Contractile and connective tissue protein content of human skeletal muscle: effects of 35 and 90 days of simulated microgravity and exercise countermeasures

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Haus JM, Carrithers JA, Carroll CC, Tesch PA, Trappe TA. Contractile and connective tissue protein content of human skeletal muscle: effects of 35 and 90 days of simulated microgravity and exercise countermeasures. Am J Physiol Regul Integr Comp Physiol 293: R1722–R1727, 2007. First published August 1, 2007; doi:10.1152/ajpregu.00292.2007.—We examined the effects of 35 and 90 days of simulated microgravity with or without resistance-exercise (RE) countermeasures on the content of the general skeletal muscle protein fractions (mixed, sarcoplasmic, and myofibrillar) and specific proteins that are critical for muscle function (myosin, actin, and collagen). Subjects from two studies, using either unilateral lower limb suspension (ULLS) or bed rest (BR), comprised four separate groups: 35 days ULLS (n = 11), 35 days ULLS+RE (n = 10), 90 days BR (n = 9), and 90 days BR+RE (n = 8). RE consisted of four sets of seven maximal concentric and eccentric repetitions of the quadriceps femoris muscles that were performed 2 or 3 times per week. Pre- and post-simulated weightlessness muscle biopsies were analyzed from the vastus lateralis of all groups and the soleus of the 35-day ULLS and 90-day BR groups. The general protein fractions and the specific proteins myosin, actin, and collagen of the vastus lateralis were unchanged (P > 0.05) in both control and countermeasures groups over 35 and 90 days, despite large changes in quadriceps femoris muscle volume (35 days ULLS: −9%, 35 days ULLS+RE: +8%; and 90 days BR: −18%, 90 days BR+RE: −1%). The soleus demonstrated a decrease in mixed (35 days ULLS: −12%, P = 0.0001; 90 days BR: −12%, P = 0.004) and myofibrillar (35 days ULLS: −12%, P = 0.009; 90 days BR: −8%, P = 0.04) protein, along with large changes in triceps surae muscle volume (35 days ULLS: −11%; 90 days BR: −29%). Despite the loss of quadriceps femoris muscle volume or preservation with RE countermeasures during simulated microgravity, the quadriceps femoris muscles are able to maintain the concentrations of the general protein pools and the main contractile and connective tissue elements. Soleus muscle protein composition appears to be disproportionately altered during long-duration simulated weightlessness.

The interactions of the proteins myosin and actin are the basis of the force-generating capacity of skeletal muscle, while a connective tissue network primarily consisting of collagen is critical for the transfer of force out to the tendon and subsequent bone (22, 28). In addition, these proteins (myosin, actin, and collagen) comprise the majority of the non-water mass of skeletal muscle. Over the past two decades, it has been clearly demonstrated that one of the hallmarks of actual or simulated spaceflight is a loss of muscle mass and a related loss of muscle function (6, 11, 13). Recently, it has been shown that resistance exercise is effective at maintaining muscle mass and function during periods of simulated spaceflight (2, 7, 33, 34, 38). However, it is unknown whether prolonged unloading of human skeletal muscle during periods of real or simulated microgravity results in a nonuniform change in muscle composition. Changes in the protein composition of skeletal muscle, especially the proteins found in the myofibril that generate force (e.g., myosin and actin) and connective tissue proteins (e.g., collagen) that facilitate the transfer of force, may contribute to the decline in whole muscle function that has been reported with unloading (2, 11, 13, 34). Conversely, it is also unknown whether preservation of muscle mass with resistance-exercise (RE) countermeasures during periods of unloading preserves muscle composition. Thus, this information is important to know as RE-countermeasure programs are being developed for astronauts on long-duration missions (e.g., multimonth missions on the International Space Station or a multiyear mission to Mars).

