Co-ingestion of protein and leucine stimulates muscle protein synthesis rates to the same extent in young and elderly lean men[1–3]

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ABSTRACT

Background: The progressive loss of skeletal muscle mass with aging is attributed to a disruption in the regulation of skeletal muscle protein turnover.

Objective: We investigated the effects on whole-body protein balance and mixed-muscle protein synthesis rates of the ingestion of carbohydrate with or without protein and free leucine after simulated activities of daily living.

Design: Eight elderly (75 ± 1 y) and 8 young (20 ± 1 y) lean men were randomly assigned to 2 crossover experiments in which they consumed either carbohydrate (CHO) or carbohydrate plus protein and free leucine (CHO+Pro+Leu) after performing 30 min of standardized activities of daily living. Primed, continuous infusions with L-[ring-$^{13}C_6$]phenylalanine and L-[ring-$^{2}H_2$]tyrosine were applied, and blood and muscle samples were collected to assess whole-body protein turnover and the protein fractional synthetic rate in the vastus lateralis muscle over a 6-h period.

Results: Whole-body phenylalanine and tyrosine flux were significantly higher in the young than in the elderly men ($P < 0.01$). Protein balance was negative in the CHO experiment but positive in the CHO+Pro+Leu experiment in both groups. Mixed-muscle protein synthesis rates were significantly greater in the CHO+Pro+Leu than in the CHO experiment in both the young (0.082 ± 0.005%/h and 0.060 ± 0.005%/h, respectively; $P < 0.01$) and the elderly (0.072 ± 0.006%/h and 0.043 ± 0.003%/h, respectively; $P < 0.01$) subjects, with no significant differences between groups.

Conclusions: Co-ingestion of protein and leucine with carbohydrate after activities of daily living improves whole-body protein balance, and the increase in muscle protein synthesis rates is not significantly different between lean young and elderly men. *Am J Clin Nutr* 2006;84:623–32.

KEY WORDS Protein metabolism, sarcopenia, muscle, aging

INTRODUCTION

Aging is associated with a slow, progressive loss of skeletal muscle mass, which is also called sarcopenia (1). Sarcopenia is generally accompanied by a reduction in strength, the loss of functional capacity, and an increased risk of developing chronic metabolic diseases such as obesity, type 2 diabetes, and osteoporosis. Sarcopenia is facilitated by a combination of factors, which include a sedentary lifestyle and a less-than-optimal diet (2). The age-related changes in skeletal muscle mass are attributed to a disruption in the regulation of skeletal muscle protein turnover (3), which results in a chronic imbalance between rates of muscle protein synthesis and breakdown. It has been reported that basal protein synthesis rates are either similar (4–7) or reduced (8–14) in the elderly compared with young adults. Furthermore, muscle protein breakdown rates tend to be greater in the elderly, which results in a gradual loss of skeletal muscle mass (6).

Protein turnover in skeletal muscle tissue is highly responsive to nutrient intake in healthy, young individuals (15). In contrast, the anabolic effect of food intake on muscle protein synthesis seems to be substantially blunted in the elderly (5, 16–18). The latter has been proposed to represent a key factor in the etiology of sarcopenia. In addition to food intake, physical activity can effectively modulate protein metabolism, because it stimulates both protein synthesis and protein breakdown (19). However, in the absence of food intake, net muscle protein balance will remain negative under these conditions (19, 20), which leads to net muscle protein loss. Carbohydrate ingestion effectively reduces the activity-induced stimulation of muscle protein degradation but does not affect protein synthesis (21, 22). As a consequence, protein balance will remain negative (21, 22) unless protein or amino acids are co-administered (23–25). Interestingly, supplementation with leucine has been proposed as an effective strategy to reduce muscle protein breakdown and to further stimulate muscle protein synthesis (24, 26–29). The latter could be attributed to the potential of leucine to stimulate protein anabolism by activating the mRNA translational machinery through the mammalian target of rapamycin (mTOR) in an insulin-dependent and insulin-independent manner (30–32). As a result, it has been suggested that the co-ingestion of a mixture

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TABLE 1
Physical characteristics of the male subjects[1]

	Young men ($n = 8$)	Elderly men ($n = 8$)
Age (y)	20 ± 1	75 ± 1[2]
Weight (kg)	73.7 ± 3.2	75.5 ± 2.1
Height (m)	1.81 ± 0.03	1.72 ± 0.01[2]
BMI (kg/m^2)	22.54 ± 1.1	25.7 ± 0.8[2]
Leg volume (L)	9.43 ± 0.39	8.23 ± 0.32[2]
HbA_{1C} (%)	5.03 ± 0.17	5.71 ± 0.11[2]
Basal glucose (mmol/L)	5.26 ± 0.12	5.51 ± 0.10
Basal insulin (mU/L)	11.15 ± 0.75	10.15 ± 0.85
HOMA-IR	2.61 ± 0.19	2.48 ± 0.20
1RM leg press (kg)	203.8 ± 7.4	151.3 ± 7.6[2]
1RM leg press (kg/BW)	2.80 ± 0.17	2.00 ± 0.06[2]
1RM leg extension (kg)	107.5 ± 3.8	78.1 ± 4.3[2]
1RM leg extension (kg/BW)	1.48 ± 0.09	1.03 ± 0.04[2]

[1] All values are $\bar{x}$ ± SEM. HbA_{1c}; glycated hemoglobin; HOMA-IR, homeostasis model assessment of insulin resistance (34); 1RM, one-repetition maximum; BW, body weight.

[2] Significantly different from young men, $P < 0.05$ (unpaired t test).

of protein and additional free leucine with carbohydrate could represent an effective strategy to augment muscle protein synthesis or inhibit protein degradation (24).

Under normal living conditions, activities of daily living (ADL) in the morning or afternoon are generally followed by food intake. As such, it is important to determine the combined effects of food intake and physical activity on skeletal muscle protein balance when studying the proposed changes in skeletal muscle protein metabolism with aging. In the present study, we investigated the differential effects of carbohydrate and carbohydrate plus protein and free leucine ingestion on skeletal muscle protein synthesis rates after simulated ADL in young (≈20 y) and elderly (≈75 y) lean men.

SUBJECTS AND METHODS

Subjects

Eight healthy, lean elderly men (75 ± 1 y) and 8 weight-matched, young controls (20 ± 1 y) with no history of participating in any regular exercise training program were selected to participate in the present study. The subjects' characteristics are shown in **Table 1**. All subjects were informed about the nature and risks of the experimental procedure before their written informed consent to participate was obtained. This study was approved by the local medical ethical committee.

