Myostatin and insulin-like growth factor-I and -II expression in the muscle of rats exposed to the microgravity environment of the NeuroLab space shuttle flight

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Abstract

The mechanism of the loss of skeletal muscle mass that occurs during spaceflight is not well understood. Myostatin has been proposed as a negative modulator of muscle mass, and IGF-I and IGF-II are known positive regulators of muscle differentiation and growth. We investigated whether muscle loss associated with spaceflight is accompanied by increased levels of myostatin and a reduction in IGF-I and -II levels in the muscle, and whether these changes correlate with an increase in muscle proteolysis and apoptosis. Twelve male adult rats sent on the 17-day NASA STS-90 NeuroLab space flight were divided upon return to earth into two groups, and killed either 1 day later (R1) or after 13 days of acclimatization (R13). Ground-based control rats were maintained for the same periods in either vivarium (R3 and R15, respectively), or flight-simulated cages (R5 and R17, respectively). RNA and protein were isolated from the tibialis anterior, biceps femoris, quadriceps, and gastrocnemius muscles. Myostatin, IGF-I, IGF-II and proteasome 2c mRNA concentrations were determined by reverse transcription/PCR; myostatin and ubiquitin mRNA were also measured by Northern blot analysis; myostatin protein was estimated by immunohistochemistry; the apoptotic index and the release of 3-methylhistidine were determined respectively by the TUNEL assay and by HPLC. Muscle weights were 19–24% lower in the R1 rats compared with the control R3 and R5 rats, but were not significantly different after the recovery period. The myostatin/β-actin mRNA ratios (means ± S.E.M.) were higher in the muscles of the R1 rats compared with the control R5 rats: 5·0-fold in tibialis (5·35 ± 1·85 vs 1·07 ± 0·26), 3·0-fold in biceps (2·46 ± 0·70 vs 0·81 ± 0·04), 1·9-fold in quadriceps (7·84 ± 1·73 vs 4·08 ± 0·52), and 2·2-fold in gastrocnemius (0·99 ± 0·35 vs 0·44 ± 0·17). These values also normalized upon acclimatization. Our antibody against a myostatin peptide was validated by detection of the recombinant human myostatin protein on Western blots, which also showed that myostatin immunostaining was increased in muscle sections from R1 rats, compared with control R3 rats, and normalized upon acclimatization. In contrast, IGF-II mRNA concentrations in the muscles from R1 rats were 64–89% lower than those in R3 animals. With the exception of the gastrocnemius, IGF-II was also decreased in R5 animals maintained in flight-simulated cages, and normalized upon acclimatization. The intramuscular IGF-I mRNA levels were not significantly different between the spaceflight rats and the controls. No increase was found in the proteolysis markers 3-methyl histidine, ubiquitin mRNA, and proteasome 2C mRNA. In conclusion, the loss of skeletal muscle mass that occurs during spaceflight is associated with increased myostatin mRNA and protein levels in the skeletal muscle, and a decrease in IGF-II mRNA levels. These alterations are normalized upon restoration of normal gravity and caging conditions. These data suggest that reciprocal changes in the expression of myostatin and IGF-II may contribute to the multifactorial pathophysiology of muscle atrophy that occurs during spaceflight.

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Introduction

Experimental animals and humans experience substantial loss of skeletal muscle mass and function during space flights (Baldwin 1996, Baldwin *et al.* 1996, Vernikos 1996, Booth & Criswell 1997, Desplanches 1997); this has emerged as a significant impediment to NASA's plans for human voyages of space exploration. Analogous but not

identical changes occur in the muscles during unloading produced by caudal suspension in animals or prolonged bed rest in humans (Ferrando *et al.* 1996, Bamman *et al.* 1998, Mozdziak *et al.* 1998, Wehling *et al.* 2000). Muscle organ cultures sent in spaceflight also demonstrate atrophy in response to microgravity (Vandenburgh *et al.* 1999).

The molecular mechanisms of sarcopenia that occurs during spaceflight are unknown. In both the spaceflight rats and the hindlimb-suspended rats, there is an early reduction in protein synthesis, accompanied by a less consistent increase in protein degradation reflected by a stimulation of the ubiquitin/proteasome pathway (Baldwin 1996, Mitch & Goldberg 1996, Both & Criswell 1997, Desplanches 1997, Stein & Schluter 1997). The astronauts and experimental animals also experience systemic changes in their hormonal milieu during spaceflight, including decreased testosterone and growth hormone levels, and increased glucocorticoid levels (Amann *et al.* 1992, Merrill *et al.* 1992, Stein & Schluter 1997, Strollo *et al.* 1998*a*,*b*). The fact that antigravity muscles are preferentially atrophied during space flights suggests that the balance of local muscle growth factors is as important in the pathophysiology of the observed sarcopenia as systemic hormonal changes.

The intramuscular concentrations of insulin-like growth factor-I (IGF-I), an important modulator of skeletal muscle growth (Husmann *et al.* 1996), have been reported to be decreased in response to hindlimb suspension, aging and glucocorticoid administration (Welle & Thornton 1997, Adam 1998, Gayan-Ramirez *et al.* 1999, Suliman *et al.* 1999), but no information is available on the changes in muscle IGF-I levels during spaceflight. Another member of the IGF family, IGF-II, is involved in muscle differentiation and regeneration (Marsh *et al.* 1997, Yoshiko *et al.* 1998, Gayan-Ramirez *et al.* 1999, Kaliman *et al.* 1999, Keller *et al.* 1999), and has been implicated in the maintenance of skeletal muscle mass in pig breeds with exceptional muscularity and strength (Jeon *et al.* 1999, Nezer *et al.* 1999). However, we do not know if IGF-II expression in the muscle is affected by spaceflight. Myostatin, a member of the transforming growth factor (TGF)-β family of growth factors, has been proposed as an inhibitor of skeletal muscle mass (McPherron *et al.* 1997, McPherron & Lee 1997, Szabo *et al.* 1998). Inactivating mutations of the myostatin gene in cattle and mice are associated with generalized increase in skeletal muscle (Kambadur *et al.* 1997, McPherron & Lee 1997, Grobet *et al.* 1998, Szabo *et al.* 1998). Similarly, mice that were made null for the myostatin gene product by homologous recombination have increased body weight (McPherron & Lee 1997). Myostatin levels are increased in the serum of HIV-infected patients with wasting (Gonzalez-Cadavid *et al.* 1998), elderly men and women with sarcopenia (Yarashesky *et al.* 1999), and in the skeletal muscle in a rat model of aging (Mallidis *et al.* 1999) and during hindlimb unloading (Baldwin *et al.* 1996). These observations have led to the speculation that increased myostatin expression might contribute to the sarcopenia observed in association with aging, chronic illness and spaceflight.

