Coordinate activation of lysosomal, Ca^{2+} -activated and ATP-ubiquitin-dependent proteinases in the unweighted rat soleus muscle

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Nine days of hindlimb suspension resulted in atrophy (55%) and loss of protein (53%) in rat soleus muscle due to a marked elevation in protein breakdown (66%, $P < 0.005$). To define which proteolytic system(s) contributed to this increase, soleus muscles from unweighted rats were incubated in the presence of proteolytic inhibitors. An increase in lysosomal and Ca^{2+} -activated proteolysis (254%, $P < 0.05$) occurred in the atrophying incubated muscles. In agreement with the measurements *in vitro*, cathepsin B, cathepsins B+L and m-calpain enzyme activities increased by 111%, 92% and 180% ($P < 0.005$) respectively in the atrophying muscles. Enhanced mRNA levels for these proteinases ($P < 0.05$ to $P < 0.001$) paralleled the increased enzyme activities, suggesting a transcriptional regulation of these enzymes. However, the lysosomal and Ca^{2+} -dependent proteolytic pathways accounted for a minor part of total proteolysis in both control (9%) and unweighted rats

(18%). Furthermore the inhibition of these pathways failed to suppress increased protein breakdown in unweighted muscle. Thus a non-lysosomal Ca^{2+} -independent proteolytic process essentially accounted for the increased proteolysis and subsequent muscle wasting. Increased mRNA levels for ubiquitin, the 14 kDa ubiquitin-conjugating enzyme E2 (involved in the ubiquitylation of protein substrates) and the C2 and C9 subunits of the 20 S proteasome (i.e. the proteolytic core of the 26 S proteasome that degrades ubiquitin conjugates) were observed in the atrophying muscles ($P < 0.02$ to $P < 0.001$). Analysis of C9 mRNA in polyribosomes showed equal distribution into both translationally active and inactive mRNA pools, in either unweighted or control rats. These results suggest that increased ATP-ubiquitin-dependent proteolysis is most probably responsible for muscle wasting in the unweighted soleus muscle.

INTRODUCTION

Microgravity results in a particular adaptation of the organism to a non-pathological situation of muscle disuse. Total nitrogen balance becomes negative in parallel with a loss of muscle protein, and muscle strength decreases during space missions [1]. However, the cost, complexity and limitations of studies performed in space have contributed to the development of alternative models of microgravity. Simulated weightlessness by hindlimb unweighting of rats is probably the best Earth-based model available for studying the metabolic disorders that occur during spaceflight [1–6]. Indeed, structural and metabolic properties of skeletal muscles are rapidly and markedly altered during hindlimb suspension (HS), and these modifications are rather similar to those observed during real weightlessness [6].

Antigravity slow-twitch muscles, such as the soleus, are preferentially atrophied during microgravity experiments. Studies both *in vivo* and *in vitro* have provided strong evidence that increased protein breakdown is mainly responsible for the soleus muscle wasting of non-weight-bearing animals [3,5–7], although there is also a decrease in protein synthesis [1,3–5]. However, the role of the different muscle proteolytic pathways during unweighting is poorly documented, as in other catabolic states [8], and contradictory results have been reported. Increased activities of cathepsin B in the extensor digitorum longus (EDL) muscle and of cathepsin D in both soleus and EDL muscles from hindlimb-suspended rats have been observed [3]. By contrast,

Tischler and co-workers [7] found no evidence for increased lysosomal proteolysis in the soleus muscle, but suggested that the increase in proteolysis between days 2 and 3 of HS was totally attributable to Ca^{2+} -activated neutral proteinases. However, the activation of either lysosomal or Ca^{2+} -dependent proteinases, or both, is unlikely to explain this muscle wasting. In many catabolic states the lysosomal and/or the Ca^{2+} -dependent proteolytic pathways account for only a minor part of increased skeletal muscle proteolysis [9–13], and the major myofibrillar proteins (actin and myosins) are not degraded by either process [10,14,15]. There is now strong evidence that the ATP-ubiquitin-dependent proteolytic pathway is the primary system responsible for the breakdown of long-lived skeletal muscle proteins, including the major contractile components [10,15]. Ubiquitin (Ub) binds covalently to protein substrates and marks them for degradation by the 26 S (1500 kDa) proteasome complex [16]. Multi-ubiquitylation of protein substrates requires ATP, the Ub-activating enzyme (E1) and specific Ub-conjugating enzymes (E2s), which are sometimes dependent on Ub protein ligase (E3s). The functioning of the 26 S proteasome, which contains a proteolytic core responsible for multiple peptidase activities (i.e. the 20 S proteasome [17]) and several regulatory components, also requires ATP [16]. To our knowledge, the role of the ATP-Ub-dependent proteolytic pathway has not been investigated in unweighted muscles, although both simulated and real weightlessness have resulted in an increase in Ub-protein conjugates in the rat soleus, EDL and adductor longus muscles [18–20].

