

Skeletal Muscle Disuse Atrophy Is Not Attenuated by Dietary Protein Supplementation in Healthy Older Men^{1,2}

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Abstract

Short successive periods of muscle disuse, due to injury or illness, can contribute significantly to the loss of muscle mass with aging (sarcopenia). It has been suggested that increasing the protein content of the diet may be an effective dietary strategy to attenuate muscle disuse atrophy. We hypothesized that protein supplementation twice daily would preserve muscle mass during a short period of limb immobilization. Twenty-three healthy older (69 ± 1 y) men were subjected to 5 d of one-legged knee immobilization by means of a full-leg cast with (PRO group; *n* = 11) or without (CON group; *n* = 12) administration of a dietary protein supplement (20.7 g of protein, 9.3 g of carbohydrate, and 3.0 g of fat) twice daily. Two d prior to and immediately after the immobilization period, single-slice computed tomography scans of the quadriceps and single-leg 1 repetition maximum strength tests were performed to assess muscle cross-sectional area (CSA) and leg muscle strength, respectively. Additionally, muscle biopsies were collected to assess muscle fiber characteristics as well as mRNA and protein expression of selected genes. Immobilization decreased quadriceps' CSAs by 1.5 ± 0.7% (*P* < 0.05) and 2.0 ± 0.6% (*P* < 0.05), and muscle strength by 8.3 ± 3.3% (*P* < 0.05) and 9.3 ± 1.6% (*P* < 0.05) in the CON and PRO groups, respectively, without differences between groups. Skeletal muscle myostatin, myogenin, and muscle RING-finger protein-1 (*MuRF1*) mRNA expression increased following immobilization in both groups (*P* < 0.05), whereas muscle atrophy F-box/atrogen-1 (*MAFBx*) mRNA expression increased in the PRO group only (*P* < 0.05). In conclusion, dietary protein supplementation (~20 g twice daily) does not attenuate muscle loss during short-term muscle disuse in healthy older men. This trial was registered at clinicaltrials.gov as NCT01588808. J. Nutr. doi: 10.3945/jn.114.194217.

Introduction

A period of prolonged (i.e., several weeks) muscle disuse because of illness or injury can lead to substantial loss of skeletal muscle mass and strength in otherwise healthy individuals. The resulting negative health consequences, such as impaired functional capacity (1–3), decreased muscle strength (4), the onset of insulin resistance (5), and a decline in basal metabolic rate (6,7), are of particular concern to older individuals, who are already functionally and/or metabolically compromised. Recently, we (8,9) and others (10) showed that even a few days of disuse can lead to substantial losses of muscle mass and strength in young and old men. These findings are of particular clinical relevance because hospitalization of older individuals with acute illness

generally results in a mean hospital stay of 5–7 d (11). It was hypothesized that such short successive periods of muscle disuse occurring throughout the lifespan may be instrumental in the progressive loss of muscle mass that occurs with aging (12,13).

Any substantial loss of skeletal muscle mass due to muscle disuse must be attributed to a chronic imbalance between muscle protein synthesis and breakdown rates. A decline in basal (post-absorptive) muscle protein synthesis rates has been reported following both bed rest (14–16) and limb immobilization (17–19). Furthermore, recent work from our laboratory (20) and others (17,21,22) showed that the muscle protein synthetic response to protein or amino acid administration becomes blunted following a period of disuse. Additionally, there is some indirect evidence that increases in muscle protein breakdown rates occur during the initial first few days of muscle disuse only (23–25). As such, it is now widely thought that declines in both post-absorptive and postprandial muscle protein synthesis rates play the biggest causal role in the loss of muscle mass during a period of disuse (26,27). Dietary protein intake stimulates muscle protein synthesis rates and inhibits muscle protein breakdown, and thereby allows net muscle protein accretion (28). Accordingly, it was speculated that maintaining or

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² Supplemental Tables 1 and 2, Supplemental Figure 1, and additional information are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at <http://jn.nutrition.org>.

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even increasing dietary protein intake can attenuate muscle loss during a period of disuse (12,27). In support, intervention studies have shown high-dose essential amino acid supplementation to attenuate muscle loss during prolonged bed rest in young (29–31) and older individuals (32). However, the potential for a practical dietary protein feeding strategy to alleviate muscle loss during short-term disuse in the older population remains to be investigated.

In the present study, we investigated our hypothesis that dietary protein supplementation attenuates muscle loss during a short period of muscle disuse in older men. To test this hypothesis, 23 healthy older men were selected to participate in a study during which they were subjected to 5 d of one-legged knee immobilization with or without dietary protein supplementation (~20 g of protein twice daily). Muscle mass and strength were assessed prior to and immediately after immobilization, and muscle biopsy samples were collected to assess muscle fiber characteristics and associated myocellular signaling.

