

Whey and casein labeled with L-[1-¹³C]leucine and muscle protein synthesis: effect of resistance exercise and protein ingestion

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Reitelseder S, Agergaard J, Doessing S, Helmark IC, Lund P, Kristensen NB, Frystyk J, Flyvbjerg A, Schjerling P, van Hall G, Kjaer M, Holm L. Whey and casein labeled with L-[1-¹³C]leucine and muscle protein synthesis: effect of resistance exercise and protein ingestion. *Am J Physiol Endocrinol Metab* 300: E231–E242, 2011. First published November 2, 2010; doi:10.1152/ajpendo.00513.2010.—Muscle protein turnover following resistance exercise and amino acid availability are relatively well described. By contrast, the beneficial effects of different sources of intact proteins in relation to exercise need further investigation. Our objective was to compare muscle anabolic responses to a single bolus intake of whey or casein after performance of heavy resistance exercise. Young male individuals were randomly assigned to participate in two protein trials ($n = 9$) or one control trial ($n = 8$). Infusion of L-[1-¹³C]leucine was carried out, and either whey, casein (0.3 g/kg lean body mass), or a noncaloric control drink was ingested immediately after exercise. L-[1-¹³C]leucine-labeled whey and casein were used while muscle protein synthesis (MPS) was assessed. Blood and muscle tissue samples were collected to measure systemic hormone and amino acid concentrations, tracer enrichments, and myofibrillar protein synthesis. Western blots were used to investigate the Akt signaling pathway. Plasma insulin and branched-chain amino acid concentrations increased to a greater extent after ingestion of whey compared with casein. Myofibrillar protein synthesis was equally increased 1–6 h postexercise after whey and casein intake, both of which were higher compared with control ($P < 0.05$). Phosphorylation of Akt and p70^{S6K} was increased after exercise and protein intake ($P < 0.05$), but no differences were observed between the types of protein except for total 4E-BP1, which was higher after whey intake than after casein intake ($P < 0.05$). In conclusion, whey and casein intake immediately after resistance exercise results in an overall equal MPS response despite temporal differences in insulin and amino acid concentrations and 4E-BP1.

milk protein; intrinsically labeled protein; myofibrillar protein synthesis

MUSCLE MASS CAN BE CONSIDERED one of the most important components of the human body by the way it serves function both on the musculoskeletal level generating force and movements but also as a major site of metabolism. Next to that, it

holds great reservoirs of amino acids (AAs) needed for crucial syntheses in stressed situations with limited exogenous supplies (58). Therefore, maintenance of muscle mass is of utmost importance during lifespan.

Resistance exercise, either alone (5, 13, 47) or in combination with AAs or whole proteins (6, 11, 44), is able to increase muscle protein synthesis (MPS), and as a consequence of repeated training sessions muscle mass and strength will increase (1, 29, 30). Furthermore, it has been shown that 20 g of protein seems to be sufficient for maximal stimulation of MPS (15, 44).

Bovine milk proteins (~20% whey and ~80% casein) are of the highest quality, holding a complete profile of essential AAs (EAA) (55), and it has been shown that milk ingestion is superior in elevating MPS compared with soy protein when ingested after resistance exercise (56). Furthermore, milk appears to be a good protein supplement over the course of a long training period (27). Whey has been characterized as “fast” protein and casein as “slow” protein because of their digestion and absorption rates, which on a whole body level influence leucine oxidation and nonoxidative leucine disposal, with casein showing the best leucine net balance during the postprandial period (8). Consequently, digestion and absorption rates can be considered independent factors regulating postprandial protein kinetics (16), which may relate to differences in systemic AA profiles after intake (12).

However, whole body measurements under resting conditions cannot be transferred directly to skeletal muscle protein turnover after exercise. Only a few studies have investigated the muscular response to whey and casein after resistance exercise, with somewhat different and inconclusive results (50, 51). The study by Tang et al. (50) observed a very different response between whey hydrolysate and micellar casein both at rest and after exercise measured with the direct incorporation technique. By contrast, the study by Tipton et al. (51) did not show any clear differences between whey and casein measured as net balances of leucine and phenylalanine across the exercised leg. Furthermore, it appears that the insulin and AA concentration responses to the casein supplements in the two mentioned studies seem to differ, although the protein amounts are very alike (50, 51).

Despite clear advantages of using stable isotope-labeled substrates, certain limitations need to be kept in mind. Thus, in

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the direct incorporation model the tracer must behave as the tracee, and the level of tracer enrichment needs to be in steady state (59). When AA concentrations increase in the circulation from exogenous sources, e.g., from feeding interventions, it becomes a methodological challenge to maintain steady state. The supply of a single bolus of protein markedly disturbs these two assumptions by diluting the tracer enrichment as a reflection to the AA uptake, making precursor enrichment levels uncertain (10). In contrast, by using intrinsically labeled proteins even large single doses will theoretically not disturb the systemic tracer enrichment as long as the infusion rate of the similarly labeled AAs is adjusted to the intrinsic enrichment of the ingested protein. Previously, milk proteins have been intrinsically labeled in sufficient amounts to allow for human experiments (9, 53).

With the present study we aimed to 1) enrich milk proteins with L-[1-¹³C]leucine in a cow model and 2) take advantage of [¹³C]whey and [¹³C]casein to compare the specific muscle anabolic responses of the two different kinds of milk proteins after a single bout of heavy resistance exercise. Our hypothesis was that a single bolus of whey would induce a rapid but transient increase in muscle protein synthesis, whereas casein on the other hand would induce a more moderate but long-lasting response.

MATERIALS AND METHODS

Subjects. Seventeen healthy male subjects were randomized to participate in either two protein trials in randomized order or one control trial {protein trial group, $n = 9$; control trial group, $n = 8$ [means \pm SE for protein and control group, respectively; age: 28 ± 2 and 26 ± 2 yr (not significant); weight: 79 ± 3 and 74 ± 2 kg (not significant); body mass index: 24.3 ± 0.7 and 22.7 ± 0.7 kg/m² (not significant); lean body mass (LBM): 58 ± 2 and 57 ± 2 kg (not significant); 1-repetition maximum (1RM): 66 ± 4 and 67 ± 4 kg (not significant)]. All participants were recruited with the criteria of being moderately active young males with no history of regular participation in aerobic or resistance training during the last 6 mo. All participants were non-smokers and on a normal Western diet that was adequate with regard to protein content (a minimum of 0.8 g·kg⁻¹·day⁻¹ was considered adequate) and energy. Furthermore, no chronic medication use or family histories of diabetes were allowed. Before inclusion, study design, purpose, and possible risks were explained to each subject. Subsequently, all subjects gave their written consent to participate in

the protocol, which adhered to the Declaration of Helsinki and was approved by the Ethics Committee of Copenhagen and Frederiksberg (H-KF 2007-0014).

Pretests and food registration. The pretests were performed on 2 separate days. The aim of the 1st day was to familiarize the subjects to test equipment and protocol, and subsequently on the second day determination of 1RM was conducted. The 2 test days were separated by ≥ 1 wk and the 1RM determination was ≥ 2 wk prior to the experiment. On the 2nd test day, all subjects were dual-energy X-ray absorptiometry scanned (Lunar DPX-IQ; GE Healthcare, Chalfont St. Giles, UK) before the strength testing. Furthermore, on the 2nd day, all subjects were carefully instructed to weigh and register their food and drink intake during the 3 consecutive days prior to the experiment; however, they had to maintain their normal daily routines and intake of nutrients. Special food registration diaries were used, and the collected data were analyzed with Dankost 3000 software (Dansk Catering Center, Herlev, Denmark). All subjects were also instructed not to perform any strenuous activity during the 3 preexperimental days.

Experimental protocol. The experiment was designed as an acute exercise trial with a preexercise baseline measurement and postexercise measurements for 6 h. All subjects arrived to the laboratory by car at 7 AM after an overnight fast (10 h). An antecubital venflon was inserted and a background sample obtained. The experiment was conducted as outlined in Fig. 1.