Abbreviations used: 14 kDa E2, 14 kDa ubiquitin-conjugating enzyme E2; EDL, extensor digitorum longus muscle; HS, hindlimb suspension; Ub, ubiquitin.

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The aims of these studies were (1) to clarify the role of the lysosomal and Ca^{2+} -dependent proteinases in muscle wasting observed in the unweighted rat soleus muscle, and (2) to determine whether the Ub-proteasome proteolytic pathway is activated in such conditions. For this purpose we used complementary but different approaches, including measurements of protein breakdown *in vitro* with and without proteolytic inhibitors, and determinations of enzyme activities and of mRNA levels for critical components of the different proteolytic processes. We also studied the distribution of the C9 proteasome subunit mRNA in polyribosomes.

MATERIALS AND METHODS

Animals and experimental design

The experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Research Council. Male Wistar rats (Iffa-Credo, L'Arbresle, France) of average body weight 120 g were randomly assigned to either a control or a hindlimb-suspended group. After 4 days of standard housing, unweighted rats were suspended by the tail as previously described [4]. All animals were maintained in a temperature-controlled room ($22 \pm 1^\circ\text{C}$) with a 12:12 h light:dark cycle. Unweighted animals were killed by cervical dislocation after 9 days of treatment and compared with age-matched controls.

Muscle atrophy and protein content

Soleus muscles were carefully dissected and weighed. Total protein contents were determined by the bicinchoninic acid procedure [21]. In another group of animals, soluble and myofibrillar proteins were extracted as previously described [22]. Briefly, muscles were homogenized in a low-salt buffer (0.1 M KCl, 10 mM Tris/maleate, pH 7.0, 1 mM EGTA, 2 mM MgCl_2 , 0.5 mM dithiothreitol) and centrifuged at 1500 g for 5 min at 4°C . The pellet, containing the myofibrillar fraction, was washed twice in the same buffer. The soluble fraction was purified from the first supernatant by centrifugation at 100000 g for 1 h at 4°C . The soluble proteins were then pelleted by 10% (w/v) trichloroacetic acid precipitation. The myofibrillar and soluble fractions were resuspended with 2% (w/v) SDS/0.2 mM dithiothreitol and 1 M NaOH respectively for subsequent determinations of protein content.

Rates of protein breakdown

Soleus muscles were carefully dissected and incubated at resting length by maintaining their tendons on stainless steel bows. All muscles were incubated at 37°C in Krebs-Henseleit buffer equilibrated and maintained under pressure with O_2/CO_2 (19:1), containing 5 mM glucose, 0.1 i.u./ml insulin, 0.17 mM leucine and 0.10 mM isoleucine, with 0.20 mM valine to improve protein balance [23]. After 1 h of preincubation, the muscles were transferred to fresh media of identical composition and incubated for a further 2 h. Muscle viability was assessed by measuring ATP, phosphocreatine and lactate concentrations in either pre-incubated and incubated or freshly excised muscles, as previously described [24].

Rates of protein breakdown were measured by following the rates of tyrosine release into the media in the presence of cycloheximide [23]. Because muscle can neither synthesize nor degrade this amino acid, tyrosine release reflects the breakdown of proteins. Tyrosine was assayed by the fluorimetric method of Waalkes and Udenfriend [25]. Non-lysosomal and Ca^{2+} -inde-

pendent proteolysis was measured in the presence of 10 mM methylamine and 50 μM E-64c in a Ca^{2+} -free medium [11–13].

Enzyme activities

Cathepsin B and L activities were measured in freshly excised soleus muscle by determining benzyloxycarbonyl-Phe-Arg-7-(4-methyl)-coumarylamide (cathepsin B+L) and benzyloxycarbonyl-Arg-Arg-7-(4-methyl)-coumarylamide (cathepsin B) hydrolysing activities of lysosomal extracts, as previously described for rat muscles [26].

The tibialis anterior muscle was used for the determination of μ - and m-calpain activities because these measurements need large samples (more than 500 mg fresh weight). This muscle also exhibited a significant weight loss (10%, $P < 0.05$) after 9 days of suspension. μ - and m-calpains were purified and the proteolytic activities were measured, with fluorescein isothiocyanate-labelled casein as substrate, as previously described [27].

