Increase in levels of polyubiquitin and proteasome mRNA in skeletal muscle during starvation and denervation atrophy

Ruth MEDINA, Simon S. WING* and Alfred L. GOLDBERG†

Department of Cell Biology, Harvard Medical School, Boston, MA 02115, U.S.A.

Most of the increased protein degradation in muscle atrophy caused by starvation and denervation is due to activation of a non-lysosomal ATP-dependent proteolytic process. To determine whether expression of the ubiquitin–proteasome-dependent pathway is activated in atrophying muscles, we measured the levels of mRNA for ubiquitin (Ub) and proteasome subunits, and Ub content. After rats had been deprived of food for 1 or 2 days, the concentration of the two polyubiquitin (polyUb) transcripts increased 2–4-fold in the pale extensor digitorum longus muscle and 1–2.5-fold in the red soleus, whereas total muscle RNA and total mRNA content fell by 50%. After denervation of the soleus, there was a progressive 2–3-fold increase in polyUb mRNA for 1–3 days, whereas total RNA content fell. On starvation or denervation, Ub concentration in the muscles also rose by 60–90%. During starvation, polyUb mRNA levels also

increased in heart, but not in liver, kidney, spleen, fat, brain or testes. Although the polyUb gene is a heat-shock gene that is induced in muscles under certain stressful conditions, the muscles of starving rats or after denervation did not express other heat-shock genes. On starvation or denervation, mRNA for several proteasome subunits (C-1, C-3, C-5, C-8 and C-9) also increased 2–4-fold in the atrophying muscles. When the food-deprived animals were re-fed, levels of Ub and proteasome mRNA in their muscles returned to control values within 1 day. In contrast, no change occurred in the levels of muscle mRNAs encoding cathepsin L, cathepsin D and calpain 1 on denervation or food deprivation. Thus polyUb and proteasome mRNAs increase in atrophying muscles in co-ordination with activation of the ATP-dependent proteolytic process.

INTRODUCTION

The rate of protein breakdown in skeletal muscle is precisely regulated by food supply, several hormones and the level of contractile activity in the muscle [1–3]. During starvation, protein breakdown in muscle increases and helps provide the organism with essential amino acids for gluconeogenesis [1,2,4,5]. In addition, proteolysis in muscle increases 2–3-fold on denervation, and this response is the primary cause of the muscle atrophy seen with denervation or prolonged disuse [6,7]. However, the biochemical pathways for degradation of muscle protein and their modes of activation under these conditions are still obscure. Like other tissues, muscle contains multiple pathways for protein breakdown, including the lysosomal, Ca^{2+} -dependent, and cytosolic ATP-dependent and independent proteolytic systems [1,8–10]. It has been widely assumed that enhanced lysosomal proteolysis is responsible for the increased protein breakdown in nutritionally poor conditions or atrophying cells [11,12]. However, recent studies with isolated muscles indicate that the large increases in proteolysis and in the degradation of myofibrillar proteins during food deprivation [5,13–15] and after denervation [6] are evident even when lysosomal and Ca^{2+} -dependent systems are blocked.

We have presented evidence that an ATP-dependent degradative process is stimulated 2–3-fold on denervation and during starvation, and is suppressed by refeeding [1,13–15]. This ATP-requiring system appears to be responsible for the bulk of protein degradation in such muscles, including the accelerated breakdown of myofibrillar components. The present studies were undertaken to define further this degradative system and the adaptations leading to its activation. Skeletal muscle, like other mammalian cells, contain the soluble ATP–ubiquitin (Ub)-dependent proteolytic system [10]. This pathway is generally

believed to catalyse the rapid breakdown of highly abnormal polypeptides and various short-lived normal proteins [16–19]. In this process, multiple Ub moieties are covalently conjugated to amino groups on protein substrates [16–20], which marks them for rapid degradation by a 26S (1500 kDa) proteasome [21–23]. The proteolytic core of this very large complex is the 20S (600 kDa) proteasome particle which contains multiple peptidase activities and at least 14 distinct subunits, many of which have now been cloned [23–26].

