Skeletal muscle adaptations to microgravity exposure in the mouse

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Submitted 11 June 2003; accepted in final form 25 July 2003

Harrison, B. C., D. L. Allen, B. Girten, L. S. Stodieck, P. J. Kostenuik, T. A. Bateman, S. Morony, D. Lacey, and L. A. Leinwand. Skeletal muscle adaptations to microgravity exposure in the mouse. J Appl Physiol 95: 2462–2470, 2003. First published July 25, 2003; 10.1152/japplphysiol.00603.2003.—To investigate the effects of microgravity on murine skeletal muscle fiber size, muscle contractile protein, and enzymatic activity, female C57BL/6J mice, aged 64 days, were divided into animal enclosure module (AEM) ground control and spaceflight (SF) treatment groups. SF animals were flown on the space shuttle Endeavour (STS-108/UP-1) and subjected to ~11 days and 19 h of microgravity. Immunohistochemical analysis of muscle fiber cross-sectional area revealed that, in each of the muscles analyzed, mean muscle fiber cross-sectional area was significantly reduced (P < 0.0001) for all fiber types for SF vs. AEM control. In the soleus, immunohistochemical analysis of myosin heavy chain (MHC) isoform expression revealed a significant increase in the percentage of muscle fibers expressing MHC IIX and MHC IIb (P < 0.05). For the gastrocnemius and plantaris, no significant changes in MHC isoform expression were observed. For the muscles analyzed, no alterations in MHC I or MHC IIA protein expression were observed. Enzymatic analysis of the gastrocnemius revealed a significant decrease in citrate synthase activity in SF vs. AEM control.

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mass, muscle fiber cross-sectional area (CSA), and tension production (27). Similarly, Carlson et al. (11) demonstrated that 7 days of hindlimb suspension reduced soleus mass by 42% but that gastrocnemius mass was only reduced 17% during this time period. With regard to MHC gene expression, McCarthy et al. (36) showed that in mice 2 wk of hindlimb suspension resulted in a decrease in the percentage of type I fibers in the soleus. Conversely, Haida et al. (27) demonstrated an increased percentage of type I fibers and a decreased percentage of type IIA fibers in the murine soleus after 2 wk of hindlimb suspension. Finally, no study to date has reported on the effects of unloading conditions on mouse skeletal muscle enzymatic makeup.

With the completion of the murine genome and the establishment of mice as the transgenic model of choice, it is important to study the adaptational response of murine skeletal muscle to a wide range of increased and decreased activity states, including the unloading conditions of microgravity. Investigation of the murine response to microgravity exposure also serves to broaden our overall understanding of muscle adaptation, because murine muscle gene expression is not identical to rat, monkey, or human gene expression. Therefore, the purpose of this study was to evaluate muscle fiber size, MHC isoform protein expression, and muscle oxidative capacity via analysis of citrate synthase (CS) activity in murine skeletal muscle subjected to nearly 12 days of microgravity exposure aboard the space shuttle Endeavour.

METHODS

Microgravity exposure and animal care. As described previously (24, 39), female C57BL/6J mice were divided into baseline, animal enclosure module (AEM) control, and SF groups (n = 12 mice/group). Baseline animals were housed in standard microfilter-topped animal cages and given access to food and water ad libitum. These animals were maintained in an approved animal care facility at the Cape Canaveral Air Force Station (Cape Canaveral, FL) on a 12-h light-dark cycle. SF mice and AEM ground control mice were housed in AEMs developed and maintained by the National Aeronautics and Space Administration (NASA) Ames Research Center (Moffett Field, CA) specifically for the transport and housing of animals in zero gravity. Mice had access to food and water ad libitum and were provided with constant airflow. Animal treatment, care, and housing density were within National Institutes of Health (NIH) guidelines with a floor area per mouse in a ground configuration, and mice were maintained on a 12-h light-dark cycle. All protocols were approved by the appropriate institutional animal care and use review groups (University of Colorado at Boulder, Amgen, NASA Ames, and NASA Kennedy Space Center). SF AEMs were located in the middeck of the space shuttle Endeavour for the duration of the mission. SF animals were flown on the space shuttle Endeavour (STS-108/UF-1) from December 5 to 17, 2001, with a total mission time of 11 days and 20 h. AEM ground control mice were housed in the orbital environmental simulator at the Cape Canaveral Air Force Station for the same duration (11 days and 20 h). During the study period, the orbital environmental simulator was configured to mimic the shuttle’s middeck temperature, humidity, and CO2 levels.

