratio of 3-MH excretion to creatinine excretion also remained unchanged throughout the protocol ( $P = 0.18$ ; Table 2).

### Blood

Serum CPK activity remained unchanged over the course of the study (day 1 =  $6.9 \pm 0.3$ ; day 2 =  $6.8 \pm 0.4$ ; day 3 =  $8.9 \pm 0.7$ ; day 4 =  $11.2 \pm 1.9$ ;  $P > 0.4$ ).

Subjects' blood  $^{15}\text{N}$ -phenylalanine and [ $^2\text{H}_5$ ]phenylalanine enrichment throughout the protocol (for day 1/rest) is shown in Fig. 2, A and B, respectively. As Fig. 2 shows, all subjects achieved a steady state in enrich-

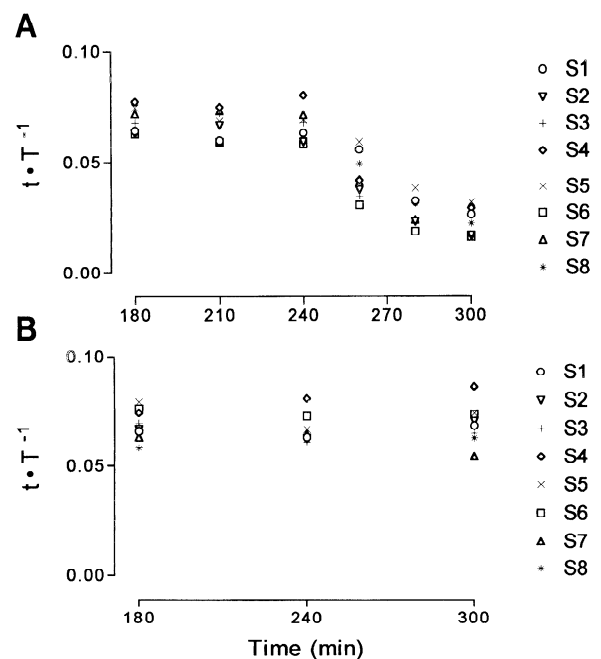


Fig. 2. Blood phenylalanine enrichment of individuals during infusion (day 1). S, subject; t, tracer; T, tracee. A: blood  $^{15}\text{N}$ -phenylalanine enrichment during infusion for each subject; B: blood [ $^2\text{H}_5$ ]phenylalanine enrichment during infusion for each subject.

ment for both tracers during the course of the study, and, more importantly, over the time course when muscle biopsies were taken. Similar patterns in enrichment were seen on all other study days (data not shown).

Whole body phenylalanine turnover was calculated from steady-state blood enrichment of [ $^2\text{H}_5$ ]phenylalanine at 180, 240, and 300 min (Fig. 2B). There was no significant difference in whole body turnover ( $R_a$ ) of phenylalanine on any study day (rest =  $0.77 \pm 0.04$ ; 3 h =  $0.77 \pm 0.04$ ; 24 h =  $0.76 \pm 0.05$ ; 48 h =  $0.78 \pm 0.04$ , all in  $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ).

### Muscle

Muscle intracellular  $^{15}\text{N}$ -phenylalanine and [ $^2\text{H}_5$ ]phenylalanine enrichment is shown in Fig. 3, A and B, respectively. The intracellular enrichments were consistently lower than arterialized enrichments, presumably because of dilution by protein breakdown (3, 4). All subjects achieved steady state in the intramuscular pool for [ $^2\text{H}_5$ ]phenylalanine enrichment; hence it appears that the 2-h infusion of  $^{15}\text{N}$ -phenylalanine was also sufficient to achieve an intracellular steady state. Intracellular enrichment of  $^{15}\text{N}$ -phenylalanine decreased in the time period between 280 and 300 min (Fig. 3A) for all subjects, allowing the calculation of FBR. Muscle intracellular phenylalanine concentration was not significantly different throughout the infusion protocol (mean of biopsies at 120, 280 and 300 min) and was not different between days (rest =  $86 \pm 16$ ; 3 h =  $93 \pm 17$ ; 24 h =  $82 \pm 10$ ; 48 h =  $76 \pm 23$  nmol/ml intracellular water;  $P > 0.6$ ).