A primary goal of these studies was to test whether the Ub–proteasome-dependent system is activated in the atrophying muscle through changes in gene expression. To learn whether Ub or proteasome production increases, we have studied the levels of mRNA encoding Ub and several proteasome subunits as well as the level of Ub itself in atrophying muscles. Ub is one of the cell's heat-shock proteins (HSP) [27–29], which are induced by heat or other types of stress [29,30]. The stress-induced gene is actually a polyUb gene, which encodes many Ub moieties in tandem [27,28]. To define the adaptations that may contribute to muscle atrophy, we have measured the levels of mRNA for polyUb, the proteasome subunits, other intracellular proteases and other HSPs under conditions in which there is enhanced ATP-dependent protein degradation in muscle. We demonstrate here a specific increase in Ub and proteasome mRNA in atrophying muscles (both skeletal and cardiac) that is not seen in non-muscle tissues, even during starvation.

EXPERIMENTAL

Muscle preparations

Young male rats (60–80 g; Charles River CD strain) were maintained on Purina Lab Chow and water *ad libitum*. One

Abbreviations used: Ub, ubiquitin; EDL, extensor digitorum longus; HSP, heat-shock protein; 1 × SSC, 0.15 M NaCl plus 0.015 M sodium citrate.

* Present address: Department of Medicine, Royal Victoria Hospital, McGill University, Montreal, Quebec, Canada H3A 1A1.

† To whom correspondence should be addressed.

group was deprived of food for 24–48 h. To denervate the soleus muscles of one hind limb, the sciatic nerve was cut about 1 cm above the popliteal fossa, while the animals were under anaesthesia [6,13]. In some experiments, the control limb received a sham-operation in which the skin was cut under anaesthesia, but the sciatic nerve, once exposed, was left intact. This sham-operation did not affect any of the parameters measured here, as observed in prior studies [6]. In all cases, the animals were killed by cervical dislocation, and the extensor digitorum longus (EDL) and soleus were dissected. For experiments involving induction of the heat-shock response, the isolated muscles were incubated at resting length in Krebs–Ringer bicarbonate buffer equilibrated with 95% O₂/5% CO₂ containing insulin (4 µg/ml), glucose (5 mM) and plasma concentrations of all amino acids [13].

RNA preparation and Northern-blot analysis

Total RNA from muscle was isolated by the guanidinium isothiocyanate/CsCl method [31]. The yield of total RNA was very reproducible within each group. In all cases, the ratio of A_{260}/A_{280} was approx. 2.0, indicating a high degree of purity for the total RNA preparations from both control and atrophying muscles. Electrophoresis of RNA was performed in 1% agarose gels containing 0.2 M formaldehyde. The RNA was transferred from the gel to nylon membranes (Gene Screen Dupont, NEN Research Products) in 20 × SSC (1 × SSC = 0.15 M NaCl plus 0.015 M sodium citrate). RNA was cross-linked to the membrane by u.v. light at 1200 µJ on a Stratalinker apparatus (Stratagene). Membranes were hybridized at 65 °C with ³²P-labelled cDNA probes prepared by the random-primer method [32]. The hybridization buffer contained polyvinylpyrrolidone-40000 (0.2%), Ficoll 400000 (0.2%), BSA (0.2%), Tris/HCl (0.05 M, pH 7.5), NaCl (1 M), sodium pyrophosphate (0.1%), SDS (1%) and salmon sperm DNA (100 µg/ml). Filters were washed in 0.5 × SSC/1% SDS at 42 °C or 65 °C. Membranes were exposed to XAR-2 film (Kodak) for autoradiography.

For dot-blot analysis, four different concentrations (2-fold dilutions from 1.5 µg) of total denatured RNA from the soleus or EDL muscle were spotted on Gene Screen membranes. The amount of RNA applied to each dot was maintained at 1.5 µg by adding *Escherichia coli* tRNA, which, in the absence of rat muscle RNA, did not show any hybridization. In dot-blot analysis, the density after hybridization with labelled probes was proportional to the concentration of total RNA (between 0.15 and 1.5 µg/well). In order to quantify the total poly(A)-containing RNA, we hybridized the dot-blots with end-labelled oligo(dT)₁₈ [33]. Harley [33] has shown that neither rRNA nor RNA reacts with this probe. For quantification, we used arbitrary densitometric units for total poly(A)-containing RNA (mRNA).