We hypothesized that the loss of muscle mass in response to microgravity might be due to a perturbation of the homeostatic balance between anabolic and catabolic factors produced locally in the muscle. We postulated that exposure to microgravity might induce the negative modulators such as myostatin and downregulate positive modulators such as IGF-I and -II, thus tilting the homeostatic balance towards loss of muscle mass. We, therefore, measured the intramuscular concentrations of myostatin, and IGF-I and -II mRNAs in rats exposed to the microgravity environment of the NeuroLab space flight, and during recovery at 1G after the animals had returned to earth (Highstein & Cohen 1999). We have compared the changes in the expression of these genes with apoptotic index and markers of muscle protein breakdown.

Materials and Methods

Experimental groups

Twelve Fischer 344 rats were sent into space aboard the NASA STS-90 NeuroLab space flight for 17 days (Highstein & Cohen 1999) and, upon return to earth, were divided into two groups of six animals each. The animals in one group, referred to as 'microgravity, non-acclimated' (R1), were killed 1 day after landing, and those in the other group, referred to as 'microgravity, acclimated', were killed 13 days after landing (R13). Weight-matched, ground-based control rats were maintained on earth, either in regular vivarium cages with a 48 h asynchronous delay ($n=12$), or in flight cages similar to those used in the space flight with a 96 h asynchronous delay ($n=12$). The asynchronous delay is the period elapsed from the launch of the mission till the time when the control animals were entered in the experimental protocol. In this way both the microgravity-exposed animals and their ground-based controls were matched for the time spent in the respective type of housing. One-half of the vivarium control rats were killed on day 3 after shuttle landing, and are referred to as 'vivarium, non-acclimated' (R3), and the remainder on day 15 after landing, and referred to as 'vivarium, acclimated' (R15). The control rats in cages similar to those used in the shuttle flight were killed either on day 5 after landing, and were referred to as 'simulation, non-acclimated' (R5), or on day 17 after landing and transfer to normal cages, named 'simulation, acclimated' (R17).

Rats were exposed to reversed 12 h:12 h light–dark cycles for 53 days before killing. All dissections were performed during the first half of the rat's dark cycle. Animals were weighed and killed at scheduled times under anesthesia, and the following skeletal muscles from the hindlegs were dissected out: tibialis anterior, biceps

femoris, quadriceps and gastrocnemius. Muscles isolated from one hindlimb were snap-frozen in liquid nitrogen and stored at −80 °C. Muscle tissue from the other hindlimb was cut into several pieces, dropped into 10% formalin, fixed for 24 h, washed in PBS, and maintained in PBS at 4 °C for subsequent immunohistochemistry.

Estimation of myostatin mRNA

Total RNA was extracted from about 100 mg muscle tissue by applying Trizol reagent. The quality of the RNA preparation was checked by agarose gel electrophoresis and ethidium bromide staining. Aliquots containing 0·1 and 0·5 μg RNA were submitted to reverse transcription (RT) with a 16-mer oligo-dT primer, as previously described (Gonzalez-Cadavid *et al.* 1998). The subsequent PCR reaction was carried out with 1/10 of the RT reaction in a total volume of 25 μl. Sense and antisense 20-mer primers (0·2 μM) were added for rat myostatin (McPherron *et al.* 1997) (nucleotides (nts) 61 and 465, respectively, in Genebank accession number AF019624), or reference genes, β-actin (nts 1552 and 2991, in Genebank accession number J00691), or 3-glyceraldehyde phosphate dehydrogenase (GADPH, nts 4219 and 4372, Ercolani *et al.* 1988). The thermal amplification of the 404 bp myostatin DNA fragments was conducted after a 3 min step of denaturation at 94 °C, by 35 cycles at 94 °C (35 s), 58 °C (45 s), and 72 °C (80 s), and a final step at 72 °C for 7 min. The 153 bp GADPH and 793 bp β-actin bands were obtained by 19 PCR cycles under the same conditions. Preliminary experiments conducted at several dilutions and times indicated that these were the optimum conditions to keep amplifications of the selected myostatin, β-actin, and GADPH DNA sequences within the linear range. Myostatin and reference gene PCR amplifications for each muscle were performed simultaneously for each individual animal. The relative intensity of the bands stained by ethidium bromide after agarose gel electrophoresis was measured by densitometry with ScanAnalysis, and values were expressed as myostatin/reference gene ratios.

In certain cases, 20 μg of total RNA were submitted to Northern blot hybridization using a 1277 bp cDNA probe obtained by PCR amplification from the cloned human myostatin cDNA (Gonzalez-Cadavid *et al.* 1998). The human probe has a 95% homology to the corresponding rat myostatin region. The filters were stripped and submitted to new hybridization with a 1233 bp cDNA probe for rat GADPH obtained by restriction enzyme digestion from the cloned fragment.

Estimation of IGF-I and -II mRNA

IGF-I and -II mRNA levels were measured by RT-PCR. Initially, IGF-I and -II cDNAs were obtained from 0·2 μg total RNA using oligo(dT)-primed reverse transcription; 1/20th of the RT reaction was submitted to PCR in the presence of five serial 10-fold dilutions starting at $8{\cdot}5 \times 10^8$ copies per reaction of competitive synthetic multiplex templates (Tarnuzzer *et al.* 1996, Pfaffl *et al.* 1998). Sequences for the IGF-I antisense and sense primers were respectively: 5′-GTAGGTCTTGTTTCC TGCAC-3′, and 5′-CACATCTCTTCTACCTGGC-3′. Sequences for the IGF-II antisense and sense primers were respectively: 5′-TCTCTGAACGCTTCGAGCTC-3′, and 5′-GAGGCTGCTTCCCGCAGCTG-3′. Amplification reactions were carried out over 40 cycles at 94 °C (1·5 min), 58 °C (2 min), and 72 °C (3 min). The IGF-I and -II product sizes were 376 and 247 bp, respectively, whereas the template product size was 517 bp. The fragments were separated on agarose gel electrophoresis and the band images were scanned using Scion Image software. The log of the ratio of band intensities within each lane was plotted against the log of the template copy number and the target mRNA copy number was determined in a region where the ratio of template and target band intensities was equal to one.