We have previously reported changes in muscle volume and function with and without RE using two separate simulated spaceflight models, unilateral lower limb suspension (ULLS) for a period of 35 days (35d ULLS) and 6° head-down tilt bed rest (BR) for a period of 90 days (90d BR) (2, 38). These studies demonstrated that quadriceps femoris muscle volume was reduced with 35d ULLS (−9%) and 90d BR alone (−18%) and increased (35d ULLS+RE: +8%) or maintained (90d BR+RE: −1%) with RE training, while triceps surae muscle volume was decreased in the 35d ULLS (−11%) and 90d BR groups (−29%) (2, 38). Given these extreme changes in muscle volume, we sought to determine whether the composition of the muscle was altered during these periods of unloading.

Previous muscle biopsy data from short-term (17 days) BR and spaceflight show disproportionate changes in the protein composition of both slow (i.e., the soleus) and mixed (i.e., the gastrocnemius) leg muscles of humans (29–31, 41). These findings are generally supported from ground-based and spaceflight studies in animals (5, 36, 43), which also show an increase in intramuscular connective tissue with unloading-induced atrophy (25, 27). On the basis of these findings, we hypothesized that the concentrations of the general skeletal muscle protein fractions (mixed, sarcoplasmic, and myofibril-
and the concentration of myosin and actin would be decreased, while collagen would increase in the vastus lateralis muscles of both the 35d ULLS and 90d BR groups. We also hypothesized that the concentrations of the general muscle protein fractions, as well as the concentrations of actin, myosin, and collagen would be maintained in the RE training groups (35d ULLS+RE and 90d BR+RE). In the 35d ULLS and 90d BR groups only, we also measured the protein composition of the soleus muscle, as this muscle appears to be the most affected muscle with unloading and spaceflight in humans and animals (2, 13, 24, 29, 41, 45).

METHODS

Overall Experimental Design

The overall experimental design and protocol from the two microgravity simulation studies presented here have been described in detail elsewhere (1, 2, 38); therefore, only a brief explanation is presented here. One study utilized the ULLS model (8, 38) for 35 days, and the other study used the 6° head-down tilt BR model (2, 14) for 90 days. The BR study was completed at the Institut de Médecine et de Physiologie Spatiales (MEDES) in Toulouse, France (1, 2). Following recruitment and screening, each subject in the 35-day study was randomly assigned to 5 wk of either ULLS only (35d ULLS) or ULLS and RE (35d ULLS+RE), and each subject in the BR study was randomly assigned to 90 days of either 6° head-down tilt BR only (90d BR) or bed rest and RE (90d BR+RE).

The RE protocols used a flywheel ergometer designed for RE in microgravity and modified for each study to isolate the quadriceps femoris muscles for training (1, 2, 37, 38). The 35d ULLS study used a seated one-joint knee extension exercise. For the 90d BR study, training was completed in the 6° head-down tilt position and used a two-joint squat exercise. The protocol used for both studies was four sets of seven maximal concentric and eccentric repetitions of knee extensions (35d ULLS study) or supine squats (90d BR study) 2 or 3 days per week, for a total of 12 training sessions over the 35d ULLS period and 29 training sessions during the 90d BR period.

Before and after the simulated weightlessness periods, all subjects underwent a muscle biopsy from the vastus lateralis and a soleus muscle biopsy was obtained from the control groups for both studies. Post-35d ULLS biopsies were taken on the final day (prior to any reloading of the suspended muscles), and post-90d BR biopsies were taken on day 84. Volume of the thigh (quadriceps femoris) and calf (triceps surae) muscles was also determined via MRI before and at the end of each study unloading period (day 35, before any loading of the suspended muscles and day 89). Detailed outlines of these measurements (2, 38, 42) and the results (2, 38) have been presented elsewhere. In addition, strength measurements were completed both statically and dynamically before and at the end of the 35d ULLS and 90d BR periods and have been reported in detail previously (2, 38).

