Glucocorticoids regulate mRNA levels for subunits of the 19 S regulatory complex of the 26 S proteasome in fast-twitch skeletal muscles

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Circulating levels of glucocorticoids are increased in many traumatic and muscle-wasting conditions that include insulin-dependent diabetes, acidosis, infection, and starvation. On the basis of indirect findings, it appeared that these catabolic hormones are required to stimulate Ub (ubiquitin)–proteasome-dependent proteolysis in skeletal muscles in such conditions. The present studies were performed to provide conclusive evidence for an activation of Ub-proteasome-dependent proteolysis after glucocorticoid treatment. In atrophying fast-twitch muscles from rats treated with dexamethasone for 6 days, compared with pair-fed controls, we found (i) increased MG132-inhibitable proteasome-dependent proteolysis, (ii) an enhanced rate of substrate ubiquitination, (iii) increased chymotrypsin-like proteasomal activity of the proteasome, and (iv) a co-ordinate increase in the mRNA expression of several ATPase (S4, S6, S7 and S8) and non-ATPase (S1, S5a and S14) subunits of the 19 S regulatory complex, which regulates the peptidase and the proteolytic activities of the 26 S proteasome. These studies provide conclusive evidence that glucocorticoids activate Ub-proteasome-dependent proteolysis and the first *in vivo* evidence for a hormonal regulation of the expression of subunits of the 19 S complex. The results suggest that adaptations in gene expression of regulatory subunits of the 19 S complex by glucocorticoids are crucial in the regulation of the 26 S muscle proteasome.

Key words: dexamethasone, glucocorticoid, muscle wasting, 26 S proteasome, skeletal muscle, ubiquitin–proteasome proteolysis.

INTRODUCTION

Circulating levels of glucocorticoids are increased in Cushing's syndrome, insulin-dependent diabetes, acidosis, infection, starvation, and various traumatic and catabolic conditions [1–3]. In rodents, administration of glucocorticoids results in an impairment of protein synthesis and increased proteolysis leading to skeletal-muscle atrophy [4,5]. However, the mechanisms underlying the increased muscle proteolysis observed in response to high levels of glucocorticoids are not yet fully understood [4–7].

Multiple proteolytic pathways, including the lysosomal, Ca^{2+}-dependent and Ub (ubiquitin)–proteasome-dependent processes and proteases (e.g. caspases and matrix metalloproteases) are responsible for skeletal-muscle proteolysis [7]. It is now established that the degradation of the most abundant contractile proteins (actin and myosins) is not directly mediated by lysosomal and Ca^{2+}-activated proteases. In contrast, overwhelming evidence that the Ub–proteasome-dependent pathway degrades these myofibrillar proteins has been provided recently [7–9]. Accordingly, this degradative process is the critical system responsible for the muscle wasting seen in different catabolic conditions [7,10]. In this pathway, Ub targets specific intracellular proteins for degradation. Ubiquitination proceeds via a thiol-ester reaction cascade involving the Ub-activating enzyme (E1), Ub-conjugating enzymes (E2) and Ub–protein ligases (E3), which possess substrate recognition sites (see [11,12] for recent reviews). Rapid degradation by the 26 S proteasome requires the formation of a poly(Ub) degradation signal that comprises at least four Ub moieties [11]. Poly(Ub) chains are generally formed by the sequential conjugation of the C-terminus of Ub to the ε-amino group of the Lys^{48} of the Ub moiety already bound to the protein. The 26 S proteasome comprises a 20 S proteasome catalytic core (a barrel-shaped particle organized as a stack of four rings of seven α- and β-subunits) and two axially positioned 19 S regulatory complexes (each containing approx. 18 subunits). The 19 S complexes recognize poly(Ub) chains, control substrate access to the catalytic chamber, and regulate both peptidase and proteolytic activities of the proteasome by ATP-dependent mechanisms [7].

In muscle cell cultures, glucocorticoids stimulate the transcription of Ub or the C3 20 S proteasome subunit via distinct signalling pathways [e.g. SP1 (stimulating protein-1) and MEK-1 (mitogen-activated protein kinase/extracellular-signal-regulated protein kinase kinase 1) or nuclear factor κB respectively] [3,13]. The glucocorticoid-dependent stimulation of skeletal-muscle proteolysis correlates with increased mRNA levels for Ub, the 14 kDa E2 (14 kDa Ub-conjugating enzyme E2), Ub-E2G, the 2E isoform of the 17 kDa E2, the muscle-specific MAFbx (muscle atrophy F-box protein) and MuRF-1 (muscle RING finger-1) E3s, and α-subunits of the 20 S proteasome [4,14,15]. In contrast, adrenalectomy suppresses the increased non-lysosomal, Ca^{2+}-independent, but ATP-dependent, proteolysis in fasted rats [16], and the increased mRNA levels for components of the Ub–proteasome pathway in fasted and acidotic rats [16–18]. Finally, treatment of rats with the glucocorticoid antagonist RU38486 blocked both the catabolic response observed in muscle from burned and septic rats [19,20], and concomitantly suppressed the increased expression of Ub [21] or muscle-specific E3s [20] that characterizes these catabolic conditions. Although these observations clearly support a role for the Ub-proteasome pathway in glucocorticoid-induced muscle atrophy, they need to be interpreted with caution. Indeed, all the information currently available in skeletal muscle pertains to very indirect observations (e.g.

Abbreviations used: DTT, dithiothreitol; DX, dexamethasone; PF, pair-fed; Ub, ubiquitin; 14 kDa E2, 14 kDa Ub-conjugating enzyme E2.

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measurements of levels of mRNA and Ub–protein conjugates; rates of proteolysis in incubated muscles in the presence of ATP depletion and of inhibitors of lysosomal/Ca^{2+}-dependent proteases). The present experiments were undertaken to determine the mechanisms of activation of Ub–proteasome-dependent proteolysis in skeletal muscles from DX (dexamethasone)-treated adult rats. For this purpose, skeletal muscles were incubated in the presence of the proteasome inhibitor MG132, and both Ub conjugation rates and the chymotrypsin-like activity of the proteasome were measured. We also investigated, for the first time in mammalian muscle, if glucocorticoids regulate mRNA and protein levels for ATPase and non-ATPase subunits of the 19 S regulatory complex of the 26 S proteasome.

EXPERIMENTAL

Animals

The animal facilities and methods were approved by the local ethical committee of the Institut National de la Recherche Agronomique (INRA, France). Adult male Wistar rats (Iffa-Credo, Lyon, France) aged 8 months were housed individually under controlled environmental conditions [room temperature (22 °C); 12 h light–dark cycle, lights on at 08:00 h]. Before the experiments were performed, they were fed *ad lib.* with a commercial laboratory chow. Animals had free access to water and were randomly divided into a control and a DX-treated group. DX (a synthetic glucocorticoid analogue that does not bind to plasma-binding proteins) was given daily at 09:00 h in the drinking water for 6 days [4]. DX concentration was adjusted every day on the basis of the amount of water that the animals drank the preceding day. To induce significant muscle atrophy, rats received 540 ± 92 μg DX · (kg body weight)$^{-1}$ · day^{-1} [4]. Since DX decreases food intake (from approx. 21 to approx. 7 g of dry matter/day between days 1 and 6 respectively), the control group was PF (pair-fed) to the DX group. Animals were killed under anaesthesia with sodium pentobarbital (6 mg/100 g of body weight) after an overnight starvation. Blood samples were collected for measuring both plasma glucose and insulin plasma levels. Gastrocnemius muscles were carefully dissected, frozen in liquid nitrogen and stored at − 80 °C until analysed.

