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Skeletal muscle proteolysis in response to short-term unloading in humans

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¹Department of Physiology and Pharmacology, ²Department of Laboratory Medicine, Karolinska Institutet, Stockholm, Sweden; ³Human Performance Laboratory, Ball State University, Muncie, Indiana; ⁴Astronaut Office, National Aeronautics and Space Administration, Johnson Space Center, Houston, Texas; and ⁵Department of Health Science, Mid Sweden University, Östersund, Sweden

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Tesch PA, von Walden F, Gustafsson T, Linnehan RM, Trappe TA. Skeletal muscle proteolysis in response to short-term unloading in humans. *J Appl Physiol* 105: 902–906, 2008. First published June 5, 2008; doi:10.1152/jappphysiol.90558.2008.—Skeletal muscle atrophy is evident after muscle disuse, unloading, or spaceflight and results from decreased protein content as a consequence of decreased protein synthesis, increased protein breakdown or both. At this time, there are essentially no human data describing proteolysis in skeletal muscle undergoing atrophy on Earth or in space, primarily due to lack of valid and accurate methodology. This particular study aimed at assessing the effects of short-term unloading on the muscle contractile proteolysis rate. Eight men were subjected to 72-h unilateral lower limb suspension (ULLS) and intramuscular interstitial levels of the naturally occurring proteolytic tracer 3-methylhistidine (3MH) were measured by means of microdialysis before and on completion of this intervention. The 3MH concentration following 72-h ULLS (2.01 ± 0.22 nmol/ml) was 44% higher ($P < 0.05$) than before ULLS (1.56 ± 0.20 nmol/ml). The present experimental model and the employed method determining 3MH in microdialysates present a promising tool for monitoring skeletal muscle proteolysis or metabolism of specific muscles during conditions resulting in atrophy caused by, e.g., disuse and real or simulated microgravity. This study provides evidence that the atrophic processes are evoked rapidly and within 72 h of unloading and suggests that countermeasures should be employed in the early stages of space missions to offset or prevent muscle loss during the period when the rate of muscle atrophy is the highest.

contractile protein breakdown; microdialysis; muscle atrophy; space-flight simulation; 3-methylhistidine

SPACEFLIGHT AND SIMULATION models of no-weight-bearing activity provoke lower limb skeletal muscle atrophy (1, 3, 4, 21, 33, 34) as a result of altered protein metabolism leading to decreased muscle contractile protein content (14, 30, 31, 35, 42). The net effect on the skeletal muscle protein pool is governed by the two opposing processes working in concert, i.e., protein synthesis and breakdown. Although it has been shown that atrophy induced by disuse is accompanied by decreased protein synthesis (6, 7, 10), based on human studies, there is less substantial evidence to support that muscle unloading provokes increased proteolytic activity.

Unfortunately, until now, feasible and viable methods that measure protein degradation in vivo in humans have not been at hand. Albeit 3-methylhistidine (3MH), through urinary excretion, has been used as a marker of muscle protein degradation following bouts of acute heavy resistance exercise (18, 25), the amount of 3MH disposed in urine reflects only changes

in the rate of contractile protein breakdown at the global level (22, 23). The arteriovenous difference across limbs has been successfully measured (26, 41) to minimize the potential interference from body regions with a high turnover rate, e.g., the gastrointestinal system (43). However, such an invasive and complex approach is not attractive for experiments involving otherwise healthy individuals subjected to interventions such as unloading or microgravity. Recently, Trappe et al. (41) adopted the “semi-invasive” microdialysis technique (16, 17, 24) for in vivo intramuscular 3MH measurements and allowing for quantitative determination of proteolysis of the contractile elements actin and myosin of individual muscles. This approach circumvents the aforementioned limitations of whole body and nonskeletal muscle contributions to the contractile proteolysis measurements, while taking advantage of the natural tracer characteristics of 3MH (i.e., 3MH cannot be reused for protein synthesis). In addition, the measurements obtained with microdialysis have been confirmed in parallel experiments using the arteriovenous balance technique (41). With the aid of microdialysis, altered skeletal myofibrillar proteolysis has been noted with aging (41) and following strenuous artificial (i.e., electrically stimulated) contractile activity (13). Hence, this methodology presents a lenient and appealing tool to be employed in healthy populations for both mechanistic and applied studies. This particular study was prompted by our long-term interest in exploring the fate of skeletal muscle in response to spaceflight.