At time -180 min (time 0 denotes the end of the exercise session), the primed (15 μ mol/kg) continuous (15 μ mol·kg⁻¹·h⁻¹) infusion of L-[1-¹³C]leucine was started. An infusion pump (AVI270 Infusion Pump, AVI; 3M Health Care, St. Paul, MN) controlled the infusion rate of tracer NaCl solution that was set to 60 ml/h. L-[1-¹³C]leucine [99 atom% excess (APE)] was purchased from Cambridge Isotope Laboratories (Andover, MA). All isotopes were dissolved into 0.9% NaCl using sterile techniques, and mixed solutions were filtered through disposable, sterile, nonpyrogenic bacteria filters with 0.20 - μ m pore size (Sartorius, Hannover, Germany). This infusion design was assumed to allow equilibration of the free leucine tracer in blood and muscle and precursor for protein synthesis after 90 min.

After the isotopic background sampling and start of tracer infusion, the femoral artery and vein of the exercise leg were cannulated under local anesthetic treatment (lidocaine, 1%). Applying the Seldinger technique, 20-gauge catheters (ES-04150; Arrow, Reading, PA) were inserted and kept patent with NaCl during the experiments. On the arterial side, a pressure bag (VBM Medizintechnik, Sulz am Neckar, Germany) was inflated to maintain a pressure of ~ 200 mmHg during the saline infusions. Catheters were secured with sutures, and the sites of insertion were observed frequently throughout the experiment. In

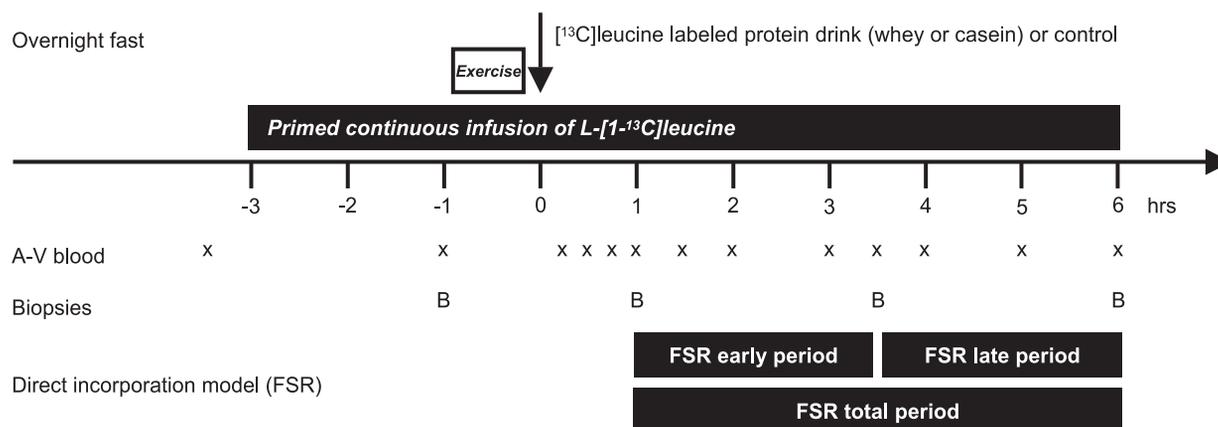


Fig. 1. Human experimental protocol. L-[1-¹³C]leucine was infused over 9 h, and a single bout of heavy resistance exercise was performed with subsequent intake of the study drink. Blood samples were collected throughout the protocol, and a total of 4 biopsies were taken in vastus lateralis of the exercised leg. A-V, arteriovenous; FSR, fractional synthesis rate.

Table 1. Amino acid concentrations in whey and casein and content in the study protein drinks

Concentration, g	100 g Protein		Protein Drink (Average)*	
	Whey	Casein	Whey	Casein
Alanine	4.66	2.84	0.82	0.50
Arginine	2.65	3.31	0.46	0.58
Asparagine	11.25	6.71	1.97	1.17
Cysteine	2.65	0.35	0.46	0.06
Glutamine	16.58	20.61	2.90	3.61
Glycine	1.78	1.73	0.31	0.30
Histidine	2.25	2.87	0.39	0.50
Isoleucine	5.72	5.03	1.00	0.88
Leucine	11.77	8.77	2.06	1.53
Lysine	9.68	7.44	1.69	1.30
Methionine	2.10	2.71	0.37	0.47
Phenylalanine	3.54	4.80	0.62	0.84
Proline	4.62	10.10	0.81	1.77
Serine	4.78	5.73	0.84	1.00
Threonine	5.05	3.97	0.88	0.70
Tryptophan	2.04	1.16	0.36	0.20
Tyrosine	3.59	5.38	0.63	0.94
Valine	5.29	6.46	0.93	1.13
Total	100	100	17.50	17.50
EAA	50.09	46.53	8.76	8.14

EAA, essential amino acids. *Protein drink amounts were adjusted to 0.30 g/kg whole body lean body mass.

five of 26 experiments (whey: $n = 3$; casein: $n = 1$; control: $n = 1$) it was not possible to insert the arterial catheter, and therefore, a second venflon was inserted into the opposite antecubital vein of the infusion.

Muscle biopsies were taken from the lateral portion of the vastus lateralis muscle in the exercise leg under local anesthetic treatment (lidocaine, 1%). The biopsies were taken with 4-mm Bergström biopsy needles (Stille, Stockholm, Sweden) with manual suction (4, 22). Blood, visible fat, and connective tissue were quickly removed from the specimen, after which the specimen was frozen in liquid nitrogen and stored at -80°C . After the frozen biopsy specimen was weighed, 25 and 20 mg were allocated to measurements of myofibrillar protein synthesis and specific proteins and protein phosphorylations in the signaling cascade downstream of the insulin/IGF-I receptor, respectively.

The acute heavy resistance exercise bout consisted of 10 sets with eight repetitions at a predetermined load corresponding to 80% of 1RM. In between sets were rest periods of 3 min. The exercise protocol was aimed at stimulating the quadriceps muscle maximally. The chosen exercise was one-legged, seated leg extension (Technogym; Super Executive Line, Gambottola, Italy) with a range of motion from 100 to 30° .

Immediately after completion of the exercise, the participants received a drink containing either water, $[^{13}\text{C}]$ whey, or $[^{13}\text{C}]$ casein. All subjects were blinded with regard to what drink they were

receiving. The amount of protein was adjusted to 0.30 g/kg LBM and was dissolved in ~ 400 ml of water. This dose was chosen because it corresponded to ~ 20 g of proteins, taking into account the individual LBM of the subjects (Table 1). The drinks were consumed within ~ 5 min.

Production of labeled milk proteins. The labeling of milk was performed in the barns at Research Centre Foulum, Department of Animal Health and Bioscience, Faculty of Agricultural Sciences, Aarhus University, Tjele, Denmark, and complied with the guidelines of the Danish Ministry of Justice (Act No. 726, 1993) with respect to animal experimentation and care of animals under study. During the production of labeled milk proteins, the cows were kept in tie stalls bedded with rubber mats. Three high-yielding Danish Holstein Friesian cows weighing 670, 617, and 767 kg were used. The cows were 49, 59, and 69 days in milk and were in their fourth, third, and third lactation, respectively. Milk and milk protein yield prior to the experiment were 52, 52, and 50 kg/day and 1,839, 1,555, and 1,610 g protein/day, respectively. Cows were offered ad libitum access to a total mixed ration that was fed at 7:30 AM, which fulfilled Danish recommendations with respect to energy and protein. Cows were fitted with 1.02-mm id, 1.78-mm od catheters (Tygon, S-54-HL; Buch & Holm, Herlev, Denmark) inserted 15 cm into both the right and left jugular veins by percutaneous venipuncture using a hypodermic needle (2.5×110 mm; Mediplast, Malmö, Sweden), followed by shaving, skin disinfection, and subcutaneous injection of 5 ml of xylocaine (20 mg/ml lidocaine; AstraZeneca, Albertslund, Denmark). The catheters were secured by skin sutures kept in place on the catheters by two cuffs (5- to 8-mm-long pieces of Tygon blue/yellow pump tubing; Buch & Holm) slid over the catheters using a pair of hemostats after removal of the hypodermic needle. Each cow received a total of 100 g of L- $[1-^{13}\text{C}]$ leucine (99 APE; Cambridge Isotope Laboratories) dissolved into 6,000 ml of 0.9% NaCl via one of the jugular vein catheters. The infusion period was scheduled for 24 h, and therefore, the infusion rate was set to 250 ml/h (Fig. 2). The other catheter was used for frequent blood sampling. Cows were separately milked in the tie stalls every 8th hour during the experiment, and a total of ~ 290 kg of $[^{13}\text{C}]$ leucine-enriched milk was obtained and further processed at the dairy company (Arla Foods, Nørre Vium, Denmark). Protein fractions of whey protein isolate and calcium caseinate were separated and purified by micro- and ultrafiltration, and a total of ~ 700 g of $[^{13}\text{C}]$ whey and $\sim 5,000$ g of $[^{13}\text{C}]$ casein was obtained. Both protein fractions were analyzed for chemical and bacteriological specifications by the dairy and showed to be suitable for human consumption. The AA compositions of the protein fractions, given by Arla Foods, are displayed in Table 1.

