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Weightlessness during spaceflight leads to functional changes in resistance arteries and loss of cancellous bone, which may be potentiated by radiation exposure. The purpose of this study was to assess the effects of hindlimb unloading (HU) and total-body irradiation (TBI) on the vasomotor responses of skeletal muscle arteries. Male C57BL/6 mice were assigned to control, HU (13–16 days), TBI (1 Gy ^{56}Fe, 600 MeV, 10 cGy/min) and HU-TBI groups. Gastrocnemius muscle feed arteries were isolated for *in vitro* study. Endothelium-dependent (acetylcholine) and -independent (Dea-NONOate) vasodilator and vasoconstrictor (KCl, phenylephrine and myogenic) responses were evaluated. Arterial endothelial nitric oxide synthase (eNOS), superoxide dismutase-1 (SOD-1) and xanthine oxidase (XO) protein content and tibial cancellous bone microarchitecture were quantified. Endothelium-dependent and -independent vasodilator responses were impaired in all groups relative to control, and acetylcholine-induced vasodilation was lower in the HU-TBI group relative to that in the HU and TBI groups. Reductions in endothelium-dependent vasodilation correlated with a lower cancellous bone volume fraction. Nitric oxide synthase inhibition abolished all group differences in endothelium-dependent vasodilation. HU and HU-TBI resulted in decreases in eNOS protein levels, while TBI and HU-TBI produced lower SOD-1 and higher XO protein content. Vasoconstrictor responses were not altered. Reductions in NO bioavailability (eNOS), lower anti-oxidant capacity (SOD-1) and higher pro-oxidant capacity (XO) may contribute to the deficits in NOS signaling in skeletal muscle resistance arteries. These findings suggest that the combination of insults experienced in spaceflight leads to impairment of vasodilator function in resistance arteries that is mediated through deficits in NOS signaling. © 2016 by Radiation Research Society

INTRODUCTION

More than 500 humans have flown into space since 1961, when Yuri Gagarin became the first man to orbit the Earth. Through their journeys, it has become apparent that space exploration comes with health risks, including cardiovascular deconditioning (*1–4*) and persistent bone loss (*5, 6*). Following long-duration spaceflight more than 80% of astronauts experience orthostatic intolerance (*3*) due to an inability to adequately raise total peripheral resistance (*1, 4*), thus implicating the resistance vasculature as a site of dysfunction. Because skeletal muscle is a major contributor to elevations in peripheral vascular resistance during standing (*7*), the skeletal muscle resistance vasculature is important for understanding microgravity-induced cardiovascular deconditioning.

Hindlimb unloading (HU) of rodents (*8, 9*) has proven to be an effective model for simulating microgravity, inducing alterations similar to those occurring in astronauts, including orthostatic hypotension (*10, 11*), reduced aerobic capacity (*9, 12, 13*), a cephalic fluid shift (*14–17*), a diminished capacity to elevate peripheral resistance (*18, 19*) and bone loss (*20, 21*). Previous assessment of vasomotor function revealed impaired vasoconstriction of gastrocnemius muscle resistance arteries from hindlimb-unloaded rats (*22*) and space-flown mice (*23*). Further, resistance arteries isolated from the soleus muscle of HU rats and gastrocnemius muscle of HU mice demonstrate impaired endotheli-

um-dependent vasodilation (*21, 24–27*) concomitant with decreased expression of endothelial nitric oxide synthase (eNOS) (*21, 24–26*) and superoxide dismutase-1 (SOD-1) (*24*).

The risks of space travel are not limited to those induced by microgravity alone. During interplanetary space travel, astronauts will be exposed to ionizing radiation from multiple sources: galactic cosmic rays, solar particle events and trapped radiation belts. Space radiation predominantly consists of high-energy, charged particles such as protons, α particles and heavier ions referred to as high (H) atomic number (Z) and energy (HZE) ions, among which protons are the most abundant and HZE ^{56}Fe ions are the most damaging (*28*). In spite of shielding, during spaceflight a typical cell in the body will be hit by an electron daily, a proton once every three days and a HZE ion once every few months (*29, 30*).

Vascular endothelial cells are especially sensitive to the effects of radiation, undergoing apoptosis (*29, 31*), reductions in the production and release of nitric oxide (NO) (*32–34*) and morphological changes (*33*). One consequence of radiation-induced endothelial dysfunction is impaired endothelium-dependent vasodilation, associated with diminished NO bioavailability in large conduit arteries (*33–39*) and resistance arteries (*21, 32*), enhanced superoxide (O_2^-) production (*32, 34*), particularly from xanthine oxidase (XO) (*34, 40*) and a down-regulation of eNOS (*33, 35*).

HZE ions are particularly relevant because they contribute to a large percentage of the absorbed dose of radiation in space (*41, 42*) and double-strand, complex DNA damage that is difficult for DNA-repair machinery to resolve (*29, 43*). Vascular endothelial cells are especially sensitive to HZE ions (*44*) and are more susceptible to apoptosis than cells exposed to an equal dose of proton radiation (*45*). Although the singular effects of HU and radiation on endothelium-dependent vasodilation have previously been reported, only one study has examined the combined effects of hindlimb unloading and low-linear energy transfer (LET) gamma radiation on arterial vasodilator function (*21*), but this study did not examine the space-relevant HZE ions. Therefore, the primary purpose of the current study was to examine the combined effects of hindlimb unloading and high-LET radiation on the vasodilator and vasoconstrictor properties of skeletal muscle resistance arteries. We hypothesized that hindlimb unloading and high-LET radiation would independently impair the vasomotor properties of resistance arteries, and that the combined effects of hindlimb unloading and HZE ions would potentiate this dysfunction. In addition, since impaired endothelium-dependent vasodilation has been previously shown to be related to bone loss in HU rats (*46*), a secondary purpose was to the determine the effects of hindlimb unloading and high-LET radiation on cancellous bone volume fraction, and to determine whether there is a relationship between endothelium-dependent vasodilation

and bone microarchitectural changes. We hypothesized that the magnitude of the impairment of endothelium-dependent vasodilation elicited through hindlimb unloading and HZE ions alone and in combination would be directly related to the magnitude of the loss of trabecular bone volume fraction.

MATERIALS AND METHODS

All experimental procedures were approved by the Institutional Animal Care and Use Committee at the National Aeronautics and Space Administration (NASA) and Brookhaven National Laboratory (BNL), conforming to the U.S. National Institutes of Health (NIH) *Guide for the Care and Use of Laboratory Animals* (Eighth edition, 2011).

Animals

Eighty-three male C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME), 16 weeks of age, were individually housed at the Brookhaven National Laboratory animal facility. Animals were maintained in a controlled environment (12:12 h light-dark cycle, 24 ± 2°C) and provided food and water *ad libitum*. Mice were randomized by body mass to one of four groups: control ($n = 23$), HU ($n = 19$), TBI ($n = 21$) and combined HU-TBI ($n = 20$).

Hindlimb Unloading

Mice were hindlimb unloaded via tail traction for 13–16 days until the time of sacrifice according to the methods of Morey-Holton *et al.* as previously described (*8, 20, 21*). Control mice were individually housed in their normal cage environment.