Given the high rate of global lower limb muscle atrophy during the initial week(s) of spaceflight, unloading, or disuse (8, 19, 20, 28, 33), altered contractile protein metabolism should also be evident early on in response to such challenges. In case changes in both protein synthesis and breakdown occur, short-term unloading protocols could readily be employed for mechanistic studies of human skeletal muscle to explore the events leading to atrophy, reducing the need for costly and demanding long-term unloading or bed rest studies.

More specifically, the main objective of the present study was to determine skeletal muscle contractile protein degradation, by means of the microdialysis technique, consequent to short-term simulated spaceflight. We hypothesized that 72-h lower limb unloading would provoke increased intramuscular interstitial levels of 3MH as a result of increased myofibrillar proteolysis.

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MATERIALS AND METHODS

Subjects. Eight men (mean $\pm$ SD; 25 ± 5 yr, 183 ± 3 cm, 76 ± 8 kg) were recruited from the greater Stockholm area. Following a medical examination, screening for any history of lower limb pathology, neuromuscular disorders, or cardiovascular disease, subjects were informed of the procedures, risks, and benefits associated with the experiments. Although some of these men were sedentary, a few took part in recreational or competitive sports programs. Written informed consent was obtained from each subject to comply with the current guidelines of the Institutional Review Board (IRB) at the Karolinska Institutet. After IRB approval, this study was conducted in accordance with the Declaration of Helsinki.

General procedures. Two weeks before the intervention, all subjects underwent four sessions of orientation and familiarization to become accustomed to the different elements of the unilateral lower limb suspension (ULLS) technique (4, 34). Three days before the onset of the ULLS regime, the subjects were required to refrain from any strenuous physical activity, yet they followed their normal dietary routines. However, beginning 24 h before the scheduled pre- and post-ULLS measurements, menus were restricted to two standardized vegetarian meals, which both consisted of pasta, tomato sauce, tomatoes, and green salad ($\sim 1,400$ kcal). Subjects were in a fasted state from 10 h before and throughout the sample collections. Water was provided ad libitum. The subjects reported to the laboratory facility in the evening, where they slept and remained through the following day's measurements. Before and at completion of the 72-h intervention, 3MH concentration, as a marker of skeletal muscle protein breakdown, was measured from the vastus lateralis muscle in vivo using the microdialysis technique. While pre ULLS samples were acquired from the right leg, post ULLS measurements were performed on the left leg (Fig. 1).

ULLS. To avoid weight bearing of the left leg at all times, the volunteers were subjected to a unilateral unloading protocol used by us in the past (34). Briefly, in any upright or ambulatory activity, short-length crutches, aided by handgrip and forearm support distal to the elbow (Swereco Rehab, Sollentuna, Sweden), were used. The right foot was equipped with a shoe having a 10-cm-thick sole. This removed weight bearing from the left unloaded limb yet allowed for movement about the hip joint. Subjects lived at home throughout the

unloading period and were encouraged to maintain normal daily routines. To ensure compliance, all subjects were interviewed daily via telephone or in person. For extended periods in the erect position subjects were provided and wore medical-graded (class 2) stockings (BSN-jobst, Rutherford College, NC).

Microdialysis. Microdialysis was performed on the vastus lateralis muscle to acquire pre- and post-ULLS data on muscle contractile protein breakdown. Between 6:00 and 7:00 AM, the CMA 60 microdialysis probes (30 mm, 20-kDa cutoff, CMA Microdialysis, Solna, Sweden) were inserted following a period of 5 min preperfusion at $10 \mu\text{l/min}$ with a sterile Ringer solution using a calibrated microinfusion pump (CMA Microdialysis, Solna, Sweden). Immediately following insertion, the probes were flushed at a perfusion rate of $10 \mu\text{l/min}$ for at least 10 min. To ensure no influence by the probe insertion on pre- and postunloading data, the catheters were continuously perfused for 3 h before collection while subjects remained at rest in the supine position (11). All dialysate samples were collected in 15-min aliquots in sealed microvials and weighed on a precision microbalance (Sartorius, Goettingen, Germany) before and after each period to determine actual dialysate volume. The microvials for each 15 min collection were immediately stored at 4°C , until completion of all acquisitions (maximum 2–3 h), and then they were weighed and stored at -80°C until assayed for 3MH.

