Divergent Modification of Low-Dose ^{56}Fe -Particle and Proton Radiation on Skeletal Muscle

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Shtifman, A., Pezone, M. J., Sasi, S. P., Agarwal, A., Gee, H., Song, J., Pereplechikov, A., Yan, X., Kishore, R., and Goukassian, D. A. Divergent Modification of Low-Dose ^{56}Fe -Particle and Proton Radiation on Skeletal Muscle. *Radiat. Res.* 180, 455–464 (2013).

It is unknown whether loss of skeletal muscle mass and function experienced by astronauts during space flight could be augmented by ionizing radiation (IR), such as low-dose high-charge and energy (HZE) particles or low-dose high-energy proton radiation. In the current study adult mice were irradiated whole-body with either a single dose of 15 cGy of 1 GeV/n ^{56}Fe -particle or with a 90 cGy proton of 1 GeV/n proton particles. Both ionizing radiation types caused alterations in the skeletal muscle cytoplasmic Ca^{2+} ($[\text{Ca}^{2+}]_i$) homeostasis. ^{56}Fe -particle irradiation also caused a reduction of depolarization-evoked Ca^{2+} release from the sarcoplasmic reticulum (SR). The increase in the $[\text{Ca}^{2+}]_i$ was detected as early as 24 h after ^{56}Fe -particle irradiation, while effects of proton irradiation were only evident at 72 h. In both instances $[\text{Ca}^{2+}]_i$ returned to baseline at day 7 after irradiation. All ^{56}Fe -particle irradiated samples revealed a significant number of centrally localized nuclei, a histologic manifestation of regenerating muscle, 7 days after irradiation. Neither unirradiated control or proton-irradiated samples exhibited such a phenotype. Protein analysis revealed significant increase in the phosphorylation of Akt, Erk1/2 and rpS6k on day 7 in ^{56}Fe -particle irradiated skeletal muscle, but not proton or unirradiated skeletal muscle, suggesting activation of pro-survival signaling. Our findings suggest that a single low-dose ^{56}Fe -particle or proton exposure is sufficient to affect Ca^{2+} homeostasis in skeletal muscle. However, only ^{56}Fe -particle irradiation led to the appearance of central nuclei and activation of pro-survival pathways, suggesting an ongoing muscle damage/recovery process. © 2013 by Radiation Research Society

INTRODUCTION

Low-dose ionizing radiation, such as that experienced by astronauts in space, could potentially augment the progressive loss of muscle mass and alterations in muscle function initially mediated by limited musculoskeletal use during exploration-type space missions (1, 2). While there is a significant amount of data demonstrating that limited musculoskeletal use is a key contributor to development of musculoskeletal functional deficits (1–9), the effects of low-dose ionizing radiation such as ^{56}Fe particles and protons, on skeletal muscle functional processes are largely unknown (10, 11). Although results of earlier histological investigations suggested that ionizing-radiation-induced skeletal muscle tissue alterations do not significantly impair skeletal muscle function, more recent studies demonstrate that certain functional aspects of adult skeletal muscle tissue stem cells (satellite cells), which are responsible for regeneration and repair of damaged skeletal muscle, are negatively affected by clinical radio-therapeutic regimens (≥ 2 Gy single and 40–60 Gy cumulative), years and decades (5–30 years) after cancer radiotherapy (12–14).

Two major sources of ionizing radiation in space include the galactic cosmic rays (GCR) and the solar particle event (SPE). One of the concerns for manned interplanetary missions is that within a few hours or days, a large SPE could subject the spacecraft and its crew to a radiation dose that is potentially equivalent to a year-long GCR exposure (15, 16). However, unlike SPEs, GCR present as a low-dose background exposure 24 h a day every day and consist of particles that range in energy from ~ 10 to $\geq 1,000$ MeV/nucleon, with fluence peak frequencies around 300–700 MeV/nucleon (15). Because of the wide energy range of GCR, effective shielding from this type of radiation is not be feasible. Therefore, GCR particles could provide a steady source of low-dose-rate radiation and this accumulated exposure might be a limiting factor for long-term exploration-type space missions. It has been determined that 99% of GCR are composed of protons and helium, while only 1% of GCR are composed of ions heavier than helium (17, 18). These heavier than helium ions are referred to as high-charge (Z) and high-energy (E) particles (HZE). During the

Editor's note. The online version of this article (DOI:10.1667/RR3329.1) contains supplementary information that is available to all authorized users.