Muscle incubation

Muscle protein breakdown was measured exactly as described previously [22]. On the day of the experiment, epitrochlearis muscles were quickly excised and rinsed in Krebs–Henseleit bicarbonate buffer (120 mM NaCl/4.8 mM KCl/25 mM $NaHCO_3$/ 1.2 mM KH_2PO_4/1.2 mM $MgSO_4$, pH 7.4) supplemented with 5 mM Hepes, 5 mM glucose and 0.1 % BSA. Muscles were then transferred to plastic tubes containing 1.5 ml of fresh buffer at 37 °C saturated with a O_2/CO_2 (19:1) gas mixture. After 30 min of preincubation, muscles were transferred to a fresh medium of identical composition and incubated further for 1 h. To date, this is the only way to evaluate the contribution of proteolytic pathways in skeletal muscles. Although isolated muscles are always in net negative nitrogen balance, such measurements quantitatively reflect changes in protein turnover rates reported in intact animals and have been validated previously [22].

The contribution of lysosomal and Ca^{2+}-dependent proteases and of the proteasome to the rates of overall proteolysis was determined as described in [23]. When measuring proteasome-dependent proteolysis, skeletal muscles were incubated in the presence of lysosomal and Ca^{2+}-dependent protease inhibitors because the proteasome inhibitor MG132 also inhibits cysteine proteases (e.g. cathepsin B, H and L, and calpains) [23]. Rates of protein breakdown were measured by following the rates of tyrosine release into the medium in the presence of 0.5 mM cycloheximide to block protein synthesis [24]. Protein degradation was expressed in nmol of tyrosine released · (mg of protein)$^{-1}$ · h^{-1} into the medium. Muscle protein content was measured according to the bicinchoninic acid procedure.

Northern-blot analysis

Tibialis anterior, epitrochlearis and gastrocnemius muscles from PF controls and DX-treated rats were rapidly excised, frozen in liquid nitrogen and stored at − 80 °C. Total RNA was extracted as described by Chomczynski and Sacchi [25], and Northern-blot analysis was performed as described in [23]. The membranes were hybridized with cDNA probes encoding poly(Ub), the 14 kDa E2, the C2, X and Z subunits of the 20 S proteasome, and the S1, S4, S5a, S6, S7, S8 and S14 subunits of the 19 S regulatory complex of the 26 S proteasome [23]. Hybridizations were performed with [^{32}P]cDNA fragments labelled by random priming. After stripping the different probes, the filters were reprobed with a cDNA fragment encoding the 18 S rRNA. The filters were autoradiographed and the signals were quantified as described in [26]. Autoradiographic signals were normalized using the corresponding 18 S rRNA signals to correct for variations in RNA loading.

Western-blot analysis

Gastrocnemius muscles were homogenized in 20 vol. of ice-cold buffer [2.5 mM Tris, pH 7.5/5 mM EDTA, pH 8.0/2 mM PMSF/4 mM DTT (dithiothreitol)/0.1 % Triton X-100]. Homogenates were centrifuged at 3000 ***g*** for 10 min at 4 °C. Protein concentrations in the resulting supernatants were determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, U.S.A.). Proteins (35 and 50 μg) were electrophoresed on 12 % polyacrylamide gels and transferred on to nitrocellulose membranes (0.45 μm; Schleicher and Schuell, Ecquevilly, France). Membranes were blocked for 1 h in 5 % (w/v) non-fat dried milk in TTNS (25 mM Tris, pH 7.5/0.1 % Tween 20/ 0.9 % NaCl) and incubated for 1 h with antibodies against the 14 kDa E2 (kindly provided by Dr Simon S. Wing, McGill University, Montréal, Canada), and the S4, S5a, S6, S7 and S14 subunits of the 19 S complex (kindly provided by Dr Martin Rechsteiner, University of Utah, Salt Lake City, UT, U.S.A. and Dr Keiji Tanaka, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan). After washing three times in TTNS, the blots were incubated for 1 h with an alkaline phosphatase-linked goat anti-mouse or anti-rabbit IgG as appropriate. The blots were washed extensively four times in TTNS for 20 min and incubated in enhanced chemifluorescence reagent (ECF; Amersham Biosciences, Orsay, France). Signals were visualized with a Phosphofluoro-Imager (Molecular Dynamics, Sunnyvale, CA, U.S.A.) and quantified using the ImageQuant software (version 1.1; Amersham Biosciences). The results were normalized using the corresponding amidoblack-staining signals to correct for variations in protein loading as described in [23].

Ubiquitination rates

Gastrocnemius muscles were excised from control PF and DX-treated rats and were homogenized individually at 4 °C with a Polytron in 50 mM Tris/HCl (pH 7.5), 1 mM DTT, 1 mM EDTA, 1 mM PMSF, 10 μg/ml pepstatin A and 10 μg/ml leupeptin (5 ml of buffer/g of muscle). The homogenates were centrifuged at

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10 000 ***g*** for 10 min at 4 °C, and the supernatants were centrifuged at 100 000 ***g*** for 60 min at 4 °C. The final supernatants were stored at − 80 °C until use. At 37 °C, rates of ubiquitination were determined by incubation of muscle extracts containing 50 μg of protein in 50 mM Tris/HCl (pH 7.5), 1 mM DTT, 2 mM $MgCl_2$, 2 mM 5′-adenylylimidodiphosphate and 5 μM ^{125}I-labelled Ub (approx. 3000 c.p.m./pmol) in a total volume of 20 μl. The reaction was stopped at 0, 15, 30, 45 and 60 min by the addition of 1× Laemmli buffer. Pilot studies showed that conjugation rates were linear for this period of time and that the concentration of exogenously labelled Ub was in significant excess of any endogenous Ub (e.g. addition of larger amounts of labelled Ub did not increase rates of ubiquitination). After incubation, Ub conjugates were resolved from free Ub by SDS/PAGE (12 % gel). After drying of the gel, high-molecular-mass radiolabelled conjugates were visualized with a PhosphofluoroImager and quantified using the ImageQuant imager software. Time courses for the accumulation of high-molecular-mass Ub conjugates were analysed by linear regression to calculate ubiquitination rates, e.g. the slopes of best fit of arbitrary densitometric units of high-molecular-mass Ub conjugates versus time.