METHODS

Participants. Twenty-three healthy older men (mean age: 69 ± 1 y) were included in the present study. Prior to inclusion, a general health questionnaire was filled out by the participants and a routine medical screening was completed to exclude individuals with the following: a BMI <18.5 or >30 kg/m²; any back, knee, or shoulder complaints that could interfere with the use of crutches; a (family) history of thrombosis; type 2 diabetes mellitus (determined by HbA1c values $>7.0\%$); severe cardiac problems; or a history of performing prolonged resistance-type exercise in the 6 mo preceding the start of the study. All participants were informed of the nature and risks of the experiment before written informed consent was obtained. The present study was approved by the Medical Ethical Committee of Maastricht University Medical Center+ in accordance with the Declaration of Helsinki.

Experimental outline. An overview of the experimental protocol is depicted in Figure 1. After inclusion into the study, participants were randomly allocated to either the control (CON)⁵ group ($n = 12$) or the group administered a dietary protein supplement (PRO group; $n = 11$). Both groups were subjected to 5 d of muscle disuse induced by way of a full-leg cast. The immobilized leg was randomly allocated and counter-balanced between left and right. Two d prior to casting and directly after cast removal, a series of measurements was performed. Single-slice computed tomography (CT) scans were performed at the mid-thigh of both legs, whole-body DXA scans were taken, a single muscle biopsy from the immobilized leg and venous blood sample were collected, and 1-legged knee extension strength (1 repetition maximum) was assessed for both legs.

Muscle mass and function tests. Forty-eight h prior to and directly after the casting period, participants visited the laboratory for 2 identical test days (i.e., test days 1 and 2). During these test days, multiple measurements of muscle mass and function were performed. First, the anatomic cross-sectional areas (CSAs) of *Musculus quadriceps femoris* and whole thigh were assessed via a single-slice CT scan (Philips Brilliance 64; Philips Medical Systems) as performed before (8). With participants placed in a supine position, their legs extended and their feet secured, a 3-mm thick axial image was taken 15-cm proximal to the top of the patella. On test day 1, the exact scanning position was marked with semi-permanent ink for replication on test day 2. ImageJ software (version 1.46r; NIH) was used to analyze CT scan images for the CSAs of all thigh muscles and the quadriceps muscle separately. Second, a DXA

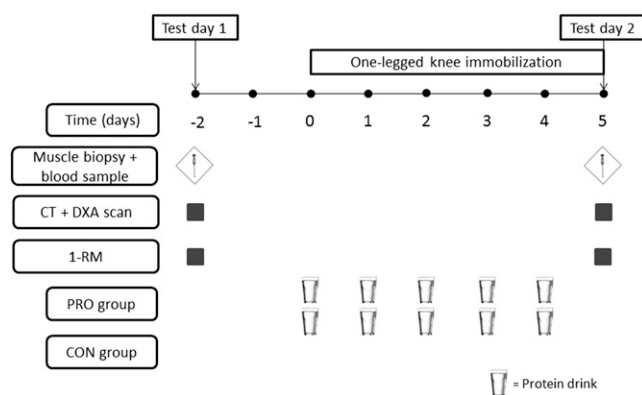


FIGURE 1 Outline of the experimental protocol. Two groups of healthy older men were assigned to undergo 5 d of one-legged knee immobilization, with (PRO group; $n = 11$) or without (CON group; $n = 12$) protein supplementation (~20 g of protein twice daily). CON, control; CT, computed tomography; PRO, dietary protein supplement; 1-RM, 1 repetition maximum.

scan (Discovery A, QDR Series; Hologic) was used to determine body composition and bone mineral content. Leg lean mass was determined by using the system's software package Apex version 2.3. Maximal muscle strength was determined for each leg individually by 1 repetition maximum strength tests on a leg extension machine (Technogym) as performed before (8,33).

Blood and muscle sampling. Fasting venous blood samples were collected for determination of basal plasma glucose and insulin concentrations on test days 1 and 2. Blood (10 mL) was collected in EDTA-containing tubes and immediately centrifuged at $1000 \times g$ for 10 min at 4°C. Aliquots of plasma were snap frozen in liquid nitrogen and stored at -80°C until further analysis. Plasma glucose, FFA, and TG concentrations were analyzed with an ABX Pentra 400 analyzer (Horiba Diagnostics) with test kits from ABX Diagnostics, whereas plasma insulin concentrations were determined by radioimmunoassay (ref. HI-14K; Millipore). Plasma amino acid concentrations were measured by using ultra-performance LC/tandem MS as described previously (34).