Whole-Body Irradiation

Mice were exposed to a single dose of radiation consisting of 1 Gy of ^{56}Fe ions (600 MeV/nucleon, LET 150 keV/μm in water) at a dose rate of 10 cGy/min at the NASA Space Radiation Laboratory beamline at Brookhaven National Laboratory, NY. Irradiation of HU-TBI mice took place 3 days after the initiation of hindlimb unloading. The control group of mice were handled in an identical fashion but were not irradiated (0 Gy).

Isolated Microvessel Studies

Mice were anesthetized with isoflurane (5%/O₂) and euthanized by excision of the heart. Gastrocnemius muscle feed arteries were isolated, cannulated and prepared for *in vitro* experimentation as previously described (*21*).

Evaluation of Vasomotor Properties

In the first set of studies, vasodilator responses of feed arteries to the endothelium-dependent vasodilator acetylcholine (ACh, 10^{-9} – 10^{-4} *M*) and endothelium-independent vasodilator Dea-NONOate (10^{-9} – 10^{-4} *M*) were assessed as previously described (*21*). ACh-induced vasodilation was also evaluated after incubation with nitric oxide synthase (NOS) inhibitor N^G-nitro-l-arginine methyl ester (L-NAME, 10^{-5} M) and following incubation with the combination of L-NAME with cyclooxygenase (COX) inhibitor indomethacin (10^{-5} M) as previously reported (*21*). Maximal diameter and medial wall thickness were determined after a 1-hr incubation period in calcium-free physiological saline buffer solution (PSS) with 10^{-4} M sodium nitroprusside (SNP) to allow complete smooth muscle cell relaxation.

In a second set of studies, vasoconstrictor responses were assessed through nonreceptor (KCl, 10–100 m*M*), myogenic (increasing

TABLE 1
Tissue and Vessel Characteristics

Tissue	Control	HU	TBI	HU-TBI
Body mass (BM) (g)	27 ± 1	27 ± 1	28 ± 1	27 ± 1
Gastrocnemius mass (mg)	219 ± 5	184 ± 9*	204 ± 8‡	158 ± 7*†
Gastrocnemius-to-BM ratio (mg/g)	7.4 ± 0.3	6.8 ± 0.3*	7.4 ± 0.2‡	5.9 ± 0.3*†
Bone volume fraction (%)	22.8 ± 0.7	21.4 ± 1.0	19.5 ± 1.0*	17.2 ± 1.4*‡
Trabecular thickness (μm)	62.8 ± 0.6	59.6 ± 0.9*	63.3 ± 0.9	56.9 ± 0.9*‡†
Trabecular separation (μm)	155 ± 4	155 ± 4	173 ± 5*‡	173 ± 7*‡
Trabecular number (1/mm)	3.6 ± 0.1	3.6 ± 0.1	3.1 ± 0.1*‡	3.0 ± 0.2*‡
Spontaneous tone (%)	28 ± 2	27 ± 3	28 ± 3	25 ± 3
Maximal diameter (μm)	182 ± 3	168 ± 4*	186 ± 3‡	175 ± 3*†
Media wall thickness (μm)	18 ± 1	17 ± 1	18 ± 1	18 ± 1

Notes. Values are mean ± SE. *Significantly different from control group, $P < 0.05$.
‡ Significantly different from HU group, $P < 0.05$.
† Significantly different from TBI group, $P < 0.05$.

intraluminal pressure from 0 cm H_2O up to 140 cm H_2O, in 20 cm H_2O increments) and receptor [α_1-adrenoreceptor agonist phenylephrine (PE, $10^{-9} - 10^{-4}$ M)] mechanisms as previously described (*23*). Passive pressure-diameter responses were recorded in a similar fashion to that of the active myogenic response after vessels were incubated in calcium-free PSS with 10^{-4} M SNP for 1 h.

Immunoblot Analysis

eNOS, SOD-1, XO and Rho-associated kinase (ROCK) protein content in gastrocnemius feed arteries were assessed via immunoblot analysis. Arteries were isolated, individually snap frozen and processed as previously described (*47*). Primary antibody dilutions were as follows: eNOS (1:150, BD Transduction, cat. no. 610296), Cu/Zn SOD-1 (1:8000, Enzo Life Sciences ADI-SOD-100-F), XO (1:1,000, Abcam, cat no. 109235), ROCK (1:2,000, Millipore, cat no. 07-1458) and β-actin (1:500 or 1:4,000, Cell Signaling, cat no. 4967). Differences in loading were normalized by expressing all data as relative densitometry units of the protein of interest versus β-actin.

Cancellous Microarchitecture of the Proximal Tibia Metaphysis

Tibiae were collected for 3D structural analyses by microcomputed tomography (SkyScan 1174, Bruker-MicroCT, Kontich, Belgium; 6.7 μm pixel size) and imaged in saline with a 3,500 ms integration time using X-rays generated by a 50 kV- and 800 μA-powered source. Images were binarized with a global threshold and the bone volume fraction (%), trabecular thickness (μm), separation (μm) and number (1/mm) were measured in the region 0.2–1.2 mm distal to the proximal metaphysis using semi-autonomous selection of the cancellous tissue (*20, 21*).

Statistical Analysis

One-way ANOVA and Fisher LSD *post-hoc* tests were used to detect differences in body and tissue masses, gastrocnemius muscle feed artery spontaneous tone, maximal diameter, medial wall thickness, protein content, maximal vasodilator responses and trabecular properties across conditions. Vasomotor responses were evaluated using repeated-measures ANOVAs to detect differences among experimental groups and drug doses or pressure changes. To examine the relationship among peak endothelium-dependent vasodilation or gastrocnemius muscle mass and trabecular bone volume, linear regression analyses were performed. All values are presented as means ± SE. A value of $P \leq 0.05$ was considered statistically significant.

RESULTS

Animal and Vessel Characteristics

Total-body mass was not different among groups. However, HU alone and the combined HU-TBI groups resulted in an 18–28% lower gastrocnemius muscle mass compared to control and TBI groups (Table 1). Similarly, the gastrocnemius-to-body mass ratio was lower by 8–20% in the HU and HU-TBI groups compared to control and TBI groups.

Media wall thickness and spontaneous tone development in gastrocnemius feed arteries were not different among groups (Table 1). Maximal intraluminal diameter, however, was lower in HU and HU-TBI groups relative to that in control and TBI mice (Table 1).