Muscle protein FSR was calculated according to the steady-state precursor-product equation outlined in

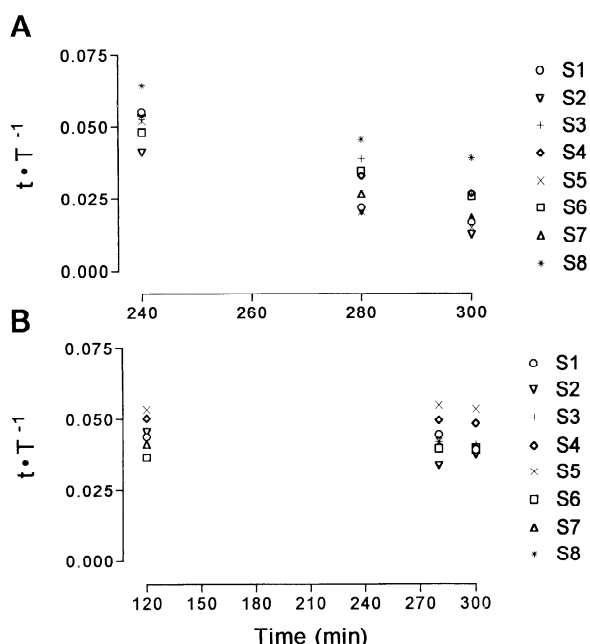


Fig. 3. Intramuscular phenylalanine enrichment of individual subjects during infusion (*day 1*). A: muscle  $^{15}\text{N}$ -phenylalanine enrichment during infusion for each subject (values at 240 min were calculated according to assumptions outlined in METHODS). B: muscle [ $^2\text{H}_5$ ]phenylalanine enrichment during infusion for each subject.

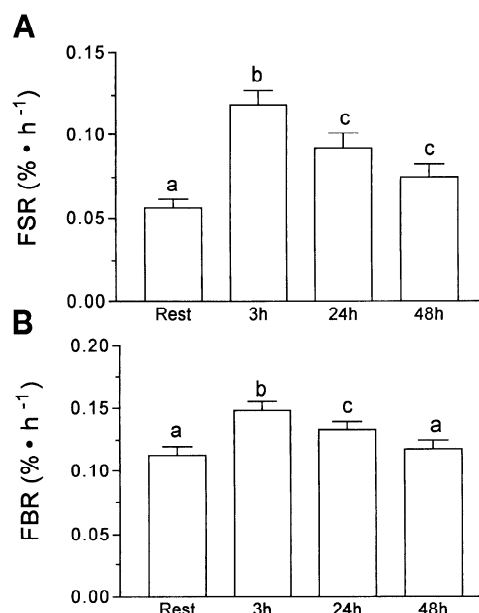


Fig. 4. Mixed muscle protein fractional synthesis rate (FSR, A) and fractional breakdown rate (FBR, B) at rest and after exercise bout. Means with different letters are statistically different ( $P < 0.05$ ). A main effect for time was found for FBR ( $P < 0.01$ ). Values are means  $\pm$  SE ( $n = 8$ ). Rest, *day 1*; 3h, 3 h postexercise; 24h, 24 h postexercise; 48h, 48 h postexercise.

METHODS (see *Eq. 1*). Muscle FSR increased from resting levels after exercise by 112% at 3 h postexercise ( $P < 0.01$ ; Fig. 4A). In addition, FSR was significantly elevated above rest at 24 and 48 h postexercise by 65 and 34% ( $P < 0.01$ ; Fig. 4A), respectively. However, there was no significant difference in FSR between 24 and 48 h postexercise (Fig. 4A).

