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Torbafylline (HWA 448) inhibits enhanced skeletal muscle ubiquitin–proteasome-dependent proteolysis in cancer and septic rats

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The development of new pharmacological approaches for preventing muscle wasting in cancer is an important goal because cachectic patients display a reduced response to chemotherapy and radiotherapy. Xanthine derivatives such as pentoxifylline inhibit tumour necrosis factor-α (TNF) production, which has been implicated in the signalling of muscle wasting. However, the effect of pentoxifylline has been inconclusive in clinical trials. We report here the first direct evidence that daily injections of torbafylline (also known as HWA 448), another xanthine derivative, had no effect by itself on muscle proteolysis in control healthy rats. In cancer rats, the drug blocked the lipopolysaccharide-induced hyperproduction of TNF and prevented muscle wasting. In these animals HWA 448 suppressed the enhanced proteasome-dependent proteolysis, which is sensitive to the proteasome inhibitor MG132, and the accumulation of high-molecular-mass ubiquitin (Ub) conjugates in the myofibrillar fraction. The drug also normalized the enhanced muscle expression of Ub, which prevails in the atrophying muscles from cancer rats. In contrast, HWA 448 did not reduce the increased expression of either the 14 kDa Ub conjugating enzyme E2 or the ATPase and non-ATPase subunits of the 19 S regulatory complex of the 26 S proteasome, including the non-ATPase subunit S5a, which recognizes polyUb degradation signals. Finally, the drug also prevented muscle wasting in septic rats (which exhibit increased TNF production), and was much more potent than pentoxifylline or other xanthine derivatives. Taken together, the data indicate that HWA 448 is a powerful inhibitor of muscle wasting that blocks enhanced Ub–proteasome-dependent proteolysis in situations where TNF production rises, including cancer and sepsis.

Key words: muscle wasting, protein breakdown, xanthine derivatives, tumour necrosis factor-α, Yoshida sarcoma.

INTRODUCTION

The anorexia–cachexia syndrome is the most common documented cause of death in cancer. Cachexia, which also prevails in trauma, burn injury, severe sepsis, and AIDS is characterized by anorexia, tissue wasting, poor performance status, and subsequent death. Thus the development of new therapies designed to prevent cancer-induced cachexia will contribute to improve the quality of life of cancer patients, as well as their life expectancy [1].

Cachexia reflects essentially a loss of adipose tissue and skeletal muscle. Muscle wasting results from increased protein breakdown in several tumour-bearing animal models and in some cancer patients [1,2]. Skeletal muscle contains three major proteolytic systems: the lysosomal, Ca^{2+}-activated and ubiquitin (Ub)–proteasome-dependent pathways. Cathepsins [3,4] and possibly calpains [5] (i.e. lysosomal and Ca^{2+}-dependent proteinases respectively) have been reported to be activated in some instances of cancer-induced cachexia. However, evidence for a systematic activation of both cathepsins [5,6] and calpains [3,6] is lacking in some models of tumour-bearing animals. Moreover, it is now well recognized that the degradation of the most abundant contractile proteins (actin and myosins) is not mediated by either lysosomal or Ca^{2+}-activated proteinases [7–10]. In contrast, overwhelming evidence that the Ub–proteasome-dependent proteolytic pathway degrades these myofibrillar proteins has

been recently provided [7–10]. Accordingly, this degradative process is the critical system responsible for the muscle wasting seen in different tumour-bearing animals [1–3,5].

In this pathway Ub targets specific protein substrates for degradation. Briefly, covalent attachment of the highly conserved 76-amino-acid peptide Ub to cellular proteins requires the formation of an isopeptide bond between its C-terminal glycine and the ϵ-amino group of a lysine residue of the acceptor protein. Ub conjugation proceeds via a thiol-ester reaction cascade involving the Ub-activating enzyme (E1), Ub-conjugating enzymes (E2), and, in general, Ub-protein ligases (E3), which possess substrate-recognition sites (for recent reviews, see [11,12]). Generally, polyUb chains are formed by the sequential conjugation of the C-terminus of Ub to the ϵ-amino group of the Lys^{48} of Ub moieties already attached to the protein. Polyubiquitinated proteins are then recognized by the 19 S regulatory complex of the 26 S proteasome and degraded in an ATP-dependent manner [13].

The precise mechanisms by which cancer results in increased muscle proteolysis are not clearly elucidated [1,2]. Circulating proteolysis-inducing factors produced by the tumour, by the host in response to the tumour, or both, have been described. They include cancer cachectic factors [1,14] and cytokines [1,2,15,16]. Indeed, in response to neoplasic and infectious diseases, a variety of cells, including macrophages and lymphocytes, secrete cytokines, which are capable of altering the host's metabolism.