Vasodilator Responses

ACh-mediated endothelium-dependent vasodilation of gastrocnemius feed arteries was impaired in all treatment groups relative to the control group (Fig. 1). There was a significant ACh dose by group interaction between the control and HU groups, resulting in lower vasodilator responses at $10^{-8} - 10^{-4}$ M ACh in arteries from HU mice. In the control and HU-TBI groups there was also a significant ACh dose by group interaction, resulting in lower vasodilator responses between $10^{-9} - 10^{-4}$ M ACh in arteries from HU-TBI animals. There was not a significant dose by group interaction between the control and TBI groups, but the maximal vasodilator response to ACh was lower in the animals whose arteries received total-body irradiation relative to that in the control animal's arteries. There was also a significant ACh dose by group interaction between the HU and HU-TBI groups (significantly different at 10^{-8} M and $10^{-6} - 10^{-4}$ M ACh) and the TBI and HU-TBI groups (significantly different at $10^{-9} - 10^{-4}$ M ACh). L-NAME reduced ACh-mediated vasodilation in all groups and abolished treatment-associated differences among groups (Fig. 2A). The combined NOS and COX inhibition
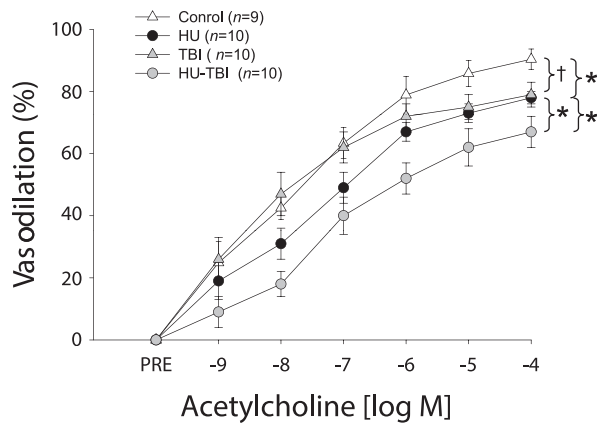
FIG. 1. Effects of hindlimb unloading (HU) and total-body irradiation (TBI), individually and combined (HU-TBI) on ACh-mediated vasodilator responses in the gastrocnemius muscle feed arteries. Values are mean $\pm$ SE. n = the number of animals studied. *Denotes significant dose by group interaction between groups, $P <$ 0.05; HU and HU-TBI responses are different from that of control (Con), and HU and TBI responses are different from that of HU-TBI group. †Denotes significant difference in the maximal vasodilator response between control and TBI groups, $P < 0.05$.

further decreased vasodilator responses with no differences among groups (Fig. 2B).

There was a significant dose by group interaction for endothelium-independent vasodilation for all treatment groups relative to control (Fig. 3). Differences were significant between control and HU arteries at $10^{-7} - 10^{-5}$ M Dea-NONOate, at $10^{-7} - 10^{-4}$ M Dea-NONOate between control and TBI arteries, and at $10^{-7} - 10^{-5}$ M Dea-NONOate between control and HU-TBI arteries. There was also a significant Dea-NONOate dose by group interaction between the HU and TBI groups (significantly different at $10^{-7} - 10^{-4}$ M Dea-NONOate) and the HU and HU-TBI groups (significantly different at $10^{-7} - 10^{-5}$ M Dea-NONOate).

Vasoconstrictor and Pressure Responses

KCl (Fig. 4A) and PE (Fig. 4B) produced dose-dependent increases in arterial vasoconstriction in all groups; there were no differences in vasoconstriction among groups. Both active myogenic vasoconstriction (Fig. 5A) and passive pressure-diameter responses (Fig. 5B) were not different among groups.

Protein Expression

There were no differences in ROCK protein content among groups (Fig. 4C). However, eNOS protein levels were lower in gastrocnemius feed arteries from the HU and HU-TBI groups compared to controls (Fig. 6). Radiation exposure alone did not alter eNOS protein levels. SOD-1 protein content was lower in TBI and HU-TBI vessels when compared to those from both control and HU mice, but unaltered by HU alone (Fig. 7A). TBI and HU-TBI vessels expressed higher XO levels than those in control (Fig. 7B).
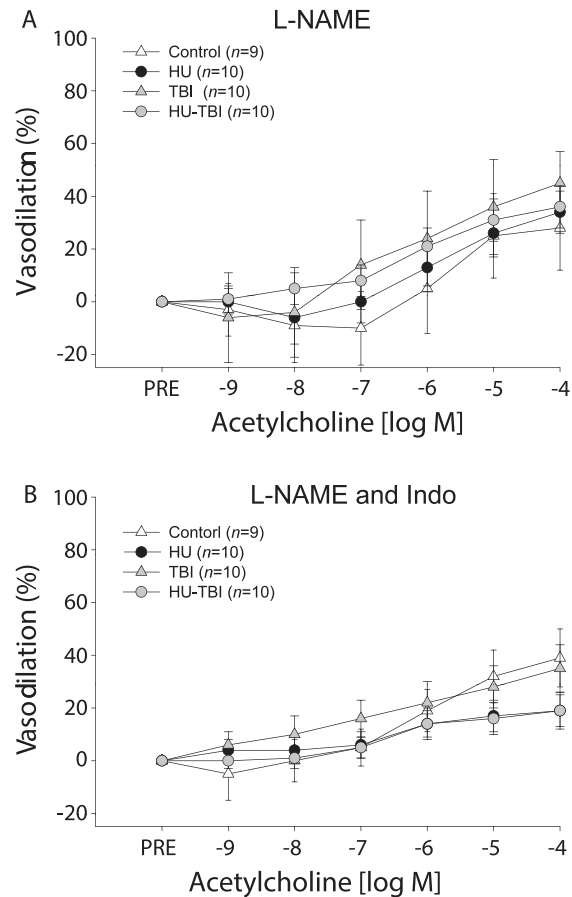

FIG. 2. Effects of HU, TBI and HU-TBI on ACh-mediated vasodilator responses in the presence of (panel A) the NOS inhibitor L-NAME, and (panel B) L-NAME and the COX inhibitor indomethacin (Indo), in gastrocnemius muscle feed arteries. Values are mean $\pm$ SE. n = the number of animals studied.

Cancellous Bone Microarchitecture

In the proximal tibial metaphysis, hindlimb unloading reduced trabecular thickness (Tb.Th) relative to that in control mice (Table 1), though bone volume fraction (BV/TV) and trabecular separation (Tb.Sp) and number (Tb.N) were not different from control animals. Radiation exposure reduced BV/TV and Tb.N and increased Tb.Sp, but did not alter Tb.Th (Table 1). The combination of HU-TBI diminished BV/TV, Tb.Th and Tb.N and increased Tb.Sp relative to that in control and HU mice (Table 1). Tb.Th was also lower in HU-TBI compared to that in the TBI group. There was a significant correlation between BV/TV and peak endothelium-dependent vasodilation (Fig. 8A) and between BV/TV and gastrocnemius muscle mass (Fig. 8B).

