Skeletal muscle disuse atrophy is not attenuated by dietary protein supplementation in healthy, older men[1,2,3]

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This manuscript contains four (4) figures and three (3) tables.

[2] Online supporting material is 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.

[3] **Abbreviations used:** CSA, cross-sectional area; CT, Computed Tomography; DEXA, Dual Energy X-ray Absorptiometry; FAK, Focal Adhesion Kinase; FOXO1, Forkhead box protein O1; FT, fiber typing; HOMA-index, Homeostatic Model Assessment Index; LAT1, Large Neutral Amino Acid Transporter 1; MAFBx, Muscle Atrophy F-box/Atrogen-1; mTOR, mammalian target of rapamycin; MuRF1, Muscle RING-finger protein-1;SC, satellite cell; PAT1, Proton-coupled amino acid transporter 1; PBS, phosphate-buffered saline; P70S6K, P70S6 kinase; 1RM, one-repetition maximum

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, elderly (69±1 y) males were subjected to 5 days 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 protein, 9.3 g carbohydrate, and 3.0 g fat) twice daily. Two days prior to and immediately after the immobilization period, single slice CT-scans of the quadriceps and single leg 1-Repetition Maximum (1RM) 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, and mRNA and protein expression of selected genes. Immobilization decreased quadriceps CSA 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 *MuRF1* mRNA expression increased following immobilization in both groups ($P<0.05$), while muscle *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. **Clinical trial registration:** NCT01588808

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Introduction

A period of prolonged (i.e. several weeks) muscle disuse, due to 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 elderly individuals, who are already functionally and/or metabolically compromised. Recently, we (8, 9) as well as others (10) have shown that even a few days of disuse can already lead to significant losses of muscle mass and strength in young and old men. These findings are of particular clinical relevance as hospitalization of elderly due to acute illness generally results in an average 5-7 day hospital stay (11). It has been 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) as well as limb immobilization (17-19). Furthermore, recent work from our laboratory (20) as well as others (17, 21, 22) has shown 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 post-prandial muscle protein synthesis rates play the major 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 has been speculated that maintaining or 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 elderly individuals (32). However, the potential for a practical dietary protein feeding strategy to alleviate muscle loss during short-term disuse in the elderly 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 elderly men were selected to participate in a study during which they were subjected to 5 days of one-legged knee immobilization with or without dietary protein supplementation (~20 g 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

Subjects

Twenty-three healthy elderly men (mean age 69±1 y) were included in the present study. Prior to inclusion, a general health questionnaire was filled in by the subjects and a routine medical screening was completed to exclude individuals with BMI below 18.5 or above 30 kg/m^2; 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 six months preceding the start of the study. All subjects were informed on 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 Centre+ 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, subjects were randomly allocated to either the control (CON, *n*=12) or the protein (PRO, *n*=11) group. Both groups were subjected to five days 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 days 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 dual energy x-ray absorptiometry (DEXA) scans were taken, a single muscle biopsy from the immobilized leg and venous blood sample were collected, and one-legged knee extension strength (1RM, one-repetition maximum) was assessed for both legs.

Muscle mass and function tests

Forty eight hours prior to, and directly after the casting period, subjects visited the laboratory for two identical test days (i.e. test days 1 and 2). During these test days, multiple measurements of muscle mass and function were performed. Firstly, the anatomical cross-sectional area (CSA) of *m. quadriceps femoris* and whole thigh were assessed via a single slice computed tomography (CT) scan (Philips Brilliance 64, Philips Medical Systems, Best, The Netherlands) as done before (8). With subjects 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, National Institute of Health, Bethesda, MD, USA) was used to analyze CT scan images for the cross-sectional area of all thigh muscles as well as the quadriceps muscle separately. Secondly, a DEXA scan (Hologic, Discovery A, QDR Series, Bradford, MA, USA) was used to determine body composition and bone mineral content. Leg lean mass was determined using the system's software package Apex version 2.3. Maximal muscle strength was determined for each leg individually by 1RM strength tests on a leg extension machine (Technogym, Rotterdam, the Netherlands) as done before (8, 33).