Northern blot analysis

Soleus muscles from control and hindlimb-suspended rats were rapidly excised, frozen in liquid nitrogen and stored at -80°C . Total RNA was extracted as described by Chomczynski and Sacchi [28]. Total RNA (10 μg) was electrophoresed in 1% (w/v) agarose gels containing formaldehyde. RNA was electrophoretically transferred to a nylon membrane (GeneScreen) and covalently bound to the membrane after UV cross-linking. The membranes were hybridized with cDNA probes encoding chicken polyUb [29], rat 14 kDa Ub-conjugating enzyme E2 (14 kDa E2) [30], C2 and C9 rat proteasome subunits [31,32], human cathepsin D [33] and human m-calpain [34]. The hybridizations were performed at 65°C with [^{32}P]cDNA fragments labelled by random priming (Ready to go, Pharmacia, Uppsala, Sweden), as previously described [12]. After washings at the same temperature, the filters were autoradiographed for 3–48 h at -80°C with intensifying screens on Hyperfilm βmax films (Amersham, Bucks., U.K.). cDNA inserts encoding rat cathepsin B [35] and murine cathepsin L [36] were sub-cloned into *EcoRI* and *PstI* sites of pGem-blue (Promega, Madison, WI, U.S.A.), respectively. The plasmids were linearized by *HindIII* (cathepsin B) and *PvuII* (cathepsin L), and [^{32}P]cRNA were synthesized with T7 RNA polymerase. Hybridizations with cathepsins B and L [^{32}P]cRNA were performed as previously described [37]. After stripping of the different probes, the filters were reprobbed with a cDNA fragment encoding β -myosin, whose expression is not altered after 7 days of HS [5]. Autoradiographic signals were quantified in arbitrary units by digital image processing and analysis (NIH Image 1.54, Apple Computer, Cupertino, CA, U.S.A.), and normalized with the corresponding β -myosin signals to correct for variations in RNA loading. Preliminary experiments showed that the intensity of the mRNAs was proportional to the amount of total RNA loaded and to the duration of exposure of the films.

Polyribosome fractionation

Polyribosome profiles were obtained by using a modified protocol of Buckingham et al. [38]. All manipulations were carried out at $0-4^\circ\text{C}$. Pooled soleus muscles ($n = 4-6$) from control and unweighted animals were frozen in liquid nitrogen and then homogenized in 3 volumes of Buffer A (80 mM Tris/HCl, pH 7.4, 10 mM MgCl_2 , 250 mM KCl, 7 mM 2-mercaptoethanol, 0.18 mM cycloheximide) with a Polytron (Kinematica, Lucerne, Switzerland) operated at half speed. The homogenate was then centrifuged at 4°C for 10 min at 4000 g. The supernatant was

placed in a new tube and the pellet was gently resuspended in 1 volume of Buffer B [Buffer A plus 1 % (w/v) Triton X-100, 0.5 % Na⁺-deoxycholate] and incubated for 5 min. The resuspended pellet was centrifuged at 4 °C for 10 min at 4000 *g*, and the resulting supernatants pooled. The pooled supernatants were centrifuged at 4 °C for 20 min at 20000 *g*. The resulting supernatant was layered onto 33 ml of 15–45 % (w/w) sucrose gradient containing a 2 ml cushion of 45 % sucrose in buffer A. The gradients were centrifuged for 4 h at 140000 *g* at 4 °C in a Beckman SW28 rotor (Beckman Instruments, Inc., Palo Alto, CA, U.S.A.). After centrifugation, the gradient was fractionated (1.5 ml) with a peristaltic pump and a fraction collector (Gilson Medical Electronics, Inc., Middleton, WI, U.S.A.). The A_{260} was monitored with a Secomam spectrophotometer (Secomam, Domont, France). Fractions of 1.5 ml were deproteinized by incubation with proteinase K (0.7 mg/ml) for 30 min at 37 °C. Total RNA from individual fractions was extracted as described by Chomczynski and Sacchi [28], except that 10 µg of tRNA was added to each fraction.

Dot-blot analysis

Dry RNA fractions were resuspended in 60 µl of buffer C [10 mM KH₂PO₄, pH 7.0, 7 % (v/v) formaldehyde, 50 % (v/v) formamide], incubated for 10 min at 65 °C and then placed in ice for 10 min. Each fraction was diluted with 340 µl of 20 × SSC (3 M NaCl, 0.34 M sodium citrate, pH 7) and applied to a nylon membrane (GeneScreen, NEN Research Products, Boston, MA, U.S.A.) with a dot-blot manifold (Schleicher & Schuell, Inc, Dassel, Germany). Hybridization with C9 rat proteasome subunit and autoradiographic signal quantification were performed as described above.

Statistical analysis

All results are expressed as means ± S.E.M. and are representative of at least two different experiments. Statistical analyses were performed with paired or unpaired Student's *t*-tests, as appropriate.

