A noninvasive analysis of urinary musculoskeletal collagen metabolism markers from rhesus monkeys subject to chronic hypergravity

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WELL OVER A CENTURY AGO, Wolff (48) proposed that “change in form and function of bone or of function alone is followed by certain definite changes in their internal architecture . . . in accordance with mathematical laws.” This proposal has been extensively studied and modified to a modern proposition, known as the Utah paradigm, that “load-bearing bones are designed to have only enough strength to keep chronically subnormal, normal or supranormal voluntary loads from causing spontaneous fractures” (15, 16). These two propositions imply that actively metabolizing tissues, such as bone, connective tissue, and muscle, should adapt to more or less loading by a change in architecture to be consistent with the load applied.

Metabolic by-products of tissue metabolism, measured in urine of astronauts, cosmonauts, and rhesus monkeys, suggest an increase in connective tissue degradation during short-duration exposure to microgravity. The increased urinary excretion of hydroxyproline (Hyp) and mineral salts in astronauts is evidence that degradation of connective tissue seems to be enhanced by an altered load environment, such as weightlessness. Also, increased urinary contents of collagen degradation metabolic by-products (collagen cross-links) are observed in patients with metabolic bone disease, in other bone disorders associated with bone remodeling, or during rapid bone growth (3, 26, 39). Connective tissues are important in maintaining the stability of joints and the body’s structural integrity (muscle, bones, cartilage, tendons, and ligaments) and also are involved in the translation of mechanical stresses to bones. Therefore, alterations in the load environment (i.e., ground reaction forces and/or muscle forces) through a decreased load history (e.g., spaceflight and immobilization) or an increased load history [e.g., hypergravity (HG)] will modify connective tissue metabolism.

Unfortunately, an assessment of collagen loss has been limited because of the technological limitations associated with sensitivity or hardware constraints and availability (MRI, bone dual-energy X-ray absorptiometry, CT scans, and intracellular metabolites). The development of new techniques to reduce the background of confounding proteins and glycoproteins in physiological media and the discovery of sensitive new technologies to measure the presence of total collagen cross-links in plasma, serum, and urine have allowed our laboratory and others to quantitate direct physiological biomarkers of collagen metabolism noninvasively. More effort needs to be directed toward the noninvasive assessment of collagen loss using markers of mature collagen degradation [hydroxylsylpyridinoline (HP) and lysylpyridinoline (LP)] to monitor the result of load histories with respect to altered gravitational environments (G forces). There has been a commitment by federal funding agencies to study the potential of a G vector as a possible countermeasure in the process of bone healing and amelioration of osteoporosis. Very little consensus information on the plasticity of bone associated with an enhanced weight-bearing stress (HG) on the skeleton has been collected. No HG

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research that involves the noninvasive measurement of collagen degradation products in animals subject to an increased gravitational force has been published. It has been shown that loading of the skeleton alters the metabolism of bone during various stages of growth (4). It is also known that many different physiological stimuli, such as physical, electrochemical, and hormonal stresses, collectively impact bone homeostasis in vivo and in vitro (43, 46, 47, 50, 51, 52). In addition, the microgravity environment of space is known to induce significant alterations in bone structure and metabolism following acute spaceflight (11, 28, 30, 40). Therefore, an alteration in the gravitational loading stress is an additional factor that affects the processes that govern the plasticity of the musculoskeletal system.

Pioneering studies of artificial gravity performed during the early years of the manned space program studying artificial gravity showed that HG can alter the length and strength of bones in a variety of rapidly growing animals (1, 14, 21, 28, 34, 40, 49). Therefore, the purpose of the present study is to further explore the consequences of HG on musculoskeletal metabolism, particularly collagen catabolism and anabolism from mineralized and nonmineralized tissue structures. We hypothesize that chronic exposure to 2 G will impact the musculoskeletal system of nonhuman primates, resulting in alterations in extracellular matrix (ECM) and inorganic mineral urinary markers of musculoskeletal metabolism from baseline levels. Results from the present study will provide important physiological collagen biomarker data that can be compared with data from future ground-based or orbiting space experiments using unique human/animal habitats and other centrifugation hardware to investigate HG as a modality to maintain musculoskeletal integrity.