Chymotrypsin-like activity of the proteasome

Soluble proteins, from pooled gastrocnemius muscles of either control PF or DX-treated animals, were extracted in 10 vol. of an ice-cold 10 % (v/v) glycerol buffer (pH 8.0) containing 50 mM Tris, 10 mM ATP, 10 mM $MgCl_2$, 1 mM DTT and protease inhibitors (10 μg/ml of antipain, aprotinin, leupeptin and pepstatin A, and 20 μM PMSF). Samples were then centrifuged at 100 000 ***g*** for 1 h at 4 °C. Supernatants were collected and submitted to 36 ml of 10–40 % glycerol gradient in the above-described buffer. Samples were centrifuged at 100 000 ***g*** for 22 h at 4 °C and 1 ml fractions were then collected from the bottom of the tubes. Each fraction was tested for the chymotrypsin-like activity of the proteasome with succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin as a substrate (Affiniti Research Products, Exeter, U.K.) as described in [23]. To estimate the proteasome-dependent activity, the difference between arbitrary fluorescence units recorded at ±40 μM of the proteasome inhibitor MG132 (Affiniti Research Products) in the reaction medium was calculated. The final data were corrected by the amount of protein loaded on to the gradient.

Blood glucose and plasma insulin

Plasma insulin was determined by RIA with INS-PR kit (CIS BIO International, Gif-sur-Yvette, France). Arterial blood glucose concentration was measured using a glucometer (Glucometer 3; Bayer Diagnostics, Marnes la Coquette, France).

Statistical analysis

All data are expressed as means ± S.E.M. and are representative of at least two different experiments. Statistical analyses were performed using the unpaired Student's *t* test. Ubiquitination rates were compared using regression analysis [27]. Significance was defined at the 0.05 level.

RESULTS

Animal characteristics

DX-treated animals lost 18.5 % of their initial body weight, whereas control PF rats lost only 8.5 % of their initial body weight (Table 1). Thus DX treatment increased the loss of body weight. At the end of the DX treatment, muscle mass of gastrocnemius and epitrochlearis in DX-treated rats was 19 and 26 % lower respectively compared with control PF animals (Table 1). In accordance with previous results [28], blood glucose increased by 109 % ($P < 0.05$) in DX-treated rats compared with control PF rats, and hyperglycaemia was associated with hyperinsulinaemia (+420 %, $P < 0.05$) (Table 1).

Table 1 Effect of DX treatment for 6 days in adult rats compared with PF animals

Data are means ± S.E.M. for $n = 9$ control PF and $n = 10$ for DX-treated rats, except for blood glucose and plasma insulin ($n = 10$).

	PF	DX
Initial body weight (g)	538 ± 13	550 ± 18
Final body weight (g)	493 ± 13	448 ± 14*
Average food intake (g)	10.2 ± 0.2	10.4 ± 0.2
Blood glucose (g/l)	0.95 ± 0.03	1.99 ± 0.18*
Plasma insulin (μU/ml)	60 ± 9	312 ± 26*
Muscle mass (g)		
Gastrocnemius	2.58 ± 0.10	2.08 ± 0.07*
Epitrochlearis	0.13 ± 0.01	0.09 ± 0.01*

* $P < 0.05$ versus control PF rats.

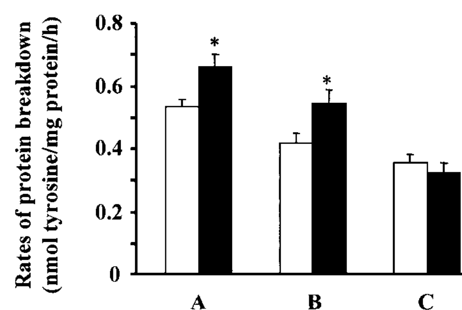

Figure 1 Effect of DX administration on proteasome-dependent rates of proteolysis in the epitrochlearis muscle

Epitrochlearis muscles were incubated in the presence of cycloheximide to block protein synthesis, and without or with different proteolytic inhibitors. White bars, control PF rats; black bars, DX-treated rats. (**A**) Total proteolysis. (**B**) Non-lysosomal, Ca^{2+}-independent proteolysis was measured in muscles incubated in a Ca^{2+}-free medium containing 10 mM methylamine and 50 μM leupeptin. (**C**) Non-lysosomal, Ca^{2+}-independent and proteasome-independent proteolysis was measured in muscles incubated in a Ca^{2+}-free medium containing 10 mM methylamine, 50 μM leupeptin and 40 μM proteasome inhibitor MG132. Data are means ± S.E.M. for nine or ten animals. Statistical differences were assessed using unpaired Student's *t* test. *$P < 0.05$ versus control PF rats.

Proteolytic pathways responsible for muscle atrophy

The DX-induced muscle atrophy resulted from increased total proteolysis in the epitrochlearis ($P < 0.05$, Figure 1A). Rates of protein breakdown in DX-treated rats were still high in the presence of inhibitors of the lysosomal and Ca^{2+}-dependent proteolytic pathways ($P < 0.05$, Figure 1B). In contrast, high rates of proteolysis in the incubated muscles from DX-treated rats were totally suppressed when the proteasome inhibitor MG132 was added to inhibitors of both lysosomal and Ca^{2+}-dependent proteases (Figure 1C). Thus the increased proteolysis in DX-treated rats is explained by a stimulation of proteasome-dependent proteolysis.

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DX administration increased rates of ubiquitination of muscle proteins

We next examined the mechanisms responsible for the activation of the MG132-sensitive proteolytic pathway in DX-treated rats. There are two main steps in the Ub–proteasome pathway: (i) Ub conjugation to protein substrates that involves the 14 kDa E2 [4] and E3α, and several other E2s [14], or recently characterized muscle-specific E3s [15], and (ii) the subsequent degradation of Ub conjugates by the 26 S proteasome.

The musculature comprises mainly fast-twitch muscles. Measurements of Ub–proteasome-dependent proteolysis (e.g. *in vitro* rates of proteolysis, mRNA and protein levels, proteasome activities etc.) often need to be performed in several muscles due to the limited amounts of material in rodents. Thus we next investigated changes in mRNA levels for components of the Ub–proteasome pathway in three different fast-twitch muscles. Figure 2(A) shows that Ub mRNA levels increased in the tibialis anterior and epitrochlearis muscles from DX-treated rats compared with control PF animals [+277 % ($P < 0.001$) and +368 % ($P < 0.01$) respectively]. mRNA levels for the 14 kDa E2 also increased significantly, but only in the epitrochlearis muscle from DX-treated animals (+150 %, $P < 0.001$). In the gastrocnemius muscle, Ub mRNA levels increased by 252 % ($P < 0.01$), whereas the 14 kDa E2 mRNA levels remained stable after DX administration (Figure 2B, left panel). Similarly, the protein level of the 14 kDa E2 in gastrocnemius muscles was also not altered by DX treatment (Figure 2B, right panel). In contrast, ubiquitination rates measured in soluble protein extracts from gastrocnemius muscles increased by 70 % ($P < 0.005$, Figure 2C). Thus the enhanced rates of proteasome-dependent proteolysis observed in epitrochlearis muscles from DX-treated rats (Figure 1B) correlated with increased expression of both the Ub and 14 kDa E2 in that muscle (Figure 2A), and reflected increased conjugation of Ub to protein substrates at least in the gastrocnemius muscle (Figure 2C).