Validation of myostatin antibody and immunohistochemistry

A cDNA construct encoding the sequence corresponding to the putative myostatin 111 amino acid protein (McPherron *et al.* 1997) was prepared with an artificial start codon ATG immediately before the Arg-Arg-Asp-Phe amino acid sequence containing the precursor cleavage site, and a pentahistidine tag at the 3′ end (Taylor *et al.* 2000). This plasmid was named pDES-Mst111(H). Drosophila DES cells were transfected with these vectors and induced with Cu^{2+} (Kirkpatrick & Shatzman 1999), and cell protein was prepared as a side-product from RNA isolation with the Trizol RNA procedure. A similar protein expressed in *E. coli*, but carrying six histidines preceded by a six amino acid stretch at the amino terminus of the fusion protein instead of the carboxy-end tag was kindly provided by Dr Fred Kull of Glaxo–Wellcome Laboratories. This plasmid was named pEC-Mst111(H).

For antibody validation, aliquots of the protein extracted from the induced and non-induced transfected DES cells or the purified recombinant myostatin protein were heated at 95 °C for 5 min in a denaturing–reducing buffer containing 1% SDS and 1·25% mercaptoethanol, and analyzed on 12% polyacrylamide gels. The proteins were detected in a two-step immunodetection assay, using rabbit polyclonal antibody B against human myostatin (Gonzalez-Cadavid *et al.* 1998) at a 1:1000 dilution and immunoperoxidase-linked anti-rabbit IgG (1:2000). Bands were visualized in a luminol-based reaction on X-ray film. Other aliquots from the cell protein extract were submitted to Western blot transfer and detected by using either a primary mouse monoclonal antibody against the polyhistidine carboxy-end tag, or a single-step immunodetection with the primary antibody linked to

immunoperoxidase. For the detection of endogenous myostatin in muscle, approximately 30–50 mg of muscle tissue was homogenized in a buffer containing protease inhibitors (Gonzalez-Cadavid *et al.* 1998). SDS was added to 1%, and aliquots containing 20 mg protein were submitted to Western blots, using only anti-myostatin antibody B. In certain cases, to verify specificity, non-immune rabbit IgG was applied to sections of the membrane blots instead of the primary anti-peptide antibody, or the primary antibody was omitted.

For immunohistochemical detection of myostatin, the formalin-fixed, paraffin-embedded skeletal muscle sections (Gonzalez-Cadavid *et al.* 1998) were deparaffinized and the peroxidase activity was blocked. Sections were incubated with primary anti-myostatin antibody (1:500) overnight, and then with biotinylated linking reagent (Signet; 1:3) for 45 min, followed by diaminobenzidine for 5 min. Sections were counterstained with hematoxylin and the staining intensity was graded visually from 0 to 4+ in four adjacent sections per tissue specimen.

Proteolysis and apoptosis markers

The free 3-methylhistidine content in muscle was measured by HPLC amino acid analysis in supernates obtained by centrifugation of muscle homogenates in 20–60% perchloric acid (Tayek 1996) at 15 000 ***g*** for 10 min. Ubiquitin mRNA was determined by hybridization of the Northern blots with a maize ubiquitin cDNA probe (Kho *et al.* 1997) that has 90% homology to the rat ubiquitin homologous region (Genebank accession number U54632). Proteasome subunit 2c mRNA was determined by RT-PCR applying 20-mer primers spanning nt 241–499 in the rat sequence (Genebank accession number M29859), using the procedure described for myostatin. Densitometric values were expressed as ratios to GAPDH.

The apoptotic index (Sinha-Hikim & Swerdloff 1995) was determined by the TUNEL method, based on the ability of terminal deoxynucleotidyl transferase to catalyze template-independent addition of digoxenin-dUTP and dATP to 3′OH ends of DNA, generated by internucleosomal cleavage. The resulting heteropolymer was recognized with an antidigoxenin antibody conjugated to peroxidase. Deparaffinized sections (5 μm) were treated with proteinase K and then with H_2O_2 to quench endogenous peroxidase, followed by the primary and secondary antisera and DAB staining. Counterstaining was done with 0·5% Methyl Green. The apoptotic index was calculated by dividing the number of apoptotic cells by the number of nuclei in the microscope field.

Statistical analysis

The data are expressed as means ± S.E.M. The Wilk–Shapiro test was used to establish whether the data were normally distributed. The outcome measures in the three groups were compared by one-way analysis of variance (ANOVA). If overall ANOVA revealed significant effect, further comparisons between groups were performed by using Tukey's *post hoc* test. The difference among groups was considered significant at $P<0·05$.

Results

Effect of spaceflight on body weight and skeletal muscle mass

The body weights of rats that were sent into space and the two groups of ground-based controls were not significantly different prior to flight (mean ± S.E.M.: 355 ± 8 g for vivarium controls, 365 ± 7 g for simulated controls and 367 ± 10 g for microgravity-exposed rats). Immediately after returning to earth, the spaceflight rats weighed on average 5% less (354 ± 9 g) than the vivarium (374 ± 7 g) and simulation control (377 ± 4 g) animals. After 13 days acclimatization on the ground, spaceflight rats still weighed significantly less (349 ± 7 g) than the two groups of control animals (372 ± 4 and 372 ± 9 g, respectively).

Exposure to microgravity during spaceflight caused a significant loss of mass in all muscle groups that we studied; the percentage decrease in muscle mass exceeded the decrease in body weight (Table 1). When compared with the simulated controls, the gastrocnemius, tibialis anterior, quadriceps and biceps femoris of the spaceflight rats had significantly less mass (23·8, 20·0, 16·5 and 22·9% reduction, respectively). After 13 days of acclimatization under ground-based conditions, the weights of the four skeletal muscle groups from the spaceflight rats were not significantly different from those of the control rats, with the exception of the gastrocnemius that weighed 18·4% less than the corresponding muscle group in the simulated controls.

