Chronic hindlimb suspension unloading markedly decreases turnover rates of skeletal and cardiac muscle proteins and adipose tissue triglycerides

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Bederman IR, Lai N, Shuster J, Henderson L, Ewart S, Cabrera ME. Chronic hindlimb suspension unloading markedly decreases turnover rates of skeletal and cardiac muscle proteins and adipose tissue triglycerides. J Appl Physiol 119: 16–26, 2015. First published April 30, 2015; doi:10.1152/japplphysiol.00004.2014.—We previously showed that a single bolus of “doubly-labeled” water ($^{2}$H$_{2}$18O) can be used to simultaneously determine energy expenditure and turnover rates (synthesis and degradation) of tissue-specific lipids and proteins by modeling labeling patterns of protein-bound alanine and triglyceride-bound glycerol (Bederman IR, Dufner DA, Alexander JC, Previs SF. Am J Physiol Endocrinol Metab 290: E1048–E1056, 2006). Using this novel method, we quantified changes in the whole body and tissue-specific energy balance in a rat model of simulated “microgravity” induced by hindlimb suspension unloading (HSU). After chronic HSU (3 wk), rats exhibited marked atrophy of skeletal and cardiac muscles and significant decrease in adipose tissue mass. For example, soleus muscle mass progressively decreased 11, 43, and 52%. We found similar energy expenditure between control (90 ± 3 kcal·kg$^{-1}$·day$^{-1}$) and hindlimb suspended (81 ± 6 kcal·kg/day) animals. By comparing food intake (~112 kcal·kg$^{-1}$·day$^{-1}$) and expenditure, we found that animals maintained positive calorie balance proportional to their body weight. From multicompartmental fitting of $^{2}$H-labeling patterns, we found significantly (P < 0.005) decreased rates of synthesis (percent decrease from control: cardiac, 25.5%; soleus, 70.3%; extensor digitorum longus, 44.9%; gastrocnemius, 52.5%; and adipose tissue, 39.5%) and rates of degradation (muscles: cardiac, 9.7%; soleus, 52.0%; extensor digitorum longus, 27.8%; gastrocnemius, 37.4%; and adipose tissue, 50.2%). Overall, HSU affected growth of young rats by decreasing the turnover rates of proteins in skeletal and cardiac muscles and adipose tissue triglycerides. Specifically, we found that synthesis rates of skeletal and cardiac muscle proteins were affected to a much greater degree compared with the decrease in degradation rates, resulting in large negative balance and significant tissue loss. In contrast, we found a small decrease in adipose tissue triglyceride synthesis paired with a large decrease in degradation, resulting in smaller negative energy balance and loss of fat mass. We conclude that HSU in rats differentially affects turnover of muscle proteins vs. adipose tissue triglycerides.

energy expenditure; deuterated water; mass spectrometry; hindlimb suspension unloading; protein turnover; triglyceride turnover

ALTERNED LOADING STATES (SPACE travel), chronic inactivity (bed rest and immobilization), and chronic diseases (congestive heart failure, acquired immune deficiency syndrome, and cancer) cause a common condition: atrophy of skeletal muscles (31, 49, 51), which is characterized by the significant decrease in muscle mass, fiber diameter, fatigue resistance, and performance. The mechanism of muscular atrophy is complex and is caused by an imbalance of synthesis and degradation rates of myofibrillar proteins, i.e., decrease in synthesis rate and concomitant increase in the degradation rate (43, 46, 48). The decrease of protein synthesis rate is mediated by combination of several mechanisms: 1) loss of mechanical stimulus; 2) impairments of insulin-mediated pathways that stimulate protein synthesis and substrate uptake/turbover (insulin resistance/mammalian target of rapamycin pathway); and 3) insufficient delivery of amino acid building blocks (protein-energy malnutrition). The rate of protein degradation is also mediated through three known pathways: calpain-calpastatin, ubiquitin-proteosomal, and lysosomal (2). Because of the complexity and variety of systems controlling protein homeostasis, one needs to consider them with respect to a particular condition. For example, as the result of zero-gravity exposure (spaceflight), type I fibers of antigravity muscles such as soleus undergo “adaptive reductive remodeling,” as coined by Stein and Wade (44), causing weakness and easy fatigue and thus loss of astronaut’s performance. In addition to loss of lean and fat body mass (24, 25), decrease in cardiac muscle mass has been documented in humans as part of adaptations to spaceflight, bed rest, or other conditions of inactivity (13, 34). However, specific changes in turnover rates of cardiac muscle proteins have not been documented in the either human or animal models of inactivity.

