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Authors
Haddad, F.
Herrick, R. E
Adams, G. R
et al.

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Myosin heavy chain expression in rodent skeletal muscle: effects of exposure to zero gravity

FADIA HADDAD, ROBERT E. HERRICK, GREGORY R. ADAMS, AND KENNETH M. BALDWIN
Department of Physiology and Biophysics, College of Medicine, University of California, Irvine, California 92717

PREVIOUS STUDIES have shown that the chronic elimination of weight-bearing activity induces both fiber atrophy and a transformation in the pattern of isomyosin expression in rodent skeletal muscle (1, 5, 8, 10, 13). Available evidence, based on 1) immunohistochemical analyses of myosin heavy chains (MHCs) in single fibers (8, 10) and 2) electrophoretic analyses of native isomyosins in whole muscles (1, 5, 13, 15), suggests that slow myosin (type I) expression is downregulated and that of the faster myosin isoforms is upregulated in muscles used extensively for posture and/or locomotion when rodents are exposed to either the ground-based model of hindlimb suspension (FL) or to a zero-gravity environment (1, 8, 10). The magnitude of these isof orm shifts is dependent on both the type of muscle affected (1, 8, 10) and the duration of unloading (1, 11, 13). However, it remains uncertain which of the faster MHCs are upregulated in response to conditions of zero gravity.

In a previous study, we reported that 14 days of rodent hindlimb suspension decreased the relative level of expression of the type I MHC while increasing the relative content of both the type Ila and IIb MHC isoforms in the antigravity vastus intermedius (VI) as studied at both the protein and mRNA levels of analyses (5). Isomyosin changes in the fast-twitch plantaris were similar to those in the VI but were of lesser magnitude (5).

To expand on these observations, a study was undertaken, as part of the integrated National Aeronautics and Space Administration (NASA) Space Life Sciences 1 mission, to ascertain whether exposure to zero gravity induces the same type of adaptational response in isomyosin expression as that reported in the ground-based models (5, 11, 13). Herein, we report that 9 days of zero-gravity exposure induced an altered pattern suggesting a reduction in both the relative and absolute content of type I and Ila MHC protein that was partially offset by small net increases in type IIb and possibly type IIx MHC content in the high-oxidative red VL and vastus lateralis (VL) skeletal muscles. However, no changes occurred in either the fast low-oxidative VL or in the non-weight-bearing tibialis anterior.

Methods

Experimental design. The rodents used in this study were part of the integrated Space Life Sciences 1 mission research project, which was a 9-day mission flown aboard the space shuttle Columbia in early June 1991. Approximately 30 days before launch, young male Sprague-Dawley rats (Taconic Farms, Germantown, NY), weighing ~80 g, were randomly assigned to either a flight (FL; n = 10) or a control (CON; n = 10) group. Each of these two groups was subdivided into an additional two groups (n = 5 each) and designated for tissue processing as recovery (R+0) or recovery plus mission length (R+9). Before launch the animals were housed in groups of five at the Kennedy Space Center vivarium and provided ad libitum with water and a Purina-type food bar diet modified for dispersion in the shuttle cages. Approximately 33 h before launch, the FL animals were transferred to the Animal Experimental Modules (AEMs), which is a facility designed for group animal housing that can be accommodated in the middeck of the shuttle. The AEMs were placed aboard the shuttle ~29 h before launch. CON

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animals were maintained in grouped housing in the Kennedy Space Center vivarium in cages of similar size as the AEMs. The logistics of the 9-day mission and subsequent postflight period were identical to those described in a companion paper (2).

**Tissue preparation.** Both at recovery (R+0) and recovery plus mission length (R+9), FL and CON animals were killed by decapitation, and the muscles were rapidly removed along with other organs by a dissecating team coordinated by the NASA Ames Research Center. For this particular experiment, we were provided with both the right and left vastus complex and tibialis anterior. The right tibialis anterior was quickly frozen. Next, the same muscles on the left side were processed by first removing connective tissue and fascia, weighing them, and then storing them in glycerol cooled to -20°C. The muscles on the left side were used for protein analyses and electrophoresis of the MHC.