DISCUSSION

The purpose of this study was to determine whether space-relevant radiation and musculoskeletal unloading, alone and in combination, alter vasomotor responses of gastrocnemius feed arteries in mice. The results demonstrate
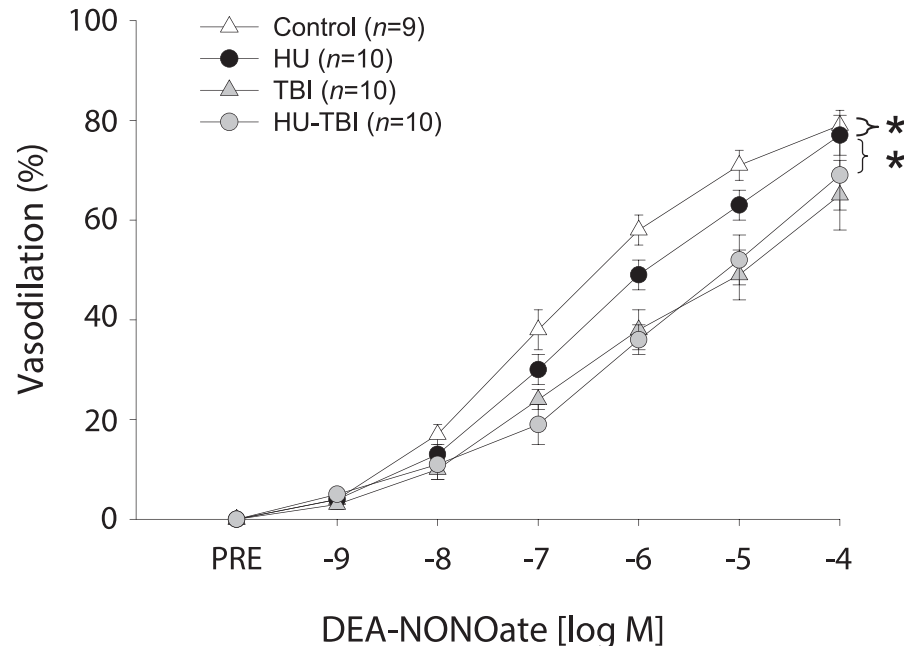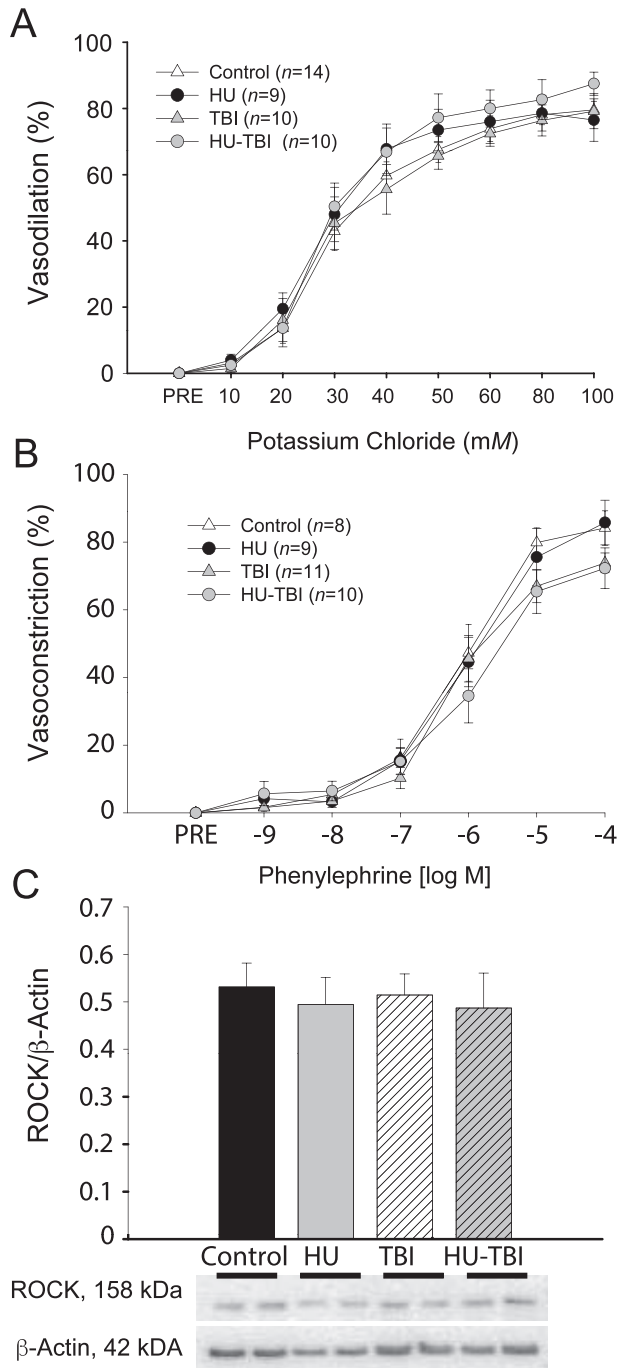
FIG. 3. Effects of HU, TBI and HU-TBI on Dea-NONOate-induced vasodilator responses in gastrocnemius muscle feed arteries. Values are mean ± SE. n = the number of animals studied. *Denotes significant dose by group interaction among groups, $P < 0.05$; HU, TBI and HU-TBI responses are different from that of control, and TBI and HU-TBI responses are different from that of HU group.

that HZE ion radiation exposure and hindlimb unloading primarily impair skeletal muscle artery vasodilation. Each treatment alone impaired endothelium-dependent vasodilation, with a potentiated effect from the combination of treatments (Fig. 1). Endothelial dysfunction occurred primarily through the NOS signaling pathway (Fig. 2A), the apparent consequence of reduced eNOS protein content with hindlimb unloading (Fig. 6), and diminished anti-oxidant capacity (Fig. 7A) and greater pro-oxidant capacity (Fig. 7B) with exposure to radiation. Vascular smooth muscle vasodilator responsiveness to exogenous NO also was diminished in all treatment groups (Fig. 3) with greater decrements evident in the two irradiated groups. Vasoconstriction mediated through voltage-gated Ca^{2+} channels (Fig. 4A), α_1-adrenergic receptors (Fig. 4B), and myogenic responses (Fig. 5A) were unaltered by HU, TBI and HU-TBI. Hindlimb unloading and HZE ion irradiation also did not alter the passive mechanical behavior of the feed arteries (Fig. 5B), although maximal intraluminal diameter was smaller in the two groups involving musculoskeletal unloading (Table 1).