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future prolonged space missions, such as those to the Moon, near Earth asteroids and Mars, approximately 40% of the dose-equivalent exposure is predicted to be from HZE particles, with iron (^{56}Fe) particles alone constituting approximately 13% of the total (19, 20). Thus, it is feasible that HZE radiation-induced skeletal muscle tissue damage may produce considerable radiobiological responses (e.g., DNA damage and repair, inflammation, changes in cellular Ca^{2+} handling and activation of intracellular signal transduction pathways, etc.) during exploration-type space missions.

Disruption of normal skeletal muscle function is frequently characterized by aberrant intracellular Ca^{2+} handling that in some instances may occur well before the appearance of histopathological and clinical symptoms (21, 22). There are two main aspects of skeletal muscle Ca^{2+} handling that may be affected by space radiation. The first aspect is the basal cytoplasmic $[\text{Ca}^{2+}]$ ($[\text{Ca}^{2+}]_i$) which is maintained by ion channels and ATP-driven Ca^{2+} pumps on the surface of the cell, as well as channels and pumps on the membrane of intracellular Ca^{2+} storing organelles, such as the sarcoplasmic reticulum (23). Alterations in $[\text{Ca}^{2+}]_i$ can have long term deleterious effects on muscle contractility and numerous other intracellular processes. The second aspect involves the process of excitation-contraction coupling. In this process, a rapid cascade of events is initiated by an action potential that activates the L-type Ca^{2+} channels, the dihydropyridine receptors (DHPRs) in the transverse tubules (TT). Activated DHPRs rapidly trigger massive release of Ca^{2+} from the sarcoplasmic reticulum by Ca^{2+} release channels, the ryanodine receptors (RyRs) that reside in the junctional region of the sarcoplasmic reticulum immediately adjacent to the TT membrane (23). The resulting release of Ca^{2+} produces a transient increase in intracellular $[\text{Ca}^{2+}]_i$, which activates the contractile apparatus of muscle fibers. Ca^{2+} is sequestered back into sarcoplasmic reticulum by sarcoplasmic reticulum Ca^{2+} ATPase (SERCA) and cleared out of the cells by plasmalemmal Ca^{2+} APTases (PMCA) and to a lesser extent by Na/Ca^{2+} exchanger (NCX) (24, 25). Alterations in each of the components of excitation-contraction coupling could result either in reduction of Ca^{2+} release that would lead to reduced activation of contractile machinery or to elevated $[\text{Ca}^{2+}]_i$, which can affect numerous downstream processes, including but not limited to, regulation of gene expression, regeneration, mitochondrial function, apoptosis and necrosis.

It remains to be elucidated whether low-dose HZE space radiation can induce adverse effect in skeletal muscle that could directly influence the functional state of the skeletal muscle tissue. Therefore, we hypothesized that space radiation-induced responses in skeletal muscle are radiation type (proton vs. iron) and time dependent. The goal of the current study was to determine whether a single, whole-body radiation exposure to either 15 cGy of 1 GeV/n ^{56}Fe particles as a model for GCR or 90 cGy of 1 GeV/n low-

dose high-energy protons as a model for SPE may lead to alterations in $[\text{Ca}^{2+}]_i$ and stimulation of sarcoplasmic reticulum Ca^{2+} release in murine skeletal muscle, as well as to determine whether these doses may induce detectable changes in the skeletal muscle.

METHODS

Animals, Muscle Fiber Preparation

Adult 8–10-month-old male C57Bl/6N mice were shipped directly from Taconic (Hudson, NY) to Brookhaven National Laboratory (BNL). Mice were fed standard laboratory chow diet (Harlan Teklad), were given water access *ad libitum* and kept in a temperature- and light-controlled environment (12 h light/dark cycles). All protocols were approved by both, the Steward St. Elizabeth's Medical Center and BNL Institutional Animal Care and Use Committees. Animals were housed at BNL and at Steward St. Elizabeth's Medical Center animal facility and were individually caged as these mice were retired male breeders that often fight and induce skin lesions.

Irradiation and Dosimetry

Both, low-LET proton (^1H) and high-LET iron (^{56}Fe) exposures were performed at Brookhaven National Laboratory in the NASA Space Radiation Laboratory according to standardized procedures. Before whole-body irradiation, the animals were placed individually into rectangular polystyrene boxes with multiple air holes (4 mm in diameter). Particle radiation (^1H and ^{56}Fe) was delivered at the entrance plateau region of the beam (20×20 cm field), at the beginning of the Bragg peak, such that linear energy transfer (LET) levels were held constant throughout the target volume. All radiation was delivered to yield a cumulative 90 cGy dose for ^1H -ion (at the average dose rate 30 ± 5 cGy/min) and 15 cGy dose for ^{56}Fe -ion (at the average dose rate 5 ± 0.5 cGy/min). Both, ^1H - and ^{56}Fe -particle irradiations were delivered at the energy of 1,000 MeV/nucleon. The irradiated mice were immediately driven to Steward St. Elizabeth's Medical Center in Boston (3–4 h) for housing and analysis.