Intramuscular myostatin mRNA concentrations

The myostatin mRNA concentrations, measured by RT-PCR, were normalized by dividing the intensity of the amplified 393 bp myostatin DNA band by the intensity of the 764 bp β-actin band. The identity of the myostatin band was confirmed by DNA sequencing of the amplified product from one of the gels. The nucleotide sequence of the amplified DNA product of the RT-PCR reaction had 100% homology to the rat myostatin DNA sequence. Mean myostatin mRNA concentrations (Table 2) were significantly higher in the gastrocnemius, tibialis anterior, quadriceps, and the biceps femoris muscle groups from the spaceflight rats than those in the simulated controls (2·3, 5·0, 1·9 and 3·0-fold higher, respectively). After 13 days of acclimatization, myostatin mRNA concentrations were normalized in three muscle groups of the spaceflight rats to values not significantly different from those in the

Table 1 Effect of microgravity on skeletal muscle mass

		Weight (g)			Percentage change
	Series	CV	CS	MG	MG/CS
Muscle					
Gastrocnemius	NA	2·02 ± 0·08[a]	2·14 ± 0·05[a]	1·63 ± 0·09[b]	− 23·8
	A	2·40 ± 0·10[a]	2·44 ± 0·17[a]	1·99 ± 0·16[b]	− 18·4
Tibialis anterior	NA	0·66 ± 0·04[a]	0·65 ± 0·02[a]	0·52 ± 0·01[b]	− 20·0
	A	0·72 ± 0·03	0·69 ± 0·04[a]	0·66 ± 0·02	− 4·3
Quadriceps	NA	3·90 ± 0·12[a]	3·76 ± 0·32[a]	3·14 ± 0·19[b]	− 16·5
	A	3·84 ± 0·20	3·98 ± 0·50	4·10 ± 0·20	+3·0
Biceps femoris	NA	2·88 ± 0·25[a]	2·84 ± 0·26[a]	2·19 ± 0·27[b]	− 22·9
	A	3·84 ± 0·13	3·55 ± 0·24	3·21 ± 0·20	− 9·6

Data are means ± S.E.M. Significantly different values ($P<0·05$) in paired comparisons between two groups within either the non-acclimated (NA) or acclimated (A) series are denoted by different letters (a vs b). CV, vivarium control rats; CS, simulated control rats; MG, microgravity-exposed rats.

Table 2 Effect of microgravity on myostatin mRNA concentrations in the muscle estimated by RT-PCR. The amplified cDNA fragments were separated by agarose gel electrophoresis and the intensities of the myostatin bands were corrected by the intensities of the respective β-actin bands

		Mst/β-actin ratios			Ratio:
	Series	CV	CS	MG	MG/CS
Muscle					
Gastrocnemius	NA	0·58 ± 0·07[a]	0·44 ± 0·17[a]	0·99 ± 0·35[b]	2·3
	A	0·80 ± 0·16[a]	1·39 ± 0·17[b]	0·66 ± 0·12[a]	0·5
Tibialis anterior	NA	1·35 ± 0·20[a]	1·07 ± 0·26[a]	5·35 ± 1·85[b]	5·0
	A	0·95 ± 0·18	1·09 ± 0·30	1·11 ± 0·09	1·0
Quadriceps	NA	6·28 ± 0·40[b]	4·08 ± 0·52[a]	7·84 ± 1·73[b]	1·9
	A	5·42 ± 0·54	7·30 ± 2·01	8·67 ± 1·57	1·2
Biceps femoris	NA	0·85 ± 0·04[a]	0·81 ± 0·04[a]	2·46 ± 0·70[b]	3·0
	A	0·65 ± 0·10	0·45 ± 0·04	0·52 ± 0·07	1·1

Data are means ± S.E.M. Significantly different values ($P<0·05$) in paired comparisons between two groups within either the non-acclimated or acclimated series are denoted by different letters (a vs b).

simulated controls; the exception was the gastrocnemius, in which myostatin mRNA concentrations in the microgravity-exposed rats were lower.

The myostatin mRNA concentrations in the tibialis anterior were also determined by Northern blot analysis (Fig. 1, left) and, when expressed as the ratio of the intensities of the myostatin and the GAPDH band, were on average 1·7-fold higher in spaceflight rats compared with vivarium controls.

Myostatin-immunoreactive protein concentrations in the skeletal muscle assessed by immunohistochemical staining

The myostatin-immunoreactive protein concentrations in the muscle cross-sections were measured by a semi-quantitative immunohistochemical procedure. As a first step, it was essential to extend the initial characterization of the specificity of the anti-myostatin antibody B (Gonzalez-Cadavid *et al.* 1998) in terms of its ability to detect recombinant human myostatin proteins. A myostatin cDNA construct that encodes the 111 amino acid carboxy-terminal protein claimed to be the mature myostatin (McPherron *et al.* 1997) was prepared in a *Drosophila* expression vector. Upon transfection into the homologous DES cells, this vector allows for a more faithful processing of recombinant animal proteins than bacterial systems, without some of the disadvantages of the baculovirus system (Taylor *et al.* 2000). The product from this cDNA construct is a fusion protein that carries a penta-histidine tag at its carboxy-terminus. Induced DES cells had the expected 1·2 kb myostatin mRNA transcribed from this construct (data not shown). The homologous 111 amino acid protein, expressed in *E. coli* and carrying the penta-histidine tag at its amino-terminus, was also tested.

In Western blots performed under denaturing and reducing conditions, our antibody B detected the 111 amino acid myostatin protein made in *E. coli* (Fig. 2, top); increasing amounts of this recombinant protein resulted in a proportionate increase in the intensity of a 17 kDa band,