Pretesting

Before selection in the study, all volunteers were subjected to an oral-glucose-tolerance test (33, 34). Leg volume was determined as described previously (35), after which all subjects participated in an orientation trial to become familiarized with the physical activity protocol and the equipment. Proper lifting technique was demonstrated and then practiced by the subjects for each of the 2 lower-limb exercises (leg press and leg extension). Subsequently, maximal strength (one-repetition maximum, or 1RM) was estimated by using the multiple repetitions testing procedure (36).

Diet and activity before testing

All subjects consumed a standardized meal (64.1 ± 2.0 kJ/kg body wt, consisting of 65% of energy as carbohydrate, 15% of energy as protein, and 20% of energy as fat) the evening before the experiments. All volunteers were instructed to refrain from any sort of heavy physical exercise and to keep their diet as constant as possible 3 d before the experiments. In addition, the subjects were asked to record their food intake for 48 h before the start of the first experiment and to consume the same diet for 48 h before the start of the second experiment.

Experiments

Each subject participated in 2 experiments, separated by 7 d, in which drinks containing carbohydrate (CHO) or carbohydrate plus protein and leucine (CHO+Pro+Leu) were administered in a randomized and double-blind fashion. Each experiment lasted ≈8 h. Repeated boluses of a given test drink were ingested after the physical activity protocol to ensure a continuous supply of glucose and amino acids. Plasma and muscle samples were collected during a 6-h period. These experiments were designed to simultaneously assess whole-body amino acid kinetics and the fractional synthetic rate (FSR) of mixed-muscle protein by the incorporation of L-[ring-$^{13}C_6$]phenylalanine in the mixed protein of muscle biopsies collected from the vastus lateralis muscle.

Protocol

At 0800, the subjects arrived at the laboratory by car or public transportation after fasting overnight, and a polytetrafluoroethylene catheter was inserted into an antecubital vein for stable-isotope infusion. A second polytetrafluoroethylene catheter was inserted in a heated dorsal hand vein of the contralateral arm, which was placed in a hot-box (60 °C), for arterialized blood sampling. After the basal blood sample collection, a single intravenous dose of L-[ring-$^{13}C_6$]phenylalanine (2 μmol/kg) and L-[ring-$^2H_2$]tyrosine (0.775 μmol/kg) was administered to prime the phenylalanine and tyrosine pool. Thereafter, tracer infusion (average infusion rate of 0.049 ± 0.001 $\mu mol \cdot kg^{-1} \cdot min^{-1}$ for L-[ring-$^{13}C_6$]phenylalanine and 0.019 ± 0.001 $\mu mol \cdot kg^{-1} \cdot min^{-1}$ for L-[ring-$^2H_2$]tyrosine) was started and the subjects rested in a supine position for 1 h before engaging in the standardized physical activity protocol. The protocol was designed to simulate 30 min of moderate-intensity physical activity (for example, garden tasks such as lawn mowing) as has been recommended by several public health authorities (37, 38). The energy consumption during such an activity pattern is estimated to be ≈650 kJ/30 min (39) and was simulated by combining low-intensity cycling and light resistance-type exercise. After 5 min of self-paced cycling, the subjects performed 6 sets of 10 repetitions on the horizontal leg press machine (Technogym BV, Rotterdam, Netherlands) and 6 sets of 10 repetitions on the leg extension machine (Technogym BV). The first 2 sets of both resistance exercises were performed at 40% of the subjects' 1RM. Sets 3–4 and 5–6 were performed at 55% and 75% of the subjects' 1RM, respectively, with 2-min rest intervals between sets. As such, the young and elderly lean men performed exercise at the same relative intensity. At the end of the exercise protocol ($t = 0$ min), the subjects rested supine and an arterialized blood sample and a muscle biopsy sample from the vastus lateralis muscle were collected. Subjects then received an initial bolus (1.33 mL/kg) of a given test drink. Repeated boluses (1.33 mL/kg) were ingested

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TABLE 2
Composition of the test drinks provided in the 2 experiments[1]

Test drink	CHO	CHO + Pro + Leu
	g/L	
Whey protein	—	60
Leucine	—	10
Glucose	92	92
Maltodextrin	92	92
Sodium saccharinate	0.2	0.2
Citric acid	1.8	1.8
Cream vanilla	5	5
Water	up to 1.00 L	up to 1.00 L

[1] CHO experiment, subjects received carbohydrate only; CHO + Pro + Leu expermint, subjects received carbohydrate, protein, and leucine.

every 30 min until $t = 330$ min. Arterialized blood samples were collected at $t = 15$, 30, 45, 60, 75, 90, 120, 150, 180, 210, 240, 270, 300, 330, and 360 min, and a second muscle biopsy sample was taken from the contralateral limb at $t = 360$ min.

Beverages

The subjects received a beverage volume of 1.33 mL/kg every 30 min to ensure a given dose of 0.49 g carbohydrate/kg (50% as glucose and 50% as maltodextrin) with or without the addition of 0.16 g/kg of a whey protein hydrolysate and 0.03 g/kg leucine every hour. The total amount of protein provided in the CHO+Pro+Leu experiment by far exceeded the calculated amount of protein needed to provide sufficient precursor substrate to sustain maximal protein synthesis rates for ≥6 h (40). Repeated boluses were administered to enable a continuous supply of amino acids in the circulation, preventing perturbations in L-[ring-$^{13}C_6$]phenylalanine and L-[ring-$^2H_2$]tyrosine enrichments. The whey protein hydrolysate (68.8% protein) contained 10.3% leucine, and consequently, the total amount of leucine administered in the CHO and CHO+Pro+Leu experiment was 0 versus 0.041 $g \cdot kg^{-1} \cdot h^{-1}$, respectively.

The compositions of all test drinks are listed in **Table 2**. Glucose and maltodextrin were obtained from AVEBE (Veendam, Netherlands). Whey protein hydrolysate was prepared by DSM Food Specialties (Delft, Netherlands). Leucine was purchased from BUFA (Uitgeest, Netherlands). To make the taste comparable in all experiments, the beverages were uniformly flavored by adding 0.2 g sodium saccharinate solution (25% w/w), 1.8 g citric acid solution (50% w/w), and 5 g of cream vanilla flavor (Numico Research, Wageningen, Netherlands) per liter of beverage. The experiments were performed in a randomized order, with the test drinks provided in a double-blind fashion.