Subjects

An overview of the subjects that participated in the 35d ULLS and 90d BR studies is presented in Table 1. Twenty-one men and women participated in the 35d ULLS investigation and seventeen men participated in the 90d BR investigation following a comprehensive medical exam and screening (1, 2, 37, 38). All procedures, risks, and benefits associated with the experimental testing were explained to the subjects before they gave signed consent. The protocols adhered to the guidelines of and was approved by the Institutional Review Board or local ethical committee of the participating institutions.

Muscle Biopsy

Muscle biopsies (9) were taken from the vastus lateralis (all subjects) and from the soleus [35d ULLS group (n = 11), and a subset of the 90d BR group (n = 3)] before and at the end of the simulated microgravity periods for the measurement of general and specific muscle protein concentrations. Following each biopsy, a portion of the muscle was immediately frozen and stored in liquid nitrogen (−190°C) until muscle protein quantification.

Muscle Protein Quantification

Mixed, sarcomplasmic, and myofibrillar muscle protein concentration. For each biopsy sample, a piece of muscle weighing ~10 mg was divided and weighed on a precision microbalance (Cahn 35, Orion Research, Beverly, MA) at −35°C. Each sample was homogenized in 40 volumes of cold homogenizing buffer (250 mM sucrose, 100 mM potassium chloride, 20 mM imidazole, and 5 mM EDTA; pH 6.8) in a ground-glass homogenizer (Radnoti Glass Technology, Monrovia, CA) (10). Samples were then centrifuged at 20,000 g for 30 min at 4°C. The supernatant was taken as the sarcoplasmic protein fraction, and the pellet was resuspended in 40 volumes of cold homogenizing buffer and taken as the myofibrillar protein fraction (20, 35). Aliquots of the homogenate (mixed protein), sarcoplasmic, and myofibrillar protein fractions were measured for protein concentration using the bicinchoninic acid assay (Sigma, St. Louis, MO) with bovine serum albumin used as the protein standard. The amount of protein in each of the three fractions was normalized to the wet weight of each muscle sample.

Myosin and actin concentration. Myosin (myosin heavy chain, MHC) and actin concentrations were determined by quantitative gel electrophoresis as previously described (20, 40, 43). Aliquots of the myofibrillar protein fraction were diluted with sodium dodecyl sulfate (SDS) buffer [2% SDS, 125 mM Tris-HCl (pH 6.8), 12.5% glycerol, 5% 2-mercaptoethanol, 0.005% bromophenol blue], and heated at 60°C for 4 min. Myofibrillar protein (800 ng) was separated by
SDS-PAGE (23). MHC was resolved with a 4% stacking gel and 10% separating gel. Actin was resolved with a 4% stacking gel and a 6–12% gradient separating gel that was allowed to polymerize overnight. For MHC and actin, electrophoresis was performed at a constant current of 20 mA per gel in the stacking gel and 25 mA per gel in the separating gel with a Tris-glycine electrode buffer at 4°C (Hoeffer SE 600, Amersham Pharmacia Biotech, Piscataway, NJ).

The separating gels were silver stained (16), digitally photographed (ChemImager 5500, Alpha Innotech, San Leandro, CA), and densitometry was completed using National Institutes of Health Image software (ver. 1.60). Each gel was loaded with five standards of MHC (Sigma, M7659) or actin (Sigma, A2522), a molecular weight standard, and subjects’ muscle biopsy samples. All standards and samples were loaded in duplicate, and an average of the duplicate densities was taken to represent each standard and sample. Each gel contained pre- and post-simulated weightlessness samples from the same individual and samples from each group for the ULLS and BR studies, respectively. Vastus lateralis and soleus samples from the same individual were also loaded on the same gel. All measurements were made in a blinded fashion by the same investigator. Unknown sample amounts of MHC and actin were determined from regression analysis of the standard curves on each gel. Correlation coefficients were 0.98–1.00 for both MHC and actin standards. Sample MHC and actin amounts were normalized to the muscle wet weight.