Gamma radiation experiments were performed at Steward St. Elizabeth's Medical Center using a Cesium (^{137}Cs) source irradiator that delivered an average dose rate of 46.6 cGy/min. Mice were placed in special polypropylene boxes ($3.25 \times 4.25 \times 2.25$ inches) with 4 mm holes drilled to produce a stress-free environment. All animals used for this study were handled the same way and were fully conscious prior to and post whole-body irradiation. The duration of restraint in plastic boxes were standardized across all radiation exposures and the total time in the boxes was no more than 3–4 min.

Skeletal Muscle Sample Collection and Myofiber Isolation

Mice were euthanized by pentobarbital overdose at respective time points of 1 h for gamma radiation (1 Gy) studies and at 24, 48, 72 h and day 7 for ^{56}Fe particle (15 cGy, 1 GeV/n) and protons (90 cGy, 1 GeV/n) studies. *Flexor digitorum brevis* muscles were removed and placed in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) containing 2 mg/ml collagenase A (Roche, Nutley, NJ) and incubated for 3 h at 37°C . Thereafter, muscles were removed from the enzyme-containing buffer and rinsed twice in Dulbecco's modified Eagle's medium. Muscle bundles were then transferred to DMEM supplemented with 10% bovine serum, 1% penicillin, 1% streptomycin and 1% glutamine and gently triturated with a polished glass pipette until a significant portion was dissociated to single cells. Myofibers were plated onto extracellular matrix (ECM) coated (Sigma, St. Louis, MO) glass-bottom dishes (MatTek, Ashland, MA) and allowed to settle to the bottom of the dish overnight in an incubator at 5% CO_2 and 37°C .

FIG. 1. Gamma radiation increases resting $[Ca^{2+}]_i$ within 1 h. Analysis of relative resting cytoplasmic $[Ca^{2+}]_i$ in dissociated muscle fibers from control and γ -irradiated mice. Cells were prepared 1 h and 7 days after irradiation (* $P < 0.05$; n -mice_{IR} = 3, n -mice_{control} = 3).

Fluorescence Recordings

All reagents, unless otherwise indicated, were purchased from Sigma-Aldrich (St. Louis, MO). Cells were bathed in a normal Ringer solution containing in [mM]: [125] NaCl, [5] KCl, [1.2] $MgSO_4$, [6] glucose, [25] HEPES, [2] $CaCl_2$, pH 7.4. For resting $[Ca^{2+}]_i$ measurements, cells were loaded with Fura-2AM (5 μM for 30 min). Relative resting $[Ca^{2+}]_i$ was assessed by ratio of 340 nm/380 nm emissions of the dye. For stimulated Ca^{2+} release measurements, myofibers were loaded at room temperature for 30 min in Ringer solution supplemented with Ca^{2+} indicator dye magFluo-4AM, 5 μM , (Molecular Probes, Eugene OR). Cells were later washed several times with Ringer solution to terminate further loading and placed in a 37°C incubator for de-esterification of the dye. To eliminate the motion artifacts due to muscle contraction, N-benzyl-p-toluene sulphonamide (BTS, 50 μM), an inhibitor of the myosin II ATPase, was added to the bathing solution. Whole cell fluorescence changes were detected using an IonOptix fluorescence system (IonOptix, Milton, MA) interfaced with an inverted Zeiss Axiovert 200 microscope equipped with a Neofluar 40× oil immersion objective. Changes in fluorescence were detected by a photo multiplier tube (PMT) recording signal at 5 kHz. Ca^{2+} transients were elicited by applying supra-threshold square wave pulses (1 ms duration) through two platinum electrodes placed on opposite sides of the experimental chamber. Changes in intracellular $[Ca^{2+}]_i$ were characterized as changes in fluorescence intensity of the dye. All experiments were conducted at room temperature (22°C). Detected changes in fluorescence within each cell were analyzed using IonOptix imaging software (IonOptix, Milton, MA). The resulting fluorescence changes were corrected for the background fluorescence within individual cells by dividing the magnitude of change in fluorescence (ΔF) by the mean baseline fluorescence intensity (F_0) to give the $\Delta F/F_0$ values.