The FBR of mixed muscle proteins was also increased after the exercise bout, at 3 h postexercise, by 31% above resting values ( $P < 0.05$ ; Fig. 4B). FBR 24 h after the exercise bout was still 18% above resting values ( $P < 0.05$ ; Fig. 4B). However, 48 h after the exercise bout, FBR had returned to resting (*day 1*) values and was not significantly different from rest (Fig. 4B).

There was a highly significant correlation ( $r = 0.88$ ;  $P < 0.001$ ) between the measured FSR and FBR (Fig. 5). Moreover, there was a significant correlation in the change in FSR and FBR between days ( $r = 0.73$ ;  $P < 0.01$ ; data not shown).

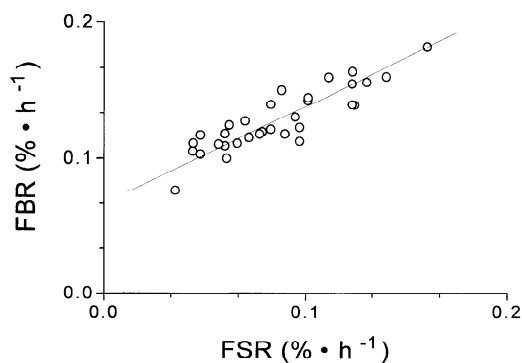


Fig. 5. Correlation between mixed muscle protein FSR and FBR ( $y = 1.22x + 0.071$ ;  $r = 0.88$ ,  $P < 0.01$ ).

Muscle protein net balance was calculated as the difference between FSR and FBR and is shown for each day during the protocol in Fig. 5. Net balance was significantly negative on all study days despite the fact that it was significantly increased at 3, 24, and 48 h postexercise vs. rest (Fig. 6). Muscle protein net balances at 3, 24, and 48 h were 48, 28, and 23% higher than at rest (Fig. 6). However, the net balance between 24 and 48 h was not significantly different (Fig. 6).

## DISCUSSION

The findings from this study have shown that a single isolated bout of concentric or eccentric resistance exercise, in untrained subjects, results in elevations in muscle FSR, FBR, and net protein balance. In addition, there was a highly significant correlation between muscle protein FSR and FBR, suggesting a tight relationship between these two processes in the fasted state. To our knowledge, this is the first study to have followed the time course of both synthesis and breakdown after a resistance exercise bout. After the exercise bout, the FBR of mixed muscle proteins was elevated at 3 and 24 h. However, FBR returned to resting levels by 48 h postexercise. In contrast, the increase in FSR persisted for  $\geq 2$  days after the exercise session. The result of the elevation in FSR was that the net protein balance within the exercised muscle increased and was significantly higher postexercise at all time points studied. It should be emphasized that all studies in the present protocol were performed in the fasted state, so it was expected that muscle net balance would be negative (4).

Previous studies have demonstrated that an isolated bout of resistance exercise resulted in an increase in the mixed muscle protein synthetic rate (4, 8, 33). Moreover, Biolo et al. (4) recently confirmed these findings by use of a different method to calculate muscle protein synthesis. The rate of biceps brachii muscle protein synthesis after an isolated bout of resistance exercise, in trained subjects, was reported to be increased 50% above resting at 4 h and 109% at 24 h postexercise (8). Subsequently, results from the same group showed that, at 36 h postexercise, muscle mixed FSR had returned to within 14% of the FSR in nonexercised muscle (19), leading the authors to postulate that FSR was initially increased but then abruptly de-