Previous work has shown that small arteries and capillaries are especially radio-sensitive (44, 48) due largely to the vulnerability of vascular endothelial cells to ionizing radiation (31, 44). Endothelial cells show signs of dysfunction within days of low-LET irradiation (38, 48). In the current study, impairment of endothelium-dependent vasodilation 10–13 days after exposure to HZE ions was due to alterations in the NOS signaling pathway, as demonstrated by the elimination of group differences following NOS inhibition (Fig. 2A). NO bioavailability could be reduced through several mechanisms, including (1.) lower NO output through reduced production and/or release and (2.) greater NO scavenging. Data from the current study point more towards this latter effect in regard to radiation. Radiation exposure increased XO and reduced SOD-1 protein levels, thus signifying greater vascular O_2^- production with a diminished capacity to neutralize O_2^-. Previous work has shown that both gamma and HZE ion irradiation decreases NO and increases ROS production in rat aortas (34) and submucosal resistance arterioles (32). Additionally, incubation of vessels with a SOD-mimetic restored NO and significantly reduced ROS production (32). A similar effect was observed in large conduit arteries incubated with an XO inhibitor (34), further implicating excess O_2^- production from XO as a contributor to reduced vascular NO bioavailability after irradiation.
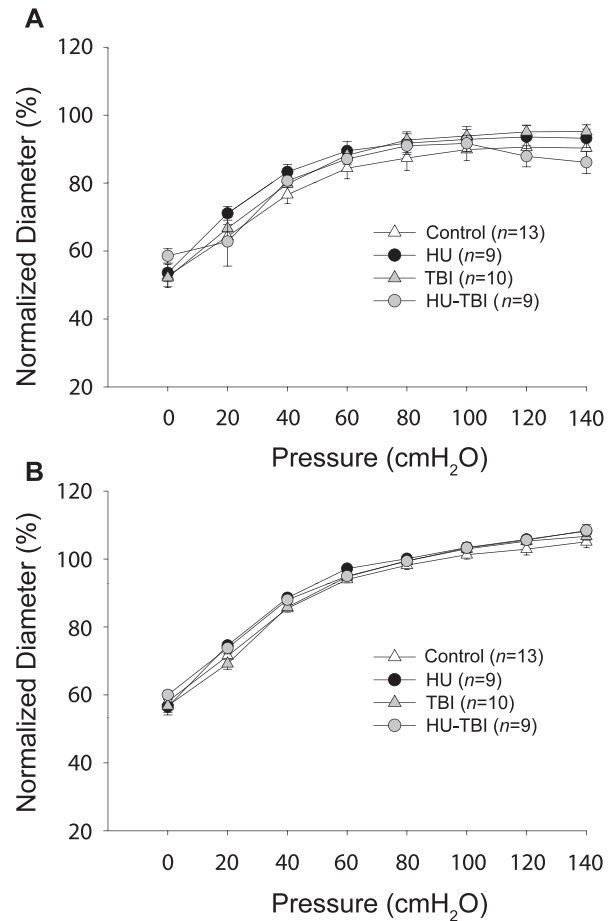
Vasodilator responses to Dea-NONOate, a direct NO donor, were also depressed postirradiation (Fig. 3). The impaired radiation-induced endothelium-independent vasodilation may likewise be a consequence of increased ROS production due to the densely ionizing quality of HZE ions, which induce higher levels of ROS than do gamma or X rays (32, 49, 50). For instance, thoracic aortas isolated from atherosclerotic rabbits display poor SNP-induced vasodilation and high levels of O_2^- (51). In the current study, greater XO and lower SOD-1 protein content after irradiation provide evidence of potentially greater ROS levels within the smooth muscle layer of gastrocnemius arteries.

FIG. 4. Effects of HU, TBI and HU-TBI on vasoconstrictor responses to (panel A) KCl and (panel B) phenylephrine and (panel C) Rho-associated kinase (ROCK) protein content in gastrocnemius muscle feed arteries. Values are mean ± SE. n = the number of animals studied. Vasoconstrictor responses and ROCK protein levels were not different among groups.


FIG. 5. Effects of HU, TBI and HU-TBI on (panel A) active myogenic vasoconstrictor responses and (panel B) passive pressure-diameter responses of gastrocnemius muscle feed arteries. Values are mean ± SE. n = the number of animals studied. Responses were not different among groups.

Consequently, scavenging of exogenous NO by ROS could reduce NO levels and impair smooth muscle relaxation.

As with radiation exposure, hindlimb unloading also diminished vasodilator responses elicited by ACh and Dea-NONOate. However, in contrast to radiation exposure, low eNOS protein levels suggest that reduced NO production rather than NO scavenging primarily contributes to a lower NO bioavailability and impairment of endothelium-dependent dilation with unloading. Hindlimb unloading has been previously reported to diminish endothelium-dependent vasodilation and eNOS mRNA and protein expression in rat soleus muscle resistance arteries (26, 27, 52), and this effect has been attributed to chronic reductions in blood flow and shear stress through the arteries of these unloaded muscles (18). Indeed, chronic decreases in blood flow result in a distinctive structural remodeling of arteries to reduce maximal diameter (26, 27, 52, 53). The smaller maximal diameter of the gastrocnemius muscle feed arteries in the HU and HU-TBI mice (Table 1) infer a chronic decrease in blood flow through the unloaded gastrocnemius muscle as the stimulus for the reductions in arterial eNOS protein levels (Fig. 6).

Studies examining the combined effects of simulated weightlessness and radiation exposure are exceedingly few (20, 21, 54–58), perhaps because the health risk associated with the potential interaction of microgravity and space radiation exposure during interplanetary space missions has
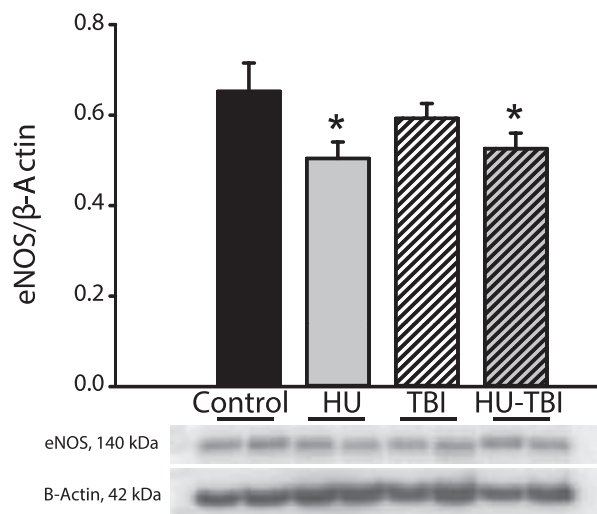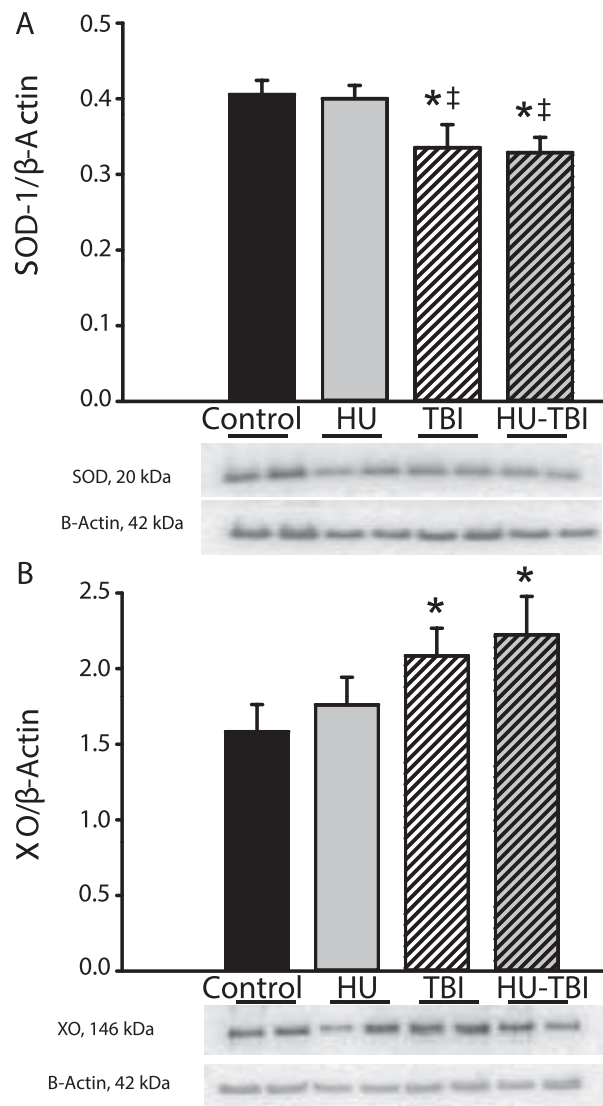
FIG. 6. Effects of HU, TBI and HU-TBI on endothelial nitric oxide synthase (eNOS) protein content in gastrocnemius muscle feed arteries. Values are mean $\pm$ SE. *Denotes significant difference from control group, $P < 0.05$.