DX administration increased the expression of 20 S proteasome subunits and chymotrypsin-like proteasomal activity

mRNA levels for non-catalytic subunits α of the 20 S proteasome (C2, C3, C8 and C9) are increased in various muscle-wasting conditions where proteolysis increases [7]. Figure 3 shows that the mRNA levels for the non-catalytic α-subunit C2 and the β catalytic subunit Z of the 20 S proteasome increased significantly ($P < 0.05$) in the three muscles studied from DX-treated rats compared with control PF animals, including the epitrochlearis muscle. In contrast, the mRNA levels for the β catalytic subunit X increased significantly in the tibialis anterior muscle from DX-treated rats ($P < 0.05$), but only tended to increase in both the epitrochlearis and gastrocnemius muscle ($P > 0.05$). However, the chymotrypsin-like activity of the proteasome increased in the gastrocnemius muscle from DX-treated rats (Figure 4). Overall, a very similar pattern of enhanced expression for α- and β-subunits of the 20 S proteasome was observed in three different fast-twitch muscles (although variations for subunit X expression were not always significant), which correlated with increased proteasome activity.

DX administration increased the expression of subunits of the 19 S regulatory complex

The binding of 19 S complexes to 20 S proteasomes regulates the peptidase and proteolytic activities of the resulting 26 S proteasomes. Thus we next investigated the effects of DX on the mRNA

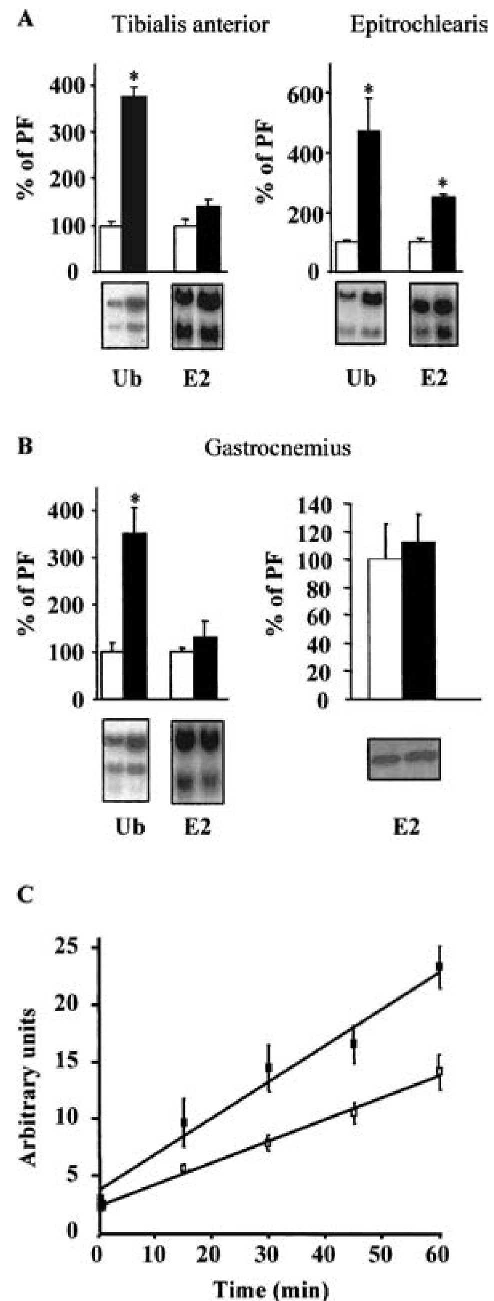

Figure 2 Effect of DX administration on the ubiquitination machinery

(**A**) Northern blots were performed as described in the Experimental section with total RNA isolated from tibialis anterior (left panel) and epitrochlearis muscles (right panel). Hybridization signals for the two transcripts of Ub and for the bottom 1.2 kb transcript of the 14 kDa E2 (which is mainly up-regulated by catabolic conditions including DX treatment, see [4]) were quantified and normalized using the corresponding 18 S rRNA signals to correct for uneven unloading. (**B**) Left panel, Northern blots were performed with total RNA extracted from gastrocnemius muscles. Right panel, Western blots were performed with proteins extracted from gastrocnemius muscles with an antibody raised against the 14 kDa E2 (see Experimental section). Signals were detected by chemifluorescence and quantified in arbitrary fluorescence units. Data are means ± S.E.M. for four to six rats. Representative Northern and Western blots are also shown. Statistical differences were assessed using unpaired Student's *t* test. *$P < 0.05$ versus control PF rats. (**C**) The time course of the formation of high-molecular-mass ^{125}I-labelled Ub conjugates was visualized with a Phosphofluoro-Imager and quantified using ImageQuant software, as described in the Experimental section. White squares, control PF rats; black squares, DX-treated animals. Data are means ± S.E.M. for five control PF animals and six DX-treated rats. The ubiquitination rate, i.e. the slope of best fit of arbitrary densitometric units of high-molecular-mass Ub conjugates versus time, was significantly increased in DX-treated rats versus control PF rats ($P < 0.01$).

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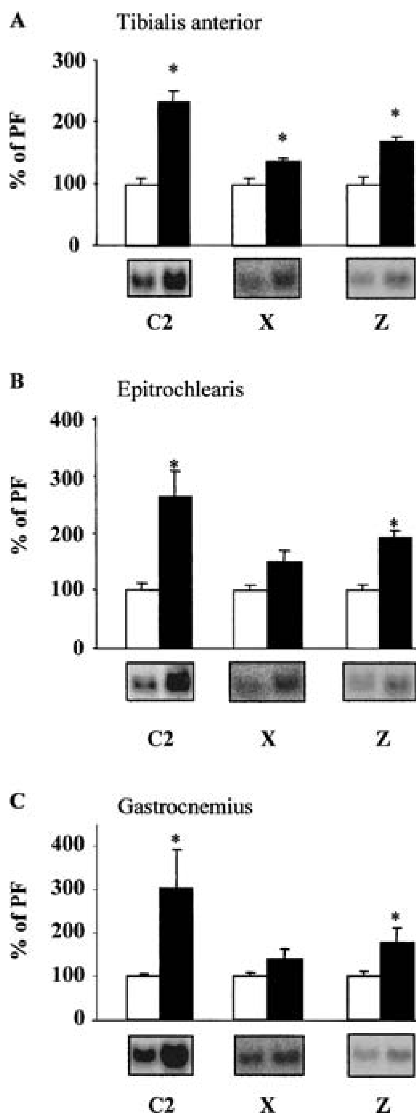

Figure 3 Effect of DX treatment on muscle mRNA levels for C2 and Z subunits of the 20 S proteasome