Hindlimb suspension unloading (HSU) was developed as a “ground-based” simulation of spaceflight conditions (29, 31). Soleus (type I fiber), gastrocnemius (mixed type I/II), and extensor digitorum longus (EDL) (type II fiber) muscles have been commonly used to represent changes in posture (soleus) and locomotor muscles (EDL and gastrocnemius) under the conditions of HSU. Using a HSU rat model, we recently reported increased hepatic gluconeogenesis, fueled by both an increased flux of amino acids from atrophying muscle and by an increased carbohydrate demand by the atrophying muscle (3). Many investigators have reported changes in protein synthesis rates caused by HSU in postural skeletal muscles with general consistency (16, 43, 45-48); however, few laboratories reported changes in degradation rates (18, 48). Based on literature in humans subjected to microgravity or bed rest, we reasoned that, in addition to changes in skeletal muscle turnover rates, rats subjected to HSU will exhibit altered turnover of cardiac muscles, adipose tissue triglycerides, and energy expenditure. Studies have demonstrated cardiac atrophy in the human subjects after bed rest and spaceflight, but quantification of protein synthesis rates was not possible due to sample limitations (13, 34). We recently demonstrated a “doubly-labeled” water method to simultaneously measure energy expenditure and tissue-specific turnover rates of skeletal muscle proteins and adipose tissue triglycerides in mice, where energy

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balance was perturbed by high-fat diet feeding (4). Loss of tissue mass resulting from inactivity conditions implies disproportional decreases in the synthesis and degradation rates, for example, a greater decrease in synthesis than degradation. Thus the goal of this work was to quantify tissue-specific turnover rates of cardiac and skeletal muscle proteins, adipose tissue triglycerides, and energy expenditure in rats subjected to HSU. We hypothesized that HSU-induced atrophy caused a greater decrease in synthesis than in degradation rates of both skeletal and cardiac muscle proteins and adipose tissue triglycerides. In this study, we applied our methodology to determine rates of synthesis and degradation of protein-bound alanine isolated from the soleus, gastrocnemius, EDL, and cardiac muscles, as well as triglyceride-bound glycerol isolated from white adipose tissue. We present our findings herein.

**MATERIALS AND METHODS**

**Chemicals and Supplies**

Unless specified, all chemicals and reagents were purchased from Sigma-Aldrich. Stable isotopes were purchased from Isotec (Miamisburg, OH). Tincture of Benzoin was purchased from Fisher Scientific (Pittsburgh, PA).

**Biological Experiments**

Forty-eight young male (6 wk of age) Sprague-Dawley rats were purchased from Taconic Farms (Hudson, NY), each weighing 150 g. Animals were randomly split into two groups: control and experimental (HSU). After brief acclimation to the animal facility, the HSU group underwent HSU procedure as described below. This study was approved by the Institutional Animal Care and Use Committee of Case Western Reserve University.

**HSU**

Individual HSU cages were custom-made from clear acrylic panels (0.22 mm thick) based on published designs (30). Cage floor space exceeded minimum requirements of 70 sq. in. (~160 sq. in.) per animal. The cage floor was made from “eggcrate” grid panel as suggested by Park and Schultz (32) to allow for animal and food waste to fall through. The suspension apparatus consisted of a swivel pulley, which glued to the smooth, stainless steel rod affixed at the top of the cage, thus allowing for 360° animal movement. A water bottle was affixed to the cage wall by a spring. Food pellets were in a small dish affixed to the cage floor. For hindlimb suspension installation, an animal was first sedated by brief inhalant anesthesia (isoflurane), and the entire tail was cleaned with tincture of Benzoin. After brief drying, the tincture became sticky, and traction tape was glued to the underside two-thirds of the tail away from the body with nonirritant adhesive. The tape was then attached to a large paper clip by the steel staples. Lastly, the glue was allowed to dry for 15 min while the rat recovered from anesthesia, and the paper clip was then attached to the swivel pulley. The height of the suspension apparatus was adjusted as needed to maintain the recommended 30° angle with the floor. Investigators previously reported that this angle provides the needed to maintain the recommended 30° angle with the floor.

**Stable Isotope Protocol**

Controls and 7-day-suspended HSU rats were briefly anesthetized with isoflurane and injected intraperitoneally with saline solution of “doubly-labeled water,” i.e., a mix of $^2$H$_2$O [99.9% atom percent excess (APE) plus $^2$H$_2^{18}$O (10% APE) stable isotopes to label body water at 2.5% $^2$H moles percent excess and 0.25% $^18$O moles percent excess, respectively. Animals recovered quickly from anesthesia and were not adversely affected by the injection of the isotopes. Controls were returned to normal cages, and HSU animals were returned to hindlimb suspension cages.

Animals from both groups were given terminal anesthesia in sets of three on the following days: days 0, 1, 2, 4, 6, 9, 12, and 16. On day 0 (injection day), animals were terminated 3 h after injection. Hindlimb muscles (soleus, EDL, and gastrocnemius) from both legs were quickly excised and freeze-clamped in liquid nitrogen. In addition, a terminal blood sample, heart, liver, and epididymal fat depots were collected and kept at ~86°C until processing. Organs were carefully weighed after freeze-clamping and total weights recorded.

**Analytic Procedures**

Isotopic enrichments were determined using an Agilent 5973N-MSD (mass spectrometric detector) equipped with an Agilent 6890 gas chromatography (GC)/MS system (GC/MS). A DB17-MS capillary column (30 m × 0.25 mm × 0.25 μm) was used in all assays with a helium flow of 1 ml/min. Samples were analyzed in selected ion monitoring mode using electron impact ionization. Ion dwell time was set to 10 ms.