creased at 36 h postexercise (19). These findings are different from those of the current study, in which FSR peaked at 3 h postexercise and was still elevated at 48 h postexercise (Fig. 3A). The discrepancy may be due to the training status of the subjects, since the time course of muscle FSR after exercise may be different in trained vs. untrained subjects. In addition, the time course reported previously (8, 19) was constructed from three independent groups of subjects, whereas the current study used a repeated-measures design. It should also be noted that the subjects studied at 4 and 24 h postexercise by Chesley et al. (8) were studied in the fed state, whereas the subjects at 36 h postexercise ate and then slept (19). Relevant to this point, we have found that an infusion of mixed amino acids after exercise, to elevate blood amino acid concentrations to postprandial levels, stimulated synthesis more than exercise alone so that, in contrast to exercise alone, net balance became positive (5). Moreover, we have shown that oral amino supplementation, with 40 g of mixed amino acids after resistance exercise, results in a positive net balance and increases in amino acid uptake by muscle (26).

The specific nature of the muscle proteins being synthesized is not distinguishable when the present technique is used to measure FSR. An elevation in mixed muscle protein FSR, which represents an average synthetic rate of all myocellular proteins, has been observed after resistance exercise (4, 8, 33; present results). However, by weight myofibrillar proteins comprise  $\sim 60\%$  of all muscle proteins (28). Hence, given the magnitude of the increase in mixed protein FSR that we (4; present results) and others (8, 33) have observed, it seems likely that an increase in myofibrillar protein synthetic rate must have occurred. In contrast to this conclusion, a report in which myofibrillar protein synthesis was examined (primarily actin and myosin, but also including troponin, tropomyosin, C protein, and titin) reported no change in myofibrillar protein synthesis at 24 h postexercise in subjects who had completed a 3-mo resistance training program (29). However, we recently reported that there was no change in mixed muscle FSR after a bout of resistance exercise in trained swimmers, all of whom were regularly engaging in both resistance and endurance exercise (27). Hence, the fact that there was no increase in mixed muscle FSR (27) and myofibrillar protein FSR (29) in trained subjects may be due to the training status of the subjects. Given the results of the present study, it may be that the response of muscle protein FBR, which would be the predominant source of amino acids for synthesis (FSR) in the fasted state, may be reduced in trained individuals.

Muscle myofibrillar protein breakdown after resistance exercise has been estimated using indirect measures such as 3-MH excretion. Because 3-MH is found exclusively in actin and myosin and cannot be reutilized once the protein is broken down, its appearance in urine serves as a marker of myofibrillar protein degradation (22, 23). Although the use of 3-MH has been criticized, because of the unknown contribution of gut

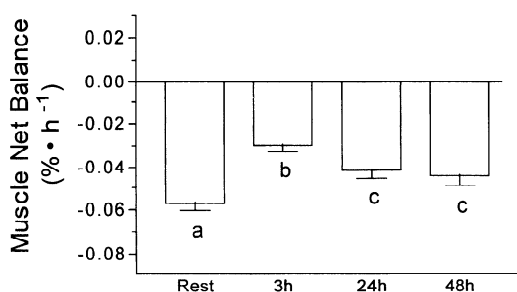


Fig. 6. Muscle protein net balance (FSR minus FBR) at rest and after exercise bout. Means with different letters are statistically different ( $P < 0.05$ ). Values are means  $\pm$  SE ( $n = 8$ ). See Fig. 4 for definitions of legend.

myofibrillar protein to 3-MH turnover, recent evidence suggests that it is a reliable index of muscle myofibrillar breakdown (22, 24). However, results from previous studies have been inconsistent, with some showing that exercise results in an increase in (11, 16) and some showing that it does not change (32, 33) 3-MH excretion. In the present study we were unable to detect a significant change in either total 3-MH excretion or 3-MH excretion expressed relative to creatinine excretion (Table 2). This is somewhat surprising, given the increase in muscle FBR that occurred at 3 and 24 h postexercise. However, it may be that a 24-h urine collection to measure 3-MH excretion is a relatively insensitive marker of myofibrillar degradation. This notion is supported by data showing that a single isolated bout of weight lifting did not increase 3-MH excretion (32), whereas daily performance of resistance exercise resulted in increased 3-MH excretion only after the third bout (16). In addition, Fielding et al. (13) did not report an increase in 3-MH excretion after an intense bout of eccentric cycling until 10 days postexercise, despite a significant elevation in leucine appearance (protein breakdown).