Muscle biopsies were taken from *Musculus vastus lateralis* of the immobilized leg prior to casting and immediately after cast removal but prior to performing any weight-bearing activities. Biopsies were taken at the same time in the morning (0830) after an overnight fast, and the same standardized meal was provided the evening prior to muscle biopsy collection. Percutaneous muscle biopsies were taken from *M. vastus lateralis*, ~15 cm above the patella with the Bergstrom technique. The collected muscle was freed from any visible nonmuscle tissue, processed immediately, and stored at -80°C until further analysis.

Leg immobilization. Two d after test day 1, at 0800, a full-leg cast (randomized and counterbalanced for left and right leg) was applied in the casting room of the Academic Hospital in Maastricht. This marked the start of the 5-d immobilization period that always included 3 weekdays and 2 weekend days. The cast extended from ~5 cm above the ankle to ~25 cm above the patella. An $\sim 30^\circ$ angle of flexion of the knee joint was established to prevent participants from performing weight-bearing activities with the immobilized leg. Participants received crutches and were instructed on the correct usage before being provided with transportation home. The cast was removed at 0800 on test day 2, after exactly 5 d of immobilization.

Protein supplementation. Participants were randomly allocated to the PRO group, which was administered a high whey protein leucine-enriched oral nutritional supplement, or the CON group, which was not administered a supplement. Participants allocated to the PRO group consumed the first drink in the laboratory the morning of casting and were instructed to consume 1 drink directly after breakfast and 1 drink immediately prior to sleep each day during immobilization (i.e., twice daily, 10 drinks in total). Each drink provided 635 kJ, 21 g of protein, 9 g

⁵ Abbreviations used: CON, control; CSA, cross-sectional area; CT, computed tomography; En%, energy percent; LAT1, large neutral amino acid transporter 1; MAFBx, muscle atrophy F-box/atrogen-1; MuRF1, muscle RING-finger protein-1; PAT1, proton-coupled amino acid transporter 1; PRO, dietary protein supplement.

of carbohydrates, 3 g of fat, and a mixture of vitamins, minerals, and fibers. **Supplemental Table 1** depicts the composition of the study product.

Dietary intake. Standardized meals, containing 2.9 MJ and providing 51 energy percent (En%) as carbohydrate, 32 En% as fat, and 17 En% as protein, were consumed the evening prior to both test days. Weighted dietary intake records were completed by the participants for the 5-d duration of the immobilization period and on a separate consecutive 5-d occasion either before or after (randomly allocated to avoid recording bias) the immobilization period. The same 5 d of the week were selected for both recording periods. DietInzicht software (35), based on NEVO table 2011, was used to analyze dietary intake records.

Muscle analysis. Muscle samples were freed from any visible nonmuscle tissue and separated into 2 sections. The first part (~30 mg) was imbedded in Tissue-Tek (Sakura Finetek), frozen in liquid nitrogen-cooled isopentane, and used to determine muscle fiber-type specific CSA and satellite cell content as performed previously (8). The second part (~15 mg) was snap frozen in liquid nitrogen and used for real-time PCR analysis to determine mRNA expression of selected genes as described before (8,20). A detailed overview of the muscle analyses is presented in the online supporting material.

Statistics. All data are expressed as means ± SEMs. Baseline values between groups were compared by means of an independent-samples *t* test. Pre- vs. post-immobilization data were analyzed by using repeated measures ANOVA with treatment (CON vs. PRO groups) as a between-subjects factor and time (pre- vs. post-immobilization) as a within-subjects factor. Fiber type (type I vs. type II) was added to the test as a within-subjects factor when performing the statistical analyses for the muscle data. In case of a significant main effect, paired-samples *t* tests were executed to determine time effects within treatment groups or within fiber types, and independent-samples *t* tests were performed to determine group differences in pre- and post-immobilization values. When a significant main effect was detected, Bonferroni's post hoc test was applied to locate the differences. A *P* value of <0.05 was used to determine statistical significance. All data were analyzed by using SPSS version 20.0 (SPSS).

RESULTS

Participants. Participants' characteristics are provided in **Table 1**. No baseline differences between the CON and PRO groups were observed for age, height, weight, BMI, glucose, insulin, HOMA, or HbA1c concentrations at baseline. Glucose, insulin, and HOMA concentrations were measured pre- and post-intervention and did not change over time in either group.

TABLE 1 Characteristics of healthy older men in the CON and PRO groups¹

	CON group (n = 12)	PRO group (n = 11)
Age, y	70 ± 1	68 ± 1
Body mass, kg	82.9 ± 3.0	79.6 ± 2.4
Height, m	1.74 ± 0.02	1.74 ± 0.02
BMI, kg/m ²	27.3 ± 0.6	26.4 ± 0.8
Leg volume, L	7.96 ± 0.28	7.90 ± 0.35
Plasma glucose, mmol/L	5.6 ± 0.1	5.7 ± 0.1
Plasma insulin, uU/mL	11.7 ± 1.4	9.9 ± 1.0
HOMA index	3.0 ± 0.4	2.6 ± 0.3
Glycated hemoglobin, %	5.4 ± 0.1	5.7 ± 0.1
Glycated hemoglobin, mmol/mol	35.9 ± 1.2	38.4 ± 1.3

¹ Values are means ± SEMs. No differences were observed between groups (*P* ≥ 0.05 for all variables). CON, control; PRO, dietary protein supplement.