RESULTS

Effects of hindlimb suspension on skeletal muscle protein breakdown

All measurements were performed at day 9 of suspension; at this stage the total rate of protein degradation was previously shown to be maximal [5] and thus the proteolytic systems were likely to be fully activated. The marked atrophy of the soleus muscle (55 %) reflected an important loss of total proteins (53 %), with an equal reduction of contractile (51 %) and soluble (50 %) fractions ($P < 0.001$; Table 1). Total protein breakdown in-

Table 1 Effect of 9 days of hindlimb suspension (HS) on muscle and protein mass in the rat soleus

Values are means ± S.E.M. for six to twelve rats. Significant difference from control (CT) animals: *, $P < 0.001$.

	Muscle mass (mg)	Total proteins (mg)	Myofibrillar fraction (mg)	Sarcoplasmic fraction (mg)
CT	80 ± 3	10.60 ± 0.68	9.50 ± 0.95	2.40 ± 0.13
HS	36 ± 1*	4.96 ± 0.20*	4.69 ± 0.22*	1.21 ± 0.06*
Change (%)	−55	−53	−51	−50

Table 2 Effect of 9 days of hindlimb suspension (HS) on total and non-lysosomal and Ca²⁺-independent proteolysis in the rat soleus muscle

Total protein breakdown was measured without inhibitors. The non-lysosomal and Ca²⁺-independent proteolysis was measured in the contralateral soleus muscle in the presence of 10 mM methylamine and 50 µM E-64c in Krebs–Henseleit buffer lacking Ca²⁺. Results are means ± S.E.M. for six to twelve rats, and are given in units of nmol of Tyr per 2 h per mg of protein. Significant differences from control (CT) animals: *, $P < 0.05$; †, $P < 0.005$. The values in parentheses are the percentages of total proteolysis.

Proteolysis	Total	Non-lysosomal and Ca ²⁺ -independent	Lysosomal and Ca ²⁺ -dependent
CT	4.12 ± 0.24	3.77 ± 0.61	0.35 ± 0.15 (9%)
HS	6.86 ± 0.20†	5.63 ± 0.50*	1.24 ± 0.54* (18%)
Difference	+2.74	+1.86	+0.89
Change (%)	+66	+50	+254

Table 3 Effect of 3 h of preincubation–incubation on ATP, phosphocreatine (PCr) and lactate concentrations in rat soleus muscle

Rat soleus muscles (85–90 mg) were assayed either immediately after excision (control) or after incubation in Krebs–Henseleit buffer (see the Materials and methods section). After 1 h of preincubation, muscles were transferred to fresh media for 2 h of incubation. Values are means ± S.E.M. for six to eight rats, and are given in units of µmol per mg of protein. Abbreviation used: NS, not significant ($P > 0.05$).

	ATP	PCr	Lactate
Controls	40.13 ± 3.28	46.15 ± 9.32	13.27 ± 2.01
Incubated	32.95 ± 3.03	50.65 ± 8.34	10.37 ± 3.21
Change (%)	−18 (NS)	+10 (NS)	−22 (NS)

creased by 66 % ($P < 0.005$) in the atrophying muscles, clearly supporting a role of proteolysis in the soleus muscle wasting (Table 2).

Muscle atrophy induced by HS is very weak in young growing rats [2]. Therefore we used older animals, with a soleus muscle weight of 80–90 mg for control rats (Tables 1 and 3). Although measurements *in vitro* revealed high proteolytic rates in these large muscles (Table 2), ATP, phosphocreatine and lactate levels were not significantly different between incubated and freshly excised soleus muscles (Table 3). These observations suggest that the viability of control muscles was not markedly altered after 3 h of preincubation and incubation. In contrast, the unweighted muscles were small enough to allow sound measurements of proteolysis [8]. In support of this, our protein breakdown values in muscles from rats hindlimb-suspended for 9 days, when expressed in fractional terms (35 % per day), are in good agreement with those of Goldspink et al. (27 % per day) obtained after only 5 days of HS [3]. Thus the higher-than-normal rates of protein breakdown in control muscles result only in an underestimation of the difference between control and hindlimb-suspended animals. Moreover the conclusions of the experiments *in vitro* were in good agreement with the independent measurements of enzyme activities and of mRNA levels for proteinases (see below).

Proteolytic pathways responsible for muscle wasting during hindlimb suspension

To identify the proteolytic pathway(s) activated in the atrophying soleus, we incubated muscles under conditions that almost totally suppressed both lysosomal and Ca²⁺-dependent proteolysis [11–

Table 4 Proteolytic activities of cathepsins B+L and B in the soleus muscle of control (CT) and hindlimb suspended (HS) rats

Activities were assessed in lysosomal extracts as previously described [26], using Z-Phe-Arg-NMec and Z-Arg-Arg-NMec as substrates for cathepsins B+L and cathepsin B respectively. Results are means $\pm$ S.E.M. for six to ten rats. Significantly different from control animals: * $P < 0.005$ and † $P < 0.001$.