Among these cytokines, tumour necrosis factor-α (TNF) was reported to induce cachexia in animal models following endogenous production by tumour cells [15,16] or exogenous administration [17]. Accordingly, anti-TNF antibodies prevented increased protein breakdown and increased expression of Ub in skeletal muscle from rats bearing the Yoshida AH-130 ascites hepatoma [18–19]. However, anti-TNF antibodies are prohibitively expensive for routine use in humans, and new pharmacological approaches must be developed to suppress the TNF-dependent activation of the Ub–proteasome pathway [1,2,20].

Pentoxifylline is a xanthine derivative, which inhibits TNF transcription [21] and has been reported to prevent muscle wasting both in tumour-bearing [20] and in septic [22] animals. However, establishing the specific effect of pentoxifylline in clinical trials has proved to be elusive. In one study, pentoxifylline-treated cachectic cancer patients gained weight [23], but the drug had no positive effect in another trial [24]. The present study was undertaken to determine whether torbafylline (also known as HWA 448), another xanthine derivative, inhibited the activation of the Ub–proteasome pathway in muscles from cachectic cancer rats. To meet this goal, we investigated whether HWA 448 was able: (i) to suppress TNF production in cancer rats; (ii) to affect proteolysis in control rats; (iii) to prevent muscle wasting and enhanced Ub–proteasome-dependent in cancer rats; and (iv) to prevent muscle wasting in another catabolic model, in which increased TNF production occurs.

MATERIALS AND METHODS

Animals and experimental design

Young male Wistar rats, weighing 50–70 g, were obtained from Iffa Credo (L'Arbresle, France) and maintained in a temperature-controlled room (22 ± 1 °C) with a 12 h light/12 h dark cycle. They were provided *ad lib.* with a standard diet (A03; UAR, Epinay sur Orge, France) during a 5 day acclimatization period and had free access to water. Animals were randomly divided into control, tumour-bearing, HWA 448-treated tumour-bearing rats and, when specified, HWA 448-treated control animals. Tumour-bearing animals were given an injection of a homogenate of Yoshida sarcoma into the left vastus lateralis muscle, as described previously [5]. The HWA 448-treated groups received a daily intraperitoneal (i.p.) injection of HWA 448 [100 mg/kg body weight in 1% (v/v) carboxymethylcellulose (CM-cellulose) solution], provided by Aventis Pharmaceuticals (Wiesbaden, Germany). The other two groups received a daily i.p. injection of the CM-cellulose vehicle. All groups were pair-fed to the tumour-bearing animals.

Rates of protein turnover

Animals were killed by cervical dislocation 9 days after tumour implantation. Extensor digitorum longus muscles (EDLs) from the non-tumour bearing leg were carefully dissected and incubated as described previously [5,20] in order to measure rates of protein synthesis and protein breakdown simultaneously. In brief, EDLs were incubated at approximate resting length by pinning the tendons to plastic supports. All tissues were incubated at 37 °C in Krebs–Henseleit buffer [120 mM NaCl/4.8 mM KCl/25 mM NaHCO$_3$/2.5 mM CaCl$_2$/1.2 mM KH$_2$PO$_4$/1.2 mM, MgSO$_4$ (pH 7.4)] containing 5 mM glucose, 0.1 unit/ml insulin, 0.17 mM leucine, 0.10 mM isoleucine and 0.20 mM valine to improve the protein balance [5,20] and equilibrated with an O$_2$/CO$_2$ mixture (19:1). After 1 h of preincubation, muscles were transferred to a fresh medium of identical composition and incubated for a further 2 h.

The rate of protein synthesis was determined by incubating muscles in a medium containing 0.5 mM [U-^{14}C]phenylalanine with a specific radioactivity in the medium of 500 d.p.m./nmol (Amersham Corp., Little Chalfont, Bucks., U.K.), as described previously [5,20]. Tissues were homogenized in 10% (w/v) trichloroacetic acid and hydrolysed in 1 M NaOH at 37 °C. Tissue protein mass was determined using the bicinchoninic acid procedure [25]. Rates of phenylalanine incorporation were converted into tyrosine equivalents, as described previously [26], and expressed as nmol of tyrosine incorporated·h^{-1}·mg of muscle^{-1}.