We recently described an isotopic technique to measure mixed muscle protein FBR (34). The results from this study show that muscle protein breakdown rate is accelerated after resistance exercise, supporting our earlier findings using a different technique (4). Muscle protein FBR was not different between eccentric and concentric contractions. This may be surprising given the myofibrillar disruption that is generally observed after eccentric work (12, 15), which one might expect to be associated with greater rate of protein breakdown. However, if we assume that there is a relationship between muscle protein breakdown and eccentric exercise, the eccentric exercise in the present study may not have been severe enough to cause damage that would result in a greater increase in FBR after the eccentric, vs. concentric, contractions. Evidence in support of this can be seen in the comparatively moderate, and heterogeneous, responses of serum CPK. In addition, electron micrographs of longitudinal muscle sections (two subjects per condition) showed no significant difference in the degree of myofibrillar damage between groups, and in fact showed very little damage at all (data not shown). Therefore, it is likely that the exercise was simply not severe enough, for these subjects, to induce different degrees of muscle damage between the two groups. This is an indication that the two groups of subjects were simply habitually performing enough eccentric leg work in other activities (cycling, running, stairmaster). It is well documented that the "protective" effect of eccentric exercise is long lasting (12, 13). Despite there being no differences between the groups, however, these data show that both FSR and FBR are increased regardless of the type of contraction performed.

A recent report indicated that muscle protein breakdown, measured as tyrosine release, after eccentric contraction-induced muscle injury did not increase significantly until 48 h postexercise and remained

elevated for  $\geq 5$  days (18). The difference in time course of muscle protein breakdown in the previous study (18), compared with the current study, likely relates to the methodology for inducing and measuring protein breakdown. In support of a more rapid response of protein breakdown after exercise, Balon et al. (1) found that tyrosine and 3-MH release were increased only 30 min after exercise. In the present study, the response of breakdown was found to be elevated by 30% at  $\sim 3$  h postexercise, and this response was attenuated at 24 h to 18% above resting levels (Fig. 3B). These results suggest a rapid postexercise activation of whatever mechanism is responsible for the increase in muscle protein degradation.

Eccentric activity has been shown to result in activation of neutral proteases such as calpain (2). It is unknown, however, whether the same is true of concentric activity. It is conceivable that these exercise-activated proteases are responsible for the muscle protein degradative response observed after exercise (4, 11, 21; present results). Eccentric contractions also result in the loss (breakdown) of muscle cytoskeletal proteins (17). If muscular contractions result in an activation of neutral proteases (2), then a sustained increase in intracellular calcium, induced by the exercise bout, could play a role in this process (12).

Despite the large increase in muscle protein FBR, there was no corresponding change in whole body protein turnover, as reflected by phenylalanine  $R_a$ . Similar disparities in muscle FSR and rates of whole body protein turnover have been documented in the postexercise period by others (4, 7, 8, 25, 27). These observations have been interpreted as indicating that whole body protein synthesis must have changed in the opposite direction from muscle protein synthesis (8, 25). This is based on the assumption that muscle protein synthesis accounts for  $\sim 25$ –30% of whole body protein synthesis (20). However, we have shown that a 50% increase in muscle protein breakdown was accompanied by only a 5% increase in whole body protein breakdown (4). This likely reflects the insensitive nature of the measurement of whole body protein turnover, rather than a change occurring in the opposite direction somewhere else in the body (splanchnic region). This interpretation is supported by our finding that exercise stimulated splanchnic protein breakdown in the dog (30). A possibility is that the lack of sensitivity of the whole body methodology, over a relatively short time interval, may be due to transient changes in tissue pool size. These changes would obscure any direct relation between protein breakdown and the  $R_a$  into plasma of essential amino acids.