Blood and muscle sampling

Fasting venous blood samples were collected for determination of basal plasma glucose and insulin concentrations on test day 1 and 2. Blood (10 mL) was collected in EDTA-containing tubes and immediately centrifuged at 1,000g 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, free fatty acids, and triglyceride concentrations were analyzed with a ABX Pentra 400 analyzer (Horiba Diagnostics, Montpellier, France) with test kits from ABX Diagnostics (Montpellier, France), whereas plasma insulin concentrations were determined by radioimmunoassay (Millipore, ref. HI-14K , Billerica, MA, USA). Plasma amino acid concentrations were measured using ultra-performance liquid chromatography tandem mass spectrometry as described previously (34).

Muscle biopsies were taken from *m. vastus lateralis* of the immobilized leg prior to casting and immediately after cast removal, prior to performing any weight bearing activities. Biopsies were taken at the same time (08.30 AM) in the morning 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* with the Bergstrom technique, approximately 15 cm above the patella. The collected muscle was freed from any visible non-muscle tissue, processed immediately, and stored at -80°C until further analysis.

Leg immobilization

Two days after performing test day 1, at 8:00 in the morning, 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 day immobilization period that always contained 3 week days and 2 weekend days. The cast extended from ~5 cm above the ankle until ~25 cm above the patella. A ~30 degree angle of flexion of the knee joint was established in order to prevent subjects from performing weight-bearing activities with the immobilized leg. Subjects received crutches and were instructed on the correct usage before being provided with transportation home. The cast was removed at 8:00 on the morning of test day 2, after exactly 5 days of immobilization.

Protein supplementation

Subjects were randomly allocated to the group receiving a high whey protein leucine-enriched oral nutritional supplement (PRO) or the control group receiving no supplement (CON). Subjects allocated to the PRO group consumed the first drink in the laboratory on the morning of casting and were instructed to consume one drink directly after breakfast and one drink immediately prior to sleep on each day during immobilization (i.e. twice-daily, 10 drinks in total). Each drink provided 635 kJ, 21 g protein, 9 g carbohydrates, 3 g 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% (En%) as carbohydrate, 32 En% as fat, and 17 En% as protein, were consumed on the evening prior to both test days. Weighted dietary intake records were completed by the subjects for the 5 day duration of the immobilization period as well as on a separate consecutive 5 day occasion either before or after (randomly allocated to avoid recording bias) the immobilization period. The same 5 days of the week were selected for both recording periods. DieetInzicht software (35), based on the NEVO table 2011, was used to analyze dietary intake records.

Muscle analysis

Muscle samples were freed from any visible non-muscle tissue and separated into two sections. The first part (~30 mg) was imbedded in Tissue-Tek (Sakura Finetek, Zoeterwoude, the Netherlands), frozen on liquid nitrogen cooled isopentane and used to determine muscle fiber-type specific cross-sectional area (CSA) and satellite cell content as done 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 mean±SEM. Baseline values between groups were compared by means of an independent samples t-test. Pre- versus post-immobilization data were analyzed using Repeated Measures ANOVA with treatment (CON vs PRO) as between-subjects factor and time (pre- vs post-immobilization) as 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 using SPSS version 20.0 (SPSS Inc., Chicago, IL, USA).

Results

Subjects

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

Muscle mass and strength

Quadriceps muscle cross-sectional area (CSA) is displayed in **Figure 2A**. At baseline, no differences were observed in quadriceps or whole leg muscle CSA between groups ($P>0.05$ for both parameters). Five days of immobilization caused significant muscle atrophy of the quadriceps (time effect, $P<0.001$; see **Figure 2A**) and the whole leg (time effect, $P<0.05$; from 13.3±5.4 to 13.2±5.3 cm^2 (-0.7±0.6%) in CON and from 12.6±4.2 to 12.4±4.6 cm^2 (-1.6±0.6%) in PRO) with no differences between groups (*P*-interaction>0.05 for both parameters). 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 group (time effect, $P<0.001$), with no differences between groups (*P*-interaction>0.05).