Muscle mass and strength. Quadriceps muscle CSAs are displayed in **Figure 2A**. At baseline, no differences were observed in quadriceps or whole-leg muscle CSAs between groups (*P* ≥ 0.05 for both variables). Five d of immobilization caused significant muscle atrophy of the quadriceps in both groups (time effect, *P* < 0.001; see Fig. 2A) and the whole leg in the CON group [time effect, *P* < 0.05; from 13.3 ± 5.4 to 13.2 ± 5.3 cm² (-0.7 ± 0.6%)] and PRO group [from 12.6 ± 4.2 to 12.4 ± 4.6 cm² (-1.6 ± 0.6%)], with no differences between groups (*P*-interaction ≥ 0.05 for both variables). Immobilization did not affect whole-body or leg lean mass in either group (data not shown; both *P* ≥ 0.05). Leg muscle strength data are presented in **Figure 2B**. Maximal leg muscle strength had decreased following immobilization in the CON and PRO groups (time effect, *P* < 0.001), with no differences between groups (*P*-interaction ≥ 0.05).

Dietary intake. **Table 2** shows data for participants' habitual diets for 5 d under free-living conditions and during the 5-d immobilization period. No differences in habitual diet were observed between groups (all measured variables, *P* ≥ 0.05). Habitual diet did not change because of immobilization in the CON group (*P* ≥ 0.05), whereas in the PRO group, twice-daily ingestion of the protein drink significantly increased protein

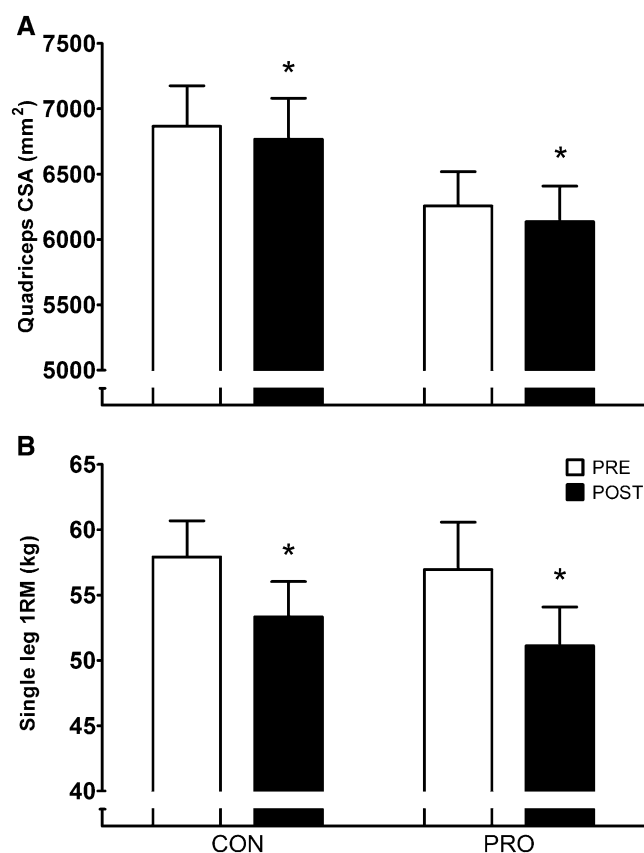


FIGURE 2 CSAs of *Musculus quadriceps femoris* in healthy older men in the CON (*n* = 12) and PRO (*n* = 11) groups, measured by a single-slice CT scan 48 h prior to (PRE) and immediately following (POST) 5 d of leg immobilization (A). Leg muscle strength as measured by 1 repetition maximum in both the CON and PRO groups (B). Data are expressed as means ± SEMs. **P* < 0.05, significantly different when compared with pre-immobilization values. CON, control; CSA, cross-sectional area; CT, computed tomography; PRO, dietary protein supplement; 1RM, 1 repetition maximum.