Given the rapid nature of the increase in muscle protein FSR seen in the present study, it is likely a posttranscriptionally regulated response to the exercise stimulus. This is supported by the finding that an increase in the synthetic response after exercise is unrelated to the concentration of muscle RNA, which remains unchanged even up to 24 h postexercise (8). It should be noted, however, that large increases in specific mRNAs could occur quite rapidly after exercise

without a significant change in total RNA concentration. Large increases in mRNA are not always accompanied, however, by equivalent, or even proportional, increases in protein synthesis. The results of the present study are consistent with our previous postulation, that at least some of the control of the protein synthetic response after exercise is related to increased intracellular amino acid availability (4).

Previously, we reported that, after exercise subjects had an increased inward transport of alanine, leucine, and lysine, but not phenylalanine, from blood to the muscle intracellular pool (4). In the fasted condition, the only available sources of amino acids for synthesis would be from inward transport from the circulation and from protein degradation. Furthermore, the inward flux of amino acids to the muscle could only be increased for a finite time; otherwise hypoaminoacidemia would result. Assuming that in the current study phenylalanine influx was unchanged after exercise (4), then the present results show that exercise increases the intracellular utilization of phenylalanine, and presumably other amino acids, for synthesis. The close correlation between FSR and FBR provides support for the idea that, in the fasted state, one process drives the other. Which process is the driving force is currently unknown and may depend on a number of factors, such as training, nutritional status, and time postexercise. It does appear, however, as if one of the primary regulators of the postexercise response of FBR and FSR is intracellular amino acid availability. Future studies will need to focus on the regulation of breakdown, synthesis, and transmembrane amino acid flux as regulators of the muscle intracellular amino acid pool and subsequent protein synthesis.

The authors thank the nurses and staff of the Clinical Research Centre (CRC) at the University of Texas Medical Branch, Galveston, for their time and competent technical assistance. The medical support and technical support and advice of Drs. A. A. Ferrando and J. Cortiela are also greatly appreciated. Thanks also to all the volunteers who participated in this project.

This study was supported by National Institutes of Health (NIH) Grant 38010 to R. R. Wolfe. The CRC at the University of Texas Medical Branch is supported by NIH grant M01-0073. S. M. Phillips is supported by a postdoctoral fellowship from the National Sciences and Engineering Research Council of Canada.

Address for reprint requests: R. R. Wolfe, Metabolism Unit, Shriners Burns Inst., 815 Market St., Galveston, TX 77550-2725.

Received 16 December 1996; accepted in final form 27 February 1997.