Dietary intake

Table 2 shows data for subjects' habitual diet for 5 days under free living conditions and during the 5 day immobilization period. No differences in habitual diet were observed between groups (all measured parameters $P>0.05$). Habitual diet did not change due to immobilization in the CON group ($P>0.05$), whereas in the PRO group, twice-daily ingestion of the protein drink significantly increased protein intake (expressed as $\text{g}\cdot\text{day}^{-1}$, $\text{g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$, and En%) compared with baseline ($P<0.05$) and the CON group ($P<0.05$). Habitual protein intake averaged 1.1 $\text{g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ and was increased to 1.6 $\text{g}\cdot\text{kg}^{-1}\cdot\text{day}^{-1}$ 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*>0.05). Immobilization, with or without protein supplementation, did not influence plasma free fatty acid (CON: from 384±33 to 354±33 µmol/L; PRO: from 446±48 to 404±46 µmol/L) or triglyceride (CON: from 1190±210 to 1270±92 µmol/L; PRO: from 968±88 to 1110±118 µmol/L) concentrations (both *P*>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*>0.05). Although no changes in myonuclear content were observed following immobilization (*P*>0.05), myonuclear domain size decreased in both fiber types in both CON and PRO (time effect, *P*<0.05). At baseline, satellite cell (SC) content expressed per muscle fiber, per millimetre 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 three parameters). No changes over time or differences between groups were observed (*P*>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* (**Figure 3A**) and *myogenin* **(Figure 3C**) increased following immobilization in both groups ($P<0.05$), whereas *myoD* (**Figure 3B**) tended towards an increase in both groups ($P=0.07$). *MAFBx* mRNA expression (**Figure 3D**) showed a significant time*treatment interaction ($P<0.05$) with a significant increase only detected in the PRO group ($P<0.05$) following immobilization. Muscle *MuRF1* mRNA (**Figure 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 greater extent in PRO ($P<0.05$) compared with CON ($P>0.05$). For the mRNA expression of both amino acid transporters *LAT1* (**Supplemental Figure 1D**) and *PAT1* (**Supplemental Figure 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 showed no significant changes between or within groups.