Whey and casein leucine enrichments. Aliquots of each protein were hydrolyzed with 6 M HCl overnight at 110°C , and subsequently, AAs were purified by cation exchange resin (Dovex AG-50W; Bio-Rad, Copenhagen, Denmark). The samples were dried under N_2 and the final whey and casein $[^{13}\text{C}]$ leucine enrichments analyzed by *tert*-butyldimethylsilyl trifluoroacetamide derivatives measuring fragment ions M (m/z 302) and M + 1 (m/z 303) by gas chromatography-mass spectrometry (GC-MS) (GC: Trace GC 2000 series; MS: Auto-

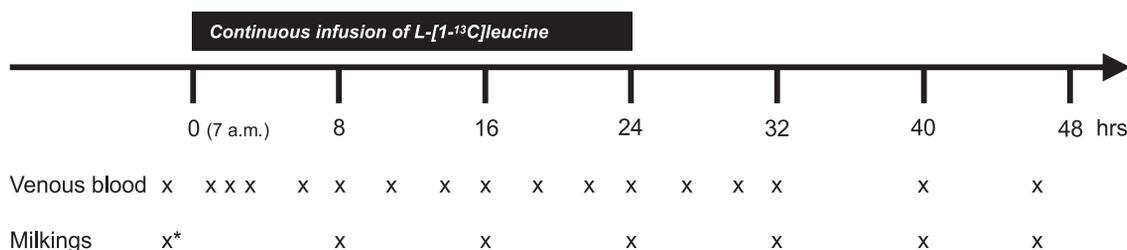


Fig. 2. Cow infusion protocol. L- $[1-^{13}\text{C}]$ leucine was infused over 24 h in 3 high-yielding Danish Holstein Friesian cows, and milk was collected every 8th hour. Venous blood samples were collected and $[1-^{13}\text{C}]$ leucine plasma enrichments measured. *Initial milking of unlabeled milk.

mass Multi; both from Thermo Quest Finnigan, Paris, France). Cow plasma samples were analyzed for [^{13}C]leucine enrichments in the same way as the protein-derived leucine. For GC-MS details, see *Stable isotope analyses*. Applying this infusion protocol, the cow plasma leucine enrichment reached a mean plateau at 22.5% [tracer/tracee ratio (TTR)] after 2 h of infusion, which lasted until the infusion was terminated. Both whey and casein showed to be 10.0% (TTR) enriched.

Blood analyses. Arterial plasma insulin concentrations were analyzed using enzyme-linked immunosorbent assay kits (cat. no. K6219; DakoCytomation, Cambridgeshire, UK), with intra-assay coefficient of variation (CV) <7.5% and interassay CV <10%. IGF-I concentrations were determined by time-resolved immunofluorometric assays (PerkinElmer, Turku, Finland), with intra- and interassay CVs <5 and 10%, respectively.

Arterial plasma AA concentrations were determined with prior phenylisothiocyanate derivatization and HPLC coupled with a UV detector [HPLC: SpectraSystem P4000, Thermo Separation Products (FinniganMat, Paris, France); column: Nova-Pak C18 60Å 4 μm , 3.9 \times 300 mm (Waters, Milford, MA); UV detector: UV6000LP PDA FINN 50 mm Cell UV photo array detector, Thermo Separation Products (FinniganMat)] (28). Norleucine was used as internal standard, and 0.05 M ammonium acetate was used as mobile phase A and 0.1 M ammonium acetate in acetonitrile-methanol-water (44:10:46) as mobile phase B (both adjusted to pH 6.8) with acetic acid and filtrated before use.

Stable isotope analyses. Determinations of plasma α -ketoisocaproate acid (KIC) enrichments were carried out on GC-MS with prior derivatization. Proteins were precipitated from 140 μl of plasma with 1 ml of ethanol (99.8%) and spun down (1,600 g, 4°C, 20 min), and the supernatant was evaporated under N_2 at 50°C. With 200- μl acidified 2% wt/vol *o*-phenylenediamine in 200 μl of Millipore water, the substrates were prepared for the subsequent derivatization. One milliliter of ethylacetate was added, and the ethylacetate phase was dried under N_2 after centrifugation (1,300 g, 4°C, 10 min). Pyridine and *N,O*-bis(trimethylsilyl) trifluoroacetamide + 1% trimethylchlorosilane (no. 38831; Pierce, Bie & Berntsen & VWR International, Rødovre, Denmark) were mixed 1:1 using 50 μl and left for 30 min at room temperature to allow the derivatization. Using PTV (programmed temperature vaporization) injection mode, 1 μl of a derivatized sample was injected and carried by a constant helium flow (1.8 ml/min) into the GC-MS system and separated by a capillary column (CP-Sil 8 CB low bleed 30 m \times 0.32 mm, coating 0.25 μm , ChromPack; Varian, Palo Alto, CA). In the MS, electron ionization was applied and the profile mode used to determine the quinoxalinol-trimethylsilyl derivative fragment ions M (m/z 232) and M + 1 (m/z 233) (43).

Muscle tissues were lyophilized under vacuum and subsequently dissected free of blood and connective tissue visualized under microscope. Dry and clean tissue was homogenized in ice-cold buffer containing 0.15 M NaCl, 5 mM EDTA, 0.1% Triton X-100, and 0.02 M Tris (pH 7.4) using an automatic homogenizer (FastPrep 120A-230; Thermo Savant, Holbrook, NY) and centrifuged at 1,600 g at 4°C for 20 min to pellet structural proteins. To dissolve the myofibrillar proteins, 1.8 ml of 0.7 M KCl was added, vortexed, left for 30 min at 4°C, and finally centrifuged (1,600 g, 4°C, 20 min). The myofibrillar supernatant was given excess ethanol (99%) to precipitate proteins and subsequently centrifuged (1,600 g, 4°C, 20 min). The supernatant was discarded, and the myofibrillar protein pellet was hydrolyzed overnight in 6 M HCl at 110°C. The constituent AAs were purified by cation exchange resin (Dowex AG-50W; Bio-Rad) and dried in a SpeedVac (Vapornet, UVS400Al Savant Instruments, Farmingdale, NY). Finally, the myofibrillar protein-bound leucine enrichments were determined as their *N*-acetyl *n*-propyl derivatives by GC-combustion-isotope ratio MS (Delta^{Plus} XL; Thermo Finnigan, Bremen, Germany) using PTV inlet and helium as the carrier gas to the capillary column (CP-Sil 19 CB 60 m \times 0.25 mm, coating 1.5 μm ,

Chrompack; Varian). The isotopic enrichment of CO_2 was calculated from the δ -difference between ^{13}C and ^{12}C of the sample and a known laboratory reference standard related to Pee Dee Belemnite limestone. The enrichment of CO_2 was converted to moles tracer leucine by multiplying the ratio between total C in the derivatised analyte and number of labeled C.

Calculations. Myofibrillar fractional synthesis rate (FSR) was calculated on the basis of the incorporation of [^{13}C]leucine into muscle protein using the standard precursor-product model: $\text{FSR} (\%/h^{-1}) = [(\Delta E_P) \times (E_{KIC} \times \Delta t)^{-1}] \times 100\%$, where ΔE_P is the change in protein-bound [^{13}C]leucine enrichment between two biopsies, Δt is the incorporation time between the two biopsies, and E_{KIC} is the mean [^{13}C]KIC enrichment in that time period (59). Venous plasma [^{13}C]KIC enrichment was used as a validated surrogate measurement of leucyl-tRNA, which is the true precursor for protein synthesis but is difficult to assess (41, 43, 52).