## REFERENCES

- Balon, T. W., A. Zorzano, J. L. Treadway, M. N. Goodman, and N. B. Ruderman. Effect of insulin on protein synthesis and degradation in skeletal muscle after exercise. *Am. J. Physiol.* 258 (*Endocrinol. Metab.* 21): E92-E97, 1990.
- Belcastro, A. N. Skeletal muscle calcium-activated neutral protease (calpain) with exercise. *J. Appl. Physiol.* 74: 1381-1386, 1993.
- Biolo, G., R. Y. D. Fleming, S. P. Maggi, and R. R. Wolfe. Transmembrane transport and intracellular kinetics of amino acids in human skeletal muscle. *Am. J. Physiol.* 268 (*Endocrinol. Metab.* 31): E75-E84, 1995.
- Biolo, G., S. P. Maggi, B. D. Williams, K. D. Tipton, and R. R. Wolfe. Increased rates of muscle protein turnover and amino acid transport after resistance exercise in humans. *Am. J. Physiol.* 268 (*Endocrinol. Metab.* 31): E514-E520, 1995.
- Biolo, G., K. D. Tipton, S. Klein, and R. R. Wolfe. An abundant supply of balanced amino acids synergistically enhances protein muscle anabolism after exercise. *Am. J. Physiol.* 273 (*Endocrinol. Metab.* 36): E122-E129, 1997.
- Brooks, D. C., P. R. Black, M. A. Arcangeli, T. T. Aoki, and D. W. Wilmore. The heated dorsal hand vein: an alternative arterial sampling site. *J. Parenter. Enteral Nutr.* 13: 102-105, 1989.
- Carraro, F., C. A. Stuart, W. H. Hartl, J. Rosenblatt, and R. R. Wolfe. Effect of exercise and recovery on muscle protein synthesis in human subjects. *Am. J. Physiol.* 259 (*Endocrinol. Metab.* 22): E470-E476, 1990.
- Chesley, A., J. D. MacDougall, M. A. Tarnopolsky, S. A. Atkinson, and K. Smith. Changes in human muscle protein synthesis after resistance exercise. *J. Appl. Physiol.* 73: 1383-1388, 1992.
- Devlin, J. T., I. Brodsky, A. Scrimgeour, S. Fuller, and D. M. Bier. Amino acid metabolism after intense exercise. *Am. J. Physiol.* 258 (*Endocrinol. Metab.* 21): E249-E255, 1990.
- Dohm, G. L., R. G. Israel, R. I. Breedlove, R. T. Williams, and E. W. Askew. Biphasic changes in 3-methylhistidine excretion in humans after exercise. *Am. J. Physiol.* 248 (*Endocrinol. Metab.* 11): E588-E592, 1985.
- Dohm, G. L., R. T. Williams, G. J. Kasperek, and A. M. van Rij. Increased excretion of urea and  $N^7$ -methylhistidine by rats and humans after a bout of exercise. *J. Appl. Physiol.* 52: 27-33, 1982.
- Evans, W. J., and J. G. Cannon. The metabolic effects of exercise-induced muscle damage. In: *Exercise and Sport Sciences Reviews*, edited by J. O. Holloszy. Baltimore, MD: Williams and Wilkins, 1991, p. 99-125.
- Fielding, R. A., C. N. Meredith, K. P. O'Reilly, W. R. Frontera, J. G. Cannon, and W. J. Evans. Enhanced protein breakdown after eccentric exercise in young and older men. *J. Appl. Physiol.* 71: 674-679, 1991.
- Friden, J., and R. L. Lieber. Structural and mechanical basis of exercise induced muscle injury. *Med. Sci. Sports Exercise* 24: 521-530, 1992.
- Gibala, M. J., J. D. MacDougall, M. A. Tarnopolsky, W. T. Stauber, and A. Elorriaga. Changes in human skeletal muscle ultrastructure and force production after acute resistance exercise. *J. Appl. Physiol.* 78: 702-708, 1995.
- Hickson, J. F., I. Wolinsky, G. P. Rodriguez, J. M. Pivarnick, M. C. Kent, and N. W. Shier. Failure of weight training to affect urinary indices of protein metabolism in men. *Med. Sci. Sports Exercise* 18: 563-567, 1986.
- Lieber, R. L., M. C. Schmitz, D. K. Mishra, and J. Fridén. Contractile and cellular remodeling in rabbit skeletal muscle after cyclic eccentric contractions. *J. Appl. Physiol.* 77: 1926-1934, 1994.
- Lowe, D. A., G. L. Warren, C. P. Ingalis, D. B. Boorstein, and R. B. Armstrong. Muscle function and protein metabolism after initiation of eccentric contraction-induced injury. *J. Appl. Physiol.* 79: 1260-1270, 1995.
- MacDougall, J. D., M. J. Gibala, M. A. Tarnopolsky, J. R. MacDonald, S. A. Interisano, and K. E. Yarasheski. The time course for elevated muscle protein synthesis following heavy resistance exercise. *Can. J. Appl. Physiol.* 20: 480-486, 1995.
- Nair, K. S., D. Halliday, and R. C. Griggs. Leucine incorporation into mixed skeletal muscle protein in humans. *Am. J. Physiol.* 254 (*Endocrinol. Metab.* 17): E208-E213, 1988.
- Pivarnick, J. M., J. F. Hickson, and I. Wolinsky. Urinary 3-methylhistidine excretion increases with repeated weight training exercise. *Med. Sci. Sports Exercise* 21: 283-287, 1989.
- Rathmacher, J. A., P. J. Flakoll, and S. L. Nissen. A compartmental model of 3-methylhistidine metabolism in humans. *Am. J. Physiol.* 269 (*Endocrinol. Metab.* 32): E193-E198, 1995.
- Rathmacher, J. A., G. A. Link, P. J. Flakoll, and S. L. Nissen. Gas chromatographic/mass spectrometric analysis of stable isotopes of 3-methylhistidine in biological fluids: application to plasma kinetics in vivo. *Biomed. Mass. Spectrom.* 21: 560-566, 1992.
- Sjölin, J., H. Stjernström, S. Henneberg, E. Andersson, J. Martensson, G. Friman, and J. Larsson. Splanchnic and