Collagen concentration. Skeletal muscle collagen concentration was determined via measurement of the collagen-specific amino acid, hydroxyproline (HYP). The concentration of HYP was quantified by HPLC and fluorometric detection (1100 Series, Agilent Technologies, Wilmington, DE) via the precolumn derivatization method described by Hutson et al. (19) with modifications for human skeletal muscle. Muscle samples −10–15 mg wet weight were weighed at −35°C on a precision microbalance (AD-2Z Autobalance, Perkin-Elmer, Wellesey, MA) and hydrolyzed in 1 ml of 6 M HCl at 110°C for 30 h. In addition, a sarcosine internal standard (Sigma, S7672) prepared in water was added to each vial. The hydrolyzates were allowed to cool to room temperature and neutralized with 6 M NaOH. HYP standards (Sigma, 56250) of 1, 10, 25, 50, 75, 100, and 125 µM were prepared along with the 2 mM sarcosine internal standard and were used to generate a standard curve.

Derivatization was verified by combining 900-µl aliquots of muscle hydrolyzate or standard with 200 µl of borate buffer (0.7 M boric acid, pH 9.5) and 100 µl of o-phthalaldehyde (OPA) solution (50 mg OPA dissolved in 1 ml acetonitrile containing 26 µl of β-mercaptoethanol), followed 60 s later by 100 µl of iodoacetamide reagent (140 mg/ml of iodoacetamide in acetonitrile). One minute later, 300 µl of 5 mM 9-fluorenylmethylchloroformate (FMOC) in acetonitrile were added. The vials were capped and vortexed between each addition of reagent. One minute after the addition of FMOC reagent, 2 ml of ethyl ether was added to each reaction vial. The vial was shaken vigorously for 30 s to wash the contents of the vial. The organic layer was discarded, and the wash was repeated twice for a total of three washes. Five microliters of the remaining aqueous phase was injected with an autosampler (Agilent Technologies). Separation was achieved through an X Terra RP 18, 5 µm, 250 mm × 4.6 mm column (Waters, Milford, MA) using an isocratic mobile phase of 65% acetic acid: 35% acetonitrile (3% glacial acetic acid, sodium acetate buffered to pH 4.3) at a 1.0 ml/min flow rate. Peaks were monitored at 260/316 nm (Ex/Em) with a gain of 8 and integrated with chromatography software (ChemStation, Agilent Technologies). The HYP signals were normalized to the internal standard for each injection, and the HYP concentration was determined from standard curves of HYP (correlation coefficient of 0.998). All samples and standards were run in triplicate with a mean coefficient of variation of 0.42% and 0.77%, respectively. Collagen content was calculated from the HYP concentration, assuming collagen weighs 7.5 times the measured HYP weight and the molecular weight of collagen is 300,000 (12, 15). Samples were normalized to muscle wet weight, and data are expressed as micrograms collagen per milligram wet weight muscle.

Statistics. A two-way (group and time) ANOVA with repeated measures on the time factor was used to compare all variables except the soleus biopsy data from the 35d ULLS and 90d BR groups, in which case a paired t-test was used. When necessary, post hoc comparisons were completed with a Tukey honestly significant difference test for equal or unequal sample sizes, depending on the data set. Significance was accepted at P < 0.05. Data are presented as means ± SE.

RESULTS

Concentrations of the general (mixed, sarcoplasmic, and myofibrillar) and specific (myosin, actin, and collagen) protein fractions from the vastus lateralis were unaltered (P > 0.05) with 35d ULLS or 35d ULLS + RE (Fig. 1), or 90d BR or 90d BR + RE (Fig. 2), despite large changes in muscle volume. Mixed protein concentration (micrograms per milligram muscle wet weight) of the vastus lateralis averaged 152 ± 2 presimulated and 150 ± 2 postsimulated weightlessness, sarcoplasmic protein concentration (micrograms per milligram muscle wet weight) averaged 63 ± 2 presimulated and 62 ± 1 postsimulated weightlessness, and myofibrillar protein concentration (micrograms per milligram muscle wet weight) averaged 95 ± 1 presimulated and 93 ± 2 postsimulated weightlessness across all experimental groups. Myosin concentration (micrograms per milligram muscle wet weight) of the vastus lateralis averaged 46 ± 3 presimulated and 48 ± 3 postsimulated weightlessness across all four experimental groups. Actin concentration (micrograms per milligram muscle wet weight) of the vastus lateralis averaged 17 ± 1 pre- and 17 ± 1 post-simulated weightlessness, and collagen concentration (micrograms per milligram muscle wet weight) averaged 6 ± 1 presimulated and 6 ± 1 postsimulated weightlessness.