**Myofibril isolation and gel electrophoresis.** The muscle samples were first rinsed free of glycerol and then homogenized in 250 mM sucrose, 100 mM KCl, and 5 mM EDTA (pH 6.8). An aliquot was used for total protein determination (6). Myofibrils were then prepared and purified by a detergent washing technique as described previously (13, 14). After protein measurements (6), myofibrils were stored at a protein concentration of 1 mg/ml in a storage buffer consisting of 50% glycerol, 100 mM Na_2PO_4, 5 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N,N-tetraacetic acid, and 2 mM 2-mercaptoethanol (pH 8.8). MHCs were separated using sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis according to a modification of the method of Danielli-Betto et al. (4), as described in detail previously (5). After staining and destaining, the gels were scanned densitometrically at 630 nm in a Zeineh soft-laser densitometer (Biomedical Instruments, Fullerton, CA) interfaced with an IBM PC computer equipped with software to perform peak-area integration analysis. The areas of the peaks corresponding to each band were summed, and then the area for each peak was expressed as a percentage of the total area. In this way, MHC isoform was expressed as a percentage of the total myosin or total MHC present in the gel. In the system used for the MHC separation of these samples, we were not capable of consistently separating the so-called type IIx MHC from the type IIa MHC, as the two proteins comigrate closely together; thus, what we refer to as the type IIa MHC band is in reality a combined type IIa-IIx MHC complex (see Discussion).

In previous studies, we reported that myofibrils can be quantitatively extracted from skeletal muscle and purified with relatively little protein loss during the procedure (13-15). Also, we have provided evidence that the myosin content relative to the other proteins making up the myofibril pool remains relatively constant at ~41-45% of the subcellular fraction in normal, atrophying, and hypertrophying skeletal muscle (1, 13-15). When these factors as well as the data reported in Tables 1-3 are taken into consideration, it is possible to estimate the absolute content of a given MHC (mg/muscle) in a particular muscle by

\[
\text{muscle wt (g) } \times \text{ myofibril yield (mg/g)}
\]

\[
\times \% \text{myosin in myofibril pool (0.45)}
\]

\[
\times \text{ relative % of MHC isoform in myosin pool (1)}
\]

\[
= \text{ MHC isoform (mg)/muscle type}
\]

This approach was used in the present study to ascertain the impact of altered muscle weight on the average amount of MHCs maintained in FL and CON muscles.

**RNA isolation and slot-blot analysis.** Total RNA was isolated from frozen muscle samples according to the RNAzol method (Tel-Test), which is based on the procedure published by Chomczynski and Sacchi (3). In our hands, this technique is favored over the cesium chloride ultracentrifugation technique because it provides greater yields of undegraded RNA, free of DNA and proteins, on the basis of analyses using agarose gel-ethidium bromide stain as well as on the basis of the ratio of optical density at 260 nm to the optical density at 280 nm (~2.0).

MHC mRNA-specific oligonucleotides, 20 bases in length, were purchased from Chemgenes (Waltham, MA). These oligo probes are complementary to the 3' untranslated regions of MHC mRNA and are highly specific to the type I, IIa, and IIb MHCs (7). The probes were 5' end labeled with ^32P to a specific activity of 4-5 x 10^6 counts.min^-1.pmol^-1 by the T4-polynucleotide kinase reaction (9). Labeled oligonucleotides were separated from unlabeled nucleotides and from unincorporated [gamma-^32P]ATP by urea polyacrylamide gel electrophoresis. The probes were tested for specificity by using Northern blot analysis with both positive and negative controls for each probe.

For slot-blot analysis, total RNA (1-2 μg) was blotted onto a Nytran nylon membrane (Schleicher & Schuell, Keene, NH) by using a standard slot-blot procedure. The filters were subsequently fixed with ultraviolet irradiation. Prehybridization, hybridization, and washing procedures for the blots were carried out according to the manufacturer's recommendations. After the final wash, the membranes were autoradiographed with an intensifying screen at -70°C for 1-4 days. After hybridization with the oligo probe, the membrane was washed with 1% SDS at 100°C for 15 min to completely remove the probe, rinsed with RNAse free water, prehybridized as above, and rehybridized with a ^32P end-labeled oligo(dt) probe, which hybridizes to poly(A) RNA (total mRNA). mRNA levels were quantified by densiometric scanning of the autoradiogram, and each specific band was normalized to its corresponding oligo(dt) signal. The quantified amount of each MHC mRNA in a given muscle for each of the experimental groups was then normalized to the level found in the combined two CON groups, which were not different from one another. These control values were set at 100%, and the experimental values were expressed as a percentage of the normal control levels.

**Statistical analysis.** The data are presented as means ± SE. All data were analyzed using a one way analysis of variance with a post hoc Newman-Keul's test being performed.
formed when \( F \) ratios were significant. MHC relative content values were arcsine transferred before the statistical analysis was performed. Because MHC mRNA and protein content did not differ between the two CON groups, both the protein and mRNA data bases of the two CON groups were combined into one group, and they were used for comparisons with the two FL groups. The 0.05 level of confidence was selected for statistical significance.