Analysis

Blood samples were collected in EDTA-containing tubes and were centrifuged at 1000 × *g* and 4 °C for 5 min. Aliquots of plasma were frozen in liquid nitrogen and stored at −80 °C. Plasma glucose (Uni Kit III, 07367204; Roche, Basel, Switzerland) concentrations were analyzed with the COBAS-FARA semiautomatic analyzer (Roche). Insulin was analyzed by radioimmunoassay (Insulin RIA kit; LINCO Research Inc, St. Charles, MO, USA). Plasma (500 μL) for amino acid analyses was deproteinized on ice with 100 μL of 24% (wt:vol) 5-sulfosalicylic acid, was mixed, and the clear supernatant fluid was collected after centrifugation. Plasma amino acid concentrations were analyzed on a dedicated amino acid analyzer (LC-A10; Shimadzu Benelux, Den Bosch, Netherlands) by using an automated precolumn derivatization procedure and a ternary solvent system (41). The exact phenylalanine and tyrosine concentrations in the infusates (4.442 ± 0.005 and 1.762 ± 0.003 mmol/L, respectively) were measured by using the same method. Plasma phenylalanine and tyrosine were derivatized to their *tert*-butyldimethylsilyl derivatives, and their ^{13}C and ^2H enrichments were determined by electron ionization gas chromatography–mass spectrometry (6890N GC/5973N MSD; Agilent, Little Falls, DE) with selected ion monitoring of masses 336 and 342 for unlabeled and labeled phenylalanine, respectively, and masses 466, 468, and 472 for unlabeled and labeled tyrosine, respectively (42).

For measurement of L-[ring-$^{13}C_6$]phenylalanine enrichment in the free amino acid pool and mixed-muscle protein, 55 mg wet muscle was freeze-dried. Collagen, blood, and other nonmuscle fiber material were removed from the muscle fibers under a light microscope. The isolated muscle fiber mass (2–3 mg) was weighed, and 8 volumes (8 × dry weight of isolated muscle fibers × wet-to-dry ratio) of ice-cold 2% perchloric acid was added. The tissue was then homogenized and centrifuged. The supernatant fluid was collected and processed in the same manner as the plasma samples, such that intracellular free L-[ring-$^{13}C_6$]phenylalanine, L-[ring-$^2H_2$]tyrosine, and L-[ring-$^{13}C_6$]-tyrosine enrichments could be measured by their *t*-butyldimethylsilyl derivatives by gas chromatography–mass spectrometry. The free amino acid concentration in the supernatant fluid was measured by using an HPLC technique, after precolumn derivatization with *o*-phthaldialdehyde (43). The protein pellet was washed with 3 additional 1.5-mL washes of 2% perchloric acid and dried, and the proteins were hydrolyzed in 6 mol HCl/L at 120 °C for 15–18 h. The hydrolyzed protein fraction was dried under a nitrogen stream while heated to 120 °C, dissolved in a 50% acetic acid solution, and passed over a Dowex exchange resin (AG 50W-X8, 100–200 mesh hydrogen form; Biorad, Hercules, CA) by using 2 mol NH_3/L. Thereafter, the eluate was dried and the purified amino acid fraction was derivatized into the *N*-acetyl-methyl-esters to determine the ^{13}C-enrichment of protein-bound phenylalanine by gas chromatography–isotope ratio mass spectrometry (Finnigan, MAT 252).

Calculations

Infusion of L-[ring-$^{13}C_6$]phenylalanine and L-[ring-$^2H_2$]tyrosine with muscle and arterialized blood sampling was used to simultaneously assess whole-body amino acid kinetics and the FSR of mixed-muscle protein. Whole-body kinetics for phenylalanine and tyrosine were calculated by using the equations described by Thompson et al (44) and Short et al (45). Briefly, phenylalanine and tyrosine turnover (flux, Q) was measured from the isotope dilution at isotopic steady state:

$$Q = i \times [(E_i/E_p) - 1] \qquad (1)$$

where i is the isotope infusion rate ($\mu mol \cdot kg\ body\ wt^{-1} \cdot h^{-1}$) and E_i and E_p correspond with the enrichments of the infusate and plasma amino acids, respectively. At isotopic steady state, protein flux (Q) equals the sum of protein synthesis (S) and oxidation (O) as well as the sum of the rate of appearance of meal protein

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from the gut (I) and protein breakdown (B); the whole-body protein synthesis rate was calculated as flux minus oxidation.

$$Q = S + O = B + I \quad (2)$$

$$S = Q - O \quad (3)$$

At isotopic steady state, whole-body phenylalanine oxidation can be determined from the conversion (hydroxylation) of L-[ring-$^{13}C_6$]phenylalanine to L-[ring-$^{13}C_6$]tyrosine. The rate of hydroxylation (Qpt) was calculated by using the following formula (45):

$$Q_{pt} = Q_t \times (E_t/E_p) \times [Q_p/(i_p - Q_p)] \quad (4)$$

where Q_t and Q_p are the flux rates for L-[ring-$^2H_2$]tyrosine and labeled phenylalanine, respectively. E_t and E_p are the L-[ring-$^{13}C_6$]tyrosine and L-[ring-$^{13}C_6$]phenylalanine enrichments in plasma, respectively, and i_p is the infusion rate of the phenylalanine tracer.

The protein FSR of mixed muscle was calculated by dividing the increment in enrichment in the product, ie, protein-bound L-[ring-$^{13}C_6$]phenylalanine, by the enrichment of the precursor. Plasma L-[ring-$^{13}C_6$]phenylalanine and free muscle L-[ring-$^{13}C_6$]phenylalanine enrichments were used to provide an estimate of the lower boundary (based on plasma precursor enrichments) and the higher boundary (based on intracellular muscle precursor enrichments) for the true fractional synthetic rate of mixed-muscle proteins. Muscle FSRs were calculated as follows (24):

$$FSR = (\Delta E_p \times 2)/(E_{precursor} \times t) \times 100 \quad (5)$$

Where ΔE_p is the delta increment of protein-bound L-[ring-$^{13}C_6$]phenylalanine during incorporation periods. $E_{precursor}$ is *1*) the average plasma L-[ring-$^{13}C_6$]phenylalanine enrichment during the time period for determination of amino acid incorporation, *2*) the free muscle L-[ring-$^{13}C_6$]phenylalanine enrichment in muscle biopsy samples taken 6 h after exercise, and *3*) the free muscle L-[ring-$^{13}C_6$]phenylalanine enrichment in muscle biopsy samples taken 6 h after exercise corrected for the contribution of extracellular water, as previously described (40). t indicates the time interval (h) between biopsies and the factor 100 is needed to express the FSR in percent per hour (%/h). The factor 2 arises because in the *N*-acetyl-methyl-ester of the L-[ring-$^{13}C_6$]phenylalanine molecule, 6 of a total of 12 carbon atoms are labeled.