Histology, Imaging and Analysis

Adductor magnus and gluteus maximus from mouse hind limbs were carefully bisected, cross sectioned through the middle and fixed in formalin. Sections (6 μm) were stained with H&E and visualized using a light microscope (Leica Microsystems GmbH, Wetzlar, Germany). Images of the full circumference of the cross sections of the above mentioned muscles were taken at 5×, 10×, 20× and 40× magnifications (Nikon Instruments, Inc., Melville, NY). Samples were coded and a single pathologist blinded to the treatment conditions evaluated muscle tissue in at least 4–5 animals of the control mice,

proton- and ^{56}Fe -particle-irradiated mice. In addition, two laboratory members were also blinded to treatment conditions independently of each other counted centrally located nuclei by delineating individual muscle fibers in 20× magnification images and identifying number of central nuclei/muscle fiber (NIH, ImageJ software; <http://rsbweb.nih.gov/ij/>). Images of each stained muscle sample were taken at 20× magnification across the board and the average number of muscle fibers per visual field was ~85 individual fibers. Results were represented as mean number of central nuclei/muscle fiber and differences were considered significant at $P < 0.05$.

Western Blot Analysis

Half of bisected adductor magnus and gluteus maximus muscles ($N = 3$ /treatment group) were snap frozen in liquid nitrogen immediately after collection. A small portion (10–15 mg) of the muscle tissue was homogenized using tissue homogenizer in RIPA lysis buffer (Fisher Scientific, Pittsburg, PA) to prepare whole tissue lysates. Samples containing 50 μg of total protein from the whole-muscle tissue lysates were mixed with equal volume of 2× sample buffer and boiled for 5 min at 95°C. Protein fractions were then separated by electrophoresis on a 10% polyacrylamide gel (Bio-Rad) and blotted onto PVDF membrane (Bio-Rad). Detection of total (T) and phosphorylated (p) Akt1 (also known as Protein Kinase B – PKB, a serine/threonine-specific kinase), Erk1/2 (Extracellular-signal-Regulated Kinase 1/2, also known as classical Mitogen-Activated Protein Kinase – MAPK) and rpS6k (ribosomal protein S6 kinase, an effector kinase downstream to mTORC1 – mammalian target of rapamycin C1) protein levels were performed using 1:1,000 dilution of rabbit monoclonal antibody against phospho-specific and total-specific mouse antibodies (p-Akt, cat. no. 9275S, T-Akt, cat. no. 4685S; p-Erk1/2, cat. no. 9101S, T-Erk1/2, cat. no. 9102S; p-rpS6k, cat. no. 2215S, T-rpS6k, cat. no. 2217S, all Cell Signaling Technology Inc., Danvers, MA). As secondary antibody, HRP-linked goat anti-rabbit IgG at 1:1000 dilution was used (cat. no. 7074S Cell Signaling Technology Inc., Danvers, MA). Gapdh (cat. no. MAB374; 1:40,000 dilution Millipore, Billerica, MA) expression was used to adjust the protein loading. As secondary antibody, HRP-linked anti-mouse IgG at 1:1,000 dilution was used (cat. no. 7076S, Cell Signaling Technology Inc.). Protein levels were revealed using enhanced chemiluminescence Western blotting method (ECL, Fisher Scientific).

Statistical Analysis

All results were expressed as mean $\pm$ SEM and plots were obtained. Statistical analysis was performed on the data by one-way ANOVA (Prism5 Software, GraphPad Software Inc., La Jolla, CA and Stat View Software, SAS Institute Inc., Gary, NC). Differences were considered significant at $P < 0.05$.

RESULTS

Effects of γ Radiation on Skeletal Muscle

We sought to determine whether exposure of mice to low-dose γ -radiation produces alterations in skeletal muscle Ca^{2+} homeostasis. We compared basal myoplasmic $[Ca^{2+}]_i$ in Flexor digitorum brevis muscle fibers isolated from 1 Gy whole-body γ -irradiated and nonirradiated animals using Fura-2 fluorescence emission ratios. Muscle fibers from mice that were sacrificed 1-h postirradiation exhibited higher Fura-2 fluorescence ratios compared to the fibers from the nonirradiated animals, suggesting an increased myoplasmic $[Ca^{2+}]_i$ (Fig. 1). To determine whether this type and dose of radiation induced prolonged effects on Ca^{2+}

FIG. 2. Low-dose ^{56}Fe -particle radiation increases $[\text{Ca}^{2+}]_i$ within 24 h and decreases Ca^{2+} transient amplitude up to 48 h after irradiation. Panel A: Analysis of relative resting cytoplasmic $[\text{Ca}^{2+}]_i$ in dissociated muscle fibers from control and ^{56}Fe -particle (15 cGy, 1 GeV/n) irradiated mice. Cells were prepared 24, 48 h and 7 days after irradiation, (* $P < 0.05$; $n\text{-mice}_{\text{IR}} = 3$, $n\text{-mice}_{\text{control}} = 3$). Panels B and C: Analysis of electrical depolarization-evoked Ca^{2+} release in dissociated muscle fibers from control and ^{56}Fe -particle (15 cGy, 1 GeV/n) irradiated mice at 24 and 48 h time point. In panel B, amplitude of transients from irradiated cells were normalized to the peak amplitude of transients in the control, nonirradiated cells obtained at each respective time point (* $P < 0.05$; $n\text{-mice}_{\text{IR}} = 3$, $n\text{-mice}_{\text{control}} = 3$).