RESULTS

Body weight, muscle weight, and protein content. Body weights were not different between FL and CON groups for the time points investigated (Table 1). The 9-day flight induced a significant reduction in muscle weight in only the VI (Table 1); this difference was greater than that reported in a companion study on this same muscle (2). Although there was a small nonsignificant reduction in the weight of the VL, this response was less than that reported in a companion project on this mission (2). We have no insight as to why the weight differential for the VI was less in this study except that the animals in this study were group housed and consequently their inherent interaction with one another may have had an impact on movement activity involving the faster muscle groups. Both total protein and myofibril protein content were not different among the muscle types for either time point (Table 2). This finding suggests that the 9-day mission produced a uniform reduction in the protein pool across the muscle to account for the difference in VI weight. However, we noticed that muscles composed predominantly of fast fibers had higher total and myofibril protein content than was previously observed (1). There are two possibilities causing this discrepancy. First, the present study involved a different rat strain than that used previously (1). Second, these particular muscles could have had a lower than normal water content.

MHC protein analyses. Figure 1 depicts representative MHC gels for the different muscles of FL and CON groups for the R+0 time point. The CON VI expresses a similar proportion of type I and Ila MHC. The red VI, expresses a bias toward the type Ila MHC, whereas the white VL and tibialis anterior are biased toward the expression of a high relative content of the type IIb MHC. As presented in Table 3, there was a small but distinct shift in the pattern of MHC distribution in both the VI and the red VL. The common response emerging in both muscles appears to be the increased relative expression of the type IIb MHC coupled with the trend for a decrease in the relative content type I MHC. The impact on the type Ila MHC pool appears to be different in these two red muscles, i.e., possibly increasing in the VI and possibly decreasing in the red VL (see DISCUSSION). No changes were observed in either the white VL or tibialis anterior (Table 3). The 9-day recovery period appeared to reverse the isomyosin shift in the red VL but not in the VI (Table 3). This differential response is surprising in view of the observation that the VL was not as responsive as the VI to the atrophying effects of zero gravity (Table 1).

In an attempt to estimate the degree that zero gravity affects the absolute content of a given MHC in different types of skeletal muscle (see METHODS), we calculated for the VI that there is on average 1.72 and 1.40 mg/muscle of type I and Ila-IIx MHC, respectively, in the CON group. In the VI of the FL group there is on average 1.04, 1.13, and 0.05 mg/muscle of type I, Ila-IIx, and IIb MHC, respectively. Thus, on an absolute basis, there appears to be a net reduction in both the type I and Ila-IIx MHC pool by 40 and 19%, respectively, whereas trace amounts of the type IIb MiIC begin to appear in this particular muscle as a result of zero-gravity exposure. With the use of this same approach for the red VL (and the muscle masses reported in Table 1), the following was observed. In the CON group, 6.4, 37.7, and 19.8 mg/muscle of type I, type Ila-IIx, and IIb MHC were expressed, whereas in the FL group this changed to 3.7, 31.3, and 28.4 for type I, Ila-IIx, and IIb MHC protein, respectively. Thus, in the red VL, the pattern of response closely resembles that observed for the VI in that there is also a net reduction in the type I and Ila-II MHC pool by 42 and 17%, respectively, whereas that of the type IIb MHC pool is increased by 33%. Therefore, although the absolute reduction in muscle mass is relatively small in the VL (Table 1), there is, nevertheless, a distinct net loss in both type I and Ila MHC protein that is largely offset by the increase in type IIb MHC protein content. Therefore, despite the lack of dramatic change in the mass of the VL, there appears to be some dramatic transformation occurring in some of the fibers in this muscle in response to both zero gravity and weight-bearing recovery (Tables 3 and 4).

MHC mRNA. The relative signals for isomyosin

| Table 1. Body weight and wet muscle weights of control and flight animals |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
|                            | R + 0                       | R + 9                       |
|                            | Control                     | Flight                      | Control                     | Flight                      |
| BW, g                      | 320±16                      | 333±29                      | 352±10                      | 344±11                      |
| VI, mg                     | 73±4                       | 77±3*                       | 86±2                        | 77±3*                       |
| VL, mg                     | 1,014±33                    | 998±33                      | 1,139±18                    | 1,083±35                    |
| TA, mg                     | 550±17                      | 548±18                      | 534±16                      | 571±2                       |