Microdialysis probe recovery. Microdialysis probe recovery for 3MH was calculated from our laboratory's previously obtained recovery data from 3MH determinations in the vastus lateralis muscle of young men during resting conditions (41). Probe recovery in this approach was determined in vivo by the internal reference technique (29). This technique measures the loss of an internal tracer placed in the perfusate and assumes the loss of the tracer to the interstitial fluid is equivalent to the recovery of the compound of interest from the interstitial fluid into the dialysate. In this case a radioactive tracer was used, and the in vivo recovery was calculated using the following formula: $(\text{perfusate}_{\text{dpm}} - \text{dialysate}_{\text{dpm}}) / \text{perfusate}_{\text{dpm}}$, where dpm is disintegrations per minute. Given that conditions known to influence probe recovery e.g., probe length, pore size, the compound of interest, pump-probe alignment and vial orientation, were identical in the present and the above study by Trappe and coworkers, we are confident recovery was the same in the two studies.

Fig. 1. General study design showing probe insertion, proteolysis measurements, and unilateral lower limb suspension (ULLS) intervention (top) and myofibrillar proteolysis measurements via microdialysis sampling (bottom). d, Day; 72H, 72 hour; Pre, before ULLS; Post, after ULLS.

3MH determination. The procedure for 3MH determination has been described in detail elsewhere (9, 15, 41, 44). In brief, in any dialysate sample 3MH concentration was determined by high-performance liquid chromatography (250 mm $\times$ 4.6 mm C-18 reverse-phase column, flow rate 1.5 ml/min) and fluorometric detection (1100 Series, Agilent Technologies, Wilmington, DE). Derivatization was completed by placing 25 μ l of dialysate or standard (M19930, Pfaltz and Bauer, Waterbury, CN), 65 μ l of borate buffer (0.4 M boric acid, adjusted to pH 12.2 with NaOH), and 65 μ l of fluorescamine reagent (160 mg fluorescamine in 100 ml acetonitrile) in a 0.6-ml microcentrifuge tube, which was then vortexed and allowed to stand at room temperature for 5 min. Ten microliters of concentrated perchloric acid was added to the tube and subsequently capped and heated at 80°C for 1 h. After cooling to room temperature, samples were neutralized with 25 μ l of 0.05 M MOPS in 3 M NaOH. Samples were injected with an autosampler (1100 Series, Agilent Technologies, Palo Alto, CA) maintained at 4°C. The mobile phase was 25% acetonitrile and 75% 10 mM Na₂HPO₄ adjusted to pH 7.5 with phosphoric acid. Peaks were monitored at 365 nm (excitation) and 460 nm (emission) and integrated with chromatography software (Chemstation, Agilent Technologies, Wilmington, DE). Dialysate concentration was determined from a standard curve of 3MH. Interstitial 3MH concentration for each sample was determined by accounting for probe recovery (see *Microdialysis probe recovery*). Calculated interstitial 3MH concentrations from all four 15-min aliquots collected (when available) were used to average each time point (pre- and post-ULLS).

Statistics. Paired *t*-test was used to compare interstitial 3MH levels and microdialysis vial weights. Significance was accepted at $P < 0.05$. Data are presented as means $\pm$ SE, unless noted.