homeostasis, we also assessed $[\text{Ca}^{2+}]_i$ at day 7 postirradiation. At day 7, $[\text{Ca}^{2+}]_i$ from irradiated muscle was not statistically different from the nonirradiated controls (data not shown). These results indicate that a single dose of 1 Gy whole-body γ radiation is sufficient to induce acute, short-duration changes in skeletal muscle Ca^{2+} homeostasis.

Effects of Low-Dose ^{56}Fe -Particle Radiation on Skeletal Muscle

To determine the effects of ^{56}Fe -particle radiation on skeletal muscle, mice were irradiated with a single dose of 15 cGy at the energy of 1,000 MeV/nucleon (1 GeV/n). The initial series of experiments assessed the potential changes in the basal $[\text{Ca}^{2+}]_i$ estimated as the Fura-2 fluorescence emission ratio as well as changes in the depolarization-evoked Ca^{2+} release from the sarcoplasmic reticulum (i.e., EC coupling) 24 h after irradiation. As demonstrated in Fig. 2, skeletal muscle fibers isolated from the ^{56}Fe -particle-irradiated animals exhibited a significant increase in $[\text{Ca}^{2+}]_i$ compared to the cells isolated from the control, nonirradiated animals 24 h postirradiation (Fig. 2A). At the same time point there was a significant decrease in the amplitude of sarcoplasmic reticulum Ca^{2+} release (Fig. 2B and C). At 48 h and day 7 time points, basal $[\text{Ca}^{2+}]_i$ was not statistically different than the values in the nonirradiated control mice (Fig. 2A), suggesting an effective recovery. Conversely, the reduced Ca^{2+} transient amplitude persisted at 48 h time

point (Fig. 2B, C) in muscle cells from the irradiated animals. At day 7, Ca^{2+} transient amplitude was similar between control and irradiated muscle. These results demonstrate that ^{56}Fe -particle irradiation results in relatively rapid changes in skeletal muscle Ca^{2+} handling and that those aspects of muscle function are able to recover by day 7.

Effect of Low-Dose Proton Radiation on Skeletal Muscle

To determine the effects of proton radiation on skeletal muscle Ca^{2+} handling we performed similar measurements in muscle cells isolated from animals irradiated with 90 cGy of 1 GeV/n protons. Unlike in the ^{56}Fe -particle irradiated muscle, proton radiation at this specific dose and energy, did not result in changes in basal $[\text{Ca}^{2+}]_i$ at the 24 h time point (Fig. 3A). However, at 72 h postirradiation there was significant increase in the $[\text{Ca}^{2+}]_i$, analogous to that observed with ^{56}Fe particle at 24 h (Fig. 2A). At one week there was no difference between the control and proton-irradiated muscle fibers (not shown). Whereas after 15 cGy of 1 GeV/n ^{56}Fe -particle irradiation, myofibers exhibited immediate decrease followed by recovery at 7 days (Fig. 2B and C), analysis of the Ca^{2+} transients in dissociated muscle fibers from 90 cGy, 1 GeV/n proton-irradiated muscle, a sixfold higher dose of proton, showed no change at either 24 or 72 h (Fig. 3B and C). These results indicate that changes in Ca^{2+} handling mediated by the proton irradiation

FIG. 3. Low-dose ^1H -particle radiation increases $[\text{Ca}^{2+}]$, and decreases Ca^{2+} transient amplitude only by 72 h after irradiation. Panel A: Analysis of relative resting cytoplasmic $[\text{Ca}^{2+}]$ in dissociated muscle fibers from control and proton (90 cGy, 1 GeV/n) irradiated mice. Cells were prepared 24 and 72 h after irradiation ($*P < 0.05$; $n\text{-mice}_{\text{IR}} = 3$, $n\text{-mice}_{\text{control}} = 3$). Panel B: Analysis of electrical depolarization-evoked Ca^{2+} release in dissociated muscle fibers from control and proton (90 cGy, 1 GeV/n) irradiated mice at 24 and 72 h time point ($*P < 0.05$; $n\text{-mice}_{\text{IR}} = 3$, $n\text{-mice}_{\text{control}} = 3$).

follow a different time course than the changes induced by exposure to the ^{56}Fe -particle irradiation.