$^2$H-labeling of body water. The $^2$H-labeling of body water was determined by the exchange with acetone, as previously described by Katanik et al. (21). Briefly, a 20-μl aliquot of blood or standard was reacted overnight with 4 μl of 10 N KOH and 4 μl of 5% acetone (vol/vol) and then extracted with chloroform and dried using sodium sulfate. A 1-μl aliquot was then injected into GC/MS, and mass-to-charge ratio (m/z) of 58 and 59 of acetone and deuterated acetone, respectively, were monitored. Body water $^2$H-labeling was determined as ratio of areas of 59/(58 + 59) mol%.

$^{18}$O-labeling of body water. The $^{18}$O-labeling of body water was determined by trimethylphosphate (TMDP) method as previously described by Brunengraber et al. (8). Briefly, TMDP was generated by reacting phosphoric acid with diazomethane. The $^{18}$O-enrichment of TMDP was assayed as follows: 5 μl of plasma sample or standard were added to a 12 × 75 mm glass tube. To this, ~3 μg of PCl₃ were added to generate phosphoric acid, and samples were left stand for 20 min. Next, ~300 μl of freshly prepared ethereal diazomethane were added to the sample and allowed to stand until the ether evaporated. Diazomethane is very hazardous and thus was made in small quantity by adding 2.5 ml of 40% KOH (wt/vol) and 8 ml of diethyl ether to a 20-ml scratch-free glass tube. To this, ~100 mg of 1-methyl-3-nitro-1-nitrosoguanidine were added, and the reaction was run until ether developed a dark green-yellow color. TEMP was extracted by the addition of 150 μl of water, 600 μl of chloroform, and sodium sulfate. A 1-μl aliquot was then injected into GC-MS under the following conditions: the temperature program was 90°C initial, increase by 30°C × min$^{-1}$ to 240°C, and hold for 1 min. The split ratio was 20:1 with helium flow 1 ml × min$^{-1}$. TEMP eluted at ~2.4 min. The $^{18}$O-enrichment was determined using electron impact ionization (70 eV) and selected ion monitoring (10-ns dwell time) of m/z 140–142. The $^{18}$O-enrichment was calculated from the signal ratio (142/142 + 140).

**Assay of $^2$H-labeling and concentration of protein-bound alanine**

Alanine from mixed-muscle protein was isolated as published previously (14). Briefly, powdered heart and skeletal muscle samples were carefully weighed and deproteinized by the addition of 6% ice-chilled perchloric acid. After centrifugation and removal of supernatant, the remaining protein pellet was redissolved in 1 ml of 6 N HCl and hydrolyzed at 100°C for 24 h. An aliquot of the hydrolyzed protein sample was evaporated to dryness and reacted with a mixture of acetoni trile, methanol, and N,N-dimethylformamide dimethyl acetate (1:2.3 vol/vol) at 60°C for 20 min to form the “methyl-8” derivative of alanine. $^2$H-labeling of protein-bound alanine was deter-
minged using GC/MS. GC/MS conditions were as follows: the oven temperature was set at 90°C and held for 5 min, then increased by 5°C/min to 130°C, and increased by 40°C/min to 240°C and held for 5 min. Alanine eluted at ~12 min, and selective ion monitoring was used to detect m/z 158 and 159. An aliquot of hydrolyzate was used to determine the concentration of protein-bound alanine enzymatically, as described by Pfeiderer et al. (35) and modified by Passonneau and Lowry (33). Briefly, alanine concentration was determined using coupled reaction of alanine transaminase and lactate dehydrogenase. Relative alanine concentration was adjusted per gram of wet muscle, and absolute concentration was computed using total muscle/heart weights.

Assay of 2H-labeling and concentration of triglyceride-bound glycerol. A ~100-mg sample of epididymal fat pad was hydrolyzed in 1 N ethanol-KOH (70% vol/vol) at 80°C for 2 h. After partial evaporation (to remove ethanol), the sample was acidified with 12 N HCl, and 1 ml of water was added. Free fatty acids were extracted with ethyl ether three times, and ether fractions were combined and evaporated to dryness. Aqueous layer containing glycerol was evaporated to dryness and reconstituted in a given amount of water. A 20-μl aliquot was used for glycerol concentration assay that was done spectrophotometrically using “Free Glycerol Reagent” from Sigma.

1H-labeling of triglyceride-bound glycerol was determined using GC/MS. A 50-μl aliquot was evaporated in a GC/MS vial and derivatized using N-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide to form the tri-tert-butyldimethylsilyl derivative of glycerol, as described by Flakoll et al. (15). The conditions for the tri-tert-butyldimethylsilyl glycerol derivative were as follows: oven temperature was set at 100°C and held for 0.5 min and then increased by 25°C/min to 300°C and held for 3 min. Glycerol eluted at ~6 min. Selective ion monitoring was done of m/z (M-57) fragment 377 and respective isotopomer species up to M4 (378–382).