RESULTS

The concentration of interstitial 3MH following 72-h ULLS (2.01 ± 0.22 nmol/ml) was 44% higher ($P < 0.05$) than before ULLS (1.56 ± 0.20 nmol/ml) (Fig. 2). The coefficient of variance for the four 15-min aliquots that were used for 3MH analysis and sampled pre- and post-ULLS were $8.9 \pm 1.4\%$ and $6.9 \pm 0.9\%$, respectively. Dialysate weights were similar ($P > 0.05$) to the expected weight of 30 mg (perfusate density 1 g/ml, dialysate volume 30 μ l) across collection periods. Dialysate sample weights averaged 29.3 ± 0.3 and 29.5 ± 0.2 mg, respectively pre and post ULLS.

DISCUSSION

This particular study was initiated as a result of our overarching long-term goal to explore how skeletal muscle and other

organ systems adapt to spaceflight and to define specific countermeasures to these effects (2, 3, 12, 14, 33, 34, 36–40, 42). The main novel finding of this study was that 72-h unloading induced increased skeletal muscle proteolysis in otherwise healthy men, as reflected in elevated interstitial 3MH concentrations. Thus it appears that both increased proteolysis and decreased protein synthesis (6, 7, 10) occur in the early phase of muscle unloading, eventually resulting in atrophy. Furthermore and not less importantly, the present 3-day unloading protocol presents a feasible and valid model for future in vivo studies exploring the atrophy process in human skeletal muscle.

Skeletal muscle atrophy at the whole muscle and single fiber level are evident with different human unloading models or spaceflight analogs within week(s) (8, 20, 33, 45), and loss of muscle seems to continue at a rather constant rate over the proceeding 3–4 wk (5, 34). Such changes are generally attributed to a net decrease in contractile protein content, resulting from an imbalance in protein metabolism (27, 31). Although studies have reported decreased skeletal muscle protein synthesis in unloaded volunteers subjected to lower limb unloading or bed rest, there are less conclusive human data showing increased contractile protein breakdown following such interventions. In the present study we showed that no more than 72-h unloading facilitates increased contractile protein breakdown. Although these results concert with the previous finding of increased 3MH plasma concentrations following short-term spaceflight, the investigators of that particular study attributed this effect to the stress response seen early on in the flight, not to the unloading per se (32). Because the present model mimics the unloading component of spaceflight with no apparent confounding factors of increased stress, we believe that the increased myosin and actin proteolysis shown in the present study must have originated from lack of weight bearing. Given that previous studies, employing the ULLS model over many weeks, showed no atrophy of the weight-bearing limb (4, 34), it is very unlikely it occurred in the present study or that our finding of increased 3MH was caused by a systemic effect.

The 3% decrease in lower limb muscle cross-sectional area, assessed by means of magnetic resonance imaging following 7 days of bed rest (8, 20), infers that molecular signs of atrophy should be evident early on after withdrawal of weight bearing. Indeed, the finding of a robust increase in 3MH concentration within the muscle in the present study implies muscle proteolysis is initiated within a 72-h period of unloading. This is in contrast to the inability of acute high intensity resistance exercise or 60 min of strenuous aerobic exercise to induce an increase in muscle proteolysis measured by interstitial 3MH concentration (13, 15, 41). These findings highlight the magnitude of the proteolytic signals evoked by unloading skeletal muscle.

In view of these findings, we believe that the experimental model introduced here could serve as a useful tool in research aimed at studying mechanisms regulating skeletal muscle size during conditions (e.g., disease, trauma, muscle disuse, or unloading), which may have implications for procedures (e.g., rehabilitation and nutritional interventions and treatment of the critically ill) employing other techniques (e.g., muscle biopsy sampling) as well.

The novel finding of the present study was the demonstration that, in otherwise healthy men, 72-h ULLS provoked increased

Fig. 2. Interstitial 3MH concentration before and after 72 h ULLS. Means are average of four 15-min acquisitions for all subjects ($n = 8$). *Difference pre- vs. post-ULLS, $P < 0.05$.

proteolytic activity of contractile proteins, evidenced by a marked increase in interstitial 3MH concentration. Thus the microdialysis technique offers a promising tool for in vivo monitoring of protein breakdown of selected muscles in humans in response to reduced muscle use, e.g., real or simulated microgravity. Furthermore, the present findings are encouraging in that they support use of short-term unloading protocols to address questions exploring mechanisms involved in the skeletal muscle atrophy process in humans.

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