Ub cDNA [34] was kindly provided by Dr. M. Schlesinger (Washington University Medical School, St. Louis, MO, U.S.A.), the HSP70 cDNA [35] by Dr. R. Voellmy (University of Miami School of Medicine, Miami, FL, U.S.A.), the HSP27 and HSP90 cDNA [36] by Dr. L. A. Weber (University of South Florida, Tampa, FL, U.S.A.) and the cDNA for five different subunits of the proteasome [26,37–39] by Dr. K. Tanaka (Tokushima University, Tokushima, Japan). Other cDNA probes used were for cathepsins D [40] and L [41] and the Ca²⁺-dependent protease (calpain I) [42]. Blots were hybridized with the different cDNAs at 65 °C and with oligo(dT) at room temperature and washed at these same temperatures. Levels of polyUb mRNA and total poly(A)-containing RNA were determined from the dot intensities of the autoradiograms by automated densitometric scanning. Unpaired Student's *t* test was used in statistical analysis to compare muscles of fed and starved animals, and the paired *t* test

was used to compare contralateral denervated and control muscles of the same animals. Measurements of total Ub content, which includes both free Ub and Ub ligated to proteins, were carried out by Dr. Arthur Haas (Medical College of Wisconsin, Milwaukee, WI, U.S.A.) using an immunochemical method described previously [43].

RESULTS

Skeletal-muscle Ub mRNA content during food deprivation

To test whether the levels of Ub mRNA increase in rat muscles when protein breakdown increases [1,13,14], we determined the levels of polyUb transcripts at different times after food deprivation. The soleus contained two transcripts of 2.4 and 1.3 kb (Figure 1), which correspond to the sizes of the polyUb genes in other species [28]. The levels of both transcripts increased progressively in the muscles of starved animals. The relative amounts of Ub mRNA in these tissues were measured by dot-blot analysis and autoradiography. Initial experiments indicated that this assay was highly reproducible and linear over the range studied. After 48 h of food deprivation, the concentration of total Ub mRNA in the EDL muscle was 4-fold greater than in muscles of control animals (Figure 1). The soleus muscle, which atrophies less than the EDL during starvation [4], showed a 2-fold increase in Ub mRNA. Consistently, the changes measured by dot-blot analysis appeared smaller than those seen on densitometric scanning of the Northern blot (Figure 1). Nevertheless, dot-blots were used routinely for quantification because of their greater reproducibility, although this approach may underestimate the actual increase in Ub mRNA. A third minor Ub transcript of 0.9 kb could also be detected by overexposing the autoradiograms. It corresponds in size to the transcript of the Ub-extension gene [28], the expression of which is associated with the biogenesis of ribosomes [44,45]. This transcript did not increase with starvation (results not shown), in contrast with polyUb mRNA.

To examine whether the increase in polyUb mRNA was reversible, some of the starved rats were then provided with food. By 24 h later, the levels of polyUb mRNA in these muscles returned to levels seen in control animals. The rise and fall in polyUb mRNA on starving/re-feeding thus parallel the changes in overall rates of protein degradation [4,5] and in the ATP-dependent degradative process [13–15]. Although the changes in polyUb mRNA were documented 24 h after food deprivation or refeeding, these responses probably occur significantly faster. For example, at the time of food deprivation, the rats had just completed their large nocturnal meal, and therefore food remained in their digestive systems for many hours.