TABLE 2 Dietary intake of healthy older men under free-living conditions and during a 5-d period of leg immobilization, with or without supplementation¹

Variable	CON group (n = 12)		PRO group (n = 11)	
	Free living	Immobilization	Free living	Immobilization
Energy intake, MJ·d ⁻¹	8.82 ± 0.62	9.03 ± 0.46	8.73 ± 0.54	9.50 ± 0.49
Protein intake, g·d ⁻¹	85 ± 9	86 ± 4	90 ± 4	125 ± 6*
Protein, g·kg ⁻¹ ·d ⁻¹	1.04 ± 0.12	1.05 ± 0.06	1.14 ± 0.07	1.60 ± 0.11*
Protein, En%	16.7 ± 1.2	16.4 ± 0.7	18.0 ± 0.9	22.9 ± 1.0*
Fat, En%	31.6 ± 1.3	32.9 ± 2.3	29.5 ± 1.8	25.8 ± 1.2*
Carbohydrate, En%	51.7 ± 2.0	50.6 ± 2.1	52.5 ± 2.1	51.3 ± 1.5

¹ Values are means ± SEMs. Data in the PRO group include the twice-daily intake of the protein supplement. *Significantly different from the free-living value ($P < 0.05$). CON, control; En%, energy percent; PRO, dietary protein supplement.

intake (expressed as g·d⁻¹, g·kg⁻¹·d⁻¹, and En%) compared with baseline ($P < 0.05$) and the CON group ($P < 0.05$). Habitual protein intake averaged 1.1 g·kg⁻¹·d⁻¹ and was increased to 1.6 g·kg⁻¹·d⁻¹ during the immobilization period in the PRO group. Energy intake in the PRO group was maintained during immobilization; a relatively higher amount of energy was received from protein, at the expense of energy from fat ($P < 0.05$).

Plasma analyses. Plasma amino acid concentrations (Supplemental Table 2) were increased in both groups for alanine, cysteine, phenylalanine, threonine, and tryptophan (all $P < 0.05$). For valine (P -interaction < 0.05), an increase following

immobilization was observed in the PRO group only ($P < 0.05$). All other measured amino acids were not changed following immobilization (all $P \geq 0.05$). Immobilization, with or without protein supplementation, did not influence plasma FFA (CON group: from 384 ± 33 to 354 ± 33 μmol/L; PRO group: from 446 ± 48 to 404 ± 46 μmol/L) or TG concentrations (CON group: from 1190 ± 210 to 1270 ± 92 μmol/L; PRO group: from 968 ± 88 to 1110 ± 118 μmol/L) (both $P \geq 0.05$).

Muscle fiber characteristics. Muscle fiber characteristics are displayed in Table 3. At baseline, no differences between groups were observed for any of the variables. No measurable decline in muscle fiber CSA was observed following immobilization in either group ($P \geq 0.05$). Although no changes in myonuclear content were observed following immobilization ($P \geq 0.05$), myonuclear domain size decreased in both fiber types in both the CON and PRO groups (time effect, $P < 0.05$). At baseline, satellite cell content expressed per muscle fiber, per millimeter squared, and as a percentage of the total number of myonuclei was higher in type I compared with type II fibers ($P < 0.05$ for all 3 variables). No changes over time or differences between groups were observed ($P \geq 0.05$).

mRNA expression. Figure 3 and Supplemental Figure 1 display the skeletal muscle mRNA expression of the selected genes of interest. Muscle mRNA expression of myostatin (Fig. 3A) and myogenin (Fig. 3C) increased following immobilization in both groups ($P < 0.05$), whereas myoD (Fig. 3B) tended toward an increase in both groups ($P = 0.07$). Muscle atrophy F-box/

TABLE 3 Muscle fiber characteristics of healthy older men before (Pre) and after (Post) 5 d of leg immobilization, with or without supplementation¹

	CON group (n = 12)		PRO group (n = 11)	
	Pre	Post	Pre	Post
Muscle fiber CSA, μm ²				
Fiber type I	5654 ± 391	5037 ± 487	5646 ± 469	5370 ± 379
Fiber type II	5592 ± 564	5000 ± 525	5131 ± 390	5027 ± 356
Fiber, %				
Fiber type I	49 ± 3	44 ± 3	48 ± 5	48 ± 5
Fiber type II	51 ± 3	56 ± 3	52 ± 5	52 ± 5
Fiber, area %				
Fiber type I	50 ± 4	45 ± 3	51 ± 5	49 ± 6
Fiber type II	50 ± 4	55 ± 3	49 ± 5	51 ± 6
Nuclei, n/fiber				
Fiber type I	2.8 ± 0.1	2.9 ± 0.3	2.8 ± 0.2	2.9 ± 0.2
Fiber type II	2.8 ± 0.1	2.8 ± 0.2	2.8 ± 0.2	2.8 ± 0.2
Myonuclear domain, μm ²				
Fiber type I	2026 ± 86	1716 ± 106*	2035 ± 89	1914 ± 97*
Fiber type II	2072 ± 112	1770 ± 126*	1843 ± 111	1791 ± 112*
SC, n/fiber				
Fiber type I	0.101 ± 0.014	0.091 ± 0.013	0.099 ± 0.010	0.099 ± 0.007
Fiber type II	0.056 ± 0.008**	0.055 ± 0.009**	0.062 ± 0.006**	0.060 ± 0.006**
SC, n/mm ²				
Fiber type I	18.1 ± 2.5	18.0 ± 2.3	17.7 ± 2.1	19.1 ± 1.6
Fiber type II	10.1 ± 1.5**	10.3 ± 1.4**	11.8 ± 1.1**	12.7 ± 1.6**
SC, ² n/myonuclei, %				
Fiber type I	3.6 ± 0.4	3.3 ± 0.5	3.7 ± 0.5	3.6 ± 0.3
Fiber type II	1.9 ± 0.2**	2.0 ± 0.3**	2.2 ± 0.2**	2.2 ± 0.2**