	Substrate hydrolysed (μ mol/min per mg of protein)	
	Cathepsins B+L	Cathepsin B
CT	1.20 $\pm$ 0.09	0.24 $\pm$ 0.03
HS	2.31 $\pm$ 0.22†	0.51 $\pm$ 0.09*

Table 5 Proteolytic activities of total, μ - and m-calpains in the tibialis anterior muscle of control (CT) and hindlimb suspended (HS) rats

Activities were assessed in purified extracts of calpains as previously described [27], using fluorescein isothiocyanate (FITC)-labelled casein. Results are means $\pm$ S.E.M. for five rats, expressed in μ mol of FITC-casein hydrolysed/min per mg of protein. Significantly different from CT, * $P < 0.05$ and † $P < 0.001$.

	FITC-casein (μ mol/min per mg of protein)		
	μ - and m-Calpain	μ -Calpain	m-Calpain
CT	1.6 $\pm$ 0.1	0.3 $\pm$ 0.1	1.0 $\pm$ 0.0
HS	3.5 $\pm$ 0.8*	0.2 $\pm$ 0.1	2.8 $\pm$ 0.4†

13]; contralateral muscles were incubated without inhibitors. We found a large increase (254 %, $P < 0.05$) in the lysosomal and Ca^{2+} -dependent proteolytic processes in unweighted rats (Table 2). To elucidate the mechanisms involved, we investigated whether this rise in proteolysis was related to any modification of the lysosomal and Ca^{2+} -activated proteinases. Table 4 shows that both cathepsin B and B+L activities increased in the soleus muscles from unweighted rats (+111 %, $P < 0.005$; +92 %, $P < 0.001$ respectively). Calpain activities were measured in the tibialis anterior muscle. Although this muscle was less affected by HS, total calpain activity was increased after 9 days of unweighting (+118 %, $P < 0.05$). This increase was mostly due to m-calpain (+180 %, $P < 0.001$) as μ -calpain activity remained unchanged (Table 5). Next we investigated whether enhanced enzyme activities were associated with modifications in mRNA levels. Increased mRNA levels for cathepsin B and L and for m-calpain (+84 %, $P < 0.05$; +220 %, $P < 0.001$; +210 %, $P < 0.05$ respectively) were indeed observed in the soleus muscle from unweighted rats (Figure 1). Figure 1 also shows that cathepsin D mRNA level was elevated (+140 %, $P < 0.005$) in unweighted rats, and that β -myosin mRNA level remained unchanged. Thus increased activities of cathepsins B and L and of m-calpain were due to specific elevation of their mRNA transcripts in unweighted animals. Taken together, these data indicate that both lysosomal and Ca^{2+} -dependent proteinases might play a role in increased skeletal muscle proteolysis during HS. However, despite a 254 % increase in lysosomal and Ca^{2+} -dependent proteolysis, the quantitative contribution of these proteinases to overall protein breakdown remained low in unweighted animals (18 % compared with 9 % in control rats) (Table 2). Furthermore the inhibition of lysosomal and Ca^{2+} -dependent proteolytic processes only partly (32 %, $P < 0.05$) suppressed the increased proteolysis in the

Figure 1 Effect of 9 days of hindlimb suspension (HS) on mRNA levels for cathepsins B, L and D, m-calpain and β -myosin in rat soleus muscle

RNA (10 μ g) samples were electrophoresed on 1% (w/v) agarose gel, transferred to nylon membrane and hybridized with [32 P]riboprobe (cathepsins B and L) or [32 P]cDNAs (cathepsin D and m-calpain). Autoradiographic signals were quantified by digital image processing. After stripping, blots were reprobated with [32 P]cDNA encoding β -myosin; data were corrected for β -myosin mRNA abundance to take into account variations in RNA loading. Values are means $\pm$ S.E.M. for five to eight animals. Representative Northern blots are also shown and transcript sizes (in kb) indicated at the left side of the blots. Significant differences from control (CT): *, $P < 0.05$; **, $P < 0.005$; †, $P < 0.001$.