Western blots. The phosphorylation status of the following target proteins downstream of the insulin/IGF-I receptor were measured with Western blot technique: v-Akt murine thymoma viral oncogene (Akt), 70-kDa ribosomal protein S6 kinase (p70^{S6K}), eukaryotic translation initiation factor 4E-binding protein-1 (4E-BP1), and glycogen synthase kinase-3 β (GSK-3 β).

Frozen muscle tissue samples were homogenized in a microvial containing one siliconcarbide crystal, five steel beads (2.3 mm), and 600 μl of homogenization buffer containing 50 mM Tris base, 1 mM EDTA, 1 mM EGTA, 10 mM β -glycerophosphate, and 50 mM NaF and adjusted to pH 7.5 with HCl. Immediately before use, mercaptoethanol to a concentration of 0.1% vol/vol, Triton-X to a concentration of 0.1% vol/vol, 0.5 mM sodium orthovanadate, and 1 protease inhibitor tablet (Complete; Roche, Basel, Switzerland) were added to the buffer. Samples were shaken three times for 15 s on a FastPrep-24 (MP Biomedicals, Solon, OH) at speed 4 with 2 min between each run and kept on ice. On the basis of Bradford assay protein quantification (Quick Start Bradford 1 \times Dye Reagent, no. 500-0205; Bio-Rad, Hercules, CA), homogenates were diluted in homogenization and Laemmli buffer to a final loading sample concentration of 0.65 $\mu\text{g}/\mu\text{l}$. Proteins (7.8 μg) were loaded and resolved by SDS-PAGE (4-12% Criterion XT Bis-Tris gel; Bio-Rad) for 1 h at 200 V standing in an ice bath at 4°C and subsequently blotted to polyvinylidene fluoride membranes (Amersham Hybond LFP; GE Healthcare, Buckinghamshire, UK) for 2 h at 400 mA in an ice bath at 4°C (Trans-blot cell; Bio-Rad) using transfer buffer (50 mM Tris base, 380 mM glycine, 80% vol/vol dH_2O , and 20% vol/vol methanol). Membranes were washed in TBS with 0.1% vol/vol Tween 20 adjusted to pH 7.4, blocked for 30 min at room temperature with Odyssey blocking buffer (Li-Cor Biosciences, Lincoln, NE), diluted 1:5 in PBS, and washed three times for 5 min in wash buffer. The membranes were then incubated with primary antibodies overnight at 4°C in a solution of 10% vol/vol Odyssey blocking buffer and 0.1% vol/vol Tween 20. The primary antibodies were p-Akt (Thr³⁰⁸; no. 2965), p-Akt (Ser⁴⁷³; no. 4060), p-p70^{S6K} (Thr³⁸⁹; no. 9206), p-4E-BP1 (Thr^{37/46}; no. 2855), p-GSK-3 β (Ser⁹; no. 9336; Cell Signaling Technology, Danvers, MA), 4E-BP1 (sc-81149; Santa Cruz Biotechnology, Santa Cruz, CA), and GSK3 β (ab31826; Abcam, Cambridge, MA). Membranes were washed as described previously and incubated for 1 h in secondary antibody [anti-mouse, Alexa 680, A21057 (Invitrogen, Carlsbad, CA); and anti-rabbit, DyLight 800 (Pierce Biotechnology, Rockford, IL)], washed, and visualized (Odyssey Infrared Imaging System; Li-Cor Biosciences). Band intensities were quantified with ImageJ (Rasband WS; National Institutes of Health, Bethesda, MD), and all measurements were normalized to the 36-kDa protein GAPDH as a housekeeping protein (primary antibody no. 4300; Ambion, Austin, TX). Because 4E-BP1 and GSK-3 β form inhibitory complexes in the unphosphorylated state, those targets are presented as totals, phosphorylated, and phosphorylated-to-total protein ratios (49), whereas Akt and p70^{S6K} are presented only as phosphorylated amounts. Due to

insufficient biopsy material, only a subset of n could be analyzed (whey: $n = 7$; casein: $n = 7$; control: $n = 7$).

Statistics. Since the aim was to compare the two different types of protein, a paired design was applied. Therefore, subjects who were randomized to participate in the protein trials were investigated twice (1 trial in each leg), and because we did not want a third trial in these subjects due to the possible effects of the first trial, a separate group of participants was randomized to a control trial. Statistical analyses were carried out as two-factor (type of protein and time point) repeated-measures ANOVA. When main effects occurred, Student-Newman-Keuls post hoc tests were performed to assess specific differences between protein type and time points. The control group data were analyzed with one-factor (time point) repeated-measures ANOVA. All area under the curve (AUC) responses between whey and casein were analyzed with paired t -tests. When appropriate, unpaired t -tests were conducted between protein and control groups. All values are presented as means \pm SE. The Western blot data were log-transformed before statistical analyses to avoid skewed data and are presented as geometric (geo) means \pm back-transformed SE. Level of significance was set at $P < 0.05$. All statistical analyses were performed with SigmaPlot 11.0 (Systat Software, San Jose, CA).

RESULTS

Systemic insulin and IGF-I concentrations. Insulin concentrations peaked at 30 min postintervention and were significantly higher in the whey group compared with the casein group from 15 to 60 min ($P < 0.05$; Fig. 3). The total insulin response, calculated as the AUC from time 15 to 360 min postintervention, was significantly higher after ingestion of whey compared with casein ($18,562 \pm 2,501$ vs. $15,585 \pm 2,211$ pmol \cdot L $^{-1}\cdot$ 345 min $^{-1}$, $P < 0.05$). Plasma IGF-I was unaffected by exercise and protein ingestion, averaging 177 ± 9 μ g/l at rest with no significant differences between groups.

Systemic AA concentrations. In general, all amino acid concentrations increased following protein intake, peaked at 45 min, and gradually returned to baseline values (Fig. 4). Leucine, isoleucine, the sum of EAA, and the sum of AA concentrations were significantly increased after intake of whey compared with casein at 30, 45, and 60 min (for leucine

furthermore at 90 min, $P < 0.05$; Fig. 4, A, C, E, and F). EAA (time point 180 min) and AA (time points 120 and 180 min) concentrations were higher after casein than after whey intake at later time points ($P < 0.05$; Fig. 4, E and F). However, AA concentrations defined as the total AUC from 15 to 360 min revealed no differences between whey and casein for leucine, isoleucine, the sum of EAA, or the sum of AA. Valine concentrations were significantly higher after whey intake only at time point 45 min ($P < 0.05$), and from 90 to 360 min casein intake resulted in significantly higher concentrations compared with whey intake ($P < 0.05$; Fig. 4B). The AUC for valine was significantly higher after casein intake than after whey intake ($P < 0.01$). For arginine concentrations, casein intake resulted in significantly higher concentrations from 60 to 360 min compared with whey intake ($P < 0.05$; Fig. 4D), with the AUC also being significantly higher ($P < 0.001$). In the control group, the AA concentrations generally declined after exercise ($P < 0.001$; Fig. 4), except for arginine and the sum of AAs. The sum of EAAs in the control group was significantly decreased at all time points after the exercise bout compared with baseline (at least $P < 0.05$).

Venous plasma KIC enrichments. KIC enrichments are reported in Fig. 5 for the baseline biopsy time point and for the three biopsy time points postintervention used for calculating FSR. There was an effect of time in the protein groups ($P < 0.001$), indicating a slight protein-dependent dilution of the 13 C label at 60 min. In the control group, KIC enrichment steady state was achieved during the FSR periods. In a subset of participants (whey: $n = 4$; casein: $n = 3$; control: $n = 2$), all samples were analyzed for KIC enrichment, which revealed that the periods between the postexercise biopsies were not affected by the intervention.