Baseline animals were killed at 64 days of age and served as a preflight, baseline control group (n = 12). SF animals and SF animals were killed at 77 days of age. After death, animal mass was recorded, and the triceps surae (gastrocnemius, plantaris, and soleus) of each mouse was dissected and frozen in liquid nitrogen-cooled isopentane.

Immunohistochemistry. To preserve resting muscle length, muscle samples were attached to corkboard with optimal cutting temperature media (Sakura Finetek, Torrance, CA) before freezing. Ten-micrometer-thick serial sections were cut from the belly of the muscle with a Cryostat Microtome Tissue Tek II, fixed to gelatin-coated slides, and stored at −20°C until use. Immunohistochemistry was performed as described previously (2). Briefly, muscle sections were air dried for 20 min and then incubated in a permeabilizing/blocking solution (0.12% BSA, 0.12% nonfat dry milk, 0.01% Triton X-100 in PBS) containing 0.05% normal goat serum. For fiber type analysis, the blocking solution also contained an antilaminin antibody (final concentration of 0.02%) to aid in fiber visualization. Blocking was performed for 1 h at 4°C in a humidifying chamber. After blocking, tissue sections were rinsed three times in PBS. Sections were placed in primary antibody solution overnight at 4°C or for 1 h at room temperature. The antibodies used were the following: MHCs (Novocastra, Newcastle upon Tyne, UK), which is reactive with type I MHC; 6H1, which is reactive with MHC IIX (34); and antibodies SC-71 and BF-F3, which are reactive with MHC IIa and MHC IIb, respectively (23). After primary incubation, sections were rinsed three times in PBS followed by three 5-min incubations in PBS. The secondary antibodies used were either goat anti-mouse IgG-peroxidase or IgG-FITC/Texas red (TR) conjugates (MHCs and SC-71), or goat anti-mouse IgM-peroxidase or IgM-FITC/TR conjugates (6H1 and BFF-3). Tissue sections were incubated in secondary antibody for 1 h at room temperature, followed by numerous washes with PBS. For bright field analysis, immunostaining was visualized by using a peroxi-
dase diaminobenzidine reaction kit (Vector labs, Burling-
ham, CA) for 5 min followed by several rinses in distilled H2O and mounting in Permount.

Muscle fiber size and MHC protein expression. Muscle fiber CSA was measured by using a video camera (Videoscope International, Dulles, VA) mounted on a Zeiss bright field microscope attached to a Macintosh-compatible PowerBase 200 computer (Power Computing, Austin, TX). Fiber CSA was determined by using a computer mouse to trace the outline of immunostained muscle fibers by using NIH Image software. The analysis software was calibrated by outlining defined areas on a slide micrometer. A total of 50 fibers of each type per muscle were analyzed from 5 to 10 randomly chosen areas in each muscle. Fiber percentages were determined from counts of 500–1,000 fibers in four to six randomly chosen muscle regions from both the deep and superficial portions of the muscle. For the gastrocnemius, where there are distinct regional differences in MHC isoform expression, fiber counts were weighted to account for the relative CSAs of these distinct muscle regions (2).

Muscle oxidative capacity. Due to collaborative tissue requirements and dissection time limitations, CS analysis was performed only on the gastrocnemius. Analysis of CS activity was performed as described previously (46). Briefly, the gas-

 trocnemius was dissected and rapidly frozen in liquid nitrogen. Frozen tissue was then weighed and homogenized with a glass homogenizer on ice in 100 mM Tris·HCl at a constant weight-to-volume ratio. Sample homogenate was then added
to a reaction mix of 100 mM Tris-HCl, 1.0 mM dithio-bis (2-nitrobenzoic acid), and 3.9 mM acetyl coenzyme A. After addition of 1.0 mM oxaloacetate, absorbance at 412 nm was recorded for a 2-min period. Mean absorbance change per minute was recorded for each sample, and CS activity in millimole per minute per gram was then calculated by using an extinction coefficient of 13.6.