Discussion

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

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 our lifespan (13). In the present study, we report that only 5 days of muscle disuse already leads to substantial loss of muscle mass (-1.5±0.7%; **Figure 2A**) and strength (-8.3±3.3%; **Figure 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.* reported significant muscle fiber atrophy after 4 days of immobilization in both young and older individuals (10). The rapid muscle atrophy observed in our older subjects after merely 5 days of leg immobilization is of important clinical significance, as successive short periods of muscle disuse due to illness or injury are highly prevalent during the later stages of our lifespan (36). In line, the average length of hospitalization for elderly patients admitted with acute illness is 5–7 days (11). The observed muscle loss is of particular relevance as the older population has difficulty to regain 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 weeks of intense supervised training (37). For these reasons, it is presently believed that the impact of short successive 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 elderly as well as more clinically compromised subpopulations. It has been 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. >2-3 weeks 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 intact protein) attenuates the loss of muscle mass (29-31). Given the clinical relevance of short, successive 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 $g \cdot kg$ body $weight^{-1} \cdot day^{-1}$ did not rescue the loss of muscle mass or strength observed during a 5 day period of leg immobilization (**Figure 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 control group. In the present study the control group retained normal habitual energy and protein intake (1.1 $g \cdot kg^{-1} \cdot day^{-1}$) whereas the protein group received additional supplementation (1.6 $g \cdot kg^{-1} \cdot day^{-1}$). 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 level no higher than 0.8 $g \cdot kg^{-1} \cdot day^{-1}$ (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 levels does not further alleviate muscle loss during disuse (38, 39). This would be of particular relevance in institutionalized or hospitalized elderly who are unable to maintain habitual dietary protein consumption during more prolonged periods of muscle disuse due to 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 as we have previously shown it leads to greater post-prandial muscle protein accretion compared with casein protein in healthy elderly men (40). We chose to supplement volunteers at breakfast time since we have previously shown that community dwelling elderly individuals generally consume inadequate amounts of protein at breakfast (41). Specifically, the supplement was consumed directly *after* breakfast to avoid volunteers compensating for the supplement by consuming less breakfast and therefore ensuring adequate protein was consumed. This was achieved given that the PRO group consumed (36±2 g at this meal compared to the CON group who only consumed 13±1 g, the latter being an amount insufficient to properly stimulate muscle protein synthesis rates (42). We opted to deliver the second supplement immediately prior to sleep, since we have recently shown 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). Though 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 (**Figure 3**) and in markers of muscle protein breakdown (i.e. increased gene expression of MAFBx and MuRF1; **Figure 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 greater extent in the PRO group (**Figure 3**), supporting the idea that increasing dietary protein intake beyond the habitual dietary protein intake level 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, **Figure 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 have 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 Large Neutral Amino Acid Transporter 1 (LAT1/SLC) and Proton-coupled amino acid transporter 1 (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 Figure 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 show that protein supplementation on top of a diet containing ample protein (1.1 $g \cdot kg^{-1} \cdot day^{-1}$) 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 due to injury or illness, low-volume physical activity (53) or even exercise surrogates (8) could be suggested. Furthermore, other nutritional compounds, such as creatine or omega-3 fatty acids, may support muscle maintenance during disuse (27). An often under-appreciated consideration is how dietary strategies could support rehabilitation following a period of disuse. This area has been comparatively under studied (54-57) but, given the opportunity to combine nutrition with re-ambulation 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 short, 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 $g \cdot kg^{-1} \cdot day^{-1}$ does not alleviate muscle disuse atrophy in healthy, elderly men.

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Author contributions

The study was performed at Maastricht University, Maastricht, the Netherlands. M.L.D., B.T.W. and L.J.C.v.L. designed the study; 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. M.L.D. had primary responsibility for the final content. All authors have read and approved the final manuscript.

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Tables

Table 1: Subjects' characteristics of healthy older men in the control (CON) and protein supplemented (PRO) groups[1,2,3]

	CON (*n*=12)	**PRO (*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±SEM, *n*=23.

[2] Abbreviations: HOMA-index, Homeostatic Model Assessment Index.

[3] No differences were observed between groups (*P*>0.05 for all variables).

Table 2: Dietary intake of healthy elderly subjects under free-living conditions and during a 5-day period of leg immobilization, with (PRO) or without (CON) supplementation.[1,2,3]

Variable	**CON (n=12)**		**PRO (n=11)**	
	Free living	**Immobilization**	**Free living**	**Immobilization**
Energy intake, ***$MJ \cdot day^{-1}$***	8.82 ± 0.62	9.03 ± 0.46	8.73 ± 0.54	9.50 ± 0.49
Protein intake, ***$g \cdot day^{-1}$***	85 ± 9	86 ± 4	90 ± 4	125 ± 6 *
Protein, ***$g \cdot kg^{-1} \cdot day^{-1}$***	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] Data represent means±SEM, *n*=23.

[2] Data in the PRO group are expressed including twice-daily intake of the protein supplement.

[3] * Significantly different from free living value ($P<0.05$)

Table 3: Muscle fiber characteristics of healthy elderly individuals before (pre) and after (post) 5 days of leg immobilization, with (PRO) or without (CON) supplementation.[1,2,3,4]