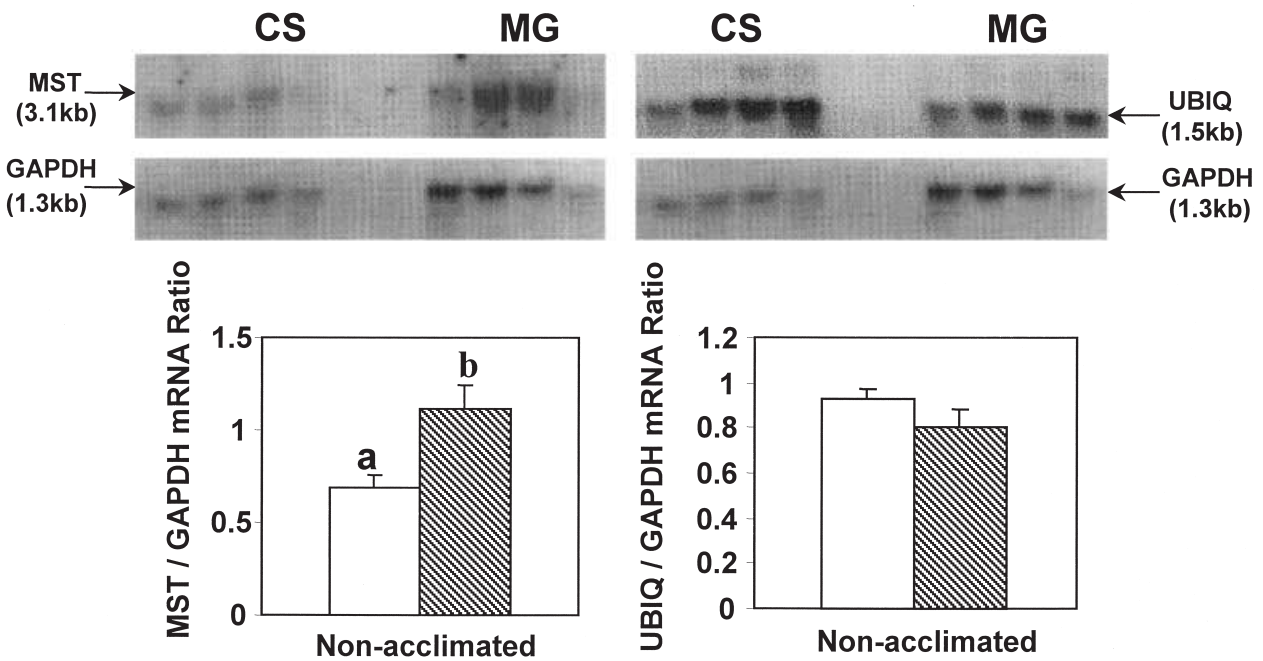

Figure 1 Effect of microgravity on myostatin (MST) and ubiquitin (UBIQ) mRNA levels in the tibialis anterior muscle measured by Northern blot analysis. RNA (20 μg) was run on 1·2% denaturing PAGE gels and subjected to Northern blot hybridization with ^{32}P-labeled cDNA probes for myostatin and glyceraldehyde phosphate dehydrogenase (GAPDH), followed by autoradiography (top panel, left). The Northern blot was stripped and rehybridized with a probe for ubiquitin (top panel, right). The respective densitometric values are shown in the bottom panel. Data are means ± S.E.M. a,b, $P<0·05$. MG, microgravity-exposed rats; SC, control rats in flight simulated cages.

which appears by its size to correspond to the monomer with the polyhistidine tag. This 17 kDa band is completely absent in the skeletal muscle preparations, which show only the 28–30 kDa band (Fig. 2, bottom). Surprisingly, a 28 kDa band is also visible in the pure recombinant protein lanes (Fig. 2, top), overlapping the endogenous 28–30 kDa band when the recombinant protein is added to the muscle extracts (Fig. 2, bottom). This endogenous band, together with a 49 kDa band, presumably corresponding to the monomeric myostatin precursor, was detected in rat skeletal muscle homogenates, but no traces of the 14–17 kDa bands seen with the recombinant myostatin were found in these preparations (Fig. 2, bottom right). The 28 kDa band detected by the antibody in skeletal muscle preparations could not be dissociated in 6 M urea (not shown). This suggests the formation of a recombinant myostatin dimer that does not fully dissociate inspite of the denaturing and reducing conditions employed in the Western blot, and that is slightly smaller than the 28–30 kDa band observed in skeletal muscle extracts, as should be the case if the endogenous protein is glycosylated (Gonzalez-Cadavid *et al.* 1998).

The electrophoretic behavior of the recombinant myostatin protein is not an artifact of the *E. coli* preparation, as shown by the detection with antibody B of identical bands in the extracts from *Drosophila* cells transfected with the cDNA encoding the 111 amino acid myostatin protein with a carboxy-terminus histidine tag. The 28 kDa band is visible in induced cells and absent in the uninduced cells (Fig. 2, middle right). This 28 kDa band is also detected with the anti-His antibody (middle left), establishing that the 28 kDa band is related to the product of the cDNA encoding the 111 amino acid myostatin carboxy-terminal protein, and may originate either by its spontaneous dimerization or its association with other protein(s).

These data validated our antibody B that was then applied to tissue sections from the tibialis anterior from the spaceflight rats (Fig. 3, top left), showing that the immunoreactivity was more intense than in the same muscle of a simulation control rat (Fig. 3, top right). The immunostaining became undetectable in the muscle from another spaceflight rat that was acclimated on the ground for 13 days (Fig. 3, bottom left); and was even lower than that in an acclimated simulation control rat (Fig. 3, bottom right). A semi-quantitative assessment based on visual inspection of three to four adjacent sections per muscle specimen with multiple observations to cover the entire field, and grading from 0 to 4 +, demonstrated a significant increase in myostatin immunoreactivity in four non-acclimated microgravity-exposed animals compared with the corresponding four simulated controls (2·5 ± 0·3 vs