To determine whether polyUb mRNA is regulated in a specific manner during starvation, we tested whether the total amount of RNA and poly(A)-containing RNA in these muscles changed after food deprivation in a similar way to polyUb mRNA. The total RNA content was measured by absorbance spectrophotometry and the total amount of poly(A)-containing RNA on the dot-blots was measured using a [³²P]oligo(dT) probe [32]. In contrast with polyUb mRNA, the total RNA content and the amount of poly(A)-containing RNA (i.e. mRNA) in the soleus and EDL decreased to approx. 50% of the levels found in muscles of fed animals. Total RNA fell from 72 ± 3.5 to 35 ± 1.6 µg/muscle and total mRNA from 2133 ± 376 to 1004 ± 20 arbitrary units/muscle in the soleus during fasting. In the EDL of starved rats, total RNA decreased from 68 ± 6 to 38.5 ± 1 µg/muscle and total mRNA from 710 ± 73 to 413 ± 11 units/muscle. The ratio of total poly(A)-containing RNA to total RNA, unlike Ub mRNA, thus did not change

Figure 1 Northern-blot analysis of Ub mRNA in muscle from starved and starved-re-fed rats

(a) Levels of polyUb mRNA in 10 μ g of total RNA/lane isolated from soleus muscles of fed rats (lane 1) and rats starved for 24 h (lane 2) or 48 h (lane 3) or starved for 48 h and re-fed for 24 h (lane 4). 28S and 18S indicate the positions of these RNAs. Northern-blot analysis was performed as described in the Experimental section. (b) Levels of polyUb mRNA and total mRNA determined by dot-blot analysis in soleus muscles of fed rats (lane 1) or starved (lane 2) or starved-re-fed rats (lane 3). Dot-blot analysis was performed with Ub cDNA or with oligo(dT) in order to measure total poly(A) content. (c) Quantification of Ub mRNA and total mRNA expressed in densitometric units. Total RNA content was determined spectrophotometrically. Dot-blot analysis was performed as described in the Experimental section. Significant difference from fed animals is indicated: * $P < 0.005$, ** $P < 0.05$.

significantly during the 24 h of food deprivation (Figure 1c). Therefore the levels of Ub mRNA varied in the opposite way to the bulk of the cellular RNA.

Ub mRNA in other tissues

Subsequent experiments tested whether the increase in polyUb mRNA on starvation is unique to skeletal muscle or whether other rat tissues show similar responses 2 days after food deprivation (results not shown). Enhanced proteolysis during food deprivation has been documented in rat liver [46], although this latter response has been attributed primarily to the activation

Figure 2 Northern-blot analysis of Ub mRNA from different tissues of rats starved for 48 h

The blots were performed as described in Figure 1 and the Experimental section. C, control; F, food-deprived.

of a lysosomal process [11,47]. In the heart (ventricle) of starved rats, an increase in polyUb mRNA similar to that in the soleus occurred (results not shown), but no such change was seen in any other tissue tested, including liver, spleen, adipose tissue, brain, testes and kidney (Figure 2). In the liver, kidney and adipose tissue, a marked loss of weight and protein occurred on starvation but, as expected, neither testes nor brain showed significant weight loss under these conditions (results not shown). Thus, during food deprivation the increase in Ub mRNA appears to be a specific adaptation in striated muscle and is not seen in other atrophying tissues.

Ub mRNA levels in denervation atrophy

A similar 2–3-fold acceleration of the ATP-dependent proteolytic process occurs in muscle during denervation atrophy [13]. To test whether the expression of polyUb genes is also activated by denervation, we analysed the levels of Ub mRNA from the soleus after denervation (Table 1). At 1 and 3 days after nerve section, the levels of polyUb transcripts increased markedly above those in the control soleus in the contralateral sham-operated limb. (The content of Ub mRNA in this control muscle did not differ from that in soleus muscles of unoperated rats.) Thus the increase in Ub mRNA was a specific consequence of nerve section (Figure 3). On denervation, no increase in the minor transcript (0.9 kb) from the Ub-extension gene occurred [28,44,45], unlike the polyUb transcripts. Dot-blot analysis of the muscles revealed a 2–3-fold increase in the polyUb mRNA content as a fraction of total mRNA after denervation. This response was greatest 1 day after nerve section, and thus preceded most of the muscle atrophy (Table 1). During the course of this experiment, the control muscles grew and their total RNA content increased in line with total body growth, and the concentrations of Ub mRNA [as ratio of total poly(A)-containing mRNA] and Ub did not change. Meanwhile, in the denervated soleus, total RNA as well as total poly(A)-containing RNA both decreased by 40% in 3 days. Thus, in the first days after denervation, as in starvation, when ATP-dependent proteolysis rose, Ub mRNA and total mRNA content increased in contrast with the bulk of cell RNA. By the fourth day after nerve section, Ub mRNA concentration (per mg of muscle or as a fraction of total RNA) returned to levels seen in control muscle (Figure 3b). By this time, the denervated muscles contained only 50–60% of the protein of the contralateral control [6].