Statistical analysis. All data are reported as means ± SE. Statistical significance was set at P < 0.05.

RESULTS

Body mass and heart mass. Preflight body mass values were not significantly different for the three groups (baseline, AEM control, and SF) (Fig. 1A). SF animals lost 5% in body mass during the 12 days of microgravity exposure, whereas AEM control animals gained 4% in body mass during this time. As reported previously (24), at the time of death, mean SF body mass was significantly reduced compared with AEM control (Fig. 1A). Absolute heart mass was significantly increased for the AEM animals compared with both baseline and SF at time of death (Fig. 1B). Normalized heart mass (heart mass/postflight body mass) was not significantly different across groups (Fig. 1B).

Muscle fiber size. In the gastrocnemius, microgravity exposure resulted in significant atrophy of muscle fibers expressing MHC I, MHC IIA, MHC IIx, and MHC IIB (Fig. 2A). In the plantaris, muscle fiber CSA was significantly reduced for SF compared with baseline and AEM control for all muscle fibers (<10 MHC I-expressing fibers were observed in any animal from any group; thus these fibers were not included in the CSA analysis; Fig. 2B). For the soleus, microgravity exposure resulted in a significant reduction in mean muscle fiber CSA of fibers expressing both MHC I and MHC IIA compared with both baseline and AEM control (Fig. 2B). The number of fibers expressing MHC IIx and MHC IIB in the soleus was insufficient to be included in the muscle fiber analyses (<20 fibers/animal). Across muscles, the soleus was more affected than the gastrocnemius and plantaris. On average, muscle fibers in the SF soleus were 22.1% smaller than muscle fibers in the baseline soleus and 26.6% smaller than muscle fibers in the AEM control soleus (Fig. 3). For the gastrocnemius, SF muscle fibers were 10.2% smaller than baseline and 17.7% smaller than AEM control (Fig. 3). Similarly, in the plantaris, SF muscle fibers were 10.8% smaller compared with baseline and 17.9% smaller than AEM control (Fig. 3). The effects of microgravity exposure on muscle fiber size were also the most dramatic for muscle fibers expressing MHC I and MHC IIA. Overall, the mean SF MHC I-expressing fiber was 19.4% smaller than baseline and 28.5% smaller than AEM control (Fig. 3). For MHC IIA-expressing fibers, the SF mean was 14.3% smaller than baseline and 18.9% smaller than AEM control (Fig. 3). For MHC Ix- and MHC IIB-expressing fibers, the SF means were 7.4 and 10.3% smaller compared with baseline and 15.9 and 16.1% smaller compared with AEM control, respectively (Fig. 3).

MHC isoform protein expression. For both the gastrocnemius and plantaris, microgravity exposure did not result in any significant alterations in the percentage of muscle fibers expressing a particular MHC isoform (Fig. 4, A and B). In the soleus, microgravity exposure resulted in a significant increase in the percentage of muscle fibers expressing both MHC IIX and MHC IIB (Fig. 4C). In the soleus, microgravity exposure more than doubled the percentage of muscle fibers expressing MHC IIX (6.0 ± 1.6, 5.1 ± 1.4, and 13.6 ± 1.8% for baseline, AEM, and SF, respectively). In the baseline and AEM control soleus, ~1% of the muscle fibers expressed the MHC IIB isoform (1.0 ± 0.8 and 1.2 ± 1.7% for baseline and AEM control, respectively). After 12 days of microgravity exposure, 16.3 ± 5.7% of soleus muscle fibers expressed MHC IIB (Fig. 4C). It is interesting to note that, for both the AEM control soleus and the SF soleus, MHC IIB appeared to always be coexpressed with MHC IIA (Fig. 5). Immunohisto-
chemical analysis did not reveal any evidence of either MHC I/MHC IIx or MHC I/MHC IIb coexpressing fibers in either control or SF animals (data not shown). Because of antibody limitations, we were unable to assess coexpression of MHC IIx and MHC IIb (i.e., both primary antibodies require IgM secondary antibodies). Overall, it is clear that the increase in expression of MHC IIb with microgravity exposure resulted in an increase in percentage of hybrid fibers (MHC isoform coexpressing) in the soleus.