been considered minimal (*59*). Results from our current study demonstrate that unloading and a single dose of high-LET radiation (1 Gy of ^{56}Fe ions) produce potentiated effects on bone (i.e., reduced trabecular thickness, Table 1) and endothelium-dependent vasodilation (Fig. 1). Previous work examining the combined effects of unloading and a single dose of low-LET radiation (2 Gy of ^{137}Cs gamma rays) demonstrated additive effects on bone loss, but not on the impairment of endothelium-dependent vasodilation (*21*). In addition, while hindlimb unloading and the low-LET irradiation impaired the NOS signaling pathway in gastrocnemius feed arteries, there was an apparent up-regulation of the COX-PGI$_2$ vasodilator pathway that served to partially compensate for the reduced NOS signaling (*21*). Such a compensatory adaptation was not apparent in the current study. Thus, the potentiated effect of HZE ion exposure with hindlimb unloading to further exacerbate the degree to which endothelium-dependent vasodilator dysfunction occurs, as well as the lack of any compensatory adaptations, suggests that the potential interaction of microgravity and space radiation to elevate astronaut health risk for circulatory diseases may be higher than initially estimated (*60*).

Emerging research indicates that the vascular system, and in particular molecules derived from vascular endothelial cells, can function to modulate bone remodeling activity. Endothelial cells in the bone vasculature can release molecules such as NO and PGI$_2$ that could serve to couple the vascular system to bone cell remodeling activity, as well as regulate skeletal blood flow and, correspondingly, bone interstitial fluid flow and pressure (*61*). Because unloading-induced decrements in endothelium-dependent vasodilation of the principal nutrient artery of the femur from HU rats (*46*) are similar to those of the gastrocnemius muscle feed artery from HU mice (*21*), we used the gastrocnemius

FIG. 7. Effects of HU, TBI and HU-TBI on (panel A) superoxide dismutase (SOD-1) and (panel B) xanthine oxidase (XO) protein levels in gastrocnemius muscle feed arteries. Values are mean $\pm$ SE. *Denotes significant difference from control group; ‡denotes significant difference from HU group, $P < 0.05$.

muscle feed artery as a surrogate for the bone vasculature to determine whether there is a relation between changes in endothelium-dependent vasodilation and cancellous bone microarchitecture in a simulated space environment. Regression analysis indicates a significant relationship between cancellous bone volume fraction and peak endothelium-dependent vasodilation (Fig. 8A). These data indicate that the effects of musculoskeletal unloading and irradiation alone and in combination may not only impact vascular health, but may also contribute to the bone loss associated with spaceflight. Other factors are also considered important determinants of cancellous bone microarchitecture, including the level of muscle force exerted on bone. The reduced gastrocnemius muscle mass and the presumed lower muscle-associated mechanical loads to the

A

R² = 0.4838
y = 4.591 + 0.198x
P<0.05

B

R² = 0.1635
y = 6.394 + 0.091x
P<0.05
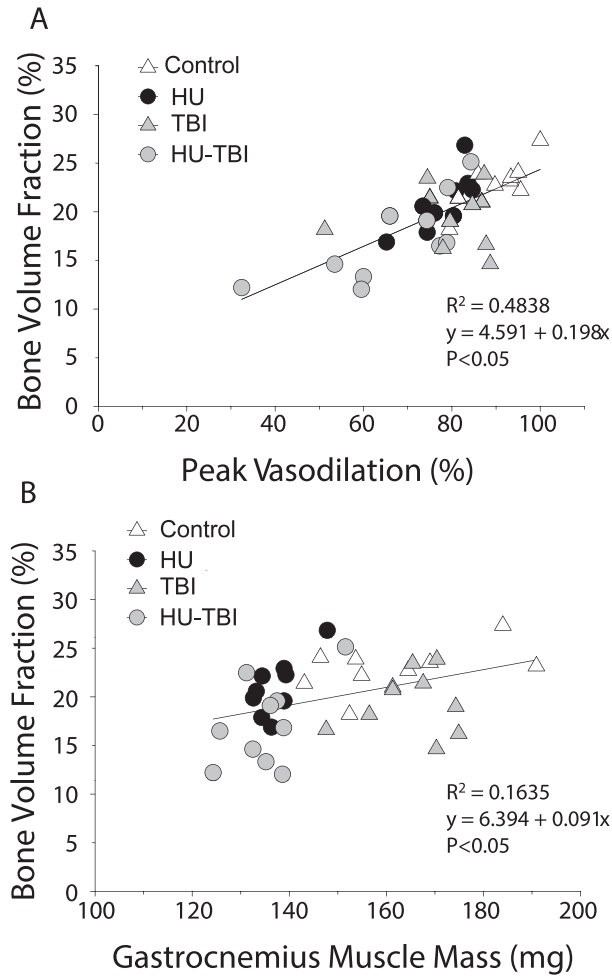
FIG. 8. The relationship between the percentage trabecular bone volume fraction in the proximal tibia metaphysis and (panel A) peak percentage endothelium-dependent vasodilation of gastrocnemius muscle feed arteries and (panel B) gastrocnemius muscle mass, from control, HU, TBI and HU-TBI mice.

tibia were significantly related to cancellous bone volume fraction (Fig. 8B), although the strength of the relationship was not as great as with endothelium-dependent vasodilation.

Coinciding with previous studies (33, 35, 36), radiation exposure did not altered gastrocnemius feed artery vasoconstrictor properties (Figs. 4A and B and 5A), suggesting that the functional responsiveness of smooth muscle cells remains intact. Contractile responses were also not affected by hindlimb unloading. In contrast, previous studies report that vasoconstrictor responses of gastrocnemius muscle resistance arteries (22) and abdominal aorta (62) are impaired in HU rats. This contractile deficit in HU rats has been attributed to a thinning of the medial wall of the artery as a result of reductions in transmural pressure concomitant with an unloading-induced cephalad fluid shift (27), as well as reductions in ROCK activity (62). The absence of contractile dysfunction in the current study is consistent with the

notion that hindlimb unloading of mice fails to generate a significant reduction in transmural pressure within the hindlimb vasculature to evoke a remodeling of arterial media wall thickness (Table 1) or alter ROCK protein content in the artery (Fig. 4C).

In summary, the purpose of this study was to test the hypothesis that space-relevant radiation and simulated weightlessness impair vasodilator and vasoconstrictor properties of skeletal muscle resistance arteries, with greater dysfunction resulting from the combination of these experimental conditions. Neither unloading nor high-LET radiation individually or in combination had any effect on arterial contractile responses or the passive pressure-diameter relationship. However, hindlimb unloading and HZE ion irradiation impaired endothelium-dependent vasodilation through the NOS signaling pathway. Endothelium-dependent vasodilator dysfunction associated with unloading appears to be due to a lower production and release of NO, as indicated by reduced eNOS protein levels. In contrast, the radiation associated endothelial dysfunction appears to be mediated primarily through greater NO scavenging by ROS, as evidenced by higher XO protein levels and lower SOD-1 protein content. The increased presence of ROS may also account for the reduction in the vasodilator response of smooth muscle cells to exogenous NO. The combination of hindlimb unloading and radiation exposure further impairs endothelium-dependent vasodilation, negatively impacting eNOS, XO and SOD-1 protein levels. The impact of HZE ions appears to have a more profoundly adverse effect on endothelial function than that mediated by low-LET radiation, given that the compensatory upregulation of the COX vasodilator signaling pathway that occurred after treatment with 2 Gy low-LET radiation (21) did not occur in the current study with 1 Gy high-LET irradiation. Impairment of endothelium-dependent vasodilation mediated by unloading, radiation and their combined effects was also strongly associated with changes in cancellous bone microarchitecture. Finally, the findings of a potentiated effect of unloading and radiation on vascular function and bone microarchitecture suggests that the interactions of these conditions may create greater risk for adverse cardiovascular and skeletal health effects in astronauts than previously estimated.