Expression of other HSP genes

As polyUb is one of the eukaryotic heat-shock proteins [30–33], we investigated whether other HSPs were also induced on

Table 1 Effect of unilateral denervation of rat soleus muscle on the content of polyUb mRNA, total RNA and weight

Dot-blot analysis was performed at different times after section of the sciatic nerve as described in the Experimental section. Levels of mRNA are expressed in arbitrary densitometric units. Values are means $\pm$ S.E.M. for six animals. Significant differences from control values are shown: * $P < 0.005$, ** $P < 0.05$.

Time after operation (days) ...	1		3	
Parameter	Control	Denervated	Control	Denervated
Ub mRNA (units/ μ g of total RNA)	2.4 $\pm$ 0.2	6.1 $\pm$ 0.6*	2.5 $\pm$ 0.2	5.4 $\pm$ 0.9**
Ub mRNA (μ g/soleus)	102 $\pm$ 0.4	224 $\pm$ 0.8**	135 $\pm$ 1.3	176 $\pm$ 1.9**
mRNA (units/ μ g of total RNA)	23.8 $\pm$ 1.4	23.1 $\pm$ 1.4	22.7 $\pm$ 0.8	23.7 $\pm$ 1.4
Total RNA (μ g/soleus)	42.3 $\pm$ 2.2	36.7 $\pm$ 1.3**	54.0 $\pm$ 6.5	32.5 $\pm$ 4.2*
Muscle weight (mg)	28.8 $\pm$ 0.7	26.3 $\pm$ 0.8**	30.7 $\pm$ 1.1	23.5 $\pm$ 2.1*
Weight loss (% of control)		-9**		-23*

Figure 3 Effect of nerve section on Ub mRNA in soleus muscle

(a) Northern-blot analysis of control (C) and denervated (D) muscles at 1 and 3 days. Each day after section of the sciatic nerve on one limb, Ub mRNA content was compared in the atrophying soleus and contralateral control muscle of the sham-operated limb. The weight of the denervated muscle at 1 day was 99%, at 3 days, 23%, and at 4 days, 36% less than that of the control, which grew continuously. (b) Quantification of Ub mRNA and total mRNA expressed in arbitrary densitometric units. The data on days 1 and 3 are taken from fed animals shown in Table 1, and unoperated controls (0 time) are from fed controls from Figure 1.

denervation or in muscles of starved animals by probing these RNA blots with labelled cDNA for human HSP27 [36], HSP70 [35], and HSP90 [36]. In contrast with polyUb mRNA, these mRNAs were not detected in muscles of fed, starved or denervated rats, despite prolonged exposure of the autoradiograms. Moreover, muscles incubated at 42 °C for 90 min showed large increases in the expression of polyUb, HSP27, HSP70 and HSP90 genes (results not shown) compared with tissues maintained at 37 °C. The muscles at 42 °C contained transcripts for these HSPs that were similar in size to the transcripts reported in HeLa cells [36]. Thus muscle tissue can show a typical heat-shock response, in which there is a co-ordinated induction of Ub and HSP genes, but this response does not occur in denervation atrophy or in food deprivation.

Table 2 Effects of starvation and denervation on Ub levels in rat skeletal muscles

Immunochemical quantification of total Ub was performed as described previously [43]. Values are means $\pm$ S.E.M. for EDL muscles from four fed or starved animals and seven paired soleus muscles 2 days after section of one of the sciatic nerves. Significant differences are shown: * $P < 0.05$, ** $P < 0.01$.