MATERIALS AND METHODS

Animal Care

Procedures were approved by the Institutional Animal Care and Use Committee of the University of California, Davis, in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

Experimental Design

A detailed description of the animal groups, centrifugation apparatus, and experimental protocol has been published by Barger (2) and Tavakol et al. (45). Briefly, six nonhuman primate rhesus monkeys (Macaca mulatta, ~3 yr 2 mo of age) were housed in individual animal enclosures located on a 4.5-m-diameter centrifuge. Tap water (0.12 ppm calcium; Davis, CA) was available ad libitum through a Lixit system. A nutritionally complete pelletized diet was provided throughout the study (5045 Monkey Tablet Diet, LabDiet, Richmond, IN). The pelletized diet contained 0.79% calcium. Twenty-four-hour urine specimen collections were obtained daily from individual monkeys via metabolic cages. The daily urine volumes (ml) were recorded, and samples were stored at −80°C. The study was divided into three phases, as shown in Fig. 1. Baseline control data were collected (basal 1 G, days 0–15); then the animals were exposed to chronic HG centrifugation for 14–15 days (chronic HG, days 16–30). Data collection continued throughout the postcentrifugation recovery period (recovery 1 G, days 31–51). The chronic HG period and the recovery 1-G period were further subdivided at or near the midpoints during the respective timelines into two subperiods: HG-1 wk (days 16–22) and HG-2 wk (days 23–30) and Rec-1 (days 31–41) and Rec-2 (days 42–51).

Biochemistry

Sample preparation. Urine aliquots were thawed, centrifuged, and filtered through a 0.45-μm nitrocellulose filter syringe (Whatman, Clifton, NJ) to clear the samples of debris. A small (1.0-ml) aliquot of urine was added to an equal volume (1.0 ml) of 12 M HCl to achieve a 6 M specimen solution. The acidified urine samples were sealed in 1-dram amber borosilicate screw-top vials with polytetrafluoroethylene-lined caps and hydrolyzed for 24 h at 110°C in the dark. Hydrolysates were subdivided into two samples: 1) an aliquot (200 μl) for the measurement of Hyp and 2) an aliquot for the evaluation of nonreducible collagen cross-links (HP and LP) by reverse-phase HPLC. The daily content of Hyp, HP, and LP for each monkey was multiplied by that monkey’s individual 24-h urine volume. The mean values for a given period (see Experimental Design) were determined from the average (n = 6) of all the daily values for that period.

Hyp determination. Amino acid analysis of Hyp was performed using dried hydrolysates of urine derivatized using Edman reagent (phenylisothiocyanate; Pierce Chemicals, Rockford, IL) according to the methods described by our laboratory (27). Dried samples were resuspended in 1.0 ml of double-distilled water (ddH2O), vortexed, and applied (950 μl) to a solid-phase extraction column (Oasis C18 Waters, Milford, MA). The samples were evaporated to dryness using a Speedvac (Savant Instruments, Farmingdale, NY). Dried samples were washed and dried twice with 20 μl of drying solution [2:2:1 (vol/vol/vol) methanol-ddH2O-triethylamine (TEA)] to neutralize excess HCl. An aliquot (20 μl) of freshly prepared derivatizing solution, methanol-ddH2O-TEA-phenylisothiocyanate [7:1:1:1 (vol/vol/vol/vol)] was added and mixed into the sample. The reaction continued for 10 min, and the excess derivatizing reagent was removed by vacuum. Hyp analysis was then executed using a column (10 cm × 4.6 mm; PicoTag, Waters) equilibrated at 38°C and eluted isocratically using 140 mM sodium acetate-TEA buffer in 6% acetonitrile at a flow rate of 1.0 ml/min. The peak for Hyp was monitored at 254 nm on an absorbance detector (450 UV/Vis, Waters) and compared with known Hyp standards (Sigma-Aldrich, St. Louis, MO). The retention time for Hyp was ~3.0 min, and the recovery of Hyp was 95%.

![Fig. 1. Experimental timeline. The study was divided into 3 loading regimens: basal 1-G period (days 0–15), chronic hypergravity (HG) period (days 16–30), and recovery 1-G period (days 31–51). Chronic HG period and recovery 1-G period were further subdivided near the midpoints during the respective timelines into 7-day (HG) and 10- to 11-day subperiods (Rec): days 16–22 (chronic HG-1 wk), days 23–30 (chronic HG-2 wk), days 31–41 (Rec-1), and days 42–51 (Rec-2).](image-url)
Nonreducible collagen cross-link analysis. An aliquot of the hydrolysate was used for extraction of the HP and LP cross-links by CF1 cellulose (Whatman) partition chromatography as previously performed by our laboratory. Processed acid hydrolysates containing the cross-link residues were filtered, dried, and redissolved in 0.01 M n-heptafluorobutyric acid (Pierce Chemicals) and loaded on a reverse-phase HPLC column (Spherisorb ODS2, Waters; 25 cm × 4.6 mm) equilibrated at 28°C. The collagen cross-links were eluted isocratically with 15% acetonitrile in 0.01 M heptafluorobutyric acid, monitored on a variable-wavelength fluorescence detector (model 470, Waters; 290-nm excitation and 395-nm emission), and compared with a known picomole amount of HP and LP standards (Metra Biosystems, Mountainview, CA). Sample recoveries for our system ranged from 93% to 97%.