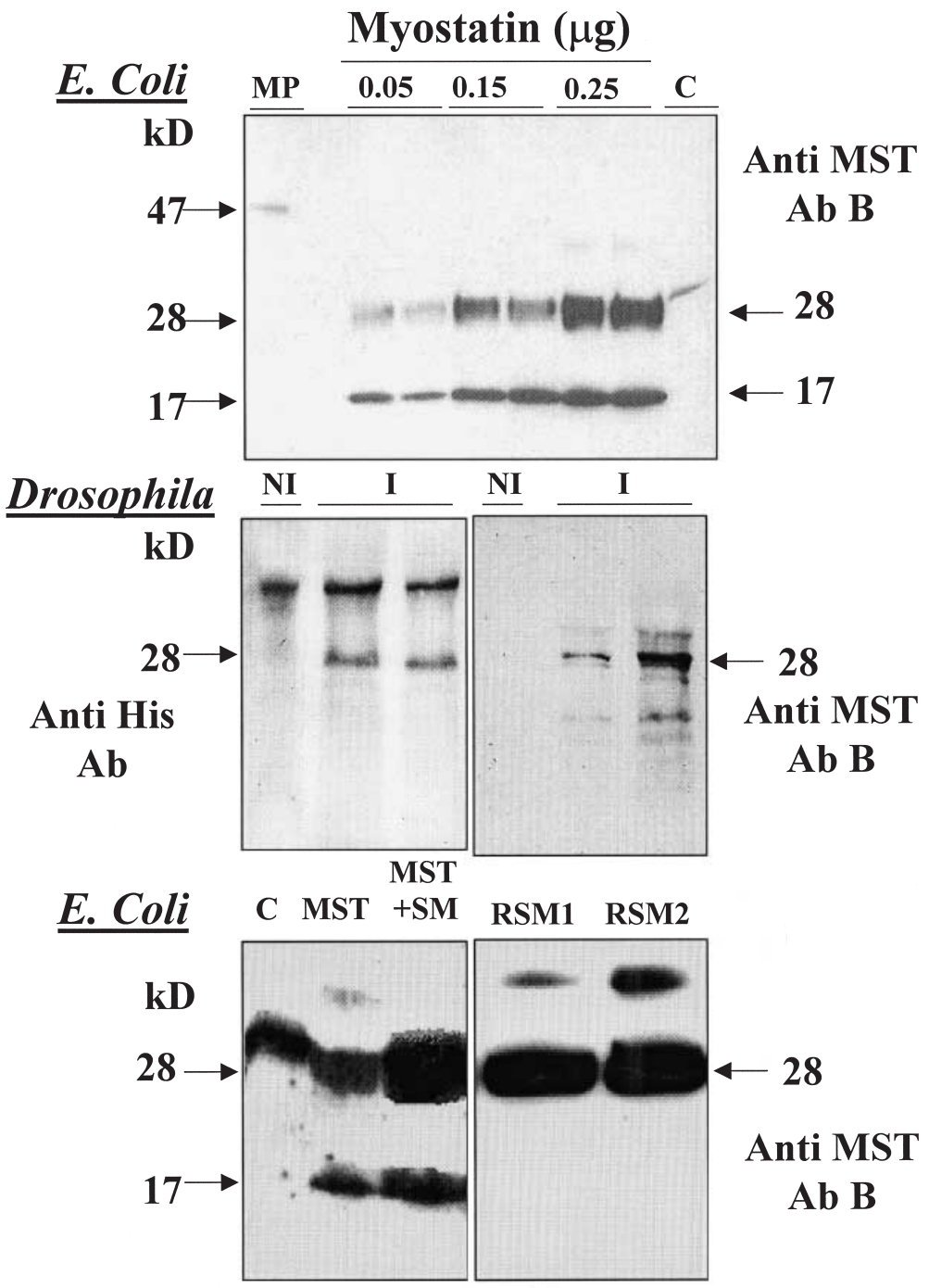

Figure 2 Validation of antibody B against a recombinant 111 amino acid, myostatin carboxy-terminal protein. Top panel: increasing amounts of a purified 111 amino acid myostatin carboxy-terminal protein made in *E. coli*, and carrying an amino-terminus-linked polyhistidine tag, were electrophoresed on 12·5% polyacrylamide gels. Western blots were performed with antibody B. MP, recombinant 375 amino acid myostatin protein produced in DES cells. C: control mouse skeletal muscle extract. Middle panel, a 111 amino acid myostatin protein containing a carboxy-terminus-linked polyhistidine tag was expressed in DES cells. Proteins were submitted to the same treatments, except that in addition to the Western blot with antibody B (right), incubation with polyHis antibody was applied (left). NI, non-induced; I, induced. Bottom panels, the 111 amino acid, myostatin protein was subjected to electrophoresis by itself (MST) or added to a mouse skeletal muscle preparation (MST+SM) before electrophoresis. C, control skeletal muscle extract alone. In another gel, two homogenates from rat tibialis anterior (RSM 1 and 2) were run separately.

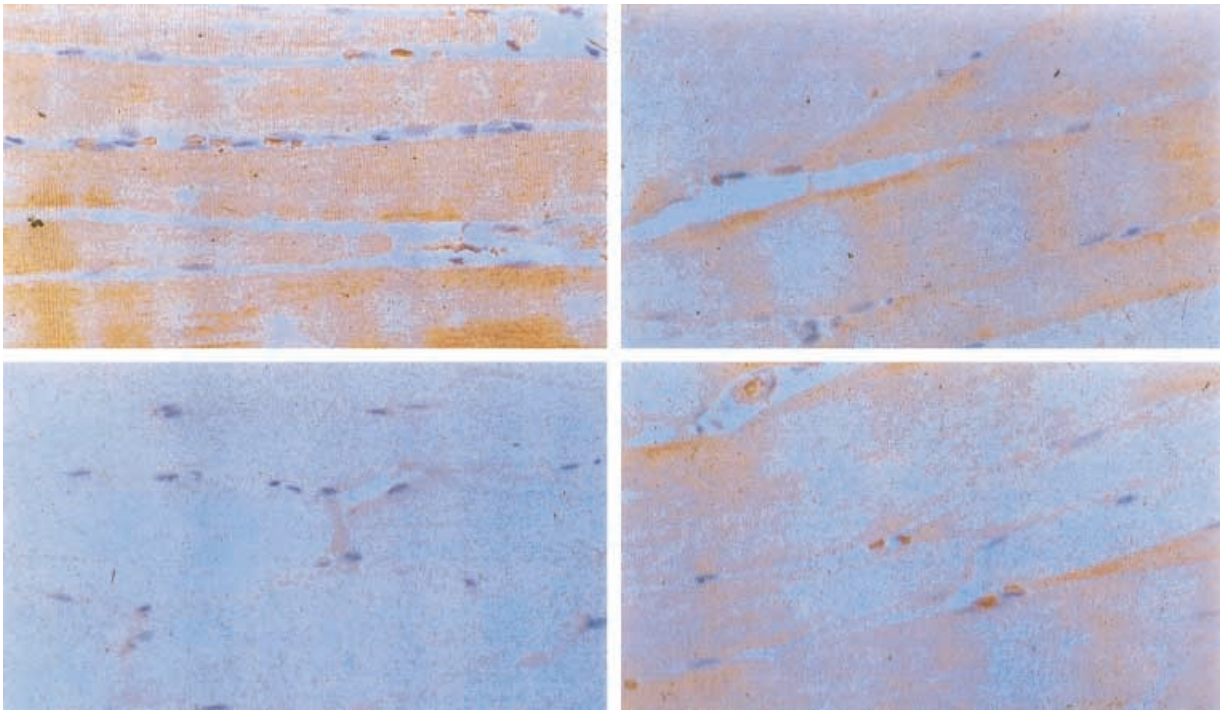

Figure 3 Immunohistochemical estimation of myostatin in skeletal muscle sections from rat exposed to microgravity. Representative sections of the tibialis anterior are shown on the top and bottom panels. Top left: microgravity-exposed animals, non-acclimated; top right: simulated control animal, non-acclimated; lower left: microgravity-exposed animal, acclimated; lower right: simulated control animal, acclimated.