REFERENCES

1. Buckey JC, Lane LD, Levine BD, Watenpaugh DE, Wright SJ, Moore WE, et al. Orthostatic intolerance after spaceflight. J Appl Physiol 1996; 81:7–18.

2. Bungo MW, Johnson PC, Jr. Cardiovascular examinations and observations of deconditioning during the space shuttle orbital flight test program. Aviat Space Environ Med 1983; 54:1001–04.

3. Meck JV, Reyes CJ, Perez SA, Goldberger AL, Ziegler MG. Marked exacerbation of orthostatic intolerance after long- vs. short-duration spaceflight in veteran astronauts. Psychosom Med 2001; 63:865–73.

4. Mulvagh SL, Charles JB, Riddle JM, Rehbein TL, Bungo MW. Echocardiographic evaluation of the cardiovascular effects of short-duration spaceflight. J Clin Pharmacol 1991; 31:1024–6.

5. LeBlanc A, Schneider V, Shackelford L, West S, Oganov V, Bakulin A, et al. Bone mineral and lean tissue loss after long duration space flight. J Musculoskelet Neuronal Interact 2000; 1:157–60.

6. Lang TF, Leblanc AD, Evans HJ, Lu Y. Adaptation of the proximal femur to skeletal reloading after long-duration spaceflight. J Bone Miner Res 2006; 21:1224–30.

7. Rowell LB, Human cardiovascular control. 1st ed. Oxford University Press; 1993.

8. Morey-Holton ER, Globus RK. Hindlimb unloading rodent model: technical aspects. J Appl Physiol 2002; 92:1367–77.

9. Powers J, Bernstein D. The mouse as a model of cardiovascular adaptations to microgravity. J Appl Physiol 2004; 97:1686–92.

10. Martel E, Champeroux P, Lacolley P, Richard S, Safar M, Cuche JL. Central hypervolemia in the conscious rat: a model of cardiovascular deconditioning. J Appl Physiol 1996; 80:1390–6.

11. Wilkerson MK, Lesniewski LA, Golding EM, Bryan RM, Amin A, Wilson E, et al. Simulated microgravity enhances cerebral artery vasoconstriction and vascular resistance through endothelial nitric oxide mechanism. Am J Physiol Heart Circ Physiol 2005; 288:H1652–61.

12. Desplanches D, Mayet MH, Semporé B, Frutoso J, Flandrois R. Effect of spontaneous recovery or retraining after hindlimb suspension on aerobic capacity. J Appl Physiol 1987; 63:1739–43.

13. Overton JM, Woodman CR, Tipton CM. Effect of hindlimb suspension on VO2 max and regional blood flow responses to exercise. J Appl Physiol 1989;66:653–9.

14. Provost SB, Tucker BJ. Effect of 14 day head-down tilt on renal function and vascular and extracellular fluid volumes in the conscious rat. Physiologist 1992; 35:S105–6.

15. Maurel D, Ixart G, Barbanel G, Mekaouche M, Assenmacher I. Effects of acute tilt from orthostatic to head-down antiorthostatic restraint and of sustained restraint on the intra-cerebroventricular pressure in rats. Brain Res 1996; 736:165–73.

16. Hargens AR, Steskal J, Johansson C, Tipton CM. Tissue fluid shift, forelimb loading, and tail tension in tail-suspended rats. Physiologist 1984; 27:S37–8.

17. Colleran PN, Wilkerson MK, Bloomfield SA, Suva LJ, Turner RT, Delp MD. Alterations in skeletal perfusion with simulated microgravity: a possible mechanism for bone remodeling. J Appl Physiol 2000; 89:1046–54.

18. McDonald KS, Delp MD, Fitts RH. Effect of hindlimb unweighting on tissue blood flow in the rat. J Appl Physiol 1992; 72:2210–8.

19. Woodman CR, Sebastian LA, Tipton CM. Influence of simulated microgravity on cardiac output and blood flow distribution during exercise. J Appl Physiol 1995; 79:1762–8.

20. Alwood JS, Yumoto K, Mojarrab R, Limoli CL, Almeida EA, Searby ND, et al. Heavy ion irradiation and unloading effects on mouse lumbar vertebral microarchitecture, mechanical properties and tissue stresses. Bone 2010; 47:248–55.

21. Prisby R, Alwood J, B B, Stabley J, McCullough D, Ghosh P, et al. Effects of hindlimb unloading and ionizing radiation on skeletal muscle resistance artery vasodilation in mice. J Appl Physiol 2016; 120:97–106.

22. Delp MD. Myogenic and vasoconstrictor responsiveness of skeletal muscle arterioles is diminished by hindlimb unloading. J Appl Physiol 1999; 86:1178–84.

23. Stabley JN, Dominguez JM 2nd, Dominguez CE, Mora Solis FR, Ahlgren J, Behnke BJ, et al. Spaceflight reduces vasoconstrictor responsiveness of skeletal muscle resistance arteries in mice. J Appl Physiol 2012; 113:1439–45.

24. Woodman CR, Schrage WG, Rush JW, Ray CA, Price EM, Hasser EM, et al. Hindlimb unweighting decreases endothelium-dependent dilation and eNOS expression in soleus not gastrocnemius. J Appl Physiol 2001; 91:1091–8.

25. Schrage WG, Woodman CR, Laughlin MH. Hindlimb unweighting alters endothelium-dependent vasodilation and ecNOS expression in soleus arterioles. J Appl Physiol 2000; 89:1483–90.

26. Jasperse JL, Woodman CR, Price EM, Hasser EM, Laughlin MH. Hindlimb unweighting decreases ecNOS gene expression and endothelium-dependent dilation in rat soleus feed arteries. J Appl Physiol 1999; 87:1476–82.

27. Delp MD, Colleran PN, Wilkerson MK, McCurdy MR, Muller-Delp J. Structural and functional remodeling of skeletal muscle microvasculature is induced by simulated microgravity. Am J Physiol Heart Circ Physiol 2000; 278:H1866–73.

28. Stephens DL, Townsend LW, Hoff JL. Interplanetary crew dose estimates for worst case solar particle events based on historical data for the Carrington flare of 1859. Acta Astronaut 2005; 56:969–74.

29. Cucinotta FA, Durante M. Cancer risk from exposure to galactic cosmic rays: implications for space exploration by human beings. Lancet Oncol 2006; 7:431–5.