Values are means ± SE; \( n = 5 \) rats each in recovery group (R + 0) and in recovery plus mission length (9 days) group (R + 9). BW, body weight; VI, vastus intermedius; VL, vastus lateralis; TA, tibialis anterior. Significantly different (\( P < 0.05 \)) from: * all other values in same group; † R + 9 control group.

| Table 2. Total muscle and myofibril protein content of control and flight animals |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
|                            | R + 0                       | R + 9                       |
|                            | Muscle protein              | Myofibril protein           |
|                            | Control                     | Flight                      | Control                     | Flight                      |
| VI                          | 200±19                      | 215±18                      | 211±16                      | 221±17                      |
| RVL                         | 300±26                      | 301±22                      | 322±18                      | 316±30                      |
| WVL                         | 297±21                      | 301±23                      | 317±17                      | 309±16                      |
| VI                          | 95±6                        | 87±10                       | 100±10                      | 96±15                       |
| RVL                         | 140±12                      | 141±13                      | 100±18                      | 154±15                      |
| WVL                         | 156±27                      | 171±35                      | 100±21                      | 164±27                      |

Values are means ± SE in mg/g; \( n = 5 \) animals each in R + 0 and R + 9 groups. RVL, red VL; WVL, white VL.
A

RECOVERY + 0

IIa ---+ I

B

C SOL C VI F C RED F C WHITE

IIa ---+ I

Fig. 1. Gel pattern of myosin heavy chains (MHCs) of soleus (SOL), vastus intermedius (VI), red vastus lateralis (VL) (RED), white VL (WHITE), and tibialis anterior (TA) of control (C) and flight (F) groups at recovery (R+0). A: VI and red VL. B: TA and white VL. Note greater appearance of type IIb MHC in VI and red VL of F group.

Table 3. Myosin heavy chain protein expression in rodent skeletal muscles in response to spaceflight

| Myosin Heavy Chain Type | VI Control | R + 0 | R + 9 |
|-------------------------|------------|------|------|
| I                       | 53.4±2.4   | 46.6±2.3 | 2.1±1.3* |
| IIa-IIx                 | 46.8±4.2   | 51.1±4.2 | 3.4±0.9* |
| IIb                     | 45.4±3.7   | 50.7±2.2 | 3.4±0.9 |
| RVL Control             | 9.7±1.7    | 61.1±1.8 | 29.3±1.3 |
| R + 0                   | 6.3±1.5    | 50.7±2.5* | 42.9±3.5* |
| R + 9                   | 5.8±1.0    | 60.7±1.7 | 33.5±2.2 |
| WVL Control             | 7.4±0.8    | 92.6±0.8 |
| R + 0                   | 7.0±0.4    | 93.0±0.4 |
| R + 9                   | 7.2±0.7    | 93.0±0.7 |
| TA Control              | 27.6±0.9   | 71.3±1.7 |
| R + 0                   | 28.9±1.2   | 71.3±1.2 |
| R + 9                   | 28.4±0.9   | 72.0±1.1 |

Values are means ± SE expressed as percentage of total myosin pool; n = 10 control rats and 5 rats each in R + 0 and R + 9 groups. Statistical analyses were performed on arcsin-transformed values to correct for binomial distribution of proportions. Significantly different (P < 0.05) from: * control value; † R + 0 value.

mRNA expression in the various muscle types are depicted in Fig. 2. Generally, the mRNA signals for the various muscles parallel, in relative intensity, the pattern presented for MHC protein expression shown in Fig. 1. Also, in those muscles responsive to MHC protein changes (VI and red VL) as a result of zero-gravity exposure, a similar pattern of response occurred for the mRNA data with the following exceptions. First, in the VI, the reduction in the type IIa mRNA signal appears to be opposite of the response seen for type IIa protein expression (Fig. 3). Second, the relative reduction in type I MHC protein for the red VL appears to exceed the reduction seen for its corresponding mRNA signal (Fig. 3). This is further illustrated (see above) by the fact that there is a net loss in the absolute amount of type I MHC protein in the red VL (due to atrophy), whereas the corresponding mRNA pool is relatively unaffected (Table 4). This suggests that protein degradation of type I MHC may be the dominating factor affecting the relative amount of this MHC isoform in the red VL. In the white VL and tibialis anterior, the mRNA data are consistent with the pattern of response noted for the MHC protein data (Table 4, Fig. 4), which further suggests that these particular muscles were insensitive to zero-gravity exposure.