**Energy Expenditure**

Total energy expenditure (TEE) was determined from the single pool model by Speakman et al. (38–40), which was validated for use in models of simulated weightlessness by Blanc et al. (6). TEE was calculated from Eq. 1:

\[
\text{TEE} = \left( \frac{15.457}{\text{FQ}} + 5.573 \right) \cdot r\text{CO}_2 \cdot 22.4 \quad (1)
\]

where 22.4 is the conversion factor for moles of CO2, and FQ is the food quotient calculated using food intakes expressed as calories (Eq. 2):

\[
\text{FQ} = (0.81 \cdot \text{protein}) + (0.71 \cdot \text{fat}) + (1 \cdot \text{carbohydrate}) \quad (2)
\]

and rCO2 is CO2 production rate determined using equation of Speakman (Eq. 3):

\[
r\text{CO}_2 = \frac{N}{2.078} \cdot (K_o - K_d) - 0.0062 \cdot K_d \cdot N \quad (3)
\]

Fractional decay constants of 2H (Kd/day) and 18O (Kd/day) were computed from isotopic enrichment decay curves (see Fig. 6A). Isotope dilution space, N, was determined from deuterium (Nd) and oxygen (N0) dilution spaces by Eq. 4:

\[
N = \left( \frac{N_0}{1.01} + \frac{N_d}{1.0} \right) / 2 \quad (4)
\]

Dilution spaces for deuterium and 18O were determined as published by Brunengraber et al. (8).

**Mathematical Modeling**

This computational model is based on the two-compartment model previously used to study isotope kinetics for different biological systems (9). The biological system, represented in Fig. 1, consists of the body water compartment (i = 6), where deuterium is homogeneously distributed, and five tissue compartments (heart, soleus, EDL, gastrocnemius, and fat, i = 1, 5) that interact with the body water compartment. Therefore, deuterium from the body water compartment is equilibrated with deuterium in each of the five tissue compartments and is incorporated into both protein-bound alanine and triglyceride-bound glycerol. Based on Previs et al. (36) and validated by Belloto et al. (5), deuterium from body water equilibrates with free alanine (tracer) and is incorporated into tissue bound protein (tracee). As shown by Bederman et al. (4), body water deuterium (tracer) incorporates into triglyceride-bound glycerol (tracee). In each muscle tissue compartment, 2H is equilibrated with free alanine, denoted as mfi, which is then incorporated into tissues via protein synthesis into protein-bound alanine, denoted as mfi (i = 1, 4). In the fat pad compartment, 2H is incorporated into triglyceride precursor glycerol via glycolysis in the adipocyte, denoted as mfi, which is then incorporated as the glycerol moiety of adipose tissue triglyceride, denoted as mfi. Deuterium-labeled free alanine and free glycerol are in equilibrium with deuterium-labeled body water. Thus the free molecules are also assumed to be “tracer” molecules, and their bound forms are “tracee” molecules. The dynamic changes of the 2H tracee present as bound forms mfi in compartment i are represented by the mass balance. In particular, the dynamic mass balance describes the relationship between free mfi and bound mfi forms of the 2H-labeled metabolite as follows:

\[
\frac{dm_i^b}{dt} = k_{i,j} m_i^f - k_{i,b} m_i^b, \quad i = 1, 5 \quad (5)
\]

where k_{i,j} and k_{i,b} are rate constants indicating synthesis and degradation rates of the bound form mfi in the compartment i. The decay of the 2H tracer in the body water is described by the dynamic mass balance:

\[
\frac{dm_6}{dt} = -k_6 m_6 \quad (6)
\]

where k6 is the decay constant of body water deuterium due to excretion. The tracee is present as free Mfi and bound Mfi forms, while
the total tracee $M'_i$ in each compartment is assumed to be constant. Thus the rates of synthesis and degradation of the tracee in each compartment can be calculated according to the following mass balances of each tracee at steady state:

$$\phi_i = k_{i,f} M'_i - k_{i,b} M'_i, \quad i = 1, 5$$

(7)

where $k_{i,f}$ and $k_{i,b}$ are rate constants of the tracer in each compartment which are equal to those of the tracee. A convenient way to express stable isotopic measurements is to express tracer mass per unit of which are equal to those of the tracee. A convenient way to express $\frac{\text{tracer mass}}{\text{metabolite time}}$, $k_i$ can be rearranged as:

$$\frac{dz'_i}{dt} = \frac{k_{i,f} M'_i}{M'_i} z_6 - k_{i,b} z'_i, \quad i = 1, 5$$

(8)

$$\frac{dz_6}{dt} = -k_6 z_6$$

(9)

where $k_{i,f} = k_{i,f} M'_i/M'_i$. The initial conditions for the simulation of the metabolite time ($t$) changes are specified at rest in both groups.

$$t = t_0, z'_i = z'_i, z_6 = z_6$$

(10)

In particular, $t_0$ is equal to the 12-day HSU period. The mass average of tracee $M'_i$ ($i = 1, 5$) used in the model equations above have been measured previously and reported in Table 1. The $K_{i,r}$, $k_{i,b}$, and $k_6$ rate constants must be estimated by using temporal experimental data profiles of $z'_i$ ratios of each compartment. For this purpose, we found the parameter values that yield the best fit of the model output dynamics to the experimental data. Specifically, for two different sets of experimental data obtained under control and HSU conditions, a least squares objective function is minimized:

$$J = \sum_{i=1}^{N_C} \sum_{m=1}^{N_D} \left[ z_{i,m}(t)_{\text{exp}} - z_{i,m}(t)_{\text{mod}} \right]^2$$

(11)

where $N_C$ and $N_D$ are the number of compartment and number of data points, respectively.