Calcium atomic absorption. Urinary calcium content was determined by atomic absorption spectrophotometry (Perkin-Elmer). An aliquot (10 μl) of urine was diluted to 1 ml in lanthanum hydrochloride stabilizing solution (0.5% lanthanum oxide and 0.03 M HCl; Sigma Chemical, St. Louis, MO). Samples were analyzed in duplicate, quantified to a relative known calcium standard (Sigma Chemical), and read at 424 nm.

Creatinine measurements. Urinary creatinines were measured using a kit assay (Sigma Diagnostics, St. Louis, MO). Creatinine was measured on a 96-well plate reader (Dynatech MRS5000, Dynex, Chantilly, VA), monitored at an absorbance of 500 nm, and compared with a known amount of creatinine using a standard curve assay (R² = 0.998).

Statistical Analysis

A repeated-measures ANOVA (SAS Statistical Software, Cary, NC) was used to detect mean difference across phases of HG. Tukey’s multiple comparison post hoc test was used to examine differences between groups when the repeated-measures ANOVA was significant. Statistical significance was set at P < 0.05.

RESULTS

As expected, food consumption decreased at the onset of centrifugation (2, 45) (Fig. 2). Food consumption was compared between basal 1 G, HG-1 wk, HG-2 wk, Rec-1, and Rec-2. Average food intake during HG-1 wk was significantly lower than that during any other phase of the experiment (P < 0.05). The food pellet consumption returned to near-basal levels by day 4 of HG-1 wk. Despite the expected decrease in food intake at the onset of HG, there was no long-term decrease in calcium intake. There were no other significant differences in food consumption between or within study phases. Also, there were no significant differences in water consumption between animals and across phases of the experiment (2, 45).

Average body mass for the six animals was 4.67 ± 0.18 (SE) kg at the onset of chronic HG, 4.61 ± 0.16 kg at the end of chronic HG-2 wk, and 5.26 ± 0.04 kg at the end of Rec-1. Urine volumes were highest during the basal period [614 ± 209 (SE) ml], decreased during HG-1 wk (175 ± 17 ml), and steadily increased during HG-2 wk (256 ± 49 ml), Rec-1 (248 ± 31 ml), and Rec-2 (550 ± 113 ml).

During HG, our results showed a nonsignificant trend toward elevated urinary Hyp, an imino acid marker for total body collagen concentration: a 27% and a 48% increase in Hyp excretion during Rec-2 compared with HG-2 wk and Rec-1, respectively (Table 1).

Urinary HP and LP collagen cross-links were elevated from basal conditions (HP from 19% to 25%, LP from 27% to 33%) during the chronic HG-1 wk and HG-2 wk periods and then significantly increased (P < 0.05) during Rec-1 and Rec-2 (HP from 33% to 63%, LP from 43% to 72%; Fig. 3). Recovery to basal 1 G collagen biomarker (Hyp, HP, and LP) levels was not achieved during the 21-day recovery period.

Urinary creatinine measurements followed the same response pattern as the collagen biomarkers during the chronic HG perturbation (Table 1). Creatinine was elevated (50%) during Rec-2 compared with all other periods (P < 0.05).

In contrast to the previous measurements, urinary calcium levels measured by atomic absorption were highest during basal 1 G (Table 1). After the onset of the chronic HG perturbation, urinary calcium levels significantly (P < 0.05) decreased 61% during HG-1 wk and remained significantly lower (between −45% and −61%) than basal 1-G levels throughout HG-2 wk.

DISCUSSION

The present study was designed to determine whether HG contributes, at least in part, to changes in urinary collagen biomarkers. Our results suggest that HG influences whole body collagen metabolism by significantly increasing the urinary output of collagen metabolic markers for bone and other connective tissues during the post-HG recovery periods.