Soleus mixed (−12%, P = 0.0001), sarcoplasmic (−9%, P = 0.015), and myofibrillar (−12%, P = 0.009) muscle protein concentrations were decreased (P < 0.05) with the 35d ULLS group and mixed (−12%, P = 0.004), and myofibrillar (−8%, P = 0.04) muscle protein concentrations were decreased with the 90d BR group along with large changes in muscle volume (Fig. 3). Soleus myosin (Pre : 52 ± 4; Post: 47 ± 3 µg/mg muscle wet weight) and actin (Pre: 18 ± 1; Post: 16 ± 1 µg/mg muscle wet weight) concentrations were unchanged (P > 0.05) from before to after the two weightlessness periods (Fig. 3). Collagen concentration was unchanged (P > 0.05) with 35d ULLS (Pre: 11 ± 3; Post: 11 ± 2 µg/mg muscle wet weight), but increased (P = 0.015) with 90d BR (Pre: 3 ± 1; Post: 18 ± 2 µg/mg muscle wet weight) (Fig. 3).

DISCUSSION

The primary findings from this investigation were twofold. First, the relative amounts of the general skeletal muscle protein pools (mixed, sarcoplasmic, and myofibrillar), as well as the specific functional and structural proteins myosin, actin, and collagen, are maintained in human quadriceps femoris muscle (vastus lateralis) under conditions of simulated weightlessness across all four experimental groups. Actin and collagen, as the specific functional and structural proteins myosin, actin, and collagen, are maintained in human quadriceps femoris muscle (vastus lateralis) under conditions of simulated weightlessness across all four experimental groups. Actin and collagen, as the specific functional and structural proteins myosin, actin, and collagen, are maintained in human quadriceps femoris muscle (vastus lateralis) under conditions of simulated weightlessness across all four experimental groups. Actin and collagen, as the specific functional and structural proteins myosin, actin, and collagen, are maintained in human quadriceps femoris muscle (vastus lateralis) under conditions of simulated weightlessness across all four experimental groups. Actin and collagen, as the specific functional and structural proteins myosin, actin, and collagen, are maintained in human quadriceps femoris muscle (vastus lateralis) under conditions of simulated weightlessness across all four experimental groups. Actin and collagen, as the specific functional and structural proteins myosin, actin, and collagen, are maintained in human quadriceps femoris muscle (vastus lateralis) under conditions of simulated weightlessness across all four experimental groups.
The two main contractile proteins that we studied are the primary proteins involved in muscle force development and comprise ~50% of all protein mass of the muscle. Given this information, along with our previous data from short-term (17 days) spaceflight and bed rest (29–31), we hypothesized that these two proteins were the logical candidates to be disproportionately altered under conditions of prolonged atrophy, countermeasures “hypertrophy”, and altered muscle function. The maintenance of myosin and actin concentrations of the vastus lateralis and soleus suggests preferential loss or gain of these two important proteins did not contribute to any impairment or improvement in muscle function during periods of unloading or RE countermeasures to unloading. Contrary to our hypothesis, it appears the quadriceps femoris muscle is able to maintain a consistent and relative amount of these two important motor proteins. Further, it appears that the quadriceps femoris muscle maintains the concentration of the general sarcoplasmic and myofibrillar protein pools, as well as the collagen fraction during simulated microgravity with and without countermeasures exercise. This maintenance is striking, given the complexity of the processes that are involved in the anabolic and catabolic pathways, coupled with the large change (and prevention of change) in muscle mass in a relatively short period of time (3 mo or less).