Muscle	Total protein (mg/muscle)	Total Ub	
		pmol/muscle	pmol/mg of protein
EDL			
Fed	4.5 ± 0.3	71 ± 6	16 ± 1
Starved for 2 days	3.1 ± 0.4	79 ± 7	26 ± 2
Difference	-1.4 ± 0.5*	+ 7 ± 9	+ 10 ± 2**
Change (%)	- 32	+ 10	+ 63
Soleus			
Innervated	3.5 ± 0.4	89 ± 5	27 ± 2
Denervated	2.7 ± 0.2	137 ± 12	51 ± 2
Difference	-0.8 ± 0.3**	+ 47 ± 11**	+ 24 ± 3**
Change (%)	- 23	+ 53	+ 91

Ubiquitin content of the muscles

To determine whether the increase in polyUb mRNA actually resulted in increased production and accumulation of Ub, we measured by immunoassay the total amount of this protein (Table 2), which includes both free Ub and Ub conjugated to cell proteins [43]. In EDL muscles from animals starved for 2 days, Ub concentration (expressed per mg of protein) was 63% higher than in muscles of fed controls. An even larger increase (91%) in Ub concentration was seen 2 days after cutting of the sciatic nerve, compared with the concentration in the contralateral innervated control. Thus total Ub levels correlated with the increase in ATP-dependent proteolysis [13] and in Ub mRNA. However, in these atrophying muscles, total protein content decreased. When these data were corrected for the fall in total muscle protein in these conditions, they indicated an increase of 53% ($P < 0.05$) in the total amount of Ub per soleus after denervation, but little or no increase on food deprivation ($P < 0.05$). Therefore the rate of synthesis of the polyUb protein must have risen on denervation, and probably also on food deprivation, as degradation of the Ub molecules is also likely to have increased along with the overall enhancement of proteolysis in such muscles. It is also noteworthy that the levels of Ub in the soleus of fed rats were higher than in the EDL, as was also found for polyUb mRNA, which appears consistent with the higher

Figure 4 (a) Expression of proteasome subunits, C-2, C-3, C-5, C-8, and C-9, in EDL muscle from control fed (C) and food-deprived (F) animals and (b) effect of starvation and refeeding on the levels of C-3 proteasome mRNA

Rats were deprived of food for 2 days and re-fed as described in Figure 1.

rate of protein turnover [4,48] and ATP-dependent proteolysis [12] in the red, predominantly slow-twitch, soleus in the fed state.

Increase in the mRNA of proteasome subunits

To examine whether production of other components of the ATP-Ub-dependent pathway also increased during starvation and denervation atrophy, we measured levels of mRNA encoding subunits of the 700 kDa (20S) proteasome. This multicatalytic protease functions as part of the 26S complex that degrades Ub-conjugated proteins [23,26,49,50]. To determine whether proteasome production in muscle increased in these catabolic states, we used cDNA probes for proteasome subunits C-2, C-3, C-5, C-8 and C-9. In the EDL muscles of rats starved for 2 days, mRNA levels of all these subunits increased in co-ordination 3–6-fold (Figure 4a). Within 1 day of refeeding, proteasome mRNA in the muscles of starved animals returned to control levels (Figure 4b). Similar, but smaller, increases in these mRNA species were found in the gastrocnemius (Table 3), which atrophies less during

starvation. Furthermore, on denervation of the soleus muscle for 3 days, there was a 2–4-fold increase in the content of mRNA encoding these proteasome subunits. Thus, in both types of muscle atrophy, the levels of mRNA for the proteasome and Ub appear to be co-ordinately regulated.