Northern blots were performed as described in Figure 2 with total RNA extracted from tibialis anterior (**A**), epitrochlearis (**B**) and gastrocnemius (**C**) muscles. Hybridization signals for subunits C2, X and Z of the 20 S proteasome were quantified and normalized using the corresponding 18 S rRNA signals to correct for uneven unloading. Data are means ± S.E.M. for four to six rats and are expressed as a percentage of PF controls. Representative Northern blots are also shown. White bars, control PF rats; black bars, DX-treated rats. Statistical differences were assessed using unpaired Student's *t* test. *$P < 0.05$ versus control PF rats.

levels for both ATPase and non-ATPase subunits of the 19 S complex. DX administration increased mRNA levels for ATPase (S4, S6, S7 and S8; Figure 5) and non-ATPase (S1, S5a and S14; Figure 6) subunits of the 19 S complex up to 6.7-fold ($P < 0.05$) in the three muscles studied. Ordway et al. [29] and Tilignac et al. [23] reported a correlation between mRNA and protein levels for some subunits of the 19 S complex in muscles where Ub–proteasome-dependent proteolysis was changed. However, in striking contrast, the enhanced mRNA levels for ATPase subunits S4, S6 and S7 and non-ATPase subunits S5a and S14 in the muscle from DX-treated rats (Figures 5 and 6) did not correlate with changes in the corresponding muscle protein levels, irrespective of the amount of total proteins loaded on the gels (e.g. 35 μg, Figure 7 or 50 μg, results not shown).

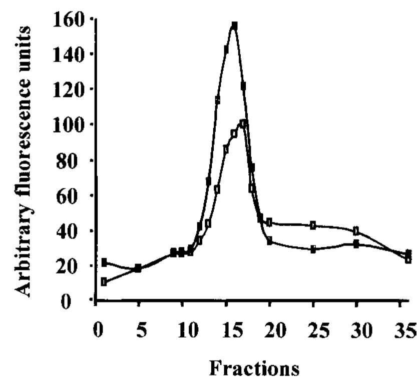

Figure 4 Effect of DX treatment on the chymotrypsin-like activity of proteasomes in the gastrocnemius muscle

The profile of arbitrary fluorescence units with succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin as a substrate in glycerol gradient fractions from pooled muscles of control PF (□) and DX-treated (■) rats. The results are representative of two distinct experiments.

DISCUSSION

In the present study, we report that glucocorticoid treatment of adult rats (i) increased proteasome-dependent proteolysis, (ii) stimulated both substrate ubiquitination and chymotrypsin-like proteasomal activity, and (iii) co-ordinately increased the expression of several ATPase and non-ATPase subunits of the 19 S regulatory complex in atrophying fast-twitch skeletal muscles. This provides strong evidence for a role of glucocorticoids in stimulating skeletal-muscle Ub–proteasome-dependent proteolysis.

Enhanced rates of proteasome-dependent proteolysis have been reported in many instances of muscle wasting [30–35]. On the basis of indirect evidence, glucocorticoids are believed to be required for this activation in several catabolic conditions that include starvation [16], metabolic acidosis [18], insulin-dependent diabetes [17], sepsis [21] and trauma [1]. We showed previously that mRNA levels for Ub, the 14 kDa E2-conjugating enzyme and the C2 subunit of the 20 S proteasome were up-regulated after glucocorticoid administration, suggesting the involvement of the Ub-proteasome pathway in the establishment of skeletal-muscle atrophy [4]. We now demonstrate, using inhibitors of the lysosomal and Ca^{2+}-dependent proteases, and the proteasome inhibitor MG132, that glucocorticoids activate proteasome-dependent proteolysis in rat skeletal muscle. We show further that this activation also reflected an increase in proteasomal chymotrypsin-like activity and a 70 % increase in rates of Ub conjugation in the gastrocnemius muscle. This increased rate of Ub conjugation reported here is consistent with similar adaptations observed in various catabolic conditions [36] and with the enhanced expression of the 14 kDa E2 in the epitrochlearis muscle (Figure 2A), as well as with increased mRNA levels for other E2 species [14] and muscle-specific E3s [15] in glucocorticoid-treated rats. Taken together, our results suggest that both ubiquitination and the subsequent breakdown of Ub conjugates by the 26 S proteasome were targets of glucocorticoid action. DX has differential effects on the proteins involved in the early steps of insulin action on skeletal muscle. Administration of this drug results in a decrease in insulin-stimulated IRS-1 (insulin receptor substrate-1)-associated

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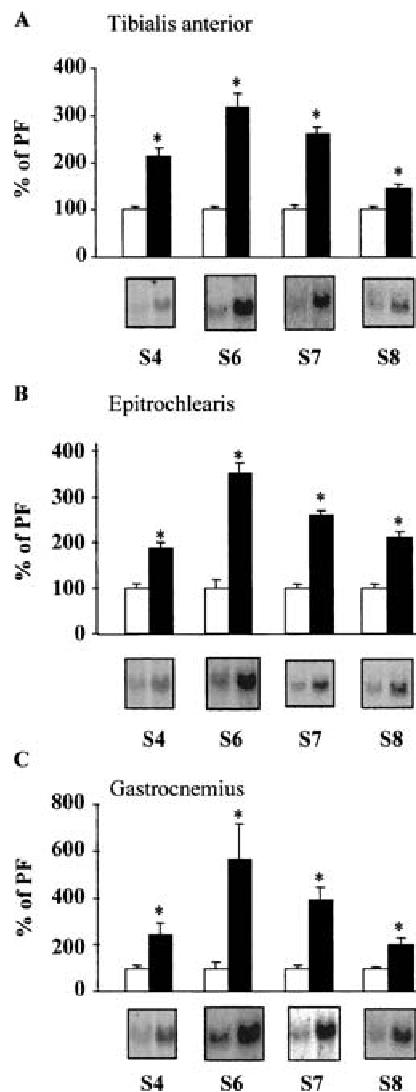

Figure 5 Effect of DX treatment on muscle mRNA levels for ATPase subunits of the 19 S regulatory complex

Northern blots were performed with total RNA extracted from tibialis anterior (**A**), epitrochlearis (**B**) and gastrocnemius (**C**) muscles. Hybridization signals for ATPase subunits S4, S6, S7 and S8 of the 19 S complex were quantified and normalized using the corresponding 18 S rRNA signals to correct for uneven unloading. Data are means ± S.E.M. for four to six rats and are expressed as a percentage of PF controls. Representative Northern blots are also shown. White bars, control PF rats; black bars, DX-treated rats. Statistical differences were assessed using unpaired Student's *t* test. $^*P < 0.05$ versus control PF rats.