Muscle oxidative capacity. Although tissue availability prevented a more in-depth analysis of SF-induced alterations in muscle enzymatic activity, analysis of CS activity in the gastrocnemius revealed no difference between baseline and AEM control groups but significantly reduced CS activity in the SF muscle samples (decreased 16.0% compared with baseline and 18.9% compared with AEM control) (Fig. 6).

DISCUSSION

The adaptational response of the musculoskeletal system to a state of reduced activity is well documented by using a number of animal models, including spinal cord transection, hindlimb suspension, and microgravity exposure (7–10, 16, 20, 30, 31, 47, 51, 52). These paradigms consistently demonstrate a reduction in muscle size attributed to muscle fiber atrophy and a...
days of microgravity exposure led to a significant decrease in body mass of the SF animals compared with AEM animals (Fig. 1A). Specifically, AEM control animals gained 4% in body mass during the 12-day study period, whereas the SF animals lost 5% in mass during this time. This finding is consistent with previous rat and human studies that have demonstrated either a reduction in body mass or decreased growth during SF (25, 45) but inconsistent with other rat studies that showed body mass was either not changed or increased with microgravity exposure (5, 12, 14, 40, 53). Of course, the relative developmental age of the animals in various studies needs to be taken into account to put the body mass change from this study in perspective. Overall, the observed loss in body mass in the present study is likely due to a combination of factors including microgravity, stress, variability in housing conditions between AEM ground control and SF (e.g., 3-dimensional vs. 2-dimensional access to AEM interior), and alterations in food and water consumption (24). Data collected from the SF and AEM revealed that the ambient temperature for the SF animals was elevated compared with AEM ground control due to unanticipated recirculation of airflow onboard the space shuttle within the individual AEM units (24). Furthermore, both water and food intake were significantly reduced for the SF animals (24), which, combined with a higher ambient temperature, likely led to animal dehydration and decreased body mass.

Muscle fiber size. It is important to note that the CSA results are, in part, confounded by the loss in body mass observed for the SF animals. It is clear that the AEM control animals gained mass during the 12-day study period (Fig. 1A). This growth is evident in the muscle fiber CSA results, which indicate larger mean fiber areas for AEM control vs. baseline (Fig. 2). This loss of body mass for the SF animals combined with the gain of mass for AEM control and the muscle fiber CSA results indicate the interplay of two factors that result in the observed mass and CSA differences: lack of growth and atrophy of existing muscle. Our data indicate that, during the 12-day study period, the SF animals did not grow larger as did the ground-based control animals. Overall, the observed reductions in muscle fiber CSA were significantly greater than those seen for body mass (10–30% decrease for fiber CSA vs. 9% for body mass), indicating that changes in body mass do not completely explain the reported decreases in muscle fiber CSA and that the SF animals lost muscle mass and muscle fiber size due to unloading and disuse.

Our results also demonstrate significant atrophy of muscle fibers expressing each of the MHC isoforms in the murine gastrocnemius, plantaris, and soleus (Fig. 2). As noted previously, microgravity-induced muscle fiber atrophy was most dramatic for the soleus and for MHC I- and MHC IIA-expressing fibers (see Fig. 3). The finding that the soleus was more affected by microgravity conditions than either the gastrocnemius or plantaris is consistent with rat and human data for both microgravity exposure and hindlimb suspension (10, 11, 16, 26, 27, 36). The mechanism by which disuse paradigms preferentially affect the soleus is unknown.
at this time. There is evidence that hindlimb suspension and microgravity exposure result in increased protein degradation via the ubiquitin-proteosome pathway in both rat and mouse models (6, 29, 41–43). Conversely, Lalani et al. (32) demonstrated no change in ubiquitin mRNA expression combined with increased myostatin mRNA and protein expression and decreased IGF-II mRNA levels in the rat tibialis anterior, gastrocnemius, biceps femoris, and quadriceps after 17 days of SF. The work of Carlson et al. (11), however, suggests that for the mouse soleus the myostatin pathway may not be directly involved, because myostatin mRNA levels are undetectable in the murine soleus, whether weight bearing or unloaded, although this evidence does not rule out possible paracrine effects of myostatin secreted by other muscles. Overall, it is clear that under normal, weight-bearing conditions, the soleus is subjected to nearly constant tonic activation patterns (28), and more research is needed to elucidate the specific pathway(s) by which reduction or loss of this tonic activation pattern results in such a rapid and marked atrophy of soleus muscle.