Figure 2 Effect of 9 days of hindlimb suspension (HS) on mRNA levels for ubiquitin, 14 kDa E2, and C2 and C9 proteasome subunits in rat soleus muscle

RNA (10 μ g) samples were electrophoresed on 1% (w/v) agarose gel, transferred to nylon membrane and hybridized with [32 P]cDNAs. Autoradiographic signals were quantified by digital image processing. After stripping, blots were reprobated with [32 P]cDNA encoding β -myosin; data were corrected for β -myosin mRNA abundance to take into account variations in RNA loading. Values are means $\pm$ S.E.M. for six to eight animals. Only the 2.6 and 1.2 kb transcripts for ubiquitin and 14 kDa E2 respectively were quantified (see the Results section). Representative Northern blots are also shown and transcript sizes (in kb) indicated at the left side of the blots. Significant differences from control (CT): *, $P < 0.02$, †, $P < 0.001$.

atrophy soleus muscle from unweighted rats (Table 2). Thus non-lysosomal and Ca^{2+} -independent proteolytic pathway(s) most probably accounted for the major part of increased protein breakdown in unweighted muscle.

Figure 3 Absorbance profiles of polyribosomes and subpolyribosomal particles fractionated on sucrose gradients (left panels), and quantitative analysis of the distribution of C9 proteasome subunit mRNA in translationally active (polyribosomes) or inactive (messenger ribonucleoparticle) fractions (right panels)

Post-mitochondrial supernatants from control (A, left panel) and unweighted (B, left panel) soleus muscles were centrifuged through a 33 ml 15–45% sucrose gradient. After centrifugation the polyribosome profile was collected; A_{260} was continuously monitored and 1.5 ml fractions were collected from the bottom to the top of the gradient. M, 60 and 40 denote monosomes and 60S and 40 S ribosomal subunits respectively. After deproteinization with proteinase K (0.7 mg/ml, 30 min at 37 °C), total RNA was extracted as described by Chomczynski and Sacchi [28]. Each fraction was diluted with 340 μ l of $20 \times$ SSC and applied to a nylon membrane with a dot-blot manifold. After hybridization with the 32 P-labelled cDNA of the C9 rat proteasome subunit, the relative amounts of C9 mRNA in the fractions were determined by densitometric scanning of the autoradiograms with digital image processing. Exposures were chosen so that the signals were within the linear range of the film. The results are expressed as a percentage of the total mRNA in each fraction for control (A, right panel) and unweighted (B, right panel) animals. The vertical broken line separates the translationally active (left) from the translationally inactive (right) mRNAs.

Recent evidence strongly suggests that the ATP-Ub-dependent proteolytic process is responsible for the degradation of the bulk of proteins in skeletal muscle [9–13,15]. To determine whether or not this pathway was activated in simulated weightlessness, we first examined Ub expression in the atrophying muscles of unweighted rats. The levels of both transcripts of Ub rose in such conditions (by 482% for the 2.6 kb mRNA species, $P < 0.001$, Figure 2; by 105% for the 1.2 kb mRNA species, $P < 0.01$, results not shown). However, Ub also has various non-proteolytic functions [16]. We therefore also probed the RNA blots with the cDNA of the 14 kDa E2, one of the few known E2 mammalian species that functions in E3-dependent Ub-conjugate formation [30]. RNA blots were also hybridized with cDNAs encoding the C2 and C9 subunits of the 20 S proteasome, which is the proteolytic core of the 26 S proteasome that degrades Ub-conjugates [16,17]. mRNA levels for both transcripts of the 14 kDa E2 rose in the atrophying muscles of unweighted rats compared with control animals, but the increase in the 1.2 kb transcript was more pronounced (+342%, $P < 0.001$; Figure 2) than for the 1.8 kb transcript (+65%, $P < 0.05$; results not

shown). Similarly, the mRNA levels for the C2 and C9 proteasome subunits increased ($P < 0.02$) by 133 and 61% respectively in the unweighted muscles (Figure 2). These co-ordinated variations strongly suggest that enhanced ATP-Ub-dependent proteolysis also contributed to the soleus muscle wasting seen in unweighted rats.

C9 mRNA distribution in polyribosome fractions

The polyribosomal profile was similar in both control and unweighted animals (Figure 3). The proportion of C9 mRNA in translationally active (polyribosome associated, 52%) and translationally inactive (free messenger ribonucleoparticles, monosomes and disomes, 49%) total mRNA was similar in control and suspended rats (Figure 3). Similar results were obtained in two different experiments, as well as for the 14 kDa E2 (results not shown). Thus these observations supported the idea that the increased mRNA levels for both C9 and 14 kDa E2 observed with Northern blot procedures (Figure 2) underwent effective translation.

DISCUSSION

There is strong evidence that increased proteolysis contributes to the wasting of soleus muscle observed during simulated or real weightlessness [3,5–7]. The present study confirms these observations (Table 2) and provides for the first time, to our knowledge, evidence that the co-ordinated activation of three major proteolytic systems (i.e. the lysosomal, Ca^{2+} -dependent and ATP-Ub-dependent pathways) is responsible for the atrophy of the unweighted soleus muscle. These findings are very similar to those previously reported in denervated muscles [10,39,40], another example of muscle disuse.