		CON (*n*=12)		**PRO (*n*=11)**	
	Fiber type	**Pre**	**Post**	**Pre**	**Post**
Muscle fiber CSA, *µm²*	I	5654 ± 391	5037 ± 487	5646 ± 469	5370 ± 379
	II	5592 ± 564	5000 ± 525	5131 ± 390	5027 ± 356
Fiber, *%*	I	49 ± 3	44 ± 3	48 ± 5	48 ± 5
	II	51 ± 3	56 ± 3	52 ± 5	52 ± 5
Fiber, *area %*	I	50 ± 4	45 ± 3	51 ± 5	49 ± 6
	II	50 ± 4	55 ± 3	49 ± 5	51 ± 6
Nuclei, *n/fiber*	I	2.8 ± 0.1	2.9 ± 0.3	2.8 ± 0.2	2.9 ± 0.2
	II	2.8 ± 0.1	2.8 ± 0.2	2.8 ± 0.2	2.8 ± 0.2
Myonuclear domain, *µm²*	I	2026 ± 86	1716 ± 106 *	2035 ± 89	1914 ± 97 *
	II	2072 ± 112	1770 ± 126 *	1843 ± 111	1791 ± 112 *
SC, *n/fiber*	I	0.101 ± 0.014	0.091 ± 0.013	0.099 ± 0.010	0.099 ± 0.007
	II	0.056 ± 0.008 #	0.055 ± 0.009 #	0.062 ± 0.006 #	0.060 ± 0.006 #
SC, *n/mm²*	I	18.1 ± 2.5	18.0 ± 2.3	17.7 ± 2.1	19.1 ± 1.6
	II	10.1 ± 1.5 #	10.3 ± 1.4 #	11.8 ± 1.1 #	12.7 ± 1.6 #
SC, *n/myonuclei, %*	I	3.6 ± 0.4	3.3 ± 0.5	3.7 ± 0.5	3.6 ± 0.3
	II	1.9 ± 0.2 #	2.0 ± 0.3 #	2.2 ± 0.2 #	2.2 ± 0.2 #

[1] Data represent means±SEM, *n*=23.

[2] Abbreviations: CSA, Cross-sectional area; SC, satellite cell; SC, n/myonuclei (%), the number of SCs as a percentage of the total number of myonuclei (i.e. number of myonuclei + number of SCs).

[3] # Significantly different from values in type I fiber ($P<0.05$)

[4] * Significantly different from pre-immobilization values ($P<0.05$)

Figure legends

Figure 1: Outline of the experimental protocol. Two groups of healthy elderly males were included to undergo 5 days of one-legged knee-immobilization, with (PRO; *n*=11) or without (CON; *n*=12) protein supplementation (~20 g protein twice daily).

Figure 2: A Cross-sectional area (CSA) of *m. quadriceps femoris* in healthy elderly participants in the CON (*n*=12) and PRO (*n*=11) groups, measured by single-slice CT scan 48h prior to and immediately following 5 days of leg immobilization. **B** Leg muscle strength as measured by 1RM, in both the CON and PRO group. Data are expressed as means±SEM. *$P<0.05$; significantly different when compared with pre-immobilization values.

Figure 3: Skeletal muscle mRNA expression of selected genes involved in myogenesis (**A-B-C**) and muscle proteolysis (**D-E-F**), measured 48h prior to and immediately following 5 days of one-legged knee immobilization in healthy elderly men in the CON (*n*=12) and PRO (*n*=11) group. *$P<0.05$; significantly different when compared with pre-immobilization values. Data are expressed as means±SEM. Abbreviations: *MAFbx*, Muscle Atrophy F-box; *MuRF1*, Muscle RING-finger protein-1; *FOXO1*, Forkhead box protein O1.

Online supporting material (OSM)

Supplemental Table 1: Composition of the study product[1,2]

Component	Unit	PRO
Energy	kcal / kJ	150 / 635
Protein	%	55
Carbohydrates	%	25
Fat	%	18
Fiber	%	2
Protein		
Total	g	20.7
Total EAA	g	10.6
Total leucine	g	2.8
Total phenylalanine	g	0.6
Carbohydrates		
Total	g	9.4
Sugars	g	4.2
Fat		
Total	g	3.0
Saturated	g	0.8
Fiber		
Total	g	1.3
Soluble	g	1.3
Minerals		
Sodium	mg	150
Potassium	mg	279
Chloride	mg	70
Calcium	mg	500
Phosphorus	mg	250
Magnesium	mg	37
Trace elements		
Iron	mg	2.4
Zinc	mg	2.2
Copper	µg	270
Manganese	mg	0.50
Fluoride	mg	0.15
Molybdenum	µg	15
Selenium	µg	15
Chromium	µg	7.5
Iodine	µg	20
Vitamins		
Vitamin A	µg-RE	152
Cholecalciferol	µg	20
Vitamin E	mg α-TE	7.5
Phylloquinone	µg	12
Thiamin	mg	0.23