Muscle protein synthesis. Myofibrillar FSRs with the mean venous plasma [13 C]KIC enrichments as precursor are presented for the two separate incorporation periods (1–3.5 and 3.5–6 h) and for the total period (1–6 h) in Fig. 6. In the early and late periods there were no effects of protein or time; however, a tendency toward an interaction was detected ($P = 0.106$). Myofibrillar FSR for whey reached 0.123 ± 0.016 and $0.071 \pm 0.016\%/h$ in the early and late periods, respectively, and myofibrillar FSR for casein reached 0.098 ± 0.011 and $0.105 \pm 0.010\%/h$, respectively. Since no effects of protein were observed in the total period, we pooled the protein groups, which were significantly elevated compared with control (0.100 ± 0.005 vs. $0.073 \pm 0.009\%/h$, $P < 0.05$).

Anabolic signaling pathways. Significant effects of time were observed for p-Akt Thr³⁰⁸, p-Akt Ser⁴⁷³, and p-p70^{S6K} Thr³⁸⁹, which were all increased at time point 60 min compared with baseline (-60 min, $P < 0.05$, p-Akt Thr³⁰⁸: $P = 0.058$; Fig. 7). Furthermore, p-p70^{S6K} Thr³⁸⁹ was increased at time point 210 min compared with baseline ($P < 0.05$). For those targets, no differences were observed between the protein trials. In the control trial, main effect of time was observed for p-p70^{S6K} ($P = 0.016$; Fig. 7C); however, the post hoc test did not reveal specific differences. Total 4E-BP1 displayed significant interaction (protein \times time), and at time point 210 and 360 min, whey was significantly higher than casein ($P < 0.05$; Fig. 8A). Phosphorylation of 4E-BP1 Thr^{37/46}/total 4E-BP1 was significantly different between the protein trials, with the casein trial showing higher ratios than the whey trial ($P = 0.030$; Fig. 8C). In the control trial, the only effect observed

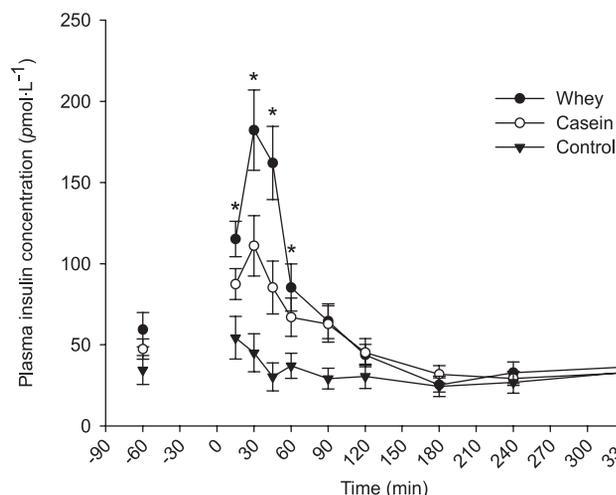


Fig. 3. Mean (\pm SE) plasma insulin concentrations (pmol/l) before (time -60 min) and after heavy resistance exercise and ingestion of study drinks at time 0. Protein groups' data were analyzed with 2-factor repeated-measures ANOVA (protein \times time). Protein effect: $P < 0.001$; time effect: $P < 0.001$; interaction (protein \times time): $P < 0.001$. *Significant difference between whey and casein (Student-Newman-Keuls test, $P < 0.05$).

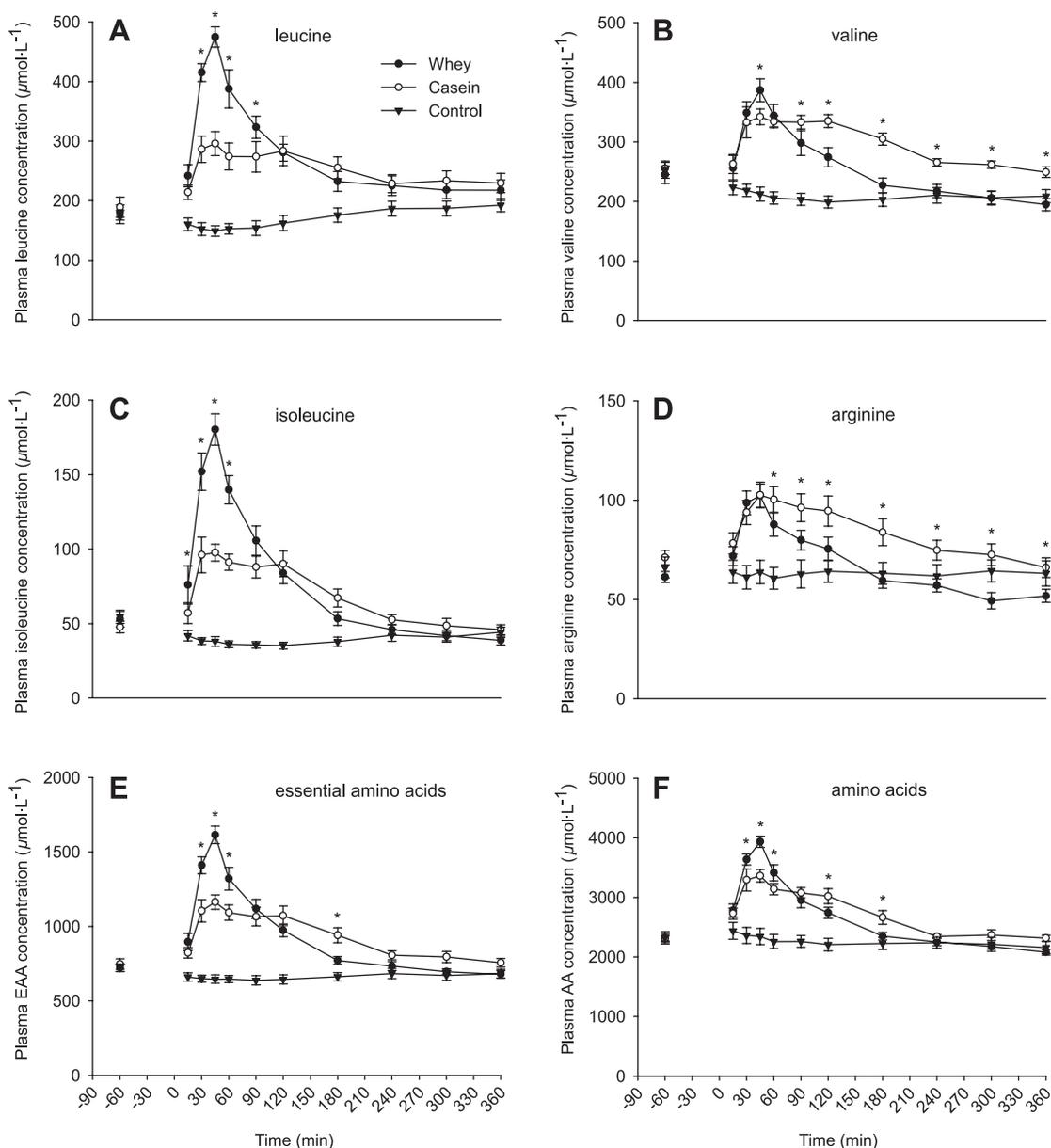


Fig. 4. Mean (\pm SE) plasma concentrations ($\mu\text{mol/l}$) of the branched-chain amino acids leucine (A), valine (B), isoleucine (C), and arginine (D), the sum of essential amino acids (EAA; E) except for Phe, Thr, and Trp, and the sum of amino acids (AAs; F) except for Asn, Asp, Cys, Phe, Thr, and Trp. Protein groups' data were analyzed with 2-factor repeated-measures ANOVA (protein \times time). For all AAs and groups of AAs, significant interactions (protein \times time) were detected; $P < 0.001$. *Significant difference between whey and casein (Student-Newman-Keuls test, $P < 0.05$).

was on p-4E-BP1 Thr^{37/46}, where time points 60 and 360 min were significantly different ($P < 0.05$; Fig. 8B). All data in relation to GSK-3 β (Fig. 8, D–F) showed no significant changes during the three recovery time points in either protein or control trials. All baseline measurements for the selected proteins and phosphorylation sites were not significantly different between whey and casein. Examples of representative Western blots are illustrated in Fig. 9.