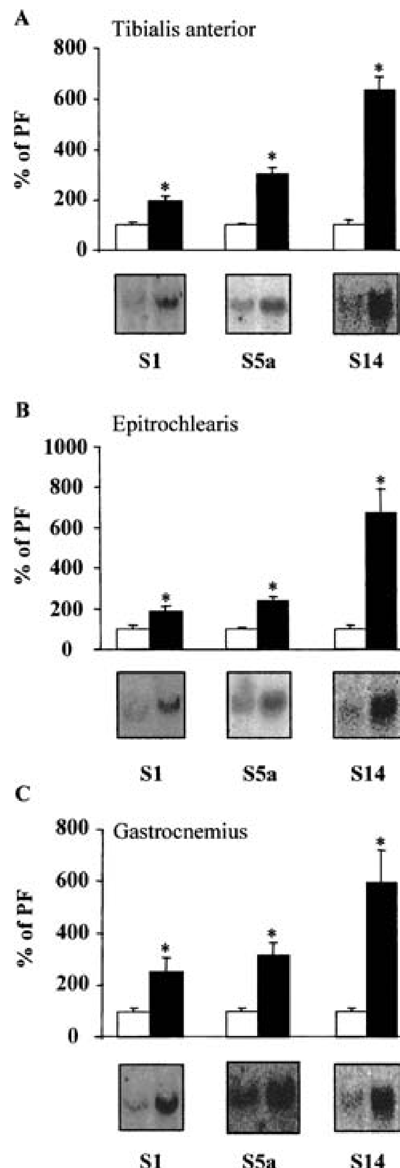

Figure 6 Effect of DX treatment on muscle mRNA levels for non-ATPase subunits of the 19 S regulatory complex

Northern blots were performed with total RNA extracted from tibialis anterior (**A**), epitrochlearis (**B**) and gastrocnemius (**C**) muscles. Hybridization signals for non-ATPase subunits S1, S5a and S14 of the 19 S complex were quantified and normalized using the corresponding 18 S rRNA signals to correct for uneven unloading. Data are means ± S.E.M. for four to six rats and are expressed as a percentage of PF controls. Representative Northern blots are also shown. White bars, control PF rats; black bars, DX-treated rats. Statistical differences were assessed using unpaired Student's *t* test. $^*P < 0.05$ versus control PF rats.

phosphoinositide 3-kinase [37,38]. The glucocorticoid-induced hyperinsulinaemia appears to be essential for the development of these alterations in insulin receptor signalling [39]. Moreover, activation of phosphoinositide 3-kinase is required for insulin-induced proteolysis inhibition [40]. Thus glucocorticoid effects on the activation of the Ub–proteasome-dependent pathway may be accounted for by abnormalities in the insulin-signalling pathway. In addition, Du et al. [13] showed that glucocorticoids induce proteasome C3 subunit expression in L6 muscle cells by opposing the suppression of its transcription by nuclear factor κB. Taken together, all these observations suggest an indirect action of glucocorticoids on Ub–proteasome-dependent proteolysis.

The second major observation of the present study is that DX treatment co-ordinately increased the levels of multiple mRNAs encoding subunits of the 19 S complex. This is the first *in vivo* demonstration of a regulation of this regulatory particle of the 26 S proteasome by glucocorticoids in mammals. Interestingly, ecdysteroid hormones also regulate the 26 S proteasome in atrophying muscles from insects [41]. The 19 S complexes bind to both outer α-rings of the 20 S proteasome in the presence of ATP to form the 26 S proteasome. They contain at least six ATPase and 12 non-ATPase subunits in human proteasomes. The ATPases provide energy for the recognition of the poly(Ub) degradation signal [42], assembly of the 26 S proteasome, gating of the 20 S proteasome channel, unfolding and injection of protein substrates

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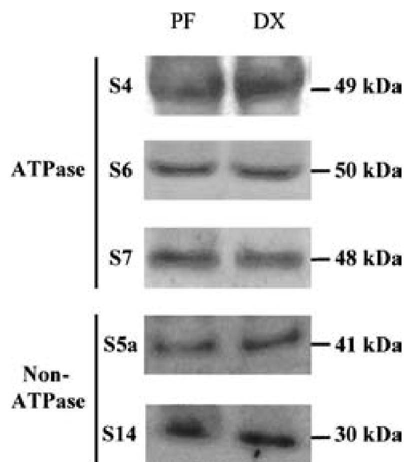

Figure 7 Effect of DX treatment on protein levels for ATPase and non-ATPase subunits of the 19 S regulatory complex

Protein (35 μg) was extracted from gastrocnemius muscles, electrophoresed, transferred on to nitrocellulose membranes; immunodetection of subunits S4, S5a, S6, S7 and S14 was performed with appropriate antibodies, as described in the Experimental section. Signals were detected by chemifluorescence, visualized with a Phosphofluoro-Imager and quantified for six control PF and five DX-treated rats using ImageQuant software. Representative Western blots are shown. There was no significant change in any protein level ($P > 0.05$) using unpaired Student's *t* test.

into the catalytic chamber of the proteasome and peptide release [43–45]. Thus the 19 S complex plays an important regulatory role in Ub–proteasome-dependent proteolysis. Unfortunately, however, the precise role of the individual subunits is still poorly understood, except for a few subunits. For example, both the non-ATPase subunit S5a [46] and the ATPase subunit S6′ [42] recognize the poly(Ub) degradation signal. In the present study, we report that mRNA levels for four ATPase (S4, S6, S7 and S8) and three non-ATPase (S1, S5a and S14) subunits of the 19 S complex increased in epitrochlearis, tibialis anterior and gastrocnemius muscles from DX-treated rats. This correlated with increased chymotrypsin-like activity of the proteasome. Conversely, both mRNA levels for the ATPase subunits S4 and S7 and the non-ATPase subunits S5a and S14, and the chymotrypsin-like proteasomal activity decreased when muscle proteolysis was down-regulated after chemotherapy [23]. Taken together, these observations suggest that changes in mRNA levels for regulatory subunits of the 19 S complex regulated the 26 S proteasome activities.

The precise significance of the DX-induced changes in the mRNA expression pattern of subunits of the 19 S complex remains to be elucidated. Since we observed no change in protein levels of subunits that were apparently overexpressed in muscle after DX treatment, we cannot rule out that DX only stabilized the mRNA half-lives of the 19 S complex subunits. However, this seems unlikely. First, increased and decreased mRNA levels for several (but not always all) subunits of this regulatory complex have been reported in various catabolic [30,47–49] and anabolic [22] conditions respectively. Secondly, these adaptations disappeared when proteasome-dependent proteolysis is normalized (e.g. by eicosapentaenoic acid treatment of starved and tumour-bearing rats; see [48,49]). Thirdly, in the reloaded unweighted soleus muscle, the increased mRNA levels for subunit S7 of the 19 S complex were actively translated [50]. Finally, in the tobacco hornworm *Manduca sexta*, eclosion is characterized by the elimination of intersegmental muscles that involves an increased chymotrypsin-like activity of the proteasome and increased protein levels for some, but not for all, ATPase subunits of the 19 S complex in such muscles [51]. Furthermore, the amount of MS73 protein (the equivalent of ATPase subunit S6) in that model did not correlate with intersegmental skeletal-muscle atrophy [41]. Thus changes in protein levels for 19 S complex subunits do not appear to correlate with stimulated proteasome activities. Further experiments are needed to support this conclusion.