Levels of mRNA of other muscle proteases

To test whether these responses were specific for components of the Ub-proteasome pathway, we also measured the levels of mRNA encoding the lysosomal enzymes, cathepsins D [40] and L [41] and the Ca^{2+} -dependent protease, calpain 1 [42]. Both of these degradative systems have been proposed to play a role in the accelerated proteolysis in atrophying muscle [5], although inhibition of these enzymes does not prevent the rise in protein degradation on denervation [6] or food deprivation [5]. No change was found in the levels of mRNA of these proteases (Table 3) in muscles from rats deprived of food for 48 h or in muscles after denervation. Thus the expression of these lysosomal enzymes and calpains is not controlled co-ordinately with Ub or the proteasome genes (Table 3). In related studies [49], we also found no change in mRNA of the neutral protein, glycerol phosphate dehydrogenase, in atrophying tissues in which levels of proteasome and Ub mRNA increased severalfold. These findings further suggest a selection increase in the capacity of the ATP-Ub pathway through increased production of Ub and proteasome (Table 3).

DISCUSSION

The changes in Ub and proteasome mRNA observed here on denervation, food deprivation and refeeding appear to be of physiological importance, as they coincide with similar alterations in overall rates of protein breakdown and degradation of myofibrillar proteins [5,6] in the same muscle [1,13,14]. These changes in overall proteolysis were shown to be due primarily to activation of a non-lysosomal ATP-dependent process, and related studies indicate that Ub conjugation to muscle proteins and thus flux through this pathway are also enhanced in atrophying tissues [51,62]. The increase in Ub and proteasome mRNA should enhance the capacity of this proteolytic system and is probably responsible for the increase in Ub concentration in atrophying muscles (Table 2), which occurred despite the marked net loss of total muscle RNA. Several recent observations also indicate that the Ub-proteasome-dependent proteolytic system is the critical degradative system in these catabolic conditions, and is precisely regulated by contractile activity and

Table 3 Comparison of mRNA levels for lysosomal proteases and calpain with those of polyUb and proteasomes in muscle on food deprivation and denervation

Significant differences are indicated: * $P < 0.005$ compared with fed control; † $P < 0.01$, †† $P < 0.05$ compared with sham-operated.

	mRNA (density units/ μg of total RNA)					
	PolyUb	C-3 proteasome	C-9 proteasome	Cathepsin L	Cathepsin D	Calpain I
Fed control	—	—	38 ± 3.4	112 ± 11.9	160 ± 20.9	181 ± 1.6
Starved for 48 h	—	—	$63 \pm 4.0^*$	97 ± 7.5	159 ± 16.6	175 ± 20.8
Sham-operated	36 ± 3.6	33 ± 4.4	25 ± 7.8	175 ± 5.8	159 ± 8.1	56 ± 9.1
Denervated	$63 \pm 3.7^\dagger$	$67 \pm 4.3^\dagger$	$74 \pm 7.8^{\dagger\dagger}$	186 ± 9.1	147 ± 17.0	52 ± 11.0

food intake. For example, the loss of muscle protein during food deprivation requires adrenal steroids [1,2], and we have found that glucocorticoids are necessary for the increases in ATP-dependent proteolysis [49] and the levels of Ub-conjugated proteins [51] and Ub mRNA [49]. Interestingly, ubiquitinated actin has been noted in insect muscles [48], and large increases in Ub mRNA have been observed in the programmed destruction of insect muscles during development [52].

The parallel changes seen in mRNA encoding all the subunits of the proteasome that we measured (C-2, C-3, C-5, C-8 and C-9) strongly suggest increased synthesis of the entire 20S particle, which contains in total about 14 distinct polypeptides, none of which can function independently [23–26]. This particle functions as the proteolytic core within the 26S proteasome complex [21,22] in degradation of Ub-conjugated proteins [23,26,50,53,54]. It will be important to learn whether mRNA of the many non-proteasomal components of the 26 S complex also increases in muscle denervation and food deprivation. Previously, the only clearly established functions of the ATP-Ub-dependent proteolytic system in differentiated cells were in the degradation of short-lived or abnormal proteins [16–20]. However, the adaptations in mRNA levels shown here and related biochemical findings parallel the changes in the breakdown of the bulk of cell proteins [1,13,14]. These data thus suggest an important role for this proteolytic system in the regulated degradation of normal muscle proteins, including the long-lived myofibrillar components.