Iron Radiation Increases the Number of Central Nuclei

Changes in the muscle intracellular Ca^{2+} handling are sometimes associated with a pathologic process or state. In skeletal muscle the regenerative processes in response to muscle damage are often manifested histologically by the appearance of nuclei in the center of the myofibers (26–28). This is in contrast to the healthy, nonregenerating myofibers, in which nuclei are located in the periphery of the cell, just beneath the cytoplasmic membrane. After a single dose of ^{56}Fe -particle-radiation, muscle cells were able to recover by 1 week with respect to Ca^{2+} homeostasis, thus suggesting a potential activation of recovery or regenerative processes. To determine whether irradiated muscle fibers were undergoing regeneration, we performed H&E staining of *abductor magnus* and *gluteus maximus* muscle cross sections from the proton and ^{56}Fe -particle irradiated mice and compared them to the nonirradiated muscle. As demonstrated in Fig. 4, at day 7 postirradiation there was a large number of myofibrils with centrally located nuclei in ^{56}Fe -particle-irradiated muscles but not proton-irradiated muscles or control muscles (5.8 ± 1.1 vs. 1.6 ± 0.6 and 0.9 ± 0.2 , ^{56}Fe particle vs. proton and iron, $P < 0.0001$ for both) (Fig. 4 A and B). All nonirradiated and the majority of proton-irradiated samples exhibited morphological proper-

ties consistent with histologically unremarkable skeletal muscle (29) with an exception of one proton sample that showed a few atrophic muscle fibers (one fiber per $40\times$ magnification field) and focal peri-capillary edema. In contrast, ^{56}Fe -particle-irradiated samples exhibited greater number of areas with muscular atrophy (see Supplementary Fig. S1A; <http://dx.doi.org/10.1667/RR3329.1.S1>) and disruption of muscle fibers (see Supplementary Fig. S1B; <http://dx.doi.org/10.1667/RR3329.1.S1>), focal cytoplasmic vacuolization (see Supplementary Fig. S1C; <http://dx.doi.org/10.1667/RR3329.1.S1>) and a significant number of muscle fibers with centrally located nuclei (Fig. 4A and B). Some muscle fibers had more than four centrally located nuclei/fiber. These results indicate that a single whole-body low-dose ^{56}Fe -particle irradiation causes damage to the muscle tissue that is sufficient to cause an initiation of the regenerative processes.

Regulation of Erk1/2, Akt and rpS6k Phosphorylation after Iron and Proton Radiation

Exposure of cells and tissues to a variety of stresses, including clinically relevant doses of ionizing radiation (30), induces compensatory activations of multiple intracellular pro-survival signaling pathways (31), such as Akt, Erk1/2 and mammalian target of rapamycin (mTOR) (32). We sought to determine the potential involvement of Akt, Erk1/2 and mTOR in the low-dose ^{56}Fe -particle- and

FIG. 4. ^{56}Fe irradiation increases the number of central nuclei in skeletal muscle tissue. Panel A: Representative images of H&E stained skeletal muscle tissue obtained 7 days after whole-body low-dose high-energy proton (90 cGy, 1 GeV/n) or iron (15 cGy, 1 GeV/n) irradiation at 40 $\times$ magnification to show central nuclei clearly. Individual muscle fibers that contain central nucleus or nuclei are outlined by white dotted lines. Panel B: Graphic representation of the number of central nuclei per $\sim 85 \pm 7$ individual muscle fibers at 20 $\times$ magnification from three different animals (at least 20 images/animal) per radiation group. Statistical significance was assigned when $P < 0.05$ ($n\text{-mice}_{\text{IR}} = 3$, $n\text{-mice}_{\text{control}} = 3$).

proton-radiation-induced responses in the skeletal muscle at day 7 post-irradiation. This time point was chosen based on the significantly increased number of central myonuclei (Fig. 4A and B) and findings of morphological alterations during histologic examination compared to other investigated time points. The basal phosphorylation levels of p-Erk1/2, p-Akt and p-rpS6k (an effector kinase downstream of mTORC1) were relatively higher and more variable in the control samples compared to proton-irradiated skeletal muscle tissue (Fig. 5A, C, E). This suggests that there may

be a requirement of some level of activity of these proteins for proper maintenance/homeostasis in adult mice skeletal muscle tissue (33, 34). On day 7, there was a significant (5 to 16-fold) increase in the expression of phosphorylated p-Erk1/2 (^{56}Fe vs. proton, $P < 0.03$), p-Akt (^{56}Fe vs. proton, $P < 0.002$) and p-rpS6k (^{56}Fe vs. proton, $P < 0.01$) in ^{56}Fe -particle-irradiated compared to proton-irradiated skeletal muscle (Fig. 5A–F). There was no significant change in total protein levels of Erk1/2, Akt and rpS6k. These results indicate that a single low-dose whole-body ^{56}Fe -particle