The objective function is minimized by numerical optimization using an adaptive, nonlinear algorithm (DN2FB, http://www.netlib.org). The $z_{i,m}(t)_{\text{mod}}$ model simulations are obtained from the solution of the model represented by Eqs. 8 and 9. Consequently, the model consists of a set of ordinary differential-difference equations that constitute an initial value problem. These ordinary differential equations were solved numerically using a robust algorithm for stiff systems (DLSODE).

Statistical Methods

Significance was determined between control and HSU groups using Student's $t$-test for independent variables.

RESULTS

Growth and Body Weight Changes During HSU

As HSU affects growth and body weight, we measured the rate of body weight gain from the onset of HSU (Fig. 2A, day $-7$). Initial body weights for the groups were similar (compare controls: $181.4 \pm 5.9$ g vs. HSU: $177.1 \pm 8.8$ g). Control animals grew at expected rate, whereas animals subjected to HSU had flat growth rate initially (from trendline regression, control: $6.5$ g/day vs. HSU: $0.2$ g/day). At the end of 7 days, controls had significantly higher body weight compared with the HSU group (controls: $230.0 \pm 7.9$ g vs. HSU: $179.3 \pm 16.4$ g, $P < 0.01$). As the animals adjusted to HSU conditions and

![Fig. 2](http://jap.physiology.org/)

**Fig. 2.** Temporal profile of body weight. **A:** "preisotope" adjustment period to hindlimb suspension unloading (HSU). **B:** "double-labeled" water administration period. HSU steady state. Values are means ± SD ($N = 3$ per time point). □, Control group; ▀, HSU group.
resumed normal food intake (after 3 days, data not shown), the rate of body weight gain markedly increased; however, body weight gain did not reach the control group (Fig. 2B, compare body weight growth rates from trendline regression, control: 6.4 g/day vs. HSU: 4.6 g/day). At the end of the experimental period, the control group still exhibited significantly higher body weight (controls: 333.3 ± 29.3 g vs. HSU: 243.0 ± 15.7 g, P < 0.01). Overall, HSU caused delayed growth at onset and slow recovery in body weight. Next we examined the effects of HSU on skeletal and cardiac muscle weights.

**HSU-induced Muscle Atrophy**

Figure 3 shows temporal profiles of absolute weights of skeletal and cardiac muscles during the experimental period (days 7–16). In the case of soleus (Fig. 3A) and EDL (Fig. 3B) muscles, average combined (right and left) muscle weight is shown. Gastrocnemius weight (Fig. 3C) is shown as a single right leg muscle. Figure 3D shows the weights of cardiac muscle. Note that HSU was initiated on “day 7” (Fig. 2A); therefore, by day 7, the “day 0” animals have been subjected to HSU for 7 days. As shown by Fig. 3A, the soleus muscle of the HSU group atrophied significantly and equaled one-half the weight of the controls (compare control: 0.09 ± 0.01 g vs. HSU: 0.05 ± 0.01 g, P < 0.001). Soleus mass of control animals maintained steady growth, whereas the soleus of HSU animals, once atrophied, changed very little (compare 6.0 vs. 0.2 mg/day, P < 0.001). At the end of the suspension period, the disparity in absolute soleus weights between controls and HSU groups was approximately threefold. The EDL muscle (Fig. 3B) is predominantly type II fiber, and expectedly, its mass/growth was not affected by the HSU to the same extent as the soleus muscle. Indeed, after 7 days of HSU, absolute EDL masses were similar between control and HSU groups (compare controls: 0.08 ± 0.01 g vs. HSU: 0.07 ± 0.01 g). The rate of change in EDL mass was lower in the case of HSU (compare 8.0 vs. 4.0 mg/day), causing EDL mass of HSU group to be significantly smaller at the end of the experimental period (compare controls: 0.15 ± 0.02 g vs. HSU: 0.09 ± 0.02 g, P < 0.01). The gastrocnemius muscle (Fig. 3C) is a mixed type I/II fiber but a large sized muscle; therefore, it exhibited marked atrophy (compare controls: 1.38 ± 0.08 g vs. HSU: 0.91 ± 0.10 g, P < 0.005). HSU caused a decreased rate of growth (compare controls: 100 mg/day vs. HSU: 49 mg/day), resulting in even greater disparity between final muscle weights (compare controls: 2.03 ± 0.14 g vs. HSU: 1.10 ± 0.20 g, P < 0.005). Cardiac muscle (Fig. 3D) showed significant atrophy after 7 days of suspension (compare controls: 0.82 ± 0.05 g vs. HSU: 0.66 ± 0.07 g, P < 0.05) and maintained the same rate of growth thereafter (compare control: 30 mg/day vs. HSU: 31 mg/day). Atrophy of cardiac muscle was still evident at the end of the experimental period (compare controls: 1.05 ± 0.11 g vs. HSU: 0.80 ± 0.09 g, P < 0.05).