In conclusion, our results provide the first evidence in mammalian muscle that glucocorticoids up-regulate Ub–proteasome-dependent proteolysis via mechanisms that include a stimulation of rates of Ub conjugation, altered proteolytic activities of the proteasome and up-regulation of the mRNA levels for ATPase and non-ATPase subunits of the 19 S complex of the 26 S proteasome. Elucidating the precise mechanisms that are responsible for changes in gene expression of regulatory subunits of the 19 S complex and that may ultimately be important in the modulation of 26 S proteasomes in catabolic and anabolic conditions is of obvious importance in designing new strategies to prevent muscle wasting.

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REFERENCES

1 Mansoor, O., Beaufrère, B., Boirie, Y., Rallière, C., Taillandier, D., Aurousseau, E., Schoeffler, P., Arnal, M. and Attaix, D. (1996) Increased mRNA levels for components of the lysosomal, Ca^{2+}-activated, and ATP-ubiquitin-dependent proteolytic pathways in skeletal muscle from head trauma patients. Proc. Natl. Acad. Sci. U.S.A. **93**, 2714–2718

2 Hasselgren, P. O. (1999) Glucocorticoids and muscle catabolism. Curr. Opin. Clin. Nutr. Metab. Care **2**, 201–205

3 Marinovic, A. C., Zheng, B., Mitch, W. E. and Price, S. R. (2002) Ubiquitin (UbC) expression in muscle cells is increased by glucocorticoids through a mechanism involving Sp1 and MEK1. J. Biol. Chem. **277**, 16673–16681

4 Dardevet, D., Sornet, C., Taillandier, D., Savary, I., Attaix, D. and Grizard, J. (1995) Sensitivity and protein turnover response to glucocorticoids are different in skeletal muscle from adult and old rats. Lack of regulation of the ubiquitin-proteasome proteolytic pathway in aging. J. Clin. Invest. **96**, 2113–2119

5 Mitch, W. E. and Goldberg, A. L. (1996) Mechanisms of muscle wasting. The role of the ubiquitin–proteasome pathway. N. Engl. J. Med. **335**, 1897–1905

6 Rallière, C., Tauveron, I., Taillandier, D., Guy, L., Boiteux, J. P., Giraud, B., Attaix, D. and Thiéblot, P. (1997) Glucocorticoids do not regulate the expression of proteolytic genes in skeletal muscle from Cushing's syndrome patients. J. Clin. Endocrinol. Metab. **82**, 3161–3164

7 Attaix, D., Combaret, L., Kee, A. J. and Taillandier, D. (2003) Mechanisms of ubiquitination and proteasome-dependent proteolysis in skeletal muscle. In Molecular Nutrition (Zempleni, J. and Daniel, H., eds.), pp. 219–235, CABI Publishing, Wallingford

8 Jagoe, R. T. and Goldberg, A. L. (2001) What do we really know about the ubiquitin–proteasome pathway in muscle atrophy? Curr. Opin. Clin. Nutr. Metab. Care **4**, 183–190

9 Hasselgren, P. O. and Fischer, J. E. (2001) Muscle cachexia: current concepts of intracellular mechanisms and molecular regulation. Ann. Surg. **233**, 9–17

10 Lecker, S. H., Solomon, V., Mitch, W. E. and Goldberg, A. L. (1999) Muscle protein breakdown and the critical role of the ubiquitin–proteasome pathway in normal and disease states. J. Nutr. **129**, 227S–237S

11 Pickart, C. M. (2001) Mechanisms underlying ubiquitination. Annu. Rev. Biochem. **70**, 503–533

12 Glickman, M. H. and Ciechanover, A. (2002) The ubiquitin–proteasome proteolytic pathway: destruction for the sake of construction. Physiol. Rev. **82**, 373–428

13 Du, J., Mitch, W. E., Wang, X. N. and Price, S. R. (2000) Glucocorticoids induce proteasome C3 subunit expression in L6 muscle cells by opposing the suppression of its transcription by NF-κB. J. Biol. Chem. **275**, 19661–19666

14 Chrysis, D. and Underwood, L. E. (1999) Regulation of components of the ubiquitin system by insulin-like growth factor I and growth hormone in skeletal muscle of rats made catabolic with dexamethasone. Endocrinology **140**, 5635–5641

15 Bodine, S. C., Latres, E., Baumhueter, S., Lai, V. K. M., Nunez, L., Clarke, B. A., Poueymirou, W. T., Panaro, F. J., Na, E. Q., Dharmarajan, K. et al. (2001) Identification of ubiquitin ligases required for skeletal muscle atrophy. Science **294**, 1704–1708