FIG. 5. ^{56}Fe -particle radiation increases survival and proliferation signaling in skeletal muscle tissue one week after irradiation. Panels A, C, E: Representative images of phospho-Erk1/2 (p-Erk1/2) and total-Erk1/2 (T-Erk1/2), p-Akt and T-Akt, p-rpS6k and T-rpS6k, and corresponding for each protein GAPDH Western blot analyses in skeletal muscle tissue homogenates in control, iron and proton whole-body irradiated mice. Panels B, D, F: Quantification of Erk1/2, Akt and rpS6k protein levels and phosphorylation using densitometric analysis of p-Erk1/2, p-Akt and p-rpS6k band intensities after adjusting for corresponding GAPDH and T-Erk1/2, T-Akt and T-rpS6k band intensities. Graphs represent data from three biological samples per radiation group. Statistical significance was assigned when $P < 0.05$ ($n\text{-mice}_{\text{IR}} = 3$, $n\text{-mice}_{\text{control}} = 3$).

irradiation activates pro-survival, protective and repair/recovery intracellular signaling pathways in skeletal muscle tissue.

DISCUSSION

There is extensive literature on the effects of weightlessness on the musculoskeletal system, however, very little is known about the effects of space radiation on muscle physiology (11, 35–41). Negative effects on musculoskeletal alterations in skeletal muscle were reported recently in mice irradiated with 20–50 cGy of simulated galactic cosmic rays, an exposure and type of radiation relevant to a space flight (11). During future missions to the Moon, near Earth asteroids and Mars, astronauts will be exposed to a complex mixture of radiation from GCRs, the currently limited studies underscore the need for further investigations using wide-range of ionizing ions (proton and HZE) with different energies. The current study sought to investigate the effects of single whole-body low-dose high-energy proton irradiation as a model for SPE (90 cGy, 1 GeV/n) and HZE irradiation as a model for GCR (e.g., 15 cGy of 1 GeV/n ^{56}Fe particle,) on skeletal muscle physiology in adult (10 months old) C57Bl/6N WT mice. We believe that this approach may allow for evaluation of biological responses of skeletal muscle to a single-ion irradiation and could provide a foundation for modeling of the complex ion-space radiation during exploration-type missions (15, 16, 19, 20).

The current data indicate that proton and ^{56}Fe -particle irradiation resulted in intra-myofiber alterations in Ca^{2+} handling. Whole-body ^{56}Fe -particle irradiation mediated a rapid cellular response, observed within 24 h postirradiation, this response was characterized by a significant rise in the resting cytoplasmic $[\text{Ca}^{2+}]_i$ and a reduction in the depolarization-evoked Ca^{2+} release from the sarcoplasmic reticulum, suggesting that these are potential functional alterations in the muscle excitation-contraction coupling. These processes are key regulators of muscle contractility, as well as other cellular functions (23). It is interesting to note that there was also a relatively effective recovery back to baseline in both of these functional processes within 7 days postirradiation.

The effects of proton irradiation followed a very different time course compared to ^{56}Fe -particle irradiation. The only changes that were observed included the increase in the $[\text{Ca}^{2+}]_i$ at 72 h post-treatment, which also recovered by day 7. However, unlike ^{56}Fe -particle irradiation, the proton irradiation did not result in alterations in the Ca^{2+} release. The functional alterations reported here suggest that ionizing radiation can indeed cause alterations in skeletal muscle function. Further investigation into the effect of both types of radiation, such as the dose response, different energies, multiple and/or fractionated exposures, are necessary to identify the underlying mechanisms and

thresholds for each type of irradiation on muscle Ca^{2+} handling.

Effects of γ radiation on skeletal muscle at high clinically-relevant doses ($>2\text{--}5$ Gy, cumulative up to 60 Gy) have been examined extensively in rodent models (42, 43). These studies have shown that at high doses γ radiation may decrease muscle adaptation to physiologic stresses (i.e., overload, exercise, etc.) and decrease skeletal muscle ability to recover after damage (i.e., repeated irradiation, DNA damaging agents, etc.). The effects of lower doses (≤ 1 Gy) of γ radiation on skeletal muscle are important to investigate because it is a principal/baseline type of ionizing radiation (44). More recently, a study in mice using 20–50 cGy of simulated GCRs (more relevant to deep-space travel radiation type and doses) have shown increase in smaller diameter fibers and more fibers containing central nuclei (11), suggesting skeletal muscle damage followed by regeneration at these low mixed ion radiation doses. Our studies, in contrast, have shown that a single dose of 15 cGy of 1 GeV/n ^{56}Fe -particles resulted in an increase in the number of central nuclei (suggestive of degeneration followed by regeneration) and activation of intracellular survival pathways (Akt/mTOR and Erk1/2), whereas proton irradiation at the dose of 90 cGy and 1 GeV/n energy had no detectable effect on either muscle regeneration or on activation of the survival pathways. In spite of very significant difference in the radiation dose (sixfold higher dose for iron) and ~ 700 -fold difference in the LET (150 keV/micron LET for iron and 0.22 keV/micron LET for proton) at 1 GeV/n energy for both ions, our findings suggest that additional studies are necessary to examine further biological responses in the skeletal muscle using various combinations of dose and fluences for proton and iron radiation. This may allow for more accurate development of predictive mathematical models for space radiation-induced degenerative risk estimates in skeletal muscle tissue.