¹ Values are means ± SEMs. *Significantly different from pre-immobilization values ($P < 0.05$). **Significantly different from values in type I fiber ($P < 0.05$). CON, control; CSA, cross-sectional area; PRO, dietary protein supplement; SC, satellite cell.

² Number of SCs as a percentage of the total number of myonuclei (i.e., number of myonuclei + number of SCs).

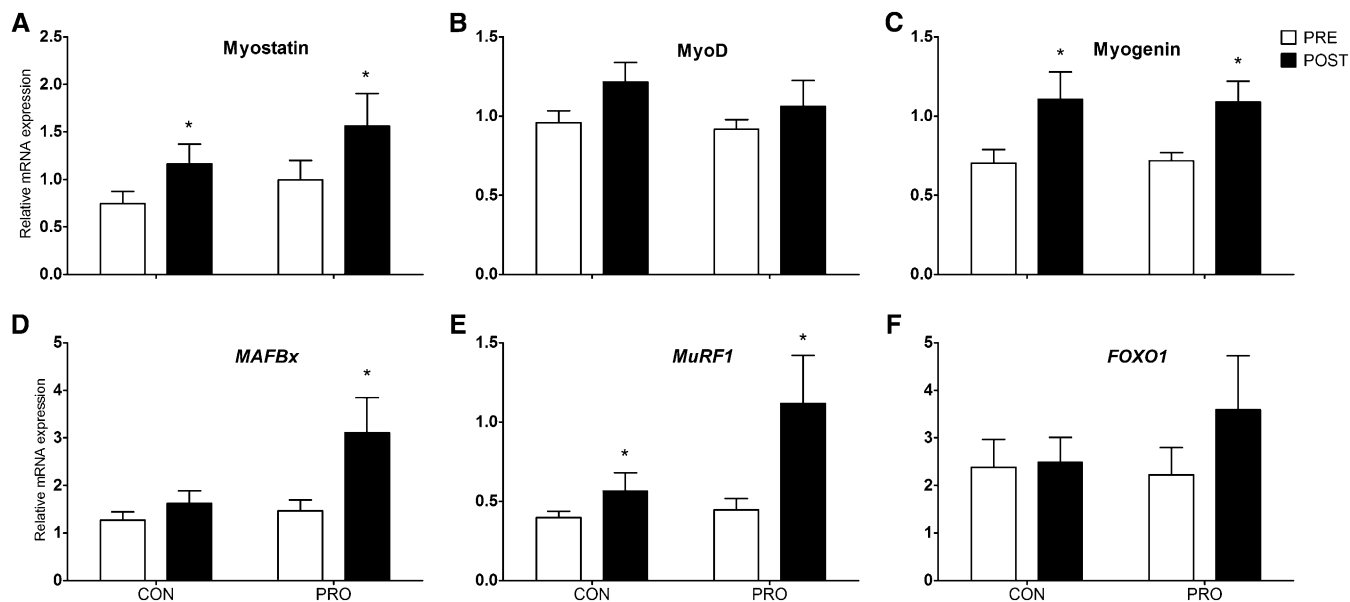


FIGURE 3 Skeletal muscle mRNA expression of selected genes involved in myogenesis (A–C) and muscle proteolysis (D–F), measured 48 h prior to (PRE) and immediately following (POST) 5 d of one-legged knee immobilization in healthy older men in the CON ($n = 12$) and PRO ($n = 11$) groups. Data are expressed as means \pm SEMs. * $P < 0.05$, significantly different when compared with pre-immobilization values. CON, control; FOXO1, forkhead box protein O1; MAFBx, muscle atrophy F-box/atrogen-1; MuRF1, muscle RING-finger protein-1; PRO, dietary protein supplement.

atrogen-1 (*MAFBx*) mRNA expression (Fig. 3D) had a significant time \times treatment interaction ($P < 0.05$) with a significant increase only detected in the PRO group ($P < 0.05$) following immobilization. Muscle RING-finger protein-1 (*MuRF1*) mRNA expression (Fig. 3E) significantly increased in both groups (time effect, $P < 0.01$). There was a trend for an interaction effect ($P = 0.07$) such that *MuRF1* mRNA expression was increased to a higher extent in the PRO group ($P < 0.05$) compared with the CON group ($P \geq 0.05$). For the mRNA expression of 2 amino acid transporters, large neutral amino acid transporter 1 (*LAT1*) (Supplemental Fig. 1D) and proton-coupled amino acid transporter 1 (*PAT1*) (Supplemental Fig. 1E), a significant time effect was found ($P < 0.01$ for both genes) such that expression was upregulated following immobilization in both groups. All other genes had no significant changes between or within groups.