Discussion

The key findings of this study indicate that, after as little as 9 days in zero gravity, both atrophy and subtle alterations in MHC expression, as examined at both the

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mRNA and protein levels, occur in rodent skeletal muscles known to be involved in both antigravity and locomotor function. Also, because both the total and myofibril protein content were not different between CON and FL groups, the net loss in muscle protein that occurred in muscles such as the VI appeared to be uniform across cellular fractions.

The findings reported herein provide further evidence that either the lowering of skeletal muscle force production and/or the altering of the activity patterns of the muscle during exposure to zero gravity induces a selective net loss of both the slow type I MHC and the faster type IIa MHC in muscles routinely used for most weight-bearing activities. Our calculations (see RESULTS) suggest that the fibers expressing an abundance of type I MHC may be more responsive than fibers expressing the type IIa-IIx MHC, which is consistent with previous reports that used histological approaches to demonstrate a greater degree of atrophy of slow fibers (10). Moreover, the data also suggest a unique process occurring to redirect the pattern of MHC expressed in these muscles and favoring an increase in expression of the fast type IIb MHC. As a result, in a muscle expressing a relatively high content of slow fibers such as the VI, which normally expresses an abundance of the type I MHC (Table 3), the fast myosins could become the functionally dominant isoform(s) expressed after extended exposure to microgravity. Furthermore, the changes reported in the present study appear to be a result of a true atrophy process, since the body weights (and presumably the muscle weights before flight) of the two groups did not differ before or after the spaceflight. Thus, the atrophy process associated with spaceflight appears to be highly specific to particular subsets of muscle fibers, i.e., those expressing either type I or type IIa MHC in regions of muscles used extensively for antigravity and locomotor function. Also, it would appear that, despite the atrophy process occurring in fibers expressing these MHCs, there is a concurrent process occurring to expand the type IIb MHC pool in some fibers, which appears to be controlled in part by pretranslational processes leading to enhanced type IIb MHC protein expression (Table 4, Fig. 3).

The findings reported herein at both the protein and mRNA level for the VI and red VL are strikingly similar to those observed in a previous flight experiment involving native myosin analysis (1) and to those observed in ground-based studies using the model of hindlimb sus-
pension to study MHC mRNA expression (5, 13). For example, Thomason et al. (13) reported a decrease in type I MHC synthesis in the soleus after 7 days of limb unweighting even though mRNA levels were not reduced. In the present study, we observed in the red VL, but not in the VI, that the relative reduction in type I MHC protein exceeded that of mRNA (Fig. 3), which further suggests that posttranslational events, possibly associated with protein degradation processes (12), are likely contributing to the reduction in net protein accumulation relative to the changes occurring in the mRNA pool. Also, Diffee et al. (5) reported a differential pattern in type IIa MHC mRNA expression compared with the relative expression of type IIa MHC protein in slow muscles of rats suspended for 14 days. In that study the type IIa MHC mRNA signal was depressed in suspended rats, whereas the (supposed) relative content of type IIa MHC protein appeared to have been increased (5). This was the identical pattern seen for these isoforms in the VI from the present study (Fig. 3).

One possible explanation to account for this particular discrepancy in MHC mRNA and protein is that the type IIa MHC protein pool may be decreasing while that of the type IIx MHC pool is increasing with an overall net increase occurring in the relative size of the type IIa-IIx MHC protein band. To resolve this issue one must separate the type IIa-IIx MHC band into its type IIa and IIx components. In an attempt to resolve this issue, we are in the process of refining our electrophoretic techniques to improve separation of the type IIa-IIx MHC band. In preliminary experiments we estimated in the CON VI samples that 75 ± 3% of the type IIa-IIx MHC band was type IIa MHC and 25 ± 3% represented type IIx MHC. In the FL samples, the type IIa MHV component accounted for only 61 ± 3% compared with 39% for type IIx MHIC. Thus, based on these distributions, it would indeed appear that there was both an absolute and relative reduction in the amount of type IIa MHC that was expressed in the VI of the FL animals, which is consistent with the mRNA data. We suspect that the same pattern occurred in the red VL.

In conclusion, the present study provides further evidence that zero gravity exerts a significant impact on both the quantity and type of MHCs expressed in muscles routinely used for both antigravity and locomotor activity. The findings further suggest that molecular events occurring at both the pretranslational and posttranslational levels of regulating MHC gene expression are likely involved in these muscle adaptations to conditions of unloading. Finally, it appears that future experiments involving exposure to zero gravity need to be conducted for longer durations to obtain a better understanding of the transformation in the steady-state pattern of isomyosin expression in both locomotor and antigravity skeletal muscle.

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Address reprint requests to K. M. Baldwin.

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