30. Durante M, Cucinotta FA. Heavy ion carcinogenesis and human space exploration. Nat Rev Cancer 2008; 8:465–72.

31. Langley RE, Bump EA, Quartuccio SG, Medeiros D, Braunhut SJ. Radiation-induced apoptosis in microvascular endothelial cells. Br J Cancer 1997; 75:666–72.

32. Hatoum OA, Otterson MF, Kopelman D, Miura H, Sukhotnik I, Larsen BT, et al. Radiation induces endothelial dysfunction in murine intestinal arterioles via enhanced production of reactive oxygen species. Arterioscler Thromb Vasc Biol 2006; 26:287–94.

33. Qi F, Sugihara T, Hattori Y, Yamamoto Y, Kanno M, Abe K. Functional and morphological damage of endothelium in rabbit ear artery following irradiation with cobalt60. Br J Pharmacol 1998; 123:653–60.

34. Soucy KG, Lim HK, Kim JH, Oh Y, Attarzadeh DO, Sevinc B, et al. HZE 56Fe-ion irradiation induces endothelial dysfunction in rat aorta: role of xanthine oxidase. Radiat Res 2011; 176:474–85.

35. Sugihara T, Hattori Y, Yamamoto Y, Qi F, Ichikawa R, Sato A, et al. Preferential impairment of nitric oxide-mediated endothelium-dependent relaxation in human cervical arteries after irradiation. Circulation 1999; 100:635–41.

36. Soloviev AI, Tishkin SM, Parshikov AV, Ivanova IV, Goncharov EV, Gurney AM. Mechanisms of endothelial dysfunction after ionized radiation: selective impairment of the nitric oxide component of endothelium-dependent vasodilation. Br J Pharmacol 2003; 138:837–44.

37. On YK, Kim HS, Kim SY, Chae IH, Oh BH, Lee MM, et al. Vitamin C prevents radiation-induced endothelium-dependent vasomotor dysfunction and de-endothelialization by inhibiting

oxidative damage in the rat. Clin Exp Pharmacol Physiol 2001; 28:816–21.

38. Menendez JC, Casanova D, Amado JA, Salas E, García-Unzueta MT, Fernandez F, et al. Effects of radiation on endothelial function. Int J Radiat Oncol Biol Phys 1998; 41:905–13.

39. Beckman JA, Thakore A, Kalinowski BH, Harris JR, Creager MA. Radiation therapy impairs endothelium-dependent vasodilation in humans. J Am Coll Cardiol 2001; 37:761–5.

40. Soucy KG, Lim HK, Benjo A, Santhanam L, Ryoo S, Shoukas AA, et al. Single exposure gamma-irradiation amplifies xanthine oxidase activity and induces endothelial dysfunction in rat aorta. Radiat Environ Biophys 2007; 46:179–86.

41. Nelson GA. Radiation in microgravity. In: Fregly, MJ, Blatteis, CM, editors. Handbook of Physiology. Sec. 4. Adaptation to the Environment. Oxford University Press, New York; 1995. pp. 785–798.

42. Durante M, Cucinotta F. Physical basis of radiation in space travel. Rev modern physics 2011; 83:1245–81.

43. Belli M, Sapora O, Tabocchini MA. Molecular targets in cellular response to ionizing radiation and implications in space radiation protection. J Radiat Res 2002; 43:S13–9.

44. Fajardo LF, Berthrong M. Vascular lesions following radiation. Pathol Annu 1988; 23 Pt 1:297–330.

45. Grabham P, Hu B, Sharma P, Geard C, Effects of ionizing radiation on three-dimensional human vessel models: differential effects according to radiation quality and cellular development. Radiat Res 2011; 175:21–8.

46. Prisby RD, Behnke BJ, Allen MR, Delp MD. Effects of skeletal unloading on the vasomotor properties of the rat femur principal nutrient artery. J Appl Physiol 2015; 118:980–88.

47. Spier SA, Delp MD, Meininger CJ, Donato AJ, Ramsey MW, Muller-Delp JM. Effects of ageing and exercise training on endothelium-dependent vasodilatation and structure of rat skeletal muscle arterioles. J Physiol 2004; 556:947–58.

48. Roth NM, Sontag MR, Kiani MF. Early effects of ionizing radiation on the microvascular networks in normal tissue. Radiat Res 1999; 151:270–7.

49. Datta K, Suman S, Kallakury BV, Fornace AJ. Exposure to heavy ion radiation induces persistent oxidative stress in mouse intestine. PLoS One 2012; 7:e42224.

50. Limoli CL, Giedzinski E, Baure J, Rola R, Fike JR. Redox changes induced in hippocampal precursor cells by heavy ion irradiation. Radiat Environ Biophys 2007; 46:167–72.

51. Miller FJ, Gutterman DD, Rios CD, Heistad DD, Davidson BL. Superoxide production in vascular smooth muscle contributes to oxidative stress and impaired relaxation in atherosclerosis. Circ Res 1998; 82:1298–305.

52. McCurdy MR, Colleran PN, Muller-Delp J, Delp MD. Effects of fiber composition and hindlimb unloading on the vasodilator properties of skeletal muscle arterioles. J Appl Physiol 2000; 89:398–405.

53. Langille BL, O'Donnell F. Reductions in arterial diameter produced by chronic decreases in blood flow are endothelium-dependent. Science 1986; 231:405–7.

54. Li M, Holmes V, Zhou Y, Ni H, Sanzari JK, Kennedy AR, et al. Hindlimb suspension and SPE-like radiation impairs clearance of bacterial infections. PLoS One 2014; 9:e85665.

55. Zhou Y, Ni H, Li M, Sanzari JK, Diffenderfer ES, Lin L, et al. Effect of solar particle event radiation and hindlimb suspension on gastrointestinal tract bacterial translocation and immune activation. PLoS One 2012; 7:e44329.

56. Xu D, Zhao X, Li Y, Ji Y, Zhang J, Wang J, et al. The combined effects of X-ray radiation and hindlimb suspension on bone loss. J Radiat Res 2014; 55:720–5.

57. Kondo H, Yumoto K, Alwood JS, Mojarrab R, Wang A, Almeida EA, et al. Oxidative stress and gamma radiation-induced cancellous bone loss with musculoskeletal disuse. J Appl Physiol 2010; 108:152–61.

58. Lloyd SA, Bandstra ER, Willey JS, Riffle SE, Tirado-Lee L, Nelson GA, et al. Effect of proton irradiation followed by hindlimb unloading on bone in mature mice: a model of long-duration spaceflight. Bone 2012; 51:756–64.

59. Radiation hazards to crews of interplanetary missions: Biological issues and research strategies. Task group on the biological effects of space radiation, space studies board, commission on physical sciences mathematics, and application, National Research Council. National Academies, Washington, D.C., 1996. (bit.ly/1OLRiMO)

60. Cucinotta FA, Kim MH, Chappell LJ, Huff JL. How safe is safe enough? Radiation risk for a human mission to Mars. PLoS One 2013; 8:e74988.

61. Parfitt AM. The mechanism of coupling: a role for the vasculature. Bone 2000; 26:319–23.

62. Summers SM, Nguyen SV, Purdy RE. Hindlimb unweighting induces changes in the RhoA-Rho-kinase pathway of the rat abdominal aorta. Vascul Pharmacol 2008; 48, 208–14.