DISCUSSION

The main finding of the present study was that ingestion of a single bolus of whey or casein immediately after one bout of heavy resistance exercise elicited a similar anabolic response in the subsequent 1- to 6-h period when measured with the solid direct-

tracer incorporation technique applicable with the L-[1-¹³C]leucine-labeled intact proteins. These similarities in the total period were observed together with differences in circulating insulin and amino acid concentrations throughout the 6 h postintervention, which were based on slower digestion and absorption of amino acids from casein compared with whey. However, whey intake resulted in the most marked increase in myofibrillar FSR in the early postintervention period, whereas casein showed a moderate but prolonged increase. The underlying molecular signaling through Akt and p70^{S6K} was also similarly affected, most likely because of the strong exercise stimulus, but the total 4E-BP1 was higher in the whey trial compared with the casein trial at 210 and 360 min. Furthermore, the phosphorylation ratio of 4E-BP1 Thr^{37/46} was higher in the casein trial compared with the whey trial.

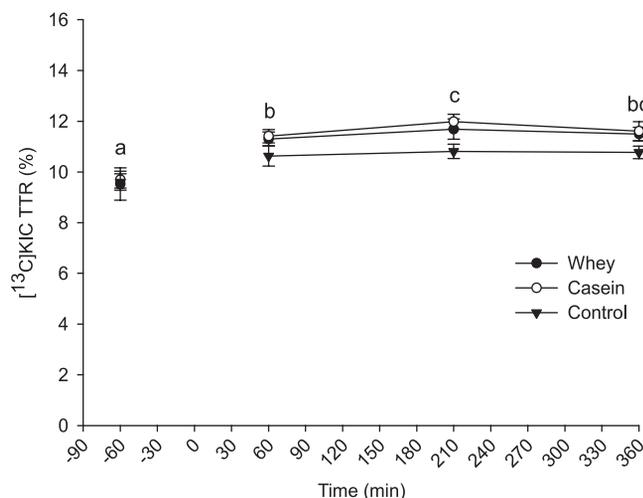


Fig. 5. Mean (\pm SE) venous [^{13}C]KIC tracer/trace ratio (TTR) %enrichments at time points of the muscle biopsies. Protein groups' data were analyzed with 2-factor repeated-measures ANOVA (protein \times time). Protein effect: $P = 0.522$; time effect: $P < 0.001$; interaction (protein \times time): $P = 0.980$. Different letters indicate significant differences in time points (Student-Newman-Keuls test, $P < 0.05$). The control group data were analyzed with 1-factor repeated-measures ANOVA (time); $P < 0.001$. Only -60 min was significantly different from the other time points (Student-Newman-Keuls test, $P < 0.05$).

Muscle protein synthesis. Whey hydrolysate and micellar casein have previously been investigated after resistance exercise, comparing MPS measured as FSRs in the 3 h after exercise (50). Whey hydrolysate was found to induce a FSR by some 0.15%/h, which is somewhat comparable with the values observed in the present study of $\sim 0.12\%/h$ in the early period (1–3.5 h). Micellar casein induced a FSR of 0.07%/h for Tang et al. (50) compared with the present FSRs of 0.10%/h in the early period and 0.10%/h in the late period (3.5–6 h). It could

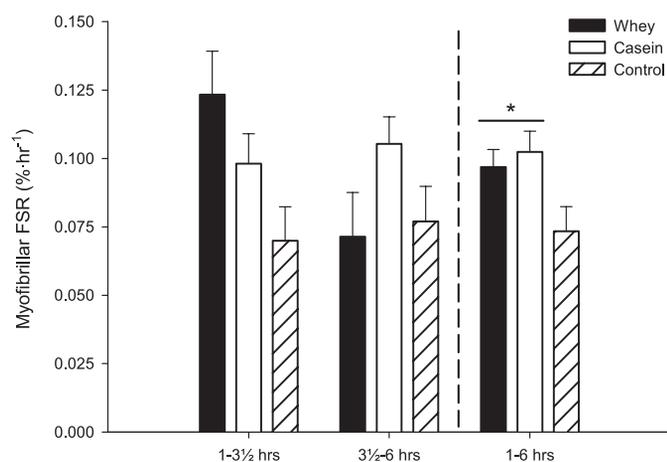


Fig. 6. Mean (\pm SE) FSR (%/h) of myofibrillar proteins after heavy resistance exercise and ingestion of the milk proteins whey or casein in the early (1–3.5 h), late (3.5–6 h), and total periods (1–6 h) in young men, using L-[^{13}C]leucine as the incorporated tracer and venous KIC as the precursor. Protein groups' data in the early and late periods were analyzed with a 2-factor repeated-measures ANOVA (protein \times time). Protein effect: $P = 0.684$; time effect: $P = 0.222$; interaction (protein \times time): $P = 0.106$. In the total period (1–6 h), FSRs of myofibrillar proteins in the pooled protein groups ($n = 9$) were higher than control ($n = 8$). *Significantly different from control (unpaired t -test, $P < 0.05$).

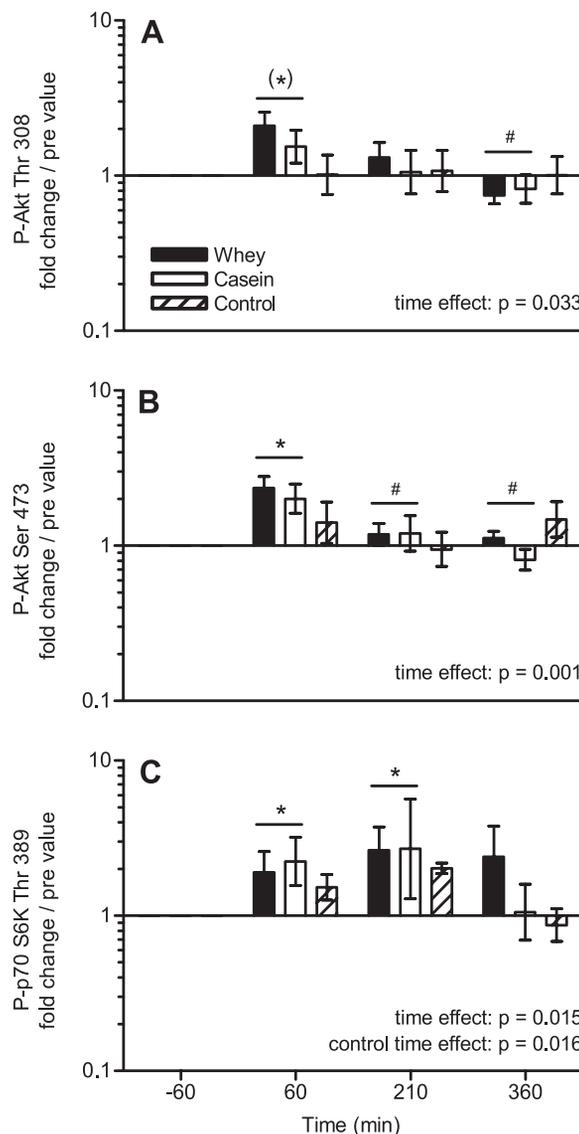


Fig. 7. Protein data shown for p-Akt Thr³⁰⁸ (A), p-Akt Ser⁴⁷³ (B), and 70-kDa ribosomal protein S6 kinase (p-p70^{S6K}) Thr³⁸⁹ (C). Values are presented for the postexercise biopsy time points 60, 210, and 360 min relative to pre values at -60 min as geo means \pm back-transformed SE. Protein groups' log-transformed data were analyzed with 2-factor repeated-measures ANOVA (protein \times time). p-Akt Thr³⁰⁸ time effect: $P = 0.033$; p-Akt Ser⁴⁷³ time effect: $P = 0.001$; p-p70^{S6K} Thr³⁸⁹ time effect: $P = 0.015$. No protein group effects were observed. *Significant difference between time points and -60 min (Student-Newman-Keuls test, $P < 0.05$). (*) $P = 0.058$. #Significant difference between time points and 60 min ($P < 0.05$). Control group log-transformed data were analyzed with 1-factor repeated-measures ANOVA (time). p-p70^{S6K}: $P = 0.016$.