DISCUSSION

In the present study, we demonstrated that merely 5 d of one-legged knee immobilization lead to substantial skeletal muscle mass and strength loss in healthy older men. Increasing dietary protein intake by supplementing with ~ 20 g of protein twice daily did not attenuate the loss of muscle mass or strength during 5 d of muscle disuse in older men.

A period of prolonged muscle disuse and the associated muscle atrophy causes numerous negative health consequences (1,2,4,6), and the occurrence of successive periods of muscle disuse likely represents a key factor responsible for the loss of muscle mass during the later decades of life (13). In the present study, we found that only 5 d of muscle disuse lead to substantial loss of muscle mass ($-1.5 \pm 0.7\%$; Fig. 2A) and strength ($-8.3 \pm 3.3\%$; Fig. 2B) in older individuals. These data are in line with recent data from our group in which we observed similar muscle mass and strength losses in younger individuals (8). Furthermore, Suetta et al. (10) reported significant muscle fiber atrophy after 4 d of

immobilization in both young and older individuals. The rapid muscle atrophy observed in our older participants after merely 5 d of leg immobilization is of important clinical significance because successive short periods of muscle disuse from illness or injury are highly prevalent during the later stages of life (36). In line, the mean length of hospitalization for older patients admitted with acute illness is 5–7 d (11). The observed muscle loss is of particular relevance because the older population has difficulty regaining skeletal muscle mass and strength following a period of disuse (37). Even when applying rehabilitative resistance-type exercise training after a period of disuse, muscle mass does not seem to be restored after 4 wk of intense supervised training (37). For these reasons, it is presently believed that the impact of successive short episodes of muscle disuse may be of key relevance in the development of sarcopenia (13).

Practical and effective interventional strategies are needed to prevent or attenuate muscle mass and strength loss during short periods of muscle disuse in healthy older individuals and more clinically compromised subpopulations. It was proposed that simply increasing the protein content of the diet may alleviate the loss of muscle tissue during a period of disuse (12,27). Indeed, studies focusing on mimicking prolonged hospitalization (i.e., >2- to 3-wk of bed rest under tightly controlled dietary conditions) have shown that supplementation with high doses of crystalline essential amino acids (~ 50 g, equivalent to ~ 100 – 150 g of intact protein) attenuates the loss of muscle mass (29–31). Given the clinical relevance of successive short periods of muscle disuse, we assessed the efficacy of a more practical and feasible dietary strategy to attenuate muscle loss during a short period of limb immobilization under free-living conditions. Increasing dietary protein intake from 1.1 to 1.6 g/kg body weight $^{-1}\cdot$ d $^{-1}$ did not attenuate the loss of muscle mass or strength observed during a 5-d period of leg immobilization (Fig. 2). The apparent discrepancy between the outcome of the present study and previous work in prolonged bed rest studies may be attributed to differences in protein intake in the CON group. In the present study, the CON group retained normal

habitual energy and protein intake ($1.1 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$), whereas the PRO group was administered additional supplementation ($1.6 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$). In contrast, in previous bed rest studies that show benefits of amino acid supplementation on muscle mass maintenance, the control groups generally consumed dietary protein at a concentration no higher than $0.8 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ (29–31). Consequently, we speculate that maintaining dietary protein intake is required to prevent muscle loss during disuse, but that increasing dietary protein intake above habitual concentrations does not further alleviate muscle loss during disuse (38,39). This would be of particular relevance in institutionalized or hospitalized older individuals who are unable to maintain habitual dietary protein consumption during more prolonged periods of muscle disuse because of illness or injury. Additional considerations of the present nutritional intervention include the type and timing of protein administered. We selected whey protein in the present study because we previously showed that it leads to higher postprandial muscle protein accretion compared with casein protein in healthy older men (40). We chose to supplement volunteers at breakfast because we previously showed that community-dwelling older individuals generally consume inadequate amounts of protein at breakfast (41). Specifically, the supplement was provided directly after breakfast to prevent volunteers from compensating for the supplement by consuming less breakfast, thereby ensuring that adequate protein was consumed. This was achieved given that the PRO group consumed $36 \pm 2 \text{ g}$ at this meal compared with the CON group, which only consumed $13 \pm 1 \text{ g}$, an amount that is insufficient to properly stimulate muscle protein synthesis rates (42). We opted to deliver the second supplement immediately prior to sleep because we recently showed that such a strategy effectively stimulates overnight muscle protein synthesis rates (43). However, it is also true that these beneficial effects on nocturnal muscle protein synthesis were obtained with the ingestion (or intragastric administration) of large amounts of casein protein to ensure a more sustained hyperaminoacidemia throughout the night (44). Accordingly, it could be speculated that future nutritional strategies aimed at attenuating muscle disuse atrophy may wish to consider incorporating large boluses of casein as a pre-bedtime meal. In contrast, it could also be hypothesized that ingestion of a large bolus of dietary protein prior to sleep increases both muscle protein synthesis and breakdown rates, without net muscle protein accretion (45). Although previous work has shown improvements in overnight whole-body protein balance following protein administration in healthy older men (43) and in young adults during overnight recovery from exercise (46), we cannot exclude that such improvements in overnight protein balance may not occur in a setting of muscle disuse.