Using different but complementary approaches, we have demonstrated that both lysosomal and Ca^{2+} -dependent proteinases contributed to muscle wasting. The lysosomal and Ca^{2+} -dependent proteolysis substantially increased (+254%) in incubated soleus from unweighted rats (Table 2). We also found increased activity of cathepsin B and B+L (Table 4) in the atrophying muscles that correlated with enhanced mRNA levels for both cathepsins (Figure 1), suggesting a transcriptional regulation of these enzymes. Furthermore we observed a significant increase in cathepsin D expression (Figure 1), suggesting that lysosomal aspartic proteinases contributed to the muscle wasting, together with the lysosomal cysteine proteinases (cathepsins B and L). These observations are consistent with those of Goldspink et al. [3], who reported increased cathepsin B and D activities after 5 days of unweighting. By contrast, Tischler et al. [7] did not find any evidence of an activation of the lysosomal pathway after 3 days of HS. A possible explanation is that significant alterations of lysosomal proteolysis are not detectable in the early stages of muscle atrophy, when protein synthesis decreases [1,5]. By contrast, the involvement of Ca^{2+} -dependent proteinases in the increased protein breakdown of the unweighted soleus muscle suggested by our observations is in agreement with the observations of Tischler et al. [7], who hypothesized an important role of calpains. These authors found that the use of thiol proteinase inhibitors or of antagonists of Ca^{2+} release decreased accelerated proteolysis in the soleus muscle after 3 days of HS. Based on these indirect observations, they concluded that Ca^{2+} -dependent proteolysis played a determinant role during unweighting atrophy. The increased activity (Table 5) and expression (Figure 1) of m-calpain observed in the muscles from unweighted rats provided strong direct evidence for an activation of calpains, and supported the hypothesis of Tischler et al. [7]. However, the effective change in m-calpain activity could be affected by changes in calpastatin (the endogenous inhibitor of Ca^{2+} -dependent proteinases), which were not measured.

It seemed, however, very unlikely that the activation of lysosomal and Ca^{2+} -activated proteinases could totally explain muscle wasting in the unweighted soleus muscle. The contribution of lysosomal and Ca^{2+} -dependent proteinases to the overall increase in protein breakdown remained low in unweighted rats (Table 2), as previously reported in fasting [13], denervation atrophy [10], acidosis [11] and cancer [12]. Furthermore lysosomal and Ca^{2+} -activated proteinases were reported not to be involved in the degradation of myofibrillar proteins [10,14,15], which represent up to 80% of total muscle proteins. For example, chloroquine, an inhibitor of lysosomal acidification, or leupeptin and E-64, two potent inhibitors of cysteine proteinases (e.g. cathepsins B, H and L, and calpains), do not affect 3-methylhistidine release in incubated muscles, indicating that myofibrillar proteins are not degraded by either proteolytic process [14,15]. The unweighted soleus muscle loses soluble components (Table 1), which are potentially target proteins for these two pathways. Cathepsins are also known to degrade endocytosed proteins, and

calpains to be involved in the degradation of cytoskeletal proteins and/or in limited proteolysis of some specific proteins [41]. A further possibility is that specific cleavage of minor contractile or cytoskeletal proteins is qualitatively important for subsequent proteolysis of actin and myosins. The various proteolytic pathways might be sequentially activated and the actions of the Ca^{2+} -activated and/or lysosomal proteolytic pathways might have a permissive role for the degradation of myofibrillar proteins by the ATP-Ub-dependent pathway (see below).

It is well known that hindlimb suspension does not create lesions in hindleg muscles, and that neither macrophages nor monocytes invade disused muscles [42,43]. Riley and co-workers have established that control and unweighted muscles have the same pattern of macrophages and monocytes, even after 12.5 days of unweighting, although myofibril transverse misalignment is observed in unweighted muscles [43]. In fact, only reloading, even after a short time (6 h), induces an invasion of hypertrophied, stellate macrophages [42,43] in muscle cells. Thus it is very unlikely that the increased proteinase activities or mRNA levels seen in the present experiments can be attributed to the invasion of non-muscle cells.