![Figure 3. Temporal profiles of absolute mass of skeletal and cardiac muscles. A: soleus. B: extensor digitorum longus. C: gastrocnemius. D: cardiac. Wet tissue weights are shown. Values are means ± SD (N = 3 per time point). Significance was determined comparing HSU to controls at each time point using independent Student’s t-test (*P < 0.005, #P < 0.001). Solid bars, control group; open bars, HSU group.](http://japp.physiology.org/doi/fig/10.1152/japplphysiol.00004.2014)
To account for changes in body weight, we normalized absolute wet muscle weights to their respective body weights, shown in Fig. 4. It is evident that temporal profiles for EDL (Fig. 4B) and cardiac (Fig. 4D) muscles were proportional to the changes in body weight, whereas profiles of soleus (Fig. 4A) and gastrocnemius (Fig. 4C) muscles were still significantly lower in HSU group compared with controls. At the end of HSU, soleus and gastrocnemius masses decreased nearly 2- and 1.3-fold, respectively.

Atrophy of Adipose Tissue

Next, we reasoned that the decrease in body weight was attributable not only to the changes in lean mass, but also to the changes in fat mass. Figure 5 shows absolute (A) and relative (B) weights of epididymal fat pads isolated from control and HSU animals. Absolute profile of control animals showed continued growth of the fat pads. During the experimental period, control epididymal fat pad weight doubled. Absolute profile and rate of growth were significantly lower in HSU animals, although, due to variability, not all experimental points were significantly different from the controls. At the end of the experimental period, absolute weights of fat pads differed by approximately twofold (compare control: 2.9 ± 0.5 g vs. HSU: 1.4 ± 0.4 g, P < 0.01). When normalized to body weight, significantly different adipose tissue mass was found on day 1 (compare control: 1.46 ± 0.09 g vs. HSU: 1.02 ± 0.54 g, P < 0.01) and day 16 of HSU (compare control: 8.6 ± 0.7 g vs. HSU: 5.6 ± 1.8 g, P < 0.05).

Energy Expenditure and Energy Balance

“Doubly-labeled” water method was used to determine carbon dioxide production and, subsequently, energy expenditure. This method is based on the difference in decay rates of $^2$H and $^{18}$O from body water. Deuterium ($^2$H) decay rates did not differ between control and HSU animals (compare $k_C$ = 0.235 and $k_{HSU}$ = 0.234, respectively). Decay rates for $^{18}$O were slightly lower for HSU group but not significantly different (compare $k_C$ = 0.415 and $k_{HSU}$ = 0.398, respectively) (Fig. 6A). From the difference in decay rates, we determined $r\text{CO}_2$ that tended to be lower in HSU animals; however, was not significant (compare 0.7 ± 0.02 vs. 0.6 ± 0.05 mmol/day, P = 0.3). Consequently, TEE also tended to be lower in HSU animals, but did not reach significance (compare 90.0 ± 2.8 vs. 81.1 ± 6.0 kcal/kg day, P = 0.4). Considering that average food intake equaled ~112 kcal·kg$^{-1}$·day$^{-1}$, all animal groups remained in positive carbon balance, despite HSU condition.

Tissue Turnover Rates

We used $^2$H-labeling patterns of protein-bound alanine to determine rates of synthesis and degradation of mixed proteins isolated from hindlimb and cardiac muscles. The triglyceride-

Fig. 4. Temporal profiles of relative mass of cardiac and skeletal muscles. A: soleus. B: extensor digitorum longus. C: gastrocnemius. D: cardiac. Wet tissue weight was normalized to the body weight on a given day. Values are means ± SD (N = 3 per time point). Significance was determined comparing HSU to controls at each time point using independent Student’s t-test ($^P < 0.001$). Solid bars, control group; open bars, HSU group.
bound glycerol $^2$H-labeling patterns were used to determine rates of synthesis and degradation of lipids isolated from epididymal fat pads. Figure 6 shows $^2$H-labeling patterns and mathematical fitting of the data. The deuterium decay curve of body water was used as the precursor enrichment for computations of turnover rates (Fig. 6A). Profiles of deuterium labeling of protein-bound alanine are shown for hindlimb and cardiac muscles in Figs. 6, B–E, respectively. Figure 6F shows labeling profiles of triglyceride-bound glycerol isolated from epididymal fat pads. Experimental data were fitted using mathematical model described in MATERIALS AND METHODS, yielding absolute rates of synthesis and degradation of tissue-specific proteins and lipids (Fig. 7). Alanine pool size was markedly lower in the cardiac and soleus muscles of HSU animals (7 and 63% lower compared with controls, respectively). Also, glycerol pool was ~50% lower in HSU animals, compared with the controls. After 3 wk of HSU,
synthesis rate of soleus (Fig. 7A) decreased by 70%, whereas degradation rate decreased by 50%, compared with the control animals. Thus controls maintained positive soleus mass balance (+17%), whereas HSU animals had negative mass balance (−34%). The rates of synthesis and degradation of EDL muscle (Fig. 7B) decreased in HSU animals by 47 and 30%, respectively. Overall, control animals had positive EDL balance, whereas hindlimb-suspended animals had negative EDL balance (+6 vs. −23%). The rates of synthesis and degradation of gastrocnemius muscle (Fig. 7C) also significantly decreased (53 and 38%, respectively) in HSU animals. Thus gastrocnemius muscle mass in the control group had positive balance (+13%), whereas HSU animals had negative mass balance (−15%). The rates of synthesis and degradation of cardiac muscle were also significantly affected by the HSU (Fig. 7D). Synthesis rate decreased by 25%, whereas degradation rate decreased by 9%. Unexpectedly, we found that cardiac muscle of control animals was in the negative balance (−26%); however, HSU animals had even greater negative balance (−53%). Lastly, HSU significantly lower both synthesis and degradation rates of triglycerides from epididymal fat pads (Fig. 7E), by 39 and 49%, respectively. Remarkably, both control and HSU animals maintained significant positive balance with respect to adipose tissue turnover (+52 and +60%, respectively).
DISCUSSION