Component	**Unit**	**PRO**
Riboflavin	mg	0.25
Niacin	mg NE	8.8
Pantothenic acid	mg	0.81
Vitamin B6	mg	0.76
Folic acid	µg	203
Vitamin B12	µg	3.0
Biotin	µg	6.1
Vitamin C	µg	32
Extra additions		
Carotenoids	mg	0.30
Choline	mg	56

[1] Data are presented as mean values, *n*=23

[2] Abbreviations used: EAA, essential amino acid; NE, niacin equivalents; RE, retinol equivalents; α-TE, α-tocopherol equivalents

Muscle analyses

Muscle samples that were mounted and frozen in Tissue-Tek were cut into 5µm thick cryosections using a cryostat at -20°C. Samples were carefully aligned for cross-sectional fiber analyses. Pre and post immobilization samples from one PRO and one CON subject were mounted together on uncoated, pre-cleaned glass slides. All biopsies were stained for muscle fiber type (FT) and satellite cell (SC) content. At the start of the staining procedure, glass slides were incubated with primary antibodies against myosin heavy chain (MHC)-I (A4.840, dilution 1:25, Developmental Studies Hybridoma Bank, Iowa City, IA), laminin (polyclonal rabbit anti-laminin, dilution 1:50; Sigma, Zwijndrecht, the Netherlands) and CD56 (dilution 1:40; BD Biosciences, San Jose, CA). CD56 has been used in previous research by ourselves (33, 58, 59) and others (60) for determination of SC content in human skeletal muscle. After washing, slides were incubated with the appropriate secondary antibodies: goat anti-rabbit IgG AlexaFluor647, goat anti-mouse IgM AlexaFluor555, and Streptavidin Alexa 488 (dilution 1:400, 1:500, and 1:200, respectively; Molecular Probes, Invitrogen, Breda, the Netherlands). Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI, 0.238 µM; Molecular Probes). All incubations steps were done at room temperature. Both primary and secondary antibodies were diluted in 0.1% Bovine Serum Albumin (BSA) in 0.1% Tween- phosphate-buffered saline (PBS). The staining procedure was done as follows. After slides were fixated in acetone for 5 min, slides were air dried and incubated for 30 min with 3% BSA in 0.1% Tween-PBS. After a 5 min washing step with PBS, slides were incubated with CD56 in 0.1% BSA in 0.1% Tween-PBS for 2 h. Afterwards slides were washed (standard washing protocol: 5 min 0.1% Tween-PBS, 2 x 5 min PBS) and incubated with goat anti-mouse Biotin (dilution 1:133, Vector Laboratories, Inc., Burlingame, CA) for 60 min. After washing, slides were incubated with Steptavidin for 30 min. Thereafter, slides were washed and incubated with primary antibodies against MHC-1 and laminin for 30 min. Slides were washed and the appropriate secondary antibodies were applied, diluted together with DAPI. After a final washing step, all slides were mounted with cover glasses using Mowiol (Calbiochem, Amsterdam, the Netherlands). Staining procedures resulted in nuclei stained in blue, CD56 in green, MHC-I in red, and laminin in far-red. Images were visualized and automatically captured at 10x magnification with a fluorescent microscope equipped with an automatic stage (IX81 motorised inverted microscope, Olympus, Hamburg, Germany) and EXi Aqua CCD camera (QImaging, Surrey, BC, Canada). Image acquisition was performed by Micro-Manager 1.4 software as done before (50). Analysis of the recorded images was performed by an investigator blinded to subject coding. To assess fiber circularity, form factors were calculated by using the following formula: $(4\pi\cdot \mathrm{CSA})/(\mathrm{perimeter})^2$. Fiber circularity did not change over time or between groups. Mean numbers of 148±12 and 151±12 fibers were analyzed in pre- and post-immobilization samples, respectively.