be speculated that a prolonged time period would favor the casein group overall; however, the amino acid concentrations in that study had all returned toward baseline 3 h postexercise (50), which somehow is not in line with our observations. In the casein trial, we observed higher concentrations of valine, arginine, the sum of EAA, and the sum of amino acids at time points after the initial whey-induced peak, a shift in concentrations not observed with micellar casein (50). Therefore, the differences observed could be dependent upon the type of casein used. In the study by Tang et al. (50), micellar casein was used, whereas we used calcium caseinate in the present

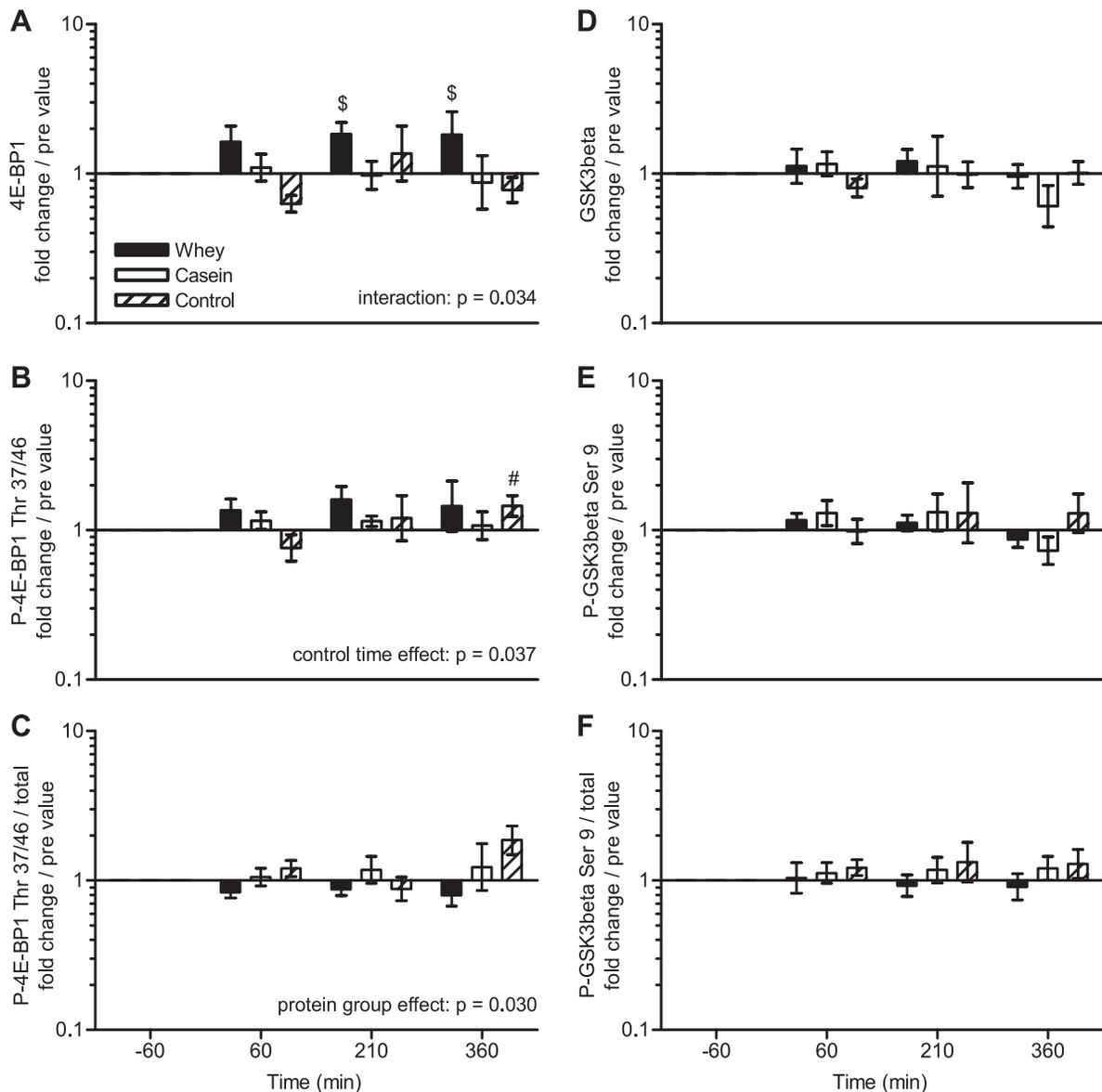


Fig. 8. Protein data shown for 4E-binding protein-1 (4E-BP1; A), p-4E-BP1 Thr^{37/46} (B), p-4E-BP1 Thr^{37/46}/total 4E-BP1 (C), GSK-3 β (D), p-GSK-3 β Ser⁹ (E), and p-GSK-3 β Ser⁹/total GSK-3 β (F). Values are presented for the postexercise biopsy time points 60, 210, and 360 min relative to pre values at -60 min as geo means \pm back-transformed SE. Protein groups' log-transformed data were analyzed with 2-factor repeated-measures ANOVA (protein \times time): $P = 0.034$; p-4E-BP1 Thr^{37/46}/total 4E-BP1 protein group effect: $P = 0.030$. \$Significant differences observed between whey and casein (Student-Newman-Keuls test, $P < 0.05$). Control group log-transformed data were analyzed with 1-factor repeated-measures ANOVA (time). p-4E-BP1 Thr^{37/46}: $P = 0.037$. #Significant difference between time points and 60 min (Student-Newman-Keuls test, $P < 0.05$). All data regarding GSK-3 β showed no effects.

study. Micellar casein is obviously the most slowly digested and absorbed of the two as reflected in the amino acid concentrations following intake. Regarding whey and whey hydrolysate, no differences in digestion and absorption have been observed (12).

It has previously been shown that a slow casein and a fast casein hydrolysate result in similar MPS in a 6-h postprandial period but with a tendency for the hydrolysate to have the largest MPS despite differences in whole body phenylalanine flux, insulin, and amino acid concentrations (37). These observations made these authors speculate that a difference between the protein types could be detected by differentiating the incorporation periods into an early and late period. Overall, we

find that whey as the fast-digested and absorbed proteins induces a strong response in MPS in the early period after intake and resistance exercise with a following value in the late period similar to controls (no protein, i.e., overnight fasting). On the other hand, casein appeared to exert a more moderate increase, equally distributed between the early and late exercise recovery periods. Therefore, whey as the fast protein seems to elicit a high but transient increase in MPS. To support these findings, milk soluble protein isolate was found to be too rapidly digested and absorbed to sustain amino acid concentrations needed for protein synthesis with casein and total milk proteins, resulting in an improved postprandial nitrogen utilization (40).

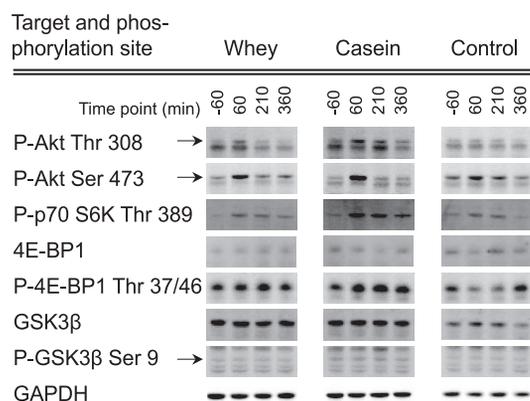


Fig. 9. Examples of representative Western blots for all measured signaling proteins and phosphorylation sites. The arrows indicate the specific bands in the case of several bands.

Taken together, it could be argued that the combination of whey and casein would be an optimal choice postexercise because whey stimulates the protein synthesis machinery with its high digestibility and consequently high peak concentrations of insulin and amino acids, including a high proportion of leucine in its composition, and casein provides amino acids for a prolonged period of time, fulfilling the amino acid need for the increased protein synthesis machinery. This is seen in studies investigating the combination in milk compared with soy (27, 56). However, a whey-based recovery supplement with its fast delivery of amino acids could be preferable immediately after exercise as long as other protein- and carbohydrate-containing nutrients are ingested within the following hours. Alternatively, the ratio of whey to casein in milk could be manipulated to 50:50, thereby supplying equal amounts of fast- and slow-digested milk protein.