The skeletal muscle collagen fraction is metabolically dynamic at rest, and in response to muscular activity (26) and acute unloading (44). Given the critical role that intramuscular collagen plays in structural integrity and force transmission, we hypothesized that alterations in the collagen fraction may contribute to the declines in muscle function observed with real and simulated spaceflight. To our knowledge, this is the first investigation to directly quantify human intramuscular collagen with simulated microgravity. Previous animal data show that atrophy of the rat hindlimb muscles as a result of real or...
Specifically, contribute to the role that collagen plays in muscle function.

Changes in collagen, independent of changes in the relative amount, may also be specific to animals and not humans. Changes in collagen, alterations of intramuscular collagen have been shown to compromise muscle function (17). When comparing the current data with the literature from animals, it appears that the changes observed in animal muscles may be a phenomenon specific to animals and not humans. Changes in collagen, independent of changes in the relative amount, may also contribute to the role that collagen plays in muscle function. Specifically, 1) the amount of cross-linking among the collagen filaments may be modified (17), 2) the type or isoform(s) of collagen may be altered (27), or 3) changes in myofiber size may alter the collagen alignment (3). Although all three of these factors have been shown to influence muscle function in lower animals, to our knowledge, there are no available data with unloading in humans.

As seen with the whole muscle atrophy response (2), the protein content of the soleus appears to be more influenced by unloading than the vastus lateralis (Fig. 3). The current data are also supported by studies of slow-twitch muscles from animals flown in space over short duration (7, 9, and 12.5 days), which are reported to have decreased total (i.e., mixed), myofibril, and myosin concentration compared with fast-twitch muscles (4, 18, 36). The reduction in the protein concentrations of the general skeletal muscle fractions of the unloaded soleus, in light of the maintenance of myosin and actin concentrations, suggests other proteins are disproportionately lost relative to the main contractile proteins. Likely candidates are the proteins found in the muscle that support the transfer of force produced by myosin and actin interactions, such as titin, nebulin, c-protein, m-protein, and various cytoskeletal proteins that connect the force-producing structures of the muscle to the sarcolema. Indeed, it has been shown that some of these proteins are damaged or degraded with simulated weightlessness (21, 39), thus supporting an increased susceptibility for muscle injury following real or simulated microgravity exposure (32). The collagen fraction of the soleus was unchanged after 35d ULLS, suggesting the concentrations of the connective tissue along with the myosin and actin proteins are well maintained in the soleus, as was seen in the vastus lateralis. However, the 90d BR group showed a large amount of collagen accumulation in the soleus, inferring the duration of unloading and/or amount of atrophy influences collagen content. These data should be viewed with caution given the relatively low preunloading values for this group and highlights the need for further investigation in this area. Given the importance of the triceps surae muscles to normal ambulation, especially for an astronaut on the surface of the Moon or Mars during emergency egress, the current data demonstrate the need for the development of effective exercise countermeasures that target the calf muscles both at the whole muscle and cellular level.

In conclusion, under conditions of atrophy and RE-countermeasures-induced hypertrophy, the quadriceps femoris muscles change size with proportional changes in protein composition, including the main functional and structural proteins myosin, actin, and collagen. The soleus is more affected than the vastus lateralis with 1 to 3 mo of simulated microgravity. The maintenance of myosin and actin concentrations in the atrophying soleus, in conjunction with a loss of the general muscle protein fractions, suggests other proteins are lost at the expense of the main contractile proteins.

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Fig. 3. General (mixed, sarcoplasmic, myofibrillar) and specific (myosin, actin, collagen) muscle protein concentrations (µg per mg muscle wet weight) in the soleus before and after 35 days of ULLS and 90 days of BR, along with changes in muscle volume (2, 38). *Significant difference from pre (P < 0.05). Data are presented as means ± SE.
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