Statistics

All data are expressed as means ± SEMs. The plasma essential amino acid, insulin, and glucose responses were calculated as aread under the curve above baseline values. A 3-factor repeated-measures ANOVA with age group, time, and treatment as factors was used to compare differences between treatments over time between groups. For non-time-dependent variables, a 2-factor ANOVA with age group and treatment as factors was used to compare differences in treatment effects between groups. In case of a significant difference between experiments, a Scheffe post hoc test was applied to locate these differences. Statistical significance was set at $P < 0.05$. All calculations were performed by using STATVIEW 5.0 (SAS Institute Inc, Cary, NC).

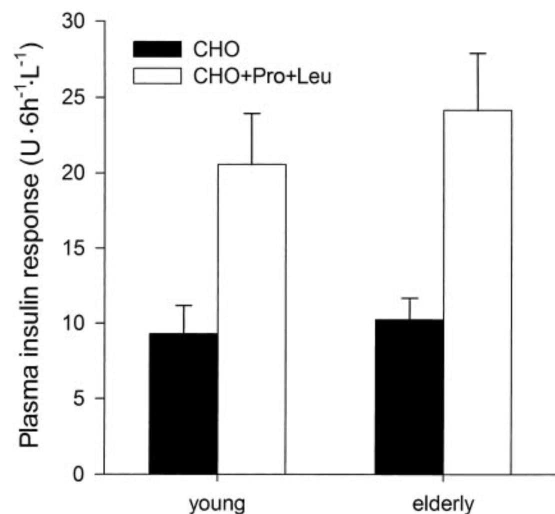

FIGURE 1. Mean (±SEM) plasma insulin responses (expressed as area under the curve minus baseline values) in lean young ($n = 8$) and elderly ($n = 8$) men while ingesting carbohydrate (0.49 g · kg^{-1} · h^{-1}; CHO) or carbohydrate plus protein and leucine (0.49, 0.16, and 0.03 g · kg^{-1} · h^{-1}, respectively; CHO+Pro+Leu). Data were analyzed by ANOVA (age group × treatment): age group effect, $P = 0.423$; treatment effect, $P < 0.001$; interaction of age group and treatment, $P = 0.636$.

RESULTS

Plasma analyses

The insulin response, expressed as area under the curve above baseline values (AUC) during the entire 6-h postexercise period is shown in **Figure 1**. The glucose response (AUC) was significantly lower in the young subjects than in the elderly (estimated marginal means: 587 ± 42 and 869 ± 39 mmol · 6 h^{-1} · L^{-1}, respectively, $P < 0.01$).

Plasma leucine, phenylalanine, and tyrosine concentrations over time are reported in **Figure 2**. Basal ($t = -120$ min) plasma leucine concentrations were significantly lower in the elderly than in the young (99.0 ± 3.8 and 119.7 ± 2.7 μmol/L, respectively; $P < 0.05$). No significant differences were observed between the young and elderly in basal ($t = -120$ min) plasma phenylalanine and tyrosine concentrations. The plasma leucine response (AUC) was negative in the CHO and positive in the CHO+Pro+Leu experiment (estimated marginal means: −13.9 ± 2.9 and 137.2 ± 2.9 mmol · 6 h^{-1} · L^{-1}, respectively; $P < 0.01$). The observed plasma insulin responses were positively correlated with the observed plasma leucine concentration ($r = 0.675$, $P < 0.001$). The plasma phenylalanine response (AUC) was negative in the CHO experiment, whereas it was positive in the CHO+Pro+Leu experiment (estimated marginal means: −3.24 ± 0.34 and 1.87 ± 0.34 mmol · 6 h^{-1} · L^{-1}, respectively; $P < 0.01$). Plasma tyrosine responses (AUC) were negative in the CHO and positive in the CHO+Pro+Leu experiment in the young (−5.8 ± 0.5 and 3.1 ± 0.6 mmol · 6 h^{-1} · L^{-1}, respectively; $P < 0.01$) and elderly (−5.6 ± 0.3 and 5.7 ± 0.8 mmol · 6 h^{-1} · L^{-1}, respectively; $P < 0.01$). The plasma tyrosine response in the CHO+Pro+Leu experiment was significantly greater in the elderly than in the young ($P < 0.05$). The plasma essential amino acid (EAA) response (area under the curve above baseline, with the exception of the supplemented leucine) was negative in the CHO and positive in the CHO+Pro+Leu experiment in the young (−67.8 ± 3.7 and 61.6 ± 4.9 mmol · 6 h^{-1} · L^{-1},

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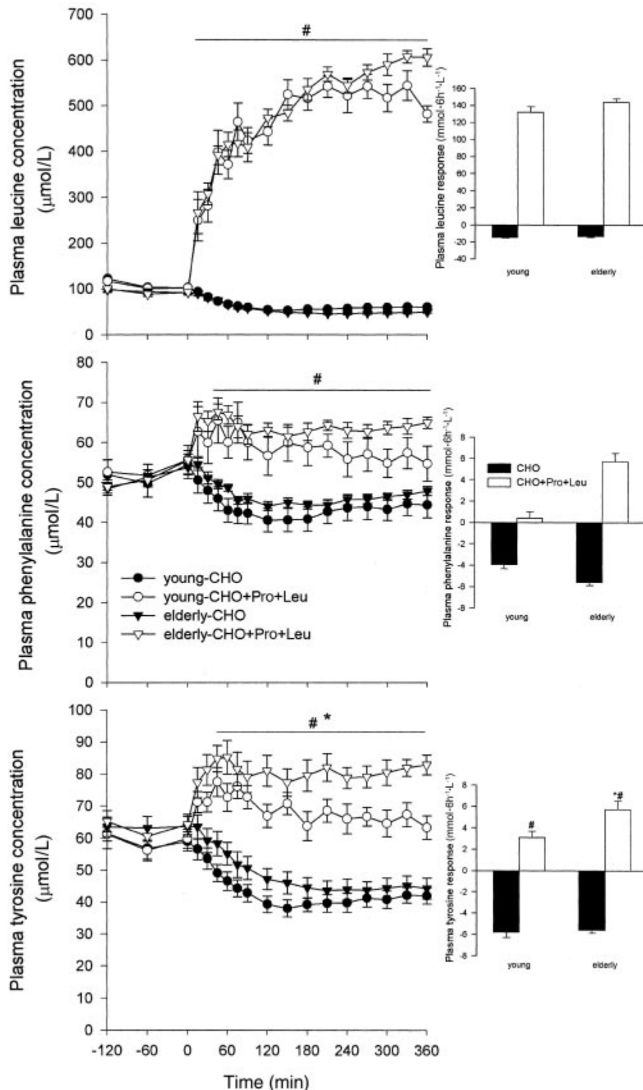