The second major observation of this study is that of increased mRNA levels for Ub, 14 kDa E2 and proteasome subunits in the atrophying soleus muscles (Figure 2) that clearly support a role for the Ub-proteasome proteolytic pathway in muscle wasting during HS. The enhanced expression of Ub is presumably essential for the increased accumulation of free Ub and Ub-protein conjugates that was reported in skeletal muscles from unweighted animals from day 3 to day 10 of suspension [18]. Furthermore the 14 kDa E2 is one of the few mammalian E2s so far cloned and sequenced known to be essential for the formation of polyUb-conjugates that are destined for rapid degradation [30]; the 1.2 kb mRNA transcript is tightly up- and down-regulated in skeletal muscle on fasting and refeeding respectively [30], and up-regulated in the atrophying muscles from tumour-bearing [12], dexamethasone-treated [44] and septic (L. Voisin, C. Obled and D. Attaix, unpublished work) rats, and from head trauma patients [45], as reported here (Figure 2). Thus the increased expression of the 14 kDa E2 observed in the unweighted soleus muscle presumably contributes to the accumulation of Ub conjugates [18], which may provide more substrates for the 26 S proteasome. To our knowledge no information is available on the role of the 26 S proteasome in muscle wasting in mammals. However, Dawson et al. [46] have recently shown that increased peptidase activities of the 26 S proteasome, and changes in associated ATPase activities, prevailed in extensive Ub-dependent proteolysis that occurs in intersegmental muscles of insects during programmed cell death. The increased mRNA levels in the atrophying soleus for subunits of the 20 S proteasome (Figure 2), which is the proteolytic core of the 26 S proteasome [16,17], supported increased proteolytic activities.

The precise significance of increased mRNA levels for proteasome subunits in muscle-wasting conditions [9,11,12,39,44,45,47] is, however, unknown at present. We provide here the first evidence that the same distribution between translationally active and inactive mRNAs for the C9 subunit of the 20 S proteasome was observed in control and unweighted muscles (Figure 3). Thus the increased abundance of mRNA for proteasome subunits in unweighted muscles (Figure 2) underwent active translation. Furthermore the distribution of the C9 proteasome subunit mRNA between light and heavy (five or more) polyribosomes was not altered in suspended rats (Figure 3). An increased proportion of muscle α -actin mRNA in heavy polyribosomes from hindlimb-suspended animals can reflect a 'traffic jam' of ribosomes, which, in turn, would slow down the

translation rate [48]. We found no evidence for such adaptation with the C9 mRNA (Figure 3), thus supporting an efficient translation of proteasome subunits in unweighted muscles.

Because the atrophy of the soleus muscle from unweighted rats resulted primarily from the activation of non-lysosomal and Ca^{2+} -independent proteolysis (Table 2), it is likely that the ATP-Ub-dependent proteolytic pathway is the critical system responsible for such adaptation. Several lines of evidence support this hypothesis. First, most of the increased muscle protein breakdown seen in various catabolic conditions is ATP-dependent [9–13]. The Ub-dependent proteolytic process is well known to degrade abnormal or short-lived proteins [16], but is also responsible for the degradation of the bulk of proteins, including the long-lived components [49]. Accordingly the degradation of myofibrillar proteins requires ATP [10,15]. Secondly, an enhanced expression of Ub [9,11–13,15,39,44,45,50] and of proteasome subunits [9,11,12,39,44,45] have been recently reported in various muscle-wasting conditions in rodents and humans [45]. An increase in Ub-protein conjugates paralleled these adaptations in cancer [50] and fasting or denervation atrophy [40], and the increase in Ub-protein conjugates occurred primarily in the myofibrillar fraction [40]. Increased ubiquitinylation of thick filaments and of disrupted myofibrils was suggestive of ubiquitin-mediated breakdown of myofibrillar proteins of rats orbited in the Cosmos 1887 and 2044 biosatellites for 12.5 and 14 days respectively [19,20]. Thirdly, proteasome preparations could degrade myofibrillar proteins *in vitro* under certain conditions [51,52], as well as intact myofibrils [53]. Finally, an activation of the Ub-proteasome proteolytic pathway has been systematically reported in many different types of muscle wasting such as fasting or denervation atrophy [39,40], acidosis [11], bacterial infections [15] and cancer [9,12]. In contrast, evidence for an activation of either lysosomal or Ca^{2+} -dependent proteolysis is lacking in several instances of muscle atrophy [9,11–13,39,47,50]. Taken together these findings suggest that the ATP-Ub-dependent pathway may be the critical system responsible for the degradation of myofibrillar proteins in unweighted rats.

In conclusion, the present experiments demonstrated that both lysosomal and Ca^{2+} -activated proteinases contribute to the increased muscle proteolysis seen during HS. They also strongly suggest that increased ATP-Ub-dependent proteolysis is mainly responsible for the wasting of the unweighted soleus muscle. The possible connection between these proteolytic pathways needs to be elucidated. Although the Ub-proteasome proteolytic process might degrade the major contractile proteins, the ubiquitinated proteins that are substrates of the 26 S proteasome remain to be identified. Polyribosome profiles showed that increased C9 mRNA levels were equally distributed between both translationally active and inactive mRNA pools, in either unweighted or control rats. However, further experiments must be designed to demonstrate whether or not the increased mRNA levels for proteasome subunits result in elevated proteolytic activities.

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