Besides assessing the impact of protein supplementation on muscle mass and strength during short-term disuse, we wished to gain insight into the underlying myocellular mechanisms involved in muscle disuse atrophy and/or muscle mass maintenance. Muscle loss during short-term muscle disuse is thought to be, at least partly, mediated by accelerated rates of muscle protein breakdown (13). Myostatin is known as a negative regulator of muscle growth in vivo (47) and acts through multiple pathways including the stimulation of muscle protein breakdown (48). Consistent with this role, we observed increases in myostatin mRNA expression (Fig. 3) and in markers of muscle protein breakdown (i.e., increased gene expression of *MAFBx* and *MuRF1*; Fig. 3). This is in line with previous findings (10) and our own work in young men (8,9), and supportive of a role for muscle protein breakdown in short-term

muscle atrophy, possibly mediated through increased myostatin transcription. Given the lack of effect of protein supplementation on muscle mass in the present study, it is not surprising that we observed no attenuation of the rise in myostatin and markers of proteolysis. In fact, we actually observed that *MAFBx* and *MuRF1* gene expression increased to a higher extent in the PRO group (Fig. 3), supporting the idea that increasing dietary protein intake beyond the habitual dietary protein intake concentration may strongly stimulate overall protein turnover rates.

Myostatin is also reported to regulate muscle size by acting via the inhibition of myogenesis through its inhibitory action on the myogenic regulatory factors (49). However, in line with our previous work (8,50), we report that the disuse-induced increase in myostatin expression does not coincide with impaired expression of the myogenic regulatory factors (i.e., MyoD and myogenin; Fig. 3). Moreover, no alterations in muscle satellite cell content were observed, suggesting that the mechanisms underlying short-term disuse atrophy do not require alterations in myogenesis or satellite cell content. Recent data suggested that the expression of specific amino acid transporters within skeletal muscle provide a site of regulation for muscle protein synthesis (51). As such, we analyzed the gene expression of *LAT1*/solute carrier and *PAT1*, which are thought to be the key transporters facilitating intramuscular transport of BCAAs particularly in response to nutrition (52). Interestingly, *LAT1* and *PAT1* mRNA expression (Supplemental Fig. 1) increased following immobilization in both groups, possibly indicating a compensatory mechanism by which atrophying muscle attempts to “scavenge” circulating amino acids as a substrate for muscle protein synthesis.

In the present study, we showed that protein supplementation on top of a diet containing ample protein ($1.1 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$) does not alleviate muscle loss during short-term single-leg disuse. This shows that besides maintaining dietary protein intake, other strategies are warranted to help maintain muscle mass. Where possible, performing some degree of exercise should be considered during disuse (39). In conditions where exercise is not feasible because of injury or illness, low-volume physical activity (53) or even exercise surrogates (8) could be suggested. Furthermore, other nutritional compounds, such as creatine or ω -3 FAs, may support muscle maintenance during disuse (27). An often underappreciated consideration is how dietary strategies could support rehabilitation following a period of disuse. This area was comparatively understudied (54–57); however, given the opportunity to combine nutrition with reambulation and/or physical exercise, future research should address how dietary protein and/or other nutritional strategies could best be used to facilitate the rapid and complete restoration of muscle mass following a period of disuse.

In sum, we conclude that short-term muscle disuse results in a substantial decline in both muscle mass and strength in older individuals. Increasing dietary protein intake during short-term muscle disuse on top of a diet providing $>1.0 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ of protein does not alleviate muscle disuse atrophy in healthy older men.

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M.L.D., B.T.W., R.N., and D.H.J.M.W. conducted the research; M.L.D., B.T.W., and R.N. analyzed the data; M.L.D., B.T.W., R.N., D.H.J.M.W., L.B.V., and L.J.C.v.L. wrote the paper; and M.L.D. had primary responsibility for the final content. All authors read and approved the final manuscript.

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