FIGURE 2. Mean (±SEM) plasma leucine, phenylalanine, and tyrosine concentrations and responses, expressed as area under the curve (AUC) above baseline, during the carbohydrate (CHO) and carbohydrate plus protein and leucine (CHO+Pro+Leu) experiments in lean young (n = 8) and elderly (n = 8) men. AUC data were analyzed with a 2-factor ANOVA (age group × treatment). Plasma leucine response: age group effect, $P = 0.1002$; treatment effect, $P < 0.001$; interaction of age group and treatment, $P = 0.186$. Plasma phenylalanine response: age group effect, $P < 0.05$; treatment effect, $P < 0.001$; interaction of age group and treatment, $P = 0.596$. Plasma tyrosine response: age group effect, $P < 0.05$; treatment effect, $P < 0.001$; interaction of age group and treatment, $P < 0.05$. #Significantly different from CHO experiment, $P < 0.05$ (Scheffe's test). *Significantly different from young men within the CHO+Pro+Leu experiment, $P < 0.05$ (Scheffe's test).

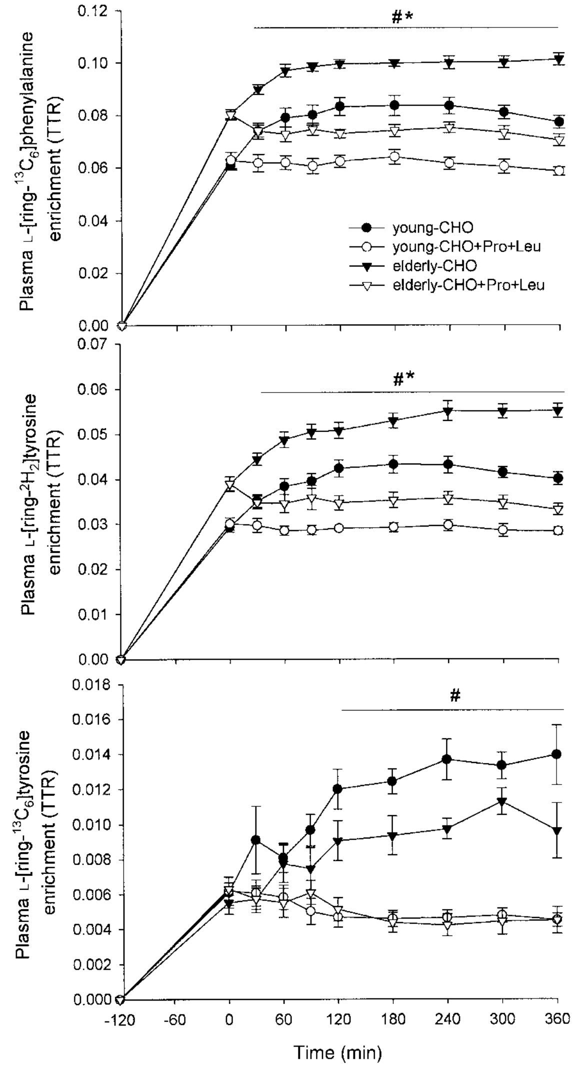

FIGURE 3. Mean (±SEM) plasma L-[ring-$^{13}C_6$]phenylalanine, L-[ring-$^{2}H_2$]tyrosine, and L-[ring-$^{13}C_6$]tyrosine enrichment (expressed as the tracer-to-tracee ratio, or TTR) during the carbohydrate (CHO) and carbohydrate plus protein and leucine (CHO+Pro+Leu) experiments in lean young (n = 8) and elderly (n = 8) men. Data were analyzed by repeated-measures ANOVA (age group × treatment × time). Plasma L-[ring-$^{13}C_6$]phenylalanine enrichment: age group effect, $P < 0.001$; treatment effect, $P < 0.001$; time effect, $P < 0.001$; interaction of age group and treatment, $P = 0.191$; interaction of age group and time, $P < 0.001$; interaction of treatment and time, $P < 0.001$; interaction of age group, treatment, and time, $P = 0.339$. Plasma L-[ring-$^{2}H_2$]tyrosine enrichment: age group effect, $P < 0.001$; treatment effect, $P < 0.001$; time effect, $P < 0.001$; interaction of age group and treatment, $P = 0.081$; interaction of age group and time, $P < 0.001$; interaction of treatment and time, $P < 0.001$; interaction of age group, treatment, and time, $P < 0.001$. Plasma L-[ring-$^{13}C_6$]tyrosine enrichment: age group effect, $P = 0.053$; treatment effect, $P < 0.001$; time effect, $P < 0.001$; interaction of age group and treatment, $P = 0.053$; interaction of age group and time, $P = 0.415$; interaction of treatment and time, $P < 0.001$; interaction of age group, treatment, and time, $P = 0.478$. *Significant differences between the young and the elderly, $P < 0.05$ (Scheffe's test). #Significant differences between treatments, $P < 0.05$ (Scheffe's test).

respectively; $P < 0.01$) and the elderly (-57.1 ± 5.0 and 74.1 ± 8.1 mmol · 6 h^{-1} · L^{-1}, respectively; $P < 0.01$), with no significant differences between the age groups.

The time course of the plasma L-[ring-$^{13}C_6$]phenylalanine, L-[ring-$^{2}H_2$]tyrosine, and L-[ring-$^{13}C_6$]tyrosine enrichments are shown in **Figure 3**. Plasma L-[ring-$^{13}C_6$]phenylalanine and L-[ring-$^{2}H_2$]tyrosine enrichments increased in the CHO experiment in both the young and elderly. In the CHO+Pro+Leu experiment, plasma L-[ring-$^{13}C_6$] phenylalanine and L-[ring-$^{2}H_2$]tyrosine enrichments did not change. Plasma L-[ring-$^{13}C_6$]phenylalanine and L-[ring-$^{2}H_2$]tyrosine enrichments were significantly greater in the CHO than in the CHO+Pro+Leu experiment ($P < 0.05$). Plasma L-[ring-$^{13}C_6$]phenylalanine and L-[ring-$^{2}H_2$]tyrosine enrichments were significantly higher in the elderly in the CHO and CHO+Pro+Leu experiments than in

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TABLE 3
Plasma and muscle tracer enrichments after the ingestion of carbhodyrate (CHO) or carbohydrate, protein, and leucine (CHO + Pro + Leu) after activities of daily living in young and elderly lean men[1]