In skeletal muscle the regenerative processes in response to muscle damage are often manifested histologically by the appearance of nuclei in the center of the myofibers (26–28). Here we report divergent regulation of skeletal muscle damage and regeneration after a single dose of ^{56}Fe -particle and proton radiation. The results of our study demonstrate that in addition to alterations in Ca^{2+} handling ^{56}Fe -particle irradiation also caused the appearance of muscle degeneration, exhibited by muscular atrophy and disruption of muscle fibers, along with centrally localized nuclei, which suggest the activation of degenerative/regenerative processes.

Ionizing radiation induces double-strand breaks (DSBs) and generates reactive oxygen species, both, leading to activation of multiple protective downstream signaling pathways, including but not limited to Erk1/2 and PI3K/Akt/mTOR/p70S6K (30). Studies have shown that PI3K/Akt/mTOR and Erk1/2 are involved in skeletal muscle hypertrophy, survival, differentiation and regeneration (32,

45–49). The increased activations of Akt (Thr308), Erk1/2 and mTOR (p-rpS6k) observed in ^{56}Fe -particle irradiated mouse skeletal muscles suggest these protective pathways were activated. In addition, the activations of these pathways combined with increase of central nuclei in ^{56}Fe -particle irradiated samples may suggest that activation of these survival pathways, perhaps, represent a protective response of the skeletal muscle to considerable ^{56}Fe -particle-radiation-induced damage.

There are two potential mechanisms of the initial radiation effects on activation of cellular membrane receptors and downstream activation of intracellular signal transduction pathways. One of these involves the radiation induced, direct DNA damage (i.e., double-strand breaks) that activates DNA damage responses, which subsequently mediate the cascade of intracellular events. These signaling events include, but are not limited to, activation of DNA repair complexes and cell cycle checkpoints, apoptosis, as well as activation of receptors/intracellular pathways (e.g., Erk1/2, Akt, mTOR, etc.) (50). This type of response is referred to as an inside-out response (30). During the inside-out response, the radiation ionizes molecules in the cytoplasm, thereby generating large amounts of reactive oxygen and nitrogen species (ROS and RNS) (51). These reactive molecules inhibit activation of receptor and nonreceptor tyrosine kinases (e.g., Erk1/2, Akt, mTOR), which are the upstream effectors of numerous signal transduction pathways. The other mechanism may involve an outside-in response with altered paracrine ligand activity and receptor reactivation (30). Therefore DNA or membrane-associated receptor activation or both could be responsible for skeletal muscle responses detected in our study. Future studies with significantly different experimental design should include equal dose and/or equal fluence comparisons for proton and iron radiation to determine the spatial and temporal effects of comparable proton and ^{56}Fe -particle irradiation on activation of various cellular and tissue biological responses.

In summary, the current results indicate that both proton and ^{56}Fe -particle radiation cause alterations in the physiological processes of skeletal muscle. The findings of this study underscore a need for further post-ionizing radiation longitudinal studies and possibly additional HZE ion studies.

ACKNOWLEDGMENTS

This work was funded by grant from NASA (NNJ10ZSA001N) to D.A.G. and in part by NIH AR054519 to A.S., NIH HL106098 and AHA-10GRNT471003 grants to X.Y., NIH HL091983 to R.K. Dr. Kishore contributed reagents to be used in our studies and edited the paper. We are grateful to Dr. Adam Rusek and all members of NASA Space Radiation Laboratory and Dr. Peter Guida and all members of Medical Department at the Brookhaven National Laboratory for their valuable support and efforts on this project. We are also grateful to Mr. Yening Zhou a member of Pathology Department and two members of Center of

Cancer Systems Biology at Steward St. Elizabeth's Medical Center, Mr. Michael Peluso and Ms. Caitlin Coelho for their technical help in automated processing of skeletal muscle samples for routine histology.

Received: February 4, 2013; accepted: July 12, 2013; published online: October 17, 2013

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