Collagen

Hyp is commonly used as a urinary marker to indicate the extent of degradation of connective tissue, including bone, muscle, and other tissues containing collagen and/or elastin. An increase in urinary Hyp excretion has been reported in astronauts after 84 days of spaceflight (42), and increases in urinary Hyp levels have also been reported in patients subjected to 7 days of bed rest (25). Increased levels of urinary Hyp suggest that microgravity and bed rest may cause an increase in bone resorption and resorption of collagen from other connective tissue sources (25, 42). Although Hyp is commonly used as a bone metabolism marker, it is not specific to bone (3, 25). Thus the increase in Hyp levels during HG may
be due to resorption in other tissues containing collagen, in addition to bone.

Although the increase in urinary Hyp content we report during the recovery phases following HG mimics elevated urinary Hyp outputs in microgravity studies, bone resorption may not be the only process occurring during HG. An increase in bone turnover resulting in net bone formation will also lead to elevated urinary Hyp levels (3, 5, 26, 39). In the present study, the levels of urinary Hyp were elevated during Rec-2, suggesting that resorption or ECM turnover was increased during the later stages of HG and through the recovery periods. In support of our study, urinary Hyp levels were increased by reduced ground reaction forces experienced by patients in bed-rest studies and remained elevated for a period after reambulation (25). It is our hypothesis that, during a decrease in load-bearing activity in the musculoskeletal system, such as immobilization and/or spaceflight, Hyp is derived from resorption from connective tissues (catabolism), whereas adaptation to a chronic HG perturbation, as shown in this HG study, may result in a delayed connective tissue loss followed by urinary Hyp excretion to a new level of basal homeostasis or, perhaps, new ECM formation (anabolism). Another hypothesis yet to be tested is that unbound Hyp could be oxidized and shunted through the citric acid cycle, thus potentially explaining the lack of differences in urinary Hyp measurements during HG. Evidence for “recycling” of Hyp has been shown in fasting northern elephant seals that were injected with [3H]Hyp, which disappeared as Hyp but reappeared as [3H]H2O (35).

### Collagen Cross-Links

Tissue contents of mature, nonreducible collagen cross-links are known to be physiological markers of mature collagen. HP and LP function to stabilize the molecular matrices of the collagen fibrils by increasing the tissue resistance to proteolytic resorption, decreasing the tissue’s solubility, and providing proper spacing of the bone collagen α-chains for normal physiological function (13, 32). HP and LP measurements in serum or urine are also useful as specific indicators of bone resorption. Increases in urinary HP and LP cross-links in 2-wk bed-rest studies have been shown to indicate bone resorption as a result of reduced ground reaction forces on long bones (23, 24, 25, 41). Long-term studies on astronauts in space have reported similar increases in urinary HP and LP urinary output, suggesting that microgravity enhances bone resorption (7, 41).

Exposure of the monkeys to HG in the present study resulted in elevated urinary Hyp levels (3, 5, 26, 39). In the present study, the levels of urinary Hyp were elevated during Rec-2, suggesting that resorption or ECM turnover was increased during the later stages of HG and through the recovery periods. In support of our study, urinary Hyp levels were increased by reduced ground reaction forces experienced by patients in bed-rest studies and remained elevated for a period after reambulation (25). It is our hypothesis that, during a decrease in load-bearing activity in the musculoskeletal system, such as immobilization and/or spaceflight, Hyp is derived from resorption from connective tissues (catabolism), whereas adaptation to a chronic HG perturbation, as shown in this HG study, may result in a delayed connective tissue loss followed by urinary Hyp excretion to a new level of basal homeostasis or, perhaps, new ECM formation (anabolism). Another hypothesis yet to be tested is that unbound Hyp could be oxidized and shunted through the citric acid cycle, thus potentially explaining the lack of differences in urinary Hyp measurements during HG. Evidence for “recycling” of Hyp has been shown in fasting northern elephant seals that were injected with [3H]Hyp, which disappeared as Hyp but reappeared as [3H]H2O (35).
from the HG environment, levels of urinary HP and LP will increase and may result in connective tissue resorption and formation during 1-G recovery. It has been shown that new collagen formation can occur in an HG environment; this finding is supported by in vitro studies. An increase in the accumulation of new collagen in response to 72 h of HG has been demonstrated in osteoblast MC3T3-E1 cells (37). These results suggest that hyperphysiological loads may accelerate collagen stabilization through the conversion of immature reducible cross-links to mature pyridinium compounds (37). Other studies of the effect of 2 wk of HG on cortical bone maturation in the growing rat, performed by our laboratory, showed an increase in middiaphyseal content of total collagen cross-links (HP and LP) (28). In future research, the relationship between collagen posttranslational modifications and increasing magnitudes of ground reaction forces should be analyzed simultaneously in bone, dense fibrous connective tissues, plasma, and urine to understand the putative mechanisms of formation and resorption of the cross-link biomarkers, especially during HG and recovery.