1·25 ± 0·3). The same trend was observed in the gastrocnemius, but it was not significant. The staining intensity in the muscles from the rats exposed to microgravity was significantly reduced upon acclimatization in comparison with the simulated controls (0·75 ± 0·2 vs 1·75 ± 0·2 in both cases). The other two muscles were not studied.

Muscle IGF-I and -II mRNA concentrations

The IGF-I and -II mRNA concentrations were measured by a RT-PCR procedure (Ercolani *et al.* 1988) based on competition with an internal template, which yields an absolute IGF mRNA copy number per μg of total RNA. IGF-I mRNA concentrations in tibialis anterior were not significantly different between the spaceflight and vivarium control rats ($24{\cdot}8 \pm 12 \times 10^{11}$ vs $29{\cdot}7 \pm 8 \times 10^{11}$ copies), but were lower in the simulated cage control animals ($16{\cdot}2 \pm 9 \times 10^{11}$) when compared with the control vivarium animals.

The IGF-II mRNA concentrations (Table 3) were significantly lower in the gastrocnemius, tibialis anterior, quadriceps and biceps femoris of the non-acclimated spaceflight rats than those in the vivarium controls (mean decrease 64–89%), but upon acclimatization were restored to normal values. In order to minimize differences due to interassay variability, all values are expressed in comparison with an intrassay control: the non-acclimated vivarium control rats. The IGF-II mRNA concentrations in the control vivarium animals varied according to muscle type, in the following order: tibialis anterior>biceps femoris>quadriceps>gastrocnemius. The IGF-II mRNA concentrations in the muscles of rats in simulated cages were also significantly lower than those in vivarium controls in all muscle groups except for the gastrocnemius. There were no significant differences in IGF-II mRNA concentrations between the three groups after 13 days of acclimatization on the ground.

Markers of protein breakdown

To determine whether the microgravity-associated loss of muscle mass is due to increased proteolysis through the ubiquitin–proteasome pathway, we measured the ubiquitin mRNA concentrations by Northern blot analysis and proteasome 2C mRNA by quantitative RT-PCR. The relative concentrations of the 2·6 and 1·2 kb ubiquitin mRNA transcripts (Fig. 1, right) were not significantly different between the non-acclimated spaceflight rats and ground-based vivarium controls. The proteasome 2C mRNA concentrations normalized by using GADPH as the reference gene, also did not differ between the flight rats and the vivarium controls (data not shown).

Table 3 Effect of microgravity on IGF-II mRNA levels in the muscle estimated by a competitive RT-PCR procedure

	Series	CV	CS	MG	Ratio: MG/CV
Muscle					
Gastrocnemius	NA	1·00 ± 0·20[a]	1·55 ± 0·03[a]	0·11 ± 0·03[b]	0·11*
	A	1·17 ± 0·21	0·98 ± 0·22	1·58 ± 0·19	1·35
Tibialis anterior	NA	1·00 ± 0·05[a]	0·19 ± 0·17[b]	0·17 ± 0·04[b]	0·17*
	A	1·07 ± 0·10	0·81 ± 0·08	0·90 ± 0·07	0·85
Quadriceps	NA	1·00 ± 0·26[a]	0·24 ± 0·04[b]	0·33 ± 0·08[b]	0·33*
	A	1·48 ± 0·41	1·88 ± 0·50	1·46 ± 0·37	0·99
Biceps femoris	NA	1·00 ± 0·31[a]	0·10 ± 0·02[b]	0·36 ± 0·14[b]	0·36*
	A	1·46 ± 0·46[a]	0·50 ± 0·08[b]	1·54 ± 0·35[a]	1·05

Values are expressed as relative amounts of IGF-II mRNA per total RNA referred to the non-acclimated control vivarium values in each muscle. Data are means ± S.E.M. Significantly different values ($P<0·05$) in paired comparisons between two groups within either the non-acclimated or acclimated series are denoted by different letters (a vs b), or with asterisks (ratios).

In agreement with these observations, the free 3-methylhistidine concentrations in the muscle, a marker of muscle protein breakdown, were not significantly different in the muscle from the non-acclimated spaceflight rats compared with their vivarium controls (0·27 ± 0·17 vs 0·23 ± 0·13 μmol/g tissue in the quadriceps). Anserine, a methylhistidine derivative, was present in the muscle at 10- to 20-fold higher concentrations, but we found no significant difference between the spaceflight and vivarium control rats, even when anserine concentrations were taken into account.

The apoptotic index, assessed by the TUNEL method, was very low in muscles of the control rats and did not exhibit any measurable increase in spaceflight rats (data not shown).

Discussion

Consistent with published data from previous missions, the rats from the NeuroLab space flight experienced significant loss of muscle mass. Our data demonstrate that this process is associated with an increase in the intramuscular myostatin mRNA concentrations and a decrease in IGF-II mRNA concentrations. The muscle mRNA concentrations of IGF-I, a more widely recognized anabolic growth factor, did not significantly change. The markers of muscle protein breakdown (3-methylhistidine, ubiquitin and proteasome 2C), and the apoptotic index, were not significantly increased in the skeletal muscle of the spaceflight rats. We postulate that the ratio between IGF-II and myostatin mRNA levels may serve as an indicator of the homeostatic balance that maintains skeletal muscle mass; a low IGF-II to myostatin mRNA ratio would reflect the alterations in muscle homeostasis that favor the loss of muscle mass in response to microgravity exposure, confinement, or other factors operating during spaceflight.

Because of the cross-sectional nature of the study and the fact that the tissue samples were obtained only at one time point after landing, we do not know whether the changes in myostatin and IGF-II mRNA concentrations were the cause or the result of the spaceflight-related sarcopenia. These changes in gene expression paralleled the decline in muscle weight and were normalized after acclimatization at ground level, suggesting that alterations in the levels of both muscle modulators might play a role in the pathogenesis of muscle wasting occurring during a spaceflight.