It is quite unlikely that the increased accumulation of polyUb *per se* stimulates protein degradation in these conditions, as the levels of Ub in muscle and other tissues appear to be well above the K_a for Ub-activating enzymes [55]. However, when overall proteolysis is accelerated, the free non-conjugated Ub level may fall, and some Ub molecules (and proteasome subunits) may also be subjected to increased degradation. Consequently, increased Ub and proteasome production may be essential to maintain these components (and therefore rates of proteolysis) at high levels. Similarly, during heat shock, increased expression of the Ub gene occurs in order to maintain a high level of free Ub, in the face of increased Ub conjugation to proteins [27]. Increasing the proteasome content of a muscle may directly enhance the maximal proteolytic rate, and studies testing this possibility are in progress.

The present studies indicate that there exists a group of genes, including those for polyUb and several subunits of the proteasome (C-2, C-3, C-5, C-8 and C-9), that are specially induced in different types of atrophying muscle when muscle mass and total RNA content are decreasing. In contrast with these mRNAs, the Ub-extension mRNA does not increase or may decrease, as would be expected, as this transcript is unrelated to proteolysis, but is involved in production of new ribosomes [44,45]. Thus polyUb and proteasome mRNA levels seem to be regulated inversely with total cellular RNA and mRNA encoding the Ub-extension protein. Ilian and Forsberg [56] have reported that, in muscles of starving rabbits, mRNA of calpains I and II and cathepsin D also increased. However, in our studies with rat muscles after food deprivation or denervation, the levels of mRNA of cathepsin D and the other major lysosomal protease, cathepsin L, and of the Ca^{2+} -dependent protease, calpain I, did not change (Table 3). Thus the expression of these proteases appears to be constitutive and unrelated to changes in overall proteolysis. These observations are in accord with previous findings that lysosomal and Ca^{2+} -dependent proteases are not essential for most of the proteolysis that occurs during starvation [49] and denervation [6]. The lack of change in these mRNAs, together with the co-ordinated increase in mRNA of Ub and

multiple proteasome subunits, further indicates the primary role for the Ub-dependent process in the atrophy.

Of particular physiological interest is the finding that the increase in Ub mRNA is restricted to striated muscle. Such changes also occur in the rat heart, which undergoes considerable weight loss during starvation [57]. Presumably, ATP-dependent proteolysis increases in cardiac muscle under such conditions as the result of similar mechanisms to those in skeletal muscle. The absence of any change in testes or brain was expected, as protein content and size are maintained in these tissues during a period of food deprivation. However, it is noteworthy that levels of Ub mRNA did not change in liver, in which overall proteolysis clearly rises in low-insulin states, but this is controlled by a lysosomal (autophagic) process [11,12,47]. In other words, in catabolic states, the lysosomal apparatus appears to be of major importance in the liver [57], whereas the Ub-proteasome pathway appears to have special significance in muscle [1,13,14].

We initially had expected that starvation and denervation might represent forms of physiological 'stress', which enhance proteolysis and increase Ub expression by provoking a heat-shock response in muscle. However, mRNA of HSPs 28, 70 and 90 did not increase co-ordinately with polyUb mRNA, even though the muscles can clearly exert a classical heat-shock response. During heat shock, the stimulus for expression of HSPs is the accumulation of abnormal proteins in cells [29,30,58], and the resulting increase in Ub expression is probably important in promoting the breakdown of damaged polypeptides. However, Ub production is clearly also subjected to other physiological regulators which function in a tissue-specific manner. For example, polyUb transcription increases specifically on DNA damage [59], and increased Ub production under these conditions may be related to the role of this protein in the repair of DNA damage [17–20,59]. In related studies, we have found that levels of polyUb and proteasome mRNA in muscle respond *in vivo* to a variety of other important physiological regulators, including hormones [49,51], monokines [60] and acidosis [61], which enhance proteolysis and cause muscle wasting. Thus the co-ordinated increase in these mRNAs suggests a specific genetic program leading to enhanced proteolytic capacity and muscle atrophy under various physiological or pathological conditions.

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