	Experiment[2]		*P* values[3]		
	CHO	CHO + Pro + Leu	Age	Treatment	Interaction
Plasma AA enrichment[4]	*TTR*				
L-[ring-$^{13}C_6$]Phenylalanine			<0.001	<0.001	0.109
Young	0.0817 ± 0.0012	0.0614 ± 0.0009			
Elderly	0.1002 ± 0.0003	0.0731 ± 0.0008			
L-[ring-$^{2}H_2$]Tyrosine			<0.001	<0.001	0.214
Young	0.0420 ± 0.0006	0.0289 ± 0.0002			
Elderly	0.0524 ± 0.0013	0.0347 ± 0.0004			
L-[ring-$^{13}C_6$]Tyrosine			<0.001	<0.001	<0.050
Young	0.0131 ± 0.0004	0.0046 ± 0.0001[5]			
Elderly	0.0098 ± 0.0004[6]	0.0045 ± 0.0002[5]			
Muscle AA enrichment[7]					
L-[ring-$^{13}C_6$]Phenylalanine			<0.001	<0.001	0.573
Young	0.0556 ± 0.0010	0.0429 ± 0.0020			
Elderly	0.0639 ± 0.0036	0.0537 ± 0.0013			
L-[ring-$^{2}H_2$]Tyrosine			0.105	<0.050	<0.050
Young	0.0226 ± 0.0026	0.0218 ± 0.0014			
Elderly	0.0297 ± 0.0016[6]	0.0209 ± 0.0010[5]			
L-[ring-$^{13}C_6$]Tyrosine			0.858	0.070	<0.05
Young	0.0214 ± 0.0024	0.0153 ± 0.0043			
Elderly	0.0214 ± 0.0020	0.0163 ± 0.0021			
Δ Enrichment muscle protein[8]					
L-[ring-$^{13}C_6$]Phenylalanine			0.398	0.149	0.442
Young	0.000297 ± 0.000030	0.000314 ± 0.000023			
Elderly	0.000255 ± 0.000016	0.000314 ± 0.000028			

[1] n = 8 elderly and 8 young men.
[2] Values are $\bar{x}$ ± SEM. Enrichments are expressed as tracer-to-tracee ratio (TTR).
[3] Data were analyzed by ANOVA (age × treatment).
[4] Mean plasma amino acid (AA) enrichments during the last 4 h of recovery.
[5] Significantly different from CHO experiment, $P < 0.05$ (Scheffe's test).
[6] Significantly different from young men, $P < 0.05$ (Scheffe's test).
[7] Muscle free AA enrichments in the 6 h after the exercise biopsy.
[8] Increments in muscle protein enrichment from 0 to 6 h after activities of daily living.

the young subjects ($P < 0.05$). Plasma L-[ring-$^{13}C_6$]tyrosine enrichment increased in the CHO but remained unchanged in the CHO+Pro+Leu experiment in the young and the elderly subjects. Plasma L-[ring-$^{13}C_6$]tyrosine enrichment was significantly higher in the CHO experiment than in the CHO+Pro+Leu experiment ($P < 0.05$).

Muscle analysis

No significant differences in free leucine and tyrosine concentrations in the muscle biopsy samples taken at $t = 0$ min were observed between the young and the elderly. Muscle free phenylalanine concentrations were lower in the elderly than in the young (77 ± 7 and 160 ± 32 μmol/L, respectively; $P < 0.05$). At $t = 360$ min, muscle leucine concentrations were significantly higher during the CHO+Pro+Leu experiment than during the CHO experiment in the young (322 ± 35 and 83 ± 11 μmol/L, respectively; $P < 0.05$) and the elderly (232 ± 38 and 103 ± 10 μmol/L, respectively; $P < 0.05$). No significant differences were observed in muscle free phenylalanine and tyrosine concentrations between experiments or between the young and elderly at $t = 360$ min.

Mean plasma amino acid enrichments during the last 4 h of recovery, muscle free amino acid enrichments in the 6 h after the exercise biopsy, and the increments in muscle protein enrichment are presented in **Table 3**. In the muscle biopsy samples collected 6 h after exercise, free L-[ring-$^{13}C_6$]phenylalanine enrichments were significantly higher in the elderly than in the young in both experiments ($P < 0.05$), whereas L-[ring-$^{2}H_2$]tyrosine enrichments were higher in the elderly in the CHO experiment only ($P < 0.05$). No significant differences in muscle free L-[ring-$^{13}C_6$]tyrosine enrichments were observed between the young and the elderly in the CHO and CHO+Pro+Leu experiments or between experiments. Free L-[ring-$^{13}C_6$]-phenylalanine enrichments in the 6 h after the exercise biopsy were significantly higher in the CHO experiment than in the CHO+Pro+Leu experiment in both the young and the elderly ($P < 0.05$), whereas L-[ring-$^{2}H_2$]tyrosine enrichments were lower in the CHO+Pro+Leu experiment in the elderly only ($P < 0.05$). No differences were observed in the increase in protein-bound L-[ring-$^{13}C_6$]phenylalanine enrichment during the CHO and CHO+Pro+Leu experiments between the young and the elderly.

Whole-body protein metabolism

Both phenylalanine and tyrosine fluxes were higher in the CHO+Pro+Leu than in the CHO experiment in the young

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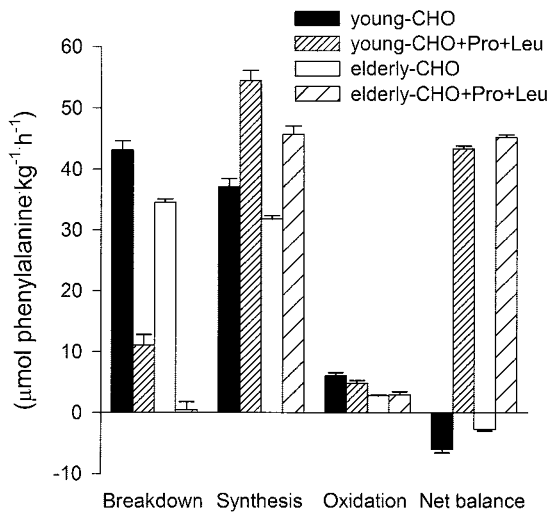

FIGURE 4. Mean (±SEM) rates of whole-body protein breakdown, synthesis, oxidation, and net protein balance during the carbohydrate (CHO) and carbohydrate plus protein and leucine (CHO+Pro+Leu) experiments in lean young (n = 8) and elderly (n = 8) men. Data were analyzed by ANOVA (age group × treatment). Breakdown: age group effect, $P < 0.001$; treatment effect, $P < 0.001$; interaction of age group and treatment, $P = 0.483$. Synthesis: age group effect, $P < 0.001$; treatment effect, $P < 0.001$; interaction of age group and treatment, $P = 0.202$. Oxidation: age group effect, $P < 0.001$; treatment effect, $P = 0.225$; interaction of age group and treatment, $P = 0.092$. Net balance: age group effect, $P < 0.001$; treatment effect, $P < 0.001$; interaction of age group and treatment, $P = 0.092$.