Online supporting material (OSM)

The part of the muscle that was directly frozen in liquid nitrogen was used to determine mRNA expression of several genes of interest. Total RNA was isolated by using Tri Reagent (Sigma-Aldrich) on 10-20 mg of frozen muscle, according to the manufacturer's protocol. Quantification of total RNA was carried out spectrophotometrically at 260 nm (NanoDrop ND-1000 Spectrophotometer, Thermo Fisher Scientific, USA), and RNA purity was determined as the ratio of readings at 260/280 nm. Subsequently, first strand cDNA was synthesized from 1 µg RNA sample using random primers (Promega) and PowerScript Reverse Transcriptase (AppliedBiosystems, USA). Taqman PCR was carried out using an ABI Prism 7000 sequence detector (AppliedBiosystems, USA), with 2 µL of cDNA, 18 $\mu l \cdot L^{-1}$ of each primer, 5 $\mu l \cdot L^{-1}$ probe, and Universal Taqman 2 × PCR mastermix (Eurogentec) in a final volume of 25 µL. Each sample was run in duplicate, in duplex reactions, with a separate standard curve included for each gene (serial dilutions of cDNA synthesized in parallel with the study sample). 18S was used as a housekeeping gene as an internal control, and similarly to previous human immobilization studies (8, 20) it seemed unaffected by treatment (i.e. mean C*t* values did not change over time in each of the intervention groups; *data not shown*). Taqman primer/probe sets (Applied Biosystems, Foster City, USA) were obtained for the following genes of interest: mammalian target of rapamycin (*mTOR*), P70S6 kinase (*P70S6K*), myogenic factor 4 (*myogenin*), *MyoD*, *myostatin*, Atrogin-1/Muscle Atrophy F-box (*MAFbx*), Muscle RING-finger protein-1 (*MuRF1*), Forkhead box protein O1 (*FOXO1*), Focal Adhesion Kinase (*FAK*), large neutral amino acid transporter 1 (*LAT1*) and Proton-coupled amino acid transporter 1 (*PAT1*). All genes of interest were labelled with the fluorescent reporter FAM. The thermal cycling conditions used were: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. C*t* values of the genes of interest were normalized to C*t* values of the housekeeping gene, and final results were calculated as relative expression against the standard curve.

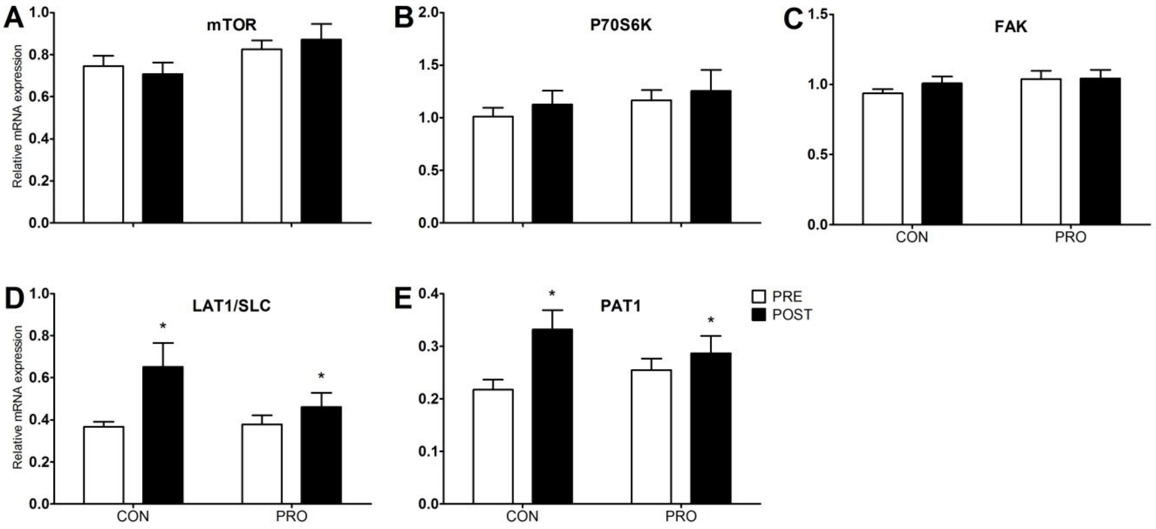
Supplemental Table 2: Plasma amino acid concentrations measured 48h prior to and immediately following 5 days of one-legged knee immobilization in healthy elderly men with (PRO; *n*=11) or without (CON; *n*=12) twice-daily protein supplementation.[1,2]