Our laboratory previously demonstrated the use of “doubly-labeled” water to simultaneously quantify energy expenditure and turnover rates of tissue-specific proteins and lipids under the conditions of positive energy balance, i.e., high-fat feeding (4). In our present work, we apply this methodology to quantify energy expenditure and tissue-specific metabolic fluxes under conditions of simulated weightlessness induced by HSU, a condition of negative energy balance (44). We studied the effects of HSU during the chronic phase of unloading (7–21 days) rather than during the acute phase (0–7 days) because, during the acute phase, rodents undergo the “adaptive” period of HSU, accompanied by sharp hormonal changes. For example, Musacchia et al. (41) found that adrenal hypertrophy returned to normal after 7 days of HSU; Jaspers et al. (19) reported large fluctuations in plasma corticosterone levels that normalized after 6 days. Thomason and Booth (49) provided an excellent review of the time course of changes in the physiological state of the suspended animal. Thus there is a general agreement in the literature that the first 3–7 days of HSU represent an acute “adaptive phase.” In agreement with others (16, 49), we show that, during the first week (Fig. 2A), there is a complete lack of weight gain of the HSU animals, partially due to decreased food intake (data not shown). However, by day 7, the food intake normalized and remained constant during the rest of the experimental period, signifying adaptation to the HSU condition and general good status of the animal’s health. As a result, HSU animals quickly regained weight and maintained growth rates similar to the controls (Fig. 2B). These findings agree with other laboratories (1, 12, 31, 53).

Studies of energy balance during early space missions revealed that astronauts required between 8 and 11.5 MJ/day while consuming between 11 and 12.5 MJ/day (23), thus keeping a positive energy balance. As length and complexity of missions has increased (e.g., extravehicular walks/work), energy requirements also increased, while energy intake remained the same. Several studies have reported negative energy balance in both humans and animals during flight (22, 42). Stein et al. (42) also reported that substrates from skeletal muscle proteins as well as adipose tissue triglycerides were being utilized during spaceflight. The use of our “doubly-labeled” water approach allowed the determination of carbon dioxide production and calculating energy expenditure during the period of HSU. Blanc et al. (6) used doubly-labeled water to assess energy expenditure in HSU-subjected rats; however, they reported no difference in energy expenditure in 10-day suspended animals. Similarly, we did not find any differences in energy expenditure (compare controls: 90.0 ± 2.8 kcal·kg⁻¹·day⁻¹ and HSU: 81.1 ± 6.0 kcal·kg⁻¹·day⁻¹). Considering that the animal’s food intake was not different between the groups (~112 kcal·kg⁻¹·day⁻¹), we concluded that the animals were indeed in the whole body positive energy balance, despite the differences in the growth rates.

In this work, a computational model based on the dynamic mass balance for tracer and tracee was used to determine protein synthesis and degradation using a bolus dose of deuterium (9). A similar approach was successfully applied in other studies on surfactant disaturated-phosphatidylcholine kinetics in the lungs (10, 11). The use of this model allowed us to analyze temporal rates of synthesis and degradation of several compartments, all related to a single precursor compartment, i.e., body water, which contained “tracer” molecule. This relationship allowed for simultaneous determination of the changes in the turnovers of different tissues during the same time period. Our methodology utilizes a single isotopic tracer, which reliably incorporates into multiple tissues, and thus provides valuable information regarding tissue turnover. Since deuterated water can be administered as drinking water (human studies), thus excluding the use of indwelling catheters for isotope infusions, our method can be extended to clinical studies to determine changes in circulating protein turnover rates affected by inactivity, bed rest, etc. Recent papers show broad application of deuterated water to measure multiple plasma components (20, 26, 52).