- peripheral release of 3-methylhistidine in relation to its urinary excretion in human infection. *Metabolism* 38: 23–29, 1989.
5. **Tarnopolsky, M. A., S. A. Atkinson, J. D. MacDougall, B. B. Senor, P. W. R. Lemon, and H. Swarczew.** Whole body leucine metabolism during and after resistance exercise in fed humans. *Med. Sci. Sports Exercise* 23: 326–333, 1991.
  6. **Tipton, K. D., A. A. Ferrando, S. M. Phillips, D. J. Doyle, J. Cortiella, and R. R. Wolfe.** Response of muscle protein metabolism to ingestion of an oral amino acid solution following resistance exercise (Abstract). *The Physiologist* 39: A-12, 1996.
  7. **Tipton, K. D., A. A. Ferrando, B. D. Williams, and R. R. Wolfe.** Muscle protein metabolism in female swimmers after a combination of resistance and endurance exercise. *J. Appl. Physiol.* 81: 2034–2038, 1996.
  8. **Welle, S., C. Thornton, R. Jozefowicz, and M. Statt.** Myofibrillar protein synthesis in young and old men. *Am. J. Physiol.* 264 (*Endocrinol. Metab.* 27): E693–E698, 1993.
  9. **Welle, S., C. Thornton, and M. Statt.** Myofibrillar protein synthesis in young and old human subjects after three months of resistance training. *Am. J. Physiol.* 268 (*Endocrinol. Metab.* 31): E422–E427, 1995.
  30. **Williams, B. D., R. R. Wolfe, D. P. Bracy, and D. H. Wasserman.** Gut proteolysis contributes essential amino acids during exercise. *Am. J. Physiol.* 270 (*Endocrinol. Metab.* 33): E85–E90, 1996.
  31. **Wolfe, R. R.** *Radioactive and Stable Isotope Tracers in Biomedicine.* New York: Wiley-Liss, 1992.
  32. **Wong, T. S., and F. W. Booth.** Protein metabolism in rat gastrocnemius muscle after stimulated chronic concentric exercise. *J. Appl. Physiol.* 69: 1709–1717, 1990.
  33. **Yarasheski, K. E., J. J. Zachwieja, and D. M. Bier.** Acute effects of resistance exercise on muscle protein synthesis rate in young and elderly men and women. *Am. J. Physiol.* 265 (*Endocrinol. Metab.* 28): E210–E214, 1993.
  34. **Zhang, X.-J., D. L. Chinkes, Y. Sakurai, and R. R. Wolfe.** An isotopic method for measurement of muscle protein fractional breakdown rate in vivo. *Am. J. Physiol.* 270 (*Endocrinol. Metab.* 33): E759–E767, 1996.