Amino acid and insulin concentrations. Leucine has been shown to be a very potent amino acid in different animal and in vitro settings investigating anabolic signaling through mammalian target of rapamycin (mTOR) (2, 36). However, compared with human studies, some controversy exists with regard to the effects of leucine on MPS (42). Addition of leucine to protein and carbohydrates creates no additional effects in mixed MPS following exercise in either young or elderly subjects, but some beneficial effects have been observed at the whole body level (38, 39). However, in the elderly, the effects of adding leucine have been shown to increase MPS, but only under resting conditions (35, 48). From these existing results, it seems, therefore, that additional or high contents of leucine in a given protein solution can be beneficial mainly for elderly subjects displaying some degree of anabolic resistance to amino acid-containing nutrients (34). For healthy young individuals, the leucine requirement for maximal protein synthetic utilization may possibly be reached with ingestion of both whey and casein. Any excess leucine appearance after ingestion of whey compared with casein seems to be directed to oxidation in muscle (8). Next to leucine concentrations, we found that arginine, a potential anabolic amino acid (46, 60), and the branched-chain amino acid valine had a significantly greater response defined as AUC following casein intake compared with whey. This may, together with the slight differences in total EAA and amino acids, explain the prolonged MPS

response seen in the casein trials since hyperaminoacemia and MPS are closely correlated (6, 11, 44).

We showed marked elevations in insulin concentrations following exercise and intake of the milk proteins, which is in agreement with other findings (51) but in contrast to findings on micellar casein, which caused no elevations in insulin concentrations following intake after resistance exercise (50). Insulin inhibits muscle proteolysis, and within physiological concentrations insulin and amino acids have additive effects on MPS (14, 45). Therefore, in the early period the high insulin peak seen after the whey intake may have added effects to the high amino acid concentrations. On the other hand, the casein trial with the longer period of hyperaminoacemia and with a more moderate insulin response resulted in an equally increased MPS as after whey in total 1–6 h postintervention. We did not observe any changes in plasma IGF-I. This finding is in agreement with numerous exercise studies and supports the concept that local IGF-I generated in skeletal muscle is more important than circulating IGF-I (17, 23, 54).

Resistance exercise and nutrient-induced molecular signaling. We have measured downstream targets of the insulin/IGF-I receptor through the Akt-mTOR-p70^{S6K} signaling cascade, which is known to influence protein synthesis (3, 7, 20, 49), and found increased activation at 60 (Akt and p70^{S6K}) and 210 min (p70^{S6K}) postexercise. This temporal pattern of increased phosphorylation is in accordance with previous findings following provision of amino acids and performed resistance exercise (24, 33). We found that resistance exercise followed by whey or casein ingestion enhanced the phosphorylation of the targets Akt and p70^{S6K}, which demonstrates that a supply of excess amino acids following exercise stimuli presumably induces an optimal stimulation of these targets. Nevertheless, the whey group displayed the highest MPS in the early period, which cannot be explained by the measured Akt-p70^{S6K} signaling. Alternatively, the divergent amino acid concentrations present during the early period after intake of either whey or casein suggests those to be responsible for the MPS responses and thus may suggest yet-unknown signaling events to, e.g., leucine transport and metabolism. Interesting concepts of amino acid transporter reception, also known as amino acid “transception,” are beginning to emerge (26, 32), and it has been shown that increased availability of extracellular EAA has upregulated mRNA expression of amino acid transporters within the L- and A-type systems (21).

In the postexercise period, we found differences between whey and casein intake in the total 4E-BP1 and the phosphorylation ratio of 4E-BP1. At all three postexercise time points, casein intake was favorable in both total and phosphorylated ratio of 4E-BP1, supporting a more constant MPS throughout the postexercise recovery after the casein intake. The differences observed in the phosphorylation ratio are based primarily on a higher total amount of 4E-BP1 after the whey intake compared with casein, and thereby the overall effect of 4E-BP1 complex to eukaryotic initiation factor 4E (eIF4E) is an increased inhibition of translation initiation. Previous findings on 4E-BP1 phosphorylation ratios have shown increases following resistance exercise and EAA feeding (18, 19), which we could not detect in the present study. This difference could possibly be caused by the fact that 20 g of EAA (19) or EAA and carbohydrate (18) were consumed compared with ~8.5 g in the form of intact proteins in the present study. Furthermore,

elevated phosphorylation of 4E-BP1 has been observed both in the fasted and fed state after resistance exercise (31, 57); however, attention should be paid to the fact that the absolute baseline levels of phosphorylated vs. total 4E-BP1 remain unknown. Because of this fact, the relative importance of total vs. phosphorylated amounts in relation to inhibitory binding of eIF4E also remains unknown. Our results on GSK-3 β did not show any significant changes, which is in line with previous findings (25) indicating minor importance of the Akt-GSK-3 β pathway (7).

Overall, based on the present result, two scenarios can be set up. One is that the exercise stimuli alone was the main trigger of increased anabolic signaling downstream of the insulin/IGF-I receptor in the post exercise recovery period, since the provision of different protein types did not have any effects except for 4E-BP1 and the related phosphorylation ratio. Alternatively, both whey and casein and their related digestion and absorption rates provide amino acids sufficient to maximally stimulate downstream signaling events to activate p70^{S6K}. Finally, the protein group differences related to total 4E-BP1 could be interpreted as a feedback inhibitory mechanism to the load of amino acids and insulin after the whey intake.

The use of labeled milk proteins. In the present project, we managed to label sufficient amounts of milk proteins with L-[1-¹³C]leucine suitable for human experiments. In this setting, the aim of the human experiments was to infuse the same stable isotope tracer as was incorporated into the intact milk proteins in the cows. Such an intrinsical labeling of proteins allows a tracer appearance from the ingested proteins equal to what is maintained through the intravenous infusion, thereby not disturbing the precursor enrichment. Thus, irrespective of protein digestion and constituent amino acid absorption rates, the precursor tracer enrichment is kept practically at steady state, which allows the study of applied protein intake with a solid tracer methodology. With our infusion protocol, we obtained slightly higher plasma enrichments than the 10% (TTR) present in the proteins. Therefore, slightly lower plasma KIC enrichments at 60 min were the result of dilution of the enriched leucine absorbed from the protein intake. However, the venous plasma KIC enrichments were only slightly disturbed, and we avoided marked tracer dilutions, which with a single bolus drink is very hard to control taking into account the different digestion and absorption rates illustrated in the amino acid and especially leucine concentrations. Furthermore, individual biological differences in these rates could also be expected. Therefore, many factors should be considered to counteract a protein-specific dilution of enrichment giving unlabeled proteins. A way to solve the precursor problem could be to add a free amino acid tracer to the protein drink, but since a free amino acid is quickly absorbed compared with digestion and absorption of intact proteins (10), the precursor enrichments would be unequally wrongly calculated. Therefore, since whey and casein show marked differences in digestion and absorption rates, with whey being closest to a free amino acid, the advantage of using labeled proteins is evident when large single boluses are given.

A major limitation of the present study is that only MPS is measured, leaving the other determinant of the total net protein balance, muscle protein breakdown, unknown. On a whole body level and in resting conditions, casein has been shown to

decrease protein breakdown and whey to increase protein synthesis (8). However, this protein-specific response is not found on arteriovenous net balance measurements of leucine and phenylalanine across an exercised leg (51). Thus, clearly more work has to be done to fully describe the muscle net anabolic effects of the fast-digested and absorbed whey protein compared with the more slowly digested and absorbed casein, and furthermore, comparisons of the combination of casein and whey in milk against whey or casein alone are needed.

Conclusion. In conclusion, immediate intake of whey and casein following heavy resistance exercise in young men results in similar MPS responses over the subsequent 6-h recovery period. However, we observed a trend toward a higher but temporally shorter MPS response with whey compared with a more moderate but prolonged MPS response with casein. These findings may have clinical relevance in situations where individuals face, e.g., restitution from rehabilitating resistance exercise, immobilization, or, in the case of an elderly individual, sarcopenia, in whom a protein-containing nutrient supplement might be needed. Milk, easily accessible and containing both whey and casein, could very likely be an optimal choice.

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DISCLOSURES

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

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