**Inorganic Mineral**

During normal mechanical loading, calcium levels are precisely regulated, and any excess calcium is excreted, leaving enough calcium to sustain bone mass (19). Several studies have shown that, during ground reaction force deprivation, such as in microgravity, bed rest, or immobilization, mammals experience hypercalciuria and bone resorption, resulting in osteopenia (9, 10, 24, 44, 51). Atomic absorption measurements of calcium showed a significant decrease in urinary calcium content during HG compared with basal levels, suggesting that HG has a pronounced effect on urinary calcium output. Our results support a previous study of the whole body calcium response to HG, in which beagle dogs exposed to 1.5–2.5 G for 3 mo showed a consistent decrease in serum calcium levels compared with noncentrifuged controls (33). It is well known that parathyroid hormone and calcitonin regulate systemic calcium levels in the body. There is little information on the effect of HG on calcium-regulating hormones, which could possibly modulate steady-state calcium levels in the urine. HG (2 G) for 60 days was shown to augment parathyroid gland secretory activity in Mongolian gerbils, suggesting that an increase in parathyroid hormone release may be necessary to preserve serum calcium levels for new bone formation (38). In contrast to HG, urinary calcium levels were higher in human patients subjected to long-term bed rest (i.e., 17 wk of limb unloading) than in non-bed-rest controls (24). We hypothesize that decreased levels of urinary calcium during HG indicate that the skeletal system is sequestering calcium to strengthen existing bone and to provide a positive calcium balance for the formation of new bone to withstand the added loading, consistent with Wolff’s law (48). New bone formation could be envisioned and would comply with Wolff’s law of bone remodeling due to the presence of added stresses placed on the skeleton during HG. Cervical spine bone mineral densities were significantly increased in Royal Australian Air Force fighter pilots completing 8 mo of training compared with age- and weight-matched control subjects, suggesting that an increase in bone mass was possibly due to HG-induced loading on the skeleton during extended training (31). Thirty days of HG (8 G) resulted in altered femoral bone geometries in young mice due to the abnormally large forces placed on the skeleton (18). These changes are similar to regional and length variations in rat femurs that were exposed to 2 G for 2 wk (28), suggesting that cortical bone can indeed remodel and adapt to varying levels of HG.

**Creatinine**

Urinary creatinine measurements were elevated during the recovery period. Elevated urinary creatinine levels can be caused by many physiological mechanisms, including renal dysfunction, but have also been attributed to the remodeling of skeletal muscles during muscle atrophy and muscle fiber turnover. The fiber type of the soleus muscles from the nonhuman primates measured after HG (recovery) demonstrated a shift in the percentage of soleus fibers expressing a fast fiber type (type I and II myosin heavy chains), while overall soleus fiber size and muscle wet weights were maintained (45). The soleus shift to “faster” myosin heavy chain during HG is opposite to the response that would be expected during chronic loading and, paradoxically, resembles muscles exhibiting atrophy caused by reduced loading during spaceflight or hindlimb unloading (12, 36). Overall nonhuman primate activity levels during HG, measured by EMG telemetry of the contralateral limbs, were 35% less than basal activity measurements and could contribute, in part, to the skeletal muscle disuse (unloading) and the creatinine levels measured in the urine. It is unknown whether longer bouts or greater magnitudes of HG would elicit similar elevated urinary creatinine responses in this animal model.

**Limitations**

A limitation of this study is the absence of serum samples from the nonhuman primates, which could have potentially provided some bone formation markers, such as osteocalcin, a late marker of osteoblast differentiation and bone mineralization. Although we could not obtain data on bone formation markers, it has been reported that HG induces a stimulatory effect on bone formation (22, 29, 37). At the cellular level, osteoblast-like cells respond variably to the magnitude of HG (2–5 G) in vitro by increasing osteoblast proliferation (2 G) and decreasing cell differentiation (5 G) (17, 29).

Our present results suggest a temporal effect of HG on musculoskeletal metabolism represented by adaptive changes in urinary collagen biomarkers and urinary calcium levels. We have shown that noninvasive collagen biomarker measurements from urine are useful indicators of whole body ECM adaptations to altered gravitational forces. However, it is unclear whether chronic exposure to HG is beneficial or detrimental to whole body musculoskeletal connective tissue. Further study is needed to test the efficacy of HG as a countermeasure against bone loss associated with long-term spaceflight.

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