MHC isoform protein expression. Immunohistochemical analysis of muscle fiber MHC isoform expression revealed no change in the percentage of muscle fibers expressing a particular MHC isoform for the gastroc-
nemius and plantaris (Fig. 4, A and B). These findings are consistent with the findings of Caiozzo et al. (10), who observed no change in the expression of MHC IIb in the rat plantaris and tibialis anterior at the protein level with 14 days of microgravity exposure. Our findings are not consistent, however, with those of Kramer et al. (31), who observed a significant decrease in the expression of MHC IIx in the rat extensor digitorum longus (EDL) and superficial gastrocnemius with 10 days of SF.

For the soleus, we observed no change in the percentage of muscle fibers expressing MHC I and MHC IIa after 12 days of SF (Fig. 4C). This is in contrast to previous rat and human studies, which reported decreased expression of these MHC isoforms with microgravity exposure (1, 8, 18), and to the work of Haida et al. (27), which demonstrated alterations in MHC I and MHC IIa expression in the mouse soleus with 2 wk of hindlimb suspension. The hindlimb suspension studies of McCarthy et al. (36, 37) demonstrated decreased expression of MHC I mRNA in mice harboring β-MHC promoter region-CAT reporter transgenes, but these studies did not explore protein level changes. Given the relatively long half-life of MHC protein (~2 wk), it may be that the time period investigated in this study (12 days) was simply not sufficient to see protein changes for MHC I.

Another possible explanation as to why the MHC adaptation to microgravity conditions seen in mice is less pronounced than that typically seen in rats or primates is that mice, in the control condition, have a significantly “faster” MHC isoform expression pattern (for example, ~40% of mouse soleus muscle fibers express MHC I, ~90% of rat soleus muscle fibers express MHC I; the mouse quadriceps consists of ~1% MHC I-expressing fibers, the human quadriceps contains ~40–50% MHC I-expressing fibers). If microgravity conditions induce a shift in MHC isoform expression toward a particular set point on the fast end of the fiber-type continuum, irregardless of body size and initial fiber composition, the adaptation seen in mice will be less than that seen in primates or rats (see Fig. 7).

It is also important to note that the immunohistochemical analysis used may not have been sensitive enough to detect small but significant changes in MHC isoform expression. Fiber-type percentage as determined by immunohistochemistry provides no quantitative information on the amount of a particular MHC isoform expressed in a given muscle fiber. It is possible that, although the percentage of muscle fibers expressing MHC I and MHC IIa in the soleus was found to be unaffected, the absolute whole muscle content of these isoforms was reduced with SF. Single muscle high-resolution gel electrophoresis or Western blot analyses may have detected such a shift in MHC isoform expression. Tissue availability, however, precluded this type of analysis, and future studies are needed to address this issue.

The percentage of soleus muscle fibers expressing MHC IIx and MHC IIb, however, was significantly increased with microgravity exposure (Fig. 4C). Specifically, there was a greater than twofold increase in the percentage of MHC IIx-expressing fibers (~5 vs. 13%) and an approximately eightfold increase in the percentage of MHC IIb-expressing fibers (~2 vs. 16%) (Fig. 4C). These results are consistent with those of Staron et al. (47), who also observed increased MHC IIx and MHC IIb expression in the rat soleus with 10 and 14 days of SF. It is interesting to note that all disuse models investigated to date have revealed an increase in expression of both MHC IIx and MHC IIb in the rodent soleus. It has even been suggested that MHC IIb, in these species, may be the “default” gene (22, 48) and that slow tonic activation patterns may be required to suppress MHC IIb gene expression in the soleus. Loughna et al. (33) demonstrated that MHC IIb is transcriptionally activated in the rat soleus within the first 24 h of disuse and showed that the expression of MHC IIb can be blocked by passive stretch. These findings support the concept that, in slow postural muscles, tonic activation patterns combined with tonic muscle activity may be required to maintain the slow MHC isoform expression patterns typically observed.