(59.2 ± 1.8 and 43.2 ± 1.5 μmol Phe · kg^{-1} · h^{-1}, respectively; $P < 0.01$) and the elderly (48.7 ± 1.35 and 34.6 ± 0.6 μmol Phe · kg^{-1} · h^{-1}, respectively; $P < 0.01$). Phenylalanine and tyrosine fluxes in the CHO experiment were 20–25% higher in the young than in the elderly subjects ($P < 0.01$). In the CHO+Pro+Leu experiment, phenylalanine and tyrosine fluxes were 30–40% higher in the young than in the elderly subjects ($P < 0.01$). Whole-body protein breakdown (**Figure 4**), calculated over the 6 h of postexercise recovery, was lower in the CHO+Pro+Leu experiment than in the CHO experiment ($P < 0.05$) in the young and elderly. Whole-body protein synthesis (Figure 4), as calculated by Qp - Qpt, was higher in the CHO+Pro+Leu than in the CHO experiment ($P < 0.01$) in both groups. The rate of postexercise whole-body phenylalanine oxidation, as calculated from the conversion of phenylalanine to tyrosine, is reported in Figure 4. Whole-body protein balance (Figure 4) was negative in the CHO experiment, whereas protein balance was positive in the CHO+Pro+Leu experiment in the young and elderly. Protein breakdown, synthesis, and oxidation rates were higher in the young than in the elderly ($P < 0.05$). Protein synthesis efficiency (whole-body protein synthesis as a percentage from phenylalanine flux) was significantly higher in the elderly than in the young subjects in the CHO ($P < 0.01$) but not the CHO+Pro+Leu experiment (NS). Protein synthesis efficiency was higher in the CHO+Pro+Leu than in the CHO experiment in the young (91.9 ± 0.7% and 86.0 ± 1.1%, respectively; $P < 0.01$) but not in the elderly subjects.

Mixed-muscle protein synthesis rates

The mixed-muscle protein FSR, with the mean plasma L-[ring-$^{13}C_6$]phenylalanine enrichment as precursor (**Figure 5**), was significantly higher in the CHO+Pro+Leu than in the CHO

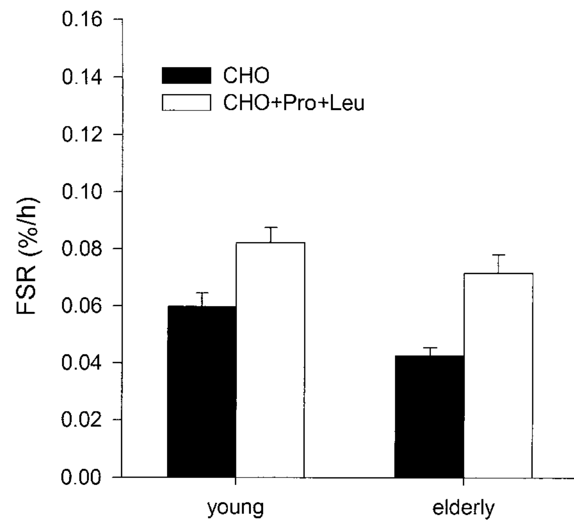

FIGURE 5. Mean (±SEM) fractional synthetic rate (FSR) of mixed-muscle protein after the ingestion of carbohydrate (CHO) or carbohydrate plus protein and leucine (CHO+Pro+Leu) in lean young (n = 8) and elderly (n = 8) men with the use of plasma phenylalanine enrichment as a precursor. Data were analyzed by ANOVA (age group × treatment). Age group effect, $P < 0.05$; treatment effect, $P < 0.001$; interaction of age group and treatment, $P = 0.583$.

experiment in the young (0.082 ± 0.005%/h and 0.060 ± 0.005%/h, respectively; $P < 0.01$) and the elderly (0.072 ± 0.006%/h and 0.043 ± 0.003%/h, respectively; $P < 0.01$). The observed FSR values in the elderly subjects were significantly lower than those in the young (P < 0.05). Net differences in the mixed-muscle protein synthesis rates between experiments were not significantly different between the young and the elderly subjects (0.022 ± 0.007%/h and 0.029 ± 0.004%/h, respectively; NS). When the free intracellular L-[ring-$^{13}C_6$]phenylalanine enrichment was used as the precursor, FSR values were higher, but showed the same intervention effect. FSR values in the young subjects averaged 0.088 ± 0.008%/h and 0.124 ± 0.011%/h for the CHO and CHO+Pro+Leu experiments, respectively ($P < 0.05$). In the elderly, FSR values averaged 0.068 ± 0.005%/h and 0.097 ± 0.008%/h for the CHO and CHO+Pro+Leu experiments respectively ($P < 0.01$). When these values were corrected for the contribution of extracellular water to the measured free muscle L-[ring-$^{13}C_6$]phenylalanine enrichment (40), FSR values averaged 0.095 ± 0.009%/h and 0.135 ± 0.014%/h for the CHO and CHO+Pro+Leu experiments, respectively ($P < 0.05$). In the elderly, FSR values averaged 0.076 ± 0.007%/h and 0.102 ± 0.008%/h for the CHO and CHO+Pro+Leu experiments, respectively ($P < 0.01$).

DISCUSSION

In the present study, we assessed whole-body protein turnover and determined mixed-muscle protein synthesis rates by measuring the incorporation of labeled phenylalanine in the vastus lateralis muscle after the ingestion of carbohydrate with or without protein and free leucine in lean young and elderly men. Co-ingestion of protein and leucine was shown to improve whole-body protein balance in both the lean young and elderly men compared with the ingestion of carbohydrate only. Direct measurement of mixed-muscle protein synthesis rates showed that protein synthesis rates are lower in the elderly than in the

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young after ADL-type activity. However, the increase in muscle protein synthesis rates did not differ significantly between the young and the elderly lean men after the co-ingestion of protein and leucine.