The changes in body weight and the weights of most skeletal muscles in the NeuroLab rats were within the range observed in other missions of the same duration (Martin *et al.* 1988, Baldwin *et al.* 1990, 1993, Chi *et al.* 1992); however, the decrease in the tibialis anterior weight during this space flight was somewhat higher than the 7% change observed in previous flights (Baldwin *et al.* 1993). A 35% decrease in fiber size for tibialis anterior has been reported in one mission (Martin *et al.* 1988).

The pathophysiology of skeletal muscle loss during spaceflight is multifactorial. The loss of gravitational pull during spaceflight is an important contributor to this process, but stress, vibration, exposure to cosmic radiation, confinement, and changes in food intake due to stress and motion sickness may also play a role. The changes in myostatin mRNA and IGF-II expression observed in this experiment could be due to the effects of one or more of these factors that are that are inevitable concomitants of spaceflight, including microgravity.

The ground-based models of microgravity provide greater flexibility and convenience, but do not replicate all the factors associated with spaceflight. In addition, rats use only the base of the simulated cage on the ground, whereas they move along all six surfaces of the cage during space flights. Therefore experimentation in space flights is necessary to examine the effects of this multifactorial process

even if constrained by the limited number of animals that can be studied and the inability to obtain samples at different time points.

The occurrence of generalized skeletal muscle hypertrophy in cattle and mice with inactivating mutations of the myostatin gene (Kambadur *et al.* 1997, McPherron *et al.* 1997, McPherron & Lee 1997, Grobet *et al.* 1998, Szabo *et al.* 1998) suggests that myostatin is a determinant of skeletal muscle mass. The increase in myostatin mRNA and protein expression in the muscles of the spaceflight rats in association with the reduction in muscle mass, and the subsequent normalization of both variables upon re-acclimatization to ground conditions, is consistent with the hypothesis that myostatin is an inhibitor of skeletal muscle growth in adult animals. Our data agree with the increase in myostatin mRNA in the gastrocnemius (Carlson *et al.* 1999) and plantaris (Wehling *et al.* 2000) muscles of rats during hindlimb suspension and with the normalization of these levels in the plantaris after reloading. The only exception to this consistent inverse correlation of myostatin mRNA levels to muscle mass is the moderate increase in myostatin mRNA we have observed in the gastrocnemius of ground-based control rats after confinement in the flight simulated cages and subsequent acclimatization. In turn, the myostatin-immunoreactive protein levels are higher in the serum and muscle of patients with the AIDS wasting syndrome or aging-related frailty than in healthy controls (Gonzalez-Cadavid *et al.* 1998, Yarashesky *et al.* 1999). Serum myostatin-immunoreactive protein concentrations correlate inversely with fat-free mass in adult humans. Taken together, these data suggest, but do not prove, that upregulation of the myostatin gene might contribute to muscle loss in postnatal life.

We observed only a modest change in IGF-I mRNA concentration in the muscle from the non-acclimated spaceflight rats that did not achieve statistical significance. IGF-I is an important regulator of muscle growth that inhibits apoptosis (Wingertzahn *et al.* 1998) and promotes cell replication and differentiation, and protein synthesis (Bark *et al.* 1998). A single previous study reported significant reduction in serum IGF-I levels during microgravity exposure (Strollo *et al.* 1998). The small sample size may have constrained our ability to detect a modest, but significant decrease in IGF-I mRNA expression.

In contrast to IGF-I, the IGF-II mRNA concentrations in all the muscle groups of the spaceflight rats were markedly lower in comparison with the vivarium controls. IGF-II mRNA concentrations were also decreased in the simulated controls, although this was not accompanied by a parallel reduction in muscle mass. It is possible that the reduction of IGF-II may be related to restriction of locomotion in the simulated cages. IGF-II regulates muscle cell differentiation through myogenic signaling pathways (Marsh *et al.* 1997, Yoshiko *et al.* 1998, Gayan-Ramirez *et al.* 1999, Kaliman *et al.* 1999, Keller *et al.* 1999). Recently, a region on the short arm of chromosome 2 containing the locus for the IGF-II gene revealed highly significant lod scores for three of the five phenotypes in pig breeds characterized by increased muscularity and leanness (Jeon *et al.* 1999, Nezer *et al.* 1999). IGF-II expression has also been associated with skeletal muscle development in double muscled cattle (Keller *et al.* 1999) and myofiber regeneration (Marsh *et al.* 1997, Keller *et al.* 1999). Our data along with these published observations suggest that IGF-II may be an important regulator of muscle mass in the rat. *In vitro* studies suggest that these effects may be mediated by the activation of satellite cells (Johnson & Allen 1995, Petrik *et al.* 1999) and/or the regulation of apoptosis (Petrik *et al.* 1999).

The absence of a significant change in the apoptotic index or in proteolytic markers in the muscle of rats exposed to microgravity contrasts with other models of wasting where these processes have been linked to muscle loss (Mitch & Goldberg 1996, Allen *et al.* 1997, Wingertzahn *et al.* 1998). Although apoptosis has been shown to contribute to the remodeling of skeletal muscle in response to hindlimb unweighting (Allen *et al.* 1997), there are no reports on the apoptotic index in the muscle of animals sent on space flights. Although we did not observe any change in the apoptotic index and proteolysis markers in the NeuroLab rats at the time point we studied, it is plausible that significant changes in muscle protein breakdown and apoptosis might have occurred earlier, preceding the observed change in muscle mass. Our data are consistent with a previous study that showed that 3-methylhistidine excretion (Stein & Schluter 1997), an indicator of myofibril degradation, is not enhanced after a 2 week space flight. It is possible that skeletal muscle loss during spaceflight might be the result of the inhibition of protein synthesis (Baldwin 1996, Caiozzo *et al.* 1996, Both & Criswell 1997, Desplanches 1997). We have shown that recombinant human myostatin protein significantly inhibits protein synthesis in C2C12 myoblasts and myotubes without affecting protein degradation rates or apoptosis (Taylor *et al.* 2000)

In summary, the loss of muscle mass during spaceflight is associated with increased myostatin and decreased IGF-II expression; these alterations are normalized upon restoration of normal gravity and caging conditions. The proposal that an alteration in the expression of myostatin and IGF-II may contribute to the multifactorial pathophysiology of sarcopenia, needs to be addressed prospectively in suitable animal models. Our data suggest that the products of the myostatin and IGF-II genes might provide potential targets for the development of counter measures for the prevention of muscle loss during space flights.

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