HSU induces characteristic muscular atrophy by primarily affecting type I fibers. Soleus is a primarily (90%) type I fiber muscle, so it was not surprising to find significant atrophy after 7 days of suspension. Our results agreed well with the vast body of literature. For list of papers, please see review by Booth and Shanely (7). Soleus mass decreased dramatically in the first week (day 7, 69% less than controls) and then the rate of decrease slowed down to 46% on day 21 (~50% across the entire time). Absolute weight of the soleus did not change after initial atrophy on day 0 (day −7 of HSU). This steady-state behavior of time course profile of soleus atrophy agrees well with findings of Thomason et al. (50). Using deuterium incorporation, we found that turnover of soleus muscle drastically decreased. Both rates of synthesis and degradation were drastically decreased by HSU; however, the decrease was not proportional (greater decrease in synthesis rates). Control animals had positive balance overall, whereas HSU animals were in negative balance with respect to soleus mass. These are in general agreement with literature findings reviewed by Bajotto and Shimomura (Ref. 2; see Table 1 for list of studies). The agreement is only general, however, since studies differ by the method used, length of HSU, age and sex of animals, and compartment sampled (e.g., myofibrillar protein). Several studies reported that degradation rate decreases significantly more than the synthesis (16, 27, 49). We report modest decrease in degradation compared with synthesis rate.

EDL muscle is typically used in HSU studies as the control muscle since it contains both type I and II fibers of about equal proportion. In this study, EDL was significantly affected, even though its absolute mass did not change as much as the soleus muscle; the time-dependent growth rate was significantly lower. In support of that, rates of synthesis and degradation were significantly decreased, and EDL mass balance changed from positive (controls) to negative (HSU). Gastrocnemius muscle, a large mixed fiber-type muscle of the hindlimbs, also significantly atrophied during HSU. Even though the balance of the muscle did not switch from positive to negative, already on day 0 (day −7 of HSU), GC muscle mass was significantly lower, as reflected in the turnover rates. This indicates that HSU-induced inactivity affects muscles, regardless of fiber type. Since gastrocnemius is a large locomotor muscle, even small changes would affect exercise performance. Current studies in our laboratory are testing the extent to which HSU-induced atrophy affects treadmill exercise capacity.

In this work, we show novel data of time course of changes to cardiac muscle mass and turnover under conditions of HSU. Ray et al. (37) studied cardiac atrophy during flight and/or HSU.
conditions and showed no changes in the heart mass and function; however, we show that animals subjected to unloading had lower absolute heart mass and altered turnover rates. Similarly to Ray et al., heart weight normalized to body weight was not different in rats subjected to HSU. To our knowledge, this is the first study where cardiac muscle turnover rates were determined under the conditions of HSU. Cardiac atrophy has significant consequences to the rest of the body, such as diminished blood volume (abnormal left ventricular function), inability to sustain changes in blood pressure and/or physical load, and even cardiac failure. Similar to skeletal muscle, we found a large decrease in protein synthesis rates paired with a small decrease in degradation rates effectively resulted in negative energy balance and loss of cardiac mass. This may have important ramifications for developing countermeasures to microgravity-induced muscular atrophy and cardiac deconditioning. For example, specific aerobic exercises can be developed to normalize cardiac function during flight or bed rest (treadmill and cycle ergometer). In addition, specific nutritional interventions (potassium-rich supplements) can lessen the burden of “zero gravity” on the loss of cardiac muscle mass and function (13). Utilization of our methodology will enable investigators to determine the effectiveness of countermeasures of preserving muscle mass during inactivity, bed rest, or flight. The simplicity of tracer administration and sample collection will allow for application of our methodology in a variety of field studies.

The other novel data in this study are findings of decreased turnover rates of adipose tissue triglycerides. We report that triglyceride pool decreased twofold, while the turnover was down by 30% in HSU animals. In contrast to changes in both skeletal and cardiac muscle turnover rates, we found a small decrease in triglyceride synthesis and deposition, paired with a large decrease in degradation rates, effectively resulting in smaller negative energy balance. The decrease in the total triglyceride pool could be a consequence of transient insulin resistance (17), and the increase in catecholamine concentrations during the initial stages of unloading would both inhibit lipoprotein lipase-mediated triglyceride uptake and stimulate hormone-sensitive lipase-mediated triglyceride release from the adipose tissue. Current studies in our laboratory are aimed to determine whether HSU induces a shift in the control of triglyceride mobilization and storage. Zderic and Hamilton (54) reported that physical inactivity downregulated muscle lipoprotein lipase, indicating that the uptake of circulating triglycerides by skeletal muscle is already affected by HSU.

Together we present another application of our methodology under the conditions of altered energy balance. Our approach uses time-dependent data to simultaneously determine tissue synthesis and breakdown rates of tissue-specific substrates. Using our approach, we show the significance of HSU effect on the turnover of skeletal muscles of various fiber-type composition, cardiac muscle, and adipose tissue. HSU-specific changes in the latter two tissues have not been presented before, and our new data will help in the understanding of the effects of HSU on broader range of tissues. In conclusion, HSU in rats caused excessively large decreases in protein synthesis rates compared with degradation rates, resulting in muscular atrophy of postural and cardiac muscles. Also, the HSU condition caused a decrease in adipose tissue turnover, thus conserving fat energy reserve. Nutritional and/or pharmacological interventions must focus on specifically supporting protein synthesis rates to prevent clinically significant losses in skeletal and cardiac muscles.

GRANT
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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