It is interesting to note that our finding of increased MHC IIb expression in the face of unaltered MHC I expression may indicate that different pathways are involved in the upregulation and/or suppression of these genes with hindlimb suspension or microgravity exposure. As mentioned previously, tonic activation of the soleal nerve may act to chronically suppress MHC IIb expression in the soleus. Our data suggest that this tonic activation may not be needed to chronically stimulate MHC I expression in the soleus, since we observed increased MHC IIb expression but no change in MHC I expression. It is also possible that the time course evaluated here (12 days of microgravity exposure) was simply not long enough to result in a significant reduction in MHC I expression and that had the experiment been of longer duration, we would have seen both increased MHC IIb expression and decreased MHC I expression.
Muscle oxidative capacity. Although the changes in MHC isoform expression in response to SF or hindlimb suspension conditions are well established for the rat, monkey, human, and, with the addition of the present study, mouse (3, 10, 13, 16, 19, 21, 31, 44, 47), the adaptive responses of the oxidative and glycolytic enzymatic pathways are less well characterized (4, 17, 35, 49). Previous studies in the rat have yielded conflicting results, with evidence that microgravity exposure results in either no change, increased muscle oxidative capacity, or decreased oxidative capacity combined with increased expression of key glycolytic enzymes such as hexokinase (4, 17, 35, 49). In this study, we demonstrate that 12 days of microgravity exposure in the mouse results in a significant decrease in CS activity in the gastrocnemius (Fig. 6). Although our findings are limited to only one muscle, this response is in contrast with the data from previous rat studies (4, 17, 49) and suggests that the adaptational response of murine enzymatic pathways to microgravity exposure may differ from that of the corresponding rat pathways. It is interesting to note that, for the gastrocnemius, no changes in MHC isoform expression were detected by the methods utilized, indicating that the microgravity-induced changes in muscle contractile and enzymatic proteins are not necessarily coordinate. It is unknown at this time whether the observed decrease in CS activity would have any functional effects on the microgravity-exposed gastrocnemius, although it might be predicted that decreased CS activity would result in a decreased capacity for ATP generation via aerobic pathways.

The data presented here provide the first analysis of the adaptational response of murine skeletal muscle to microgravity exposure. Our results demonstrate that, in the mouse, 12 days of SF results in significant muscle fiber atrophy combined with minimal alterations in MHC isoform protein expression. This muscle fiber atrophy is more pronounced in the slow postural soleus muscle and in muscle fibers expressing MHC I and MHC IIa. We have demonstrated that microgravity exposure in the mouse results in a significant increase in the expression of MHC IIX and MHC IIB protein in the soleus. Our results indicate that, in the mouse soleus, MHC IIB is always coexpressed with MHC IIA, and this pattern of coexpression is not altered with microgravity exposure. In contrast to previous work in the rat and the human, however, microgravity exposure in the mouse did not result in alterations in expression of either MHC I or MHC IIA at the protein level. Also in contrast to previous rat data, we demonstrate that, in the mouse, microgravity exposure results in a significant decrease in muscle oxidative capacity as measured by CS activity for the gastrocnemius. Overall, our findings indicate that, although the adaptation of murine skeletal muscle to SF may differ somewhat from the adaptations typically seen in other mammalian species, mice should prove valuable as a model species for understanding the specific mechanisms of microgravity-induced muscle atrophy in mammalian skeletal muscle and may prove beneficial in testing pharmaceutical treatments or other methods designed to mitigate these effects.

The authors thank all of the individuals who assisted in the very complicated process of sending animals into space. The results presented would not have been possible without the help and tissue dissection skills of many individuals from Amgen, Jackson Laboratories, Loma Linda University, BioServe Space Technologies, NASA Ames, and the Johnson Space Center.

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