It has been reported that basal muscle protein synthesis rates are similar (4–7) or reduced in the elderly (9–14, 46), whereas protein degradation rates are increased (6). In addition, there are indications that the stimulating effect of food intake on muscle protein synthesis is blunted in the elderly (5, 16–18), which may be due in part to impaired anabolic signaling (18, 47). Besides food intake, physical activity can also effectively modulate protein metabolism. Under normal living conditions, food intake generally follows ADL. Therefore, determining the combined effects of food intake and physical activity on skeletal muscle protein metabolism is of crucial importance when investigating the proposed changes in skeletal muscle protein metabolism with aging. In the present study, we investigated the anabolic response to carbohydrate (CHO) or carbohydrate, protein, and leucine (CHO+Pro+Leu) ingestion after simulated ADL-type activities in vivo in young (≈20 y) and elderly (≈75 y) men. We showed that muscle protein synthesis rates were significantly lower in the elderly than in the young under these conditions. The observed mixed-muscle FSR values after ADL were ≈30% lower in the elderly than in the young subjects (Figure 5). These observations tend to be in line with previous reports showing muscle protein synthesis rates to be 20–30% lower in elderly humans (60–84 y) than in young adults in a basal, fasted state (3, 9, 16). However, the comparison of our data with data obtained in the fasted state reported by other groups (3, 9, 16) should be regarded as speculative. From our data we can only conclude that protein synthesis rates are reduced in the elderly after ADL activities when carbohydrate with or without the addition of protein and leucine is ingested.

Our findings in muscle are in line with our observations made by using whole-body tracer methods, which showed whole-body phenylalanine and tyrosine turnover to be 20–25% lower in the elderly than in the young. In the CHO experiment, we observed lower protein oxidation rates (absolute and expressed as a percentage of total phenylalanine flux) in the elderly than in the young. The latter implies that a greater proportion of the rate of disappearance of phenylalanine is used for protein synthesis (ie, greater protein synthesis efficiency). However, because of the decreased whole-body protein turnover rates in the elderly, protein synthesis and breakdown rates were lower in the elderly than in the young, despite greater protein synthesis efficiency. In accordance with earlier findings (24), we observed that whole-body net protein balance remains negative when only carbohydrate is ingested. To our knowledge, we are the first to show that muscle protein synthesis is reduced in the elderly in practical daily living conditions in which ADL are followed by the intake of carbohydrate with or without the addition of protein and leucine.

Administration of amino acids with or without carbohydrate results in a rapid increase in muscle protein synthesis in young adults, whereas protein degradation rates are reduced (7, 23–27, 48, 49). The latter can be attributed to the role of amino acids as precursors for protein synthesis (15), the potential of amino acids to stimulate insulin secretion (50), and the proposed property of amino acids, and leucine in particular, to stimulate protein synthesis by activating the mRNA translational machinery through the mTOR pathway (51). This activation through the mTOR pathway has been reported for leucine administration in vivo in rodents (28, 29, 52). Therefore, a mixture containing carbohydrate, protein, and additional free leucine may represent an effective nutritional intervention to optimize net muscle accretion. Recently, we showed that the combined ingestion of protein and free leucine augments skeletal muscle protein synthesis rates in healthy young adults after intense resistance exercise when compared with the ingestion of carbohydrate only (24). In the present study, we aimed to investigate whether the proposed anabolic response to the combined ingestion of protein and leucine would also be present in the elderly when compared with the ingestion of carbohydrate only, and we questioned whether this response is of a similar or lower magnitude in the elderly.

In accordance with our earlier findings (24), we observed that whole-body protein breakdown rates were significantly suppressed after the co-ingestion of leucine and protein with carbohydrate, whereas protein synthesis rates were increased (Figure 4). Co-ingestion of protein and leucine with carbohydrate resulted in a 47 ± 3% and 44 ± 4% increase in whole-body protein synthesis in the young and elderly, respectively, when compared with the ingestion of carbohydrate only. As a result, whole-body net protein balance became positive in the CHO+Pro+Leu experiment, which verifies that protein and amino acid ingestion is necessary for net muscle anabolism to occur. In the CHO+Pro+Leu experiment, we observed an increase in whole-body protein synthesis efficiency in the young but not in the elderly. The latter is in part due to a reduction in whole-body protein oxidation in the young after the ingestion of leucine and protein with carbohydrate, which does not occur in the elderly. These observations indicate that whole-body protein oxidation in the CHO+Pro+Leu trial was not inhibited as much in the elderly as in the young. The co-ingestion of protein and leucine with carbohydrate increased the FSR compared with carbohydrate ingestion only (Figure 5). The net differences in the mixed-muscle protein synthesis rates between the CHO and CHO+Pro+Leu experiments did not differ significantly between the young and elderly subjects. This would agree with more recent observations showing a similar response of muscle protein turnover to an amino acid load in healthy elderly and younger adults (4, 7, 53). However, our FSR data seem to be in contrast with data presented by Volpi et al (5), who showed that the response of muscle protein synthesis to the combined ingestion of amino acids and glucose is impaired in the elderly. The latter could be related to the blunted insulin response in the elderly compared with the young controls that was reported by Volpi et al (5). In contrast, plasma insulin responses in the young and elderly volunteers were not significantly different after the co-ingestion of protein and leucine with carbohydrate. The observed differences might be attributed to the property of leucine to stimulate insulin secretion (50) or mRNA translation (52). In the present study, we investigated the potential differences in the capacity to stimulate muscle protein synthesis in the young and elderly after the ingestion of a theoretically optimal anabolic nutritional intervention. The latter shows that these properties are as effective in the young as in the elderly.

In conclusion, co-ingestion of protein and leucine improves whole-body protein balance when compared with the ingestion of carbohydrate only in lean young and elderly men. Mixed-muscle protein synthesis rates are reduced in the elderly under conditions in which ADL-type activities are followed by food intake. However, the increase in muscle protein synthesis rates is

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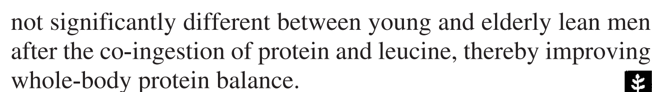
not significantly different between young and elderly lean men after the co-ingestion of protein and leucine, thereby improving whole-body protein balance.



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RK and LJCvL designed the study. RK organized and carried out the clinical experiments with the assistance of LV and RJFM. RK performed the statistical analysis and wrote the manuscript together with LJCvL and AJMW. APG performed the plasma and muscle analyses for amino acid enrichments. MG performed the plasma analyses for amino acid concentrations. EP provided medical assistance. None of the authors had a personal or financial conflict of interest

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