	CON (*n*=12)		**PRO (*n*=11)**	
	Pre	**Post**	**Pre**	**Post**
	µmol/L	µmol/L	µmol/L	µmol/L
α-aminobutyric acid	30 ± 4	25 ± 2	27 ± 2	30 ± 4
Alanine	393 ± 36	485 ± 36 *	379 ±25	423 ± 22 *
Arginine	88 ± 5	90 ± 4	84 ± 3	81 ± 4
Asparagine	47 ± 2	47 ± 2	45 ± 2	45 ± 2
Aspartic acid	5 ± 1	5 ± 1	4 ± 1	4 ± 1
Citrulline	39 ± 3	36 ± 2	35 ± 1	38 ± 3
Cysteine	42 ± 3	46 ± 2 *	44 ± 2	50 ± 2 *
Glutamic acid	69 ± 10	68 ± 7	58 ± 9	59 ± 9
Glutamine	605 ± 29	622 ± 27	575 ± 39	540 ± 31
Glycine	217 ± 11	237 ± 12	204 ± 10	194 ± 13
Histidine	86 ± 5	86 ± 2	83 ± 4	84 ± 3
Isoleucine	70 ± 5	73 ± 4	66 ± 4	76 ± 8
Leucine	138 ± 8	137 ± 5	128 ± 7	154 ± 16
Lysine	190 ± 10	191 ± 7	177 ± 9	212 ± 14
Methionine	28 ± 2	29 ± 1	27 ± 1	30 ± 2
Ornithine	60 ± 4	62 ± 3	58 ± 4	60 ± 4
Phenylalanine	59 ± 3	60 ± 2 *	56 ± 3	64 ± 2 *
Proline	218 ± 27	223 ± 22	169 ± 14	192 ± 12
Serine	90 ± 6	93 ± 5	85 ± 5	89 ± 7
Taurine	81 ± 8	89 ± 6	96 ± 10	82 ± 7
Threonine	129 ± 7	135 ± 8 *	120 ± 7	154 ± 15 *
Tryptophan	56 ± 4	59 ± 3 *	53 ± 3	62 ± 4 *
Tyrosine	67 ± 4	69 ± 3	65 ± 4	71 ± 3
Valine	261 ± 17	259 ± 9	240 ± 12	288 ± 19 *

[1] Data are presented as means ± SEM, *n*=23.

[2] * Significantly different from pre-immobilization value (P<0.05).

Supplemental Figure 1



Supplemental Figure 1: mRNA expression of anabolic genes of interest in the CON (*n*=12) and PRO (*n*=11) group 48h prior to and immediately following 5 days of leg immobilization. *$P<0.05$; significantly different when compared with pre-immobilization values. Data are expressed as means±SEM, *n*=23. Abbreviations: *FAK*, Focal Adhesion Kinase; *LAT1*, large neutral amino acid transporter; *mTOR*, mammalian target of rapamycin; *PAT1*, proton-coupled amino acid transporter 1; *P70S6K*, P70S6 kinase.

No significant time*treatment nor time effects were found for *mTOR* (**Supplemental Figure 1A**), *P706SK* (**Supplemental Figure 1B**), and *FAK* (**Supplemental Figure 1C**: all $P>0.05$).