© 2004 Biochemical Society

16 Wing, S. S. and Goldberg, A. L. (1993) Glucocorticoids activate the ATP-ubiquitin-dependent proteolytic system in skeletal muscle during fasting. Am. J. Physiol. **264**, E668–E676
17 Price, S. R., England, B. K., Bailey, J. L., Van Vreede, K. and Mitch, W. E. (1994) Acidosis and glucocorticoids concomitantly increase ubiquitin and proteasome subunit mRNAs in rat muscle. Am. J. Physiol. **266**, C955–C960
18 May, R. C., Bailey, J. L., Mitch, W. E., Masud, T. and England, B. K. (1996) Glucocorticoids and acidosis stimulate protein and amino acid catabolism *in vivo*. Kidney Int. **49**, 679–683
19 Fang, C. H., Tiao, G., James, H., Ogle, C., Fischer, J. E. and Hasselgren, P. O. (1995) Burn injury stimulates multiple proteolytic pathways in skeletal muscle, including the ubiquitin-energy dependent pathway. J. Am. Coll. Surg. **180**, 161–170
20 Wray, C. J., Mammen, J. M. V. and Hasselgren, P.-O. (2003) Sepsis upregulates the gene expression of multiple ubiquitin ligases in skeletal muscle. Int. J. Biochem. Cell Biol. **35**, 698–705
21 Tiao, G., Fagan, J., Roegner, V., Lieberman, M., Wang, J. J., Fischer, J. E. and Hasselgren, P. O. (1996) Energy-ubiquitin-dependent muscle proteolysis during sepsis in rats is regulated by glucocorticoids. J. Clin. Invest. **97**, 339–348
22 Dardevet, D., Sornet, C., Attaix, D., Baracos, V. E. and Grizard, J. (1994) Insulin-like growth factor-1 and insulin resistance in skeletal muscles of adult and old rats. Endocrinology **134**, 1475–1484
23 Tilignac, T., Temparis, S., Combaret, L., Taillandier, D., Pouch, M.-N., Cervek, M., Cardenas, D. M., Le Bricon, T., Debiton, E., Samuels, S. E. et al. (2002) Chemotherapy inhibits skeletal muscle ubiquitin-proteasome-dependent proteolysis. Cancer Res. **62**, 2771–2777
24 Tischler, M. E., Desautels, M. and Goldberg, A. L. (1982) Does leucine, leucyl-tRNA, or some metabolite of leucine regulate protein synthesis and degradation in skeletal and cardiac muscle? J. Biol. Chem. **257**, 1613–1621
25 Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol–chloroform extraction. Anal. Biochem. **162**, 156–159
26 Deval, C., Mordier, S., Obled, C., Béchet, D., Combaret, L., Attaix, D. and Ferrara, M. (2001) Identification of cathepsin L as a differentially expressed message associated with skeletal muscle wasting. Biochem. J. **360**, 143–150
27 Snedecor, G. W. and Cochran, W. G. (1967) Analysis of covariance. Statistical Methods, 6th edn, pp. 419–446, The Iowa State University Press, Ames
28 Dardevet, D., Sornet, C., Savary, I., Debras, E., Patureau-Mirand, P. and Grizard, J. (1998) Glucocorticoid effects on insulin- and IGF-I-regulated muscle protein metabolism during aging. J. Endocrinol. **156**, 83–89
29 Ordway, G. A., Neufer, P. D., Chin, E. R. and DeMartino, G. N. (2000) Chronic contractile activity upregulates the proteasome system in rabbit skeletal muscle. J. Appl. Physiol. **88**, 1134–1141
30 Combaret, L., Tilignac, T., Claustre, A., Voisin, L., Taillandier, D., Obled, C., Tanaka, K. and Attaix, D. (2002) Torbafylline (HWA 448) inhibits enhanced skeletal muscle ubiquitin–proteasome-dependent proteolysis in cancer and septic rats. Biochem. J. **361**, 185–192
31 Hobler, S. C., Tiao, G., Fischer, J. E., Monaco, J. and Hasselgren, P. O. (1998) Sepsis-induced increase in muscle proteolysis is blocked by specific proteasome inhibitors. Am. J. Physiol. **274**, R30–R37
32 Fang, C. H., Wang, J. J., Hobler, S., Li, B. G., Fischer, J. E. and Hasselgren, P. O. (1998) Proteasome blockers inhibit protein breakdown in skeletal muscle after burn injury in rats. Clin. Sci. **95**, 225–233
33 Price, S. R., Bailey, J. L., Wang, X. N., Jurkovitz, C., England, B. K., Ding, X. Y., Phillips, L. S. and Mitch, W. E. (1996) Muscle wasting in insulinopenic rats results from activation of the ATP-dependent, ubiquitin–proteasome proteolytic pathway by a mechanism including gene transcription. J. Clin. Invest. **98**, 1703–1708
34 Bailey, J. L., Wang, X. N., England, B. K., Price, S. R., Ding, X. Y. and Mitch, W. E. (1996) The acidosis of chronic renal failure activates muscle proteolysis in rats by augmenting transcription of genes encoding proteins of the ATP-dependent ubiquitin–proteasome pathway. J. Clin. Invest. **97**, 1447–1453
35 Tawa, Jr, N. E., Odessey, R. and Goldberg, A. L. (1997) Inhibitors of the proteasome reduce the accelerated proteolysis in atrophying rat skeletal muscles. J. Clin. Invest. **100**, 197–203
36 Lecker, S. H., Solomon, V., Price, S. R., Kwon, Y. T., Mitch, W. E. and Goldberg, A. L. (1999) Ubiquitin conjugation by the N-end rule pathway and mRNAs for its components increase in muscles of diabetic rats. J. Clin. Invest. **104**, 1411–1420
37 Saad, M. J., Folli, F., Kahn, J. A. and Kahn, C. R. (1993) Modulation of insulin receptor, insulin receptor substrate-1, and phosphatidylinositol 3-kinase in liver and muscle of dexamethasone-treated rats. J. Clin. Invest. **92**, 2065–2072
38 Giorgino, F. and Smith, R. J. (1995) Dexamethasone enhances insulin-like growth factor-I effects on skeletal muscle cell proliferation. Role of specific intracellular signaling pathways. J. Clin. Invest. **96**, 1473–1483
39 Giorgino, F., Almahfouz, A., Goodyear, L. J. and Smith, R. J. (1993) Glucocorticoid regulation of insulin receptor and substrate IRS-1 tyrosine phosphorylation in rat skeletal muscle *in vivo*. J. Clin. Invest. **91**, 2020–2030
40 Dardevet, D., Sornet, C., Vary, T. and Grizard, J. (1996) Phosphatidylinositol 3-kinase and p70 s6 kinase participate in the regulation of protein turnover in skeletal muscle by insulin and insulin-like growth factor I. Endocrinology **137**, 4087–4094
41 Löw, P., Bussell, K., Dawson, S. P., Billett, M. A., Mayer, R. J. and Reynolds, S. E. (1997) Expression of a 26 S proteasome ATPase subunit, MS73, in muscles that undergo developmentally programmed cell death, and its control by ecdysteroid hormones in the insect *Manduca sexta*. FEBS Lett. **400**, 345–349
42 Lam, Y. A., Lawson, T. G., Velayutham, M., Zweier, J. L. and Pickart, C. M. (2002) A proteasomal ATPase subunit recognizes the polyubiquitin degradation signal. Nature (London) **416**, 763–767
43 Voges, D., Zwickl, P. and Baumeister, W. (1999) The 26 S proteasome: a molecular machine designed for controlled proteolysis. Annu. Rev. Biochem. **68**, 1015–1068
44 Kohler, A., Cascio, P., Leggett, D. S., Woo, K. M., Goldberg, A. L. and Finley, D. (2001) The axial channel of the proteasome core particle is gated by the Rpt2 ATPase and controls both substrate entry and product release. Mol. Cell **7**, 1143–1152
45 Navon, A. and Goldberg, A. L. (2001) Proteins are unfolded on the surface of the ATPase ring before transport into the proteasome. Mol. Cell **8**, 1339–1349
46 Deveraux, Q., Ustrell, V., Pickart, C. and Rechsteiner, M. (1994) A 26 S protease subunit that binds ubiquitin conjugates. J. Biol. Chem. **269**, 7059–7061
47 Attaix, D., Taillandier, D., Combaret, L., Rallière, C., Larbaud, D., Aurousseau, E. and Tanaka, K. (1997) Expression of subunits of the 19 S complex and of the PA28 activator in rat skeletal muscle. Mol. Biol. Rep. **24**, 95–98
48 Whitehouse, A. S. and Tisdale, M. J. (2001) Downregulation of ubiquitin-dependent proteolysis by eicosapentaenoic acid in acute starvation. Biochem. Biophys. Res. Commun. **285**, 598–602
49 Whitehouse, A. S., Smith, H. J., Drake, J. L. and Tisdale, M. J. (2001) Mechanism of attenuation of skeletal muscle protein catabolism in cancer cachexia by eicosapentaenoic acid. Cancer Res. **61**, 3604–3609
50 Taillandier, D., Aurousseau, E., Combaret, L., Guezennec, C.-Y. and Attaix, D. (2003) Regulation of proteolysis during reloading of the unweighted soleus muscle. Int. J. Biochem. Cell Biol. **35**, 665–675
51 Dawson, S. P., Arnold, J. E., Mayer, N. J., Reynolds, S. E., Billett, M. A., Gordon, C., Colleaux, L., Kloetzel, P. M., Tanaka, K. and Mayer, R. J. (1995) Developmental changes of the 26 S proteasome in abdominal intersegmental muscles of *Manduca sexta* during programmed cell death. J. Biol. Chem. **270**, 1850–1858

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