Rates of protein breakdown were measured by following the rates of tyrosine release into the medium. Since muscle neither synthesizes nor degrades this amino acid, tyrosine release reflects the net breakdown of proteins. Thus rates of total protein degradation were calculated by adding the rate of protein synthesis and the net rate of tyrosine release into the medium [26]. Tyrosine was assayed by the method of Waalkes and Udenfriend [27].

To estimate the contribution of the major proteolytic processes to overall protein breakdown, total proteolysis was measured in muscles incubated without inhibitors, and non-lysosomal, Ca^{2+}-independent proteolysis was measured in muscles incubated in the presence of 10 mM methylamine and 50 μM *trans*-epoxy-succinyl-L-leucylamido-(4-guanidino)butane (E-64) in a Ca^{2+}-free medium [5,20,28]. Proteasome-independent proteolysis was measured using a medium containing 10 mM methylamine and 50 μM E-64 in a Ca^{2+}-free medium, as well as 40 μM MG132, a proteasome inhibitor (Affiniti, Mamhead Castle, Mamhead, Exeter, U.K.) [29]. Because MG 132 also inhibits cysteine proteases (e.g. cathepsins B, H and L and calpains), control muscles were incubated in this medium lacking MG 132 only. In all these experiments, proteolysis was measured in the presence of 0.5 mM cycloheximide to block protein synthesis [26], and was expressed in nmol of tyrosine released in the medium·h^{-1}·mg of muscle^{-1}.

TNF production in lipopolysaccharide (LPS)-stimulated rats

LPS (from *Escherichia coli* strain 011:B4; Sigma, St Quentin Fallavier, France) was dissolved in sterile saline solution immediately before administration, and animals received an i.p. injection of either LPS (1 mg/kg) or the saline vehicle solution. Control and Yoshida sarcoma-bearing rats were treated on a daily basis or not with HWA 448 for 9 days, as described above. At day 9, rats received LPS or saline injection 1 h after HWA 448 or vehicle injection. The eight groups were killed 180 min after the endotoxin injection (five rats in each group) for plasma TNF determination. TNF levels were determined using the Quanti-kine®M rat TNF immunoassay kit (R & D Systems, Abingdon, Oxon., U.K.), and expressed as pg of TNF·ml^{-1} of plasma.

Northern blot analysis

Tibialis anterior muscles from control, tumour-bearing and HWA 448-treated tumour-bearing rats were rapidly excised, frozen in liquid nitrogen and stored at -80 °C. Total RNA was extracted [30], and Northern blot analysis was performed as described previously [5]. The membranes were hybridized with cDNA probes encoding chicken polyUb, the rat 14 kDa Ub conjugating enzyme E2 (14 kDa E2) and the C2 subunit of the 20 S proteasome, as described before [5,20,28]. Hybridizations were also performed under similar conditions with cDNA probes encoding the human ATPase subunit S7 of the 19 S complex [31], the human non-ATPase subunits S1 [32] and S5a [33] of the 19 S complex, and the rat PA28α subunit [34]. After washing at the same temperature (65 °C), the filters were subjected to auto-

radiography for 3–72 h at $-80\,^{\circ}\mathrm{C}$ with an intensifying screen on Hyperfilms-MP films (Amersham International). After stripping of the different probes, the filters were re-probed with a cDNA fragment encoding the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [35] to confirm that changes seen were not due to non-specific changes in all mRNAs or uneven loading. Neither the implantation of the Yoshida sarcoma [5] nor HWA 448 treatment (results not shown) affected skeletal-muscle GAPDH mRNA levels.

Western blot analysis

Tibialis anterior muscles were homogenized in 4 ml of ice-cold buffer A [10 mM Tris/HCl (pH 7.5)/10 mM EDTA (pH 8.0)/2 mM PMSF/0.5 mM tosyl-lysylchloromethane/0.5 mM tosyl-phenylalanylchloromethane/10 mM N-ethylmaleimide/10 μg/ml leupeptin/10 μg/ml pepstatin A/10 μg/ml soya-bean trypsin inhibitor]. Homogenates were centrifuged for 5 min (1500 g, $4\,^{\circ}\mathrm{C}$) to pellet myofibrillar proteins, which were then washed three times in buffer A containing 1 % Triton X-100. Myofibrillar proteins were then resuspended in 8 M urea/50 mM Tris/HCl, pH 7.5. Supernatants from the 1500 g spin were recentrifuged for 10 min (10000 g, $4\,^{\circ}\mathrm{C}$), and the resulting supernatants were ultracentrifuged for 3 h (100000 g, $4\,^{\circ}\mathrm{C}$). These supernatants contained soluble proteins, and protein concentration was determined using the Bio-Rad protein assay.

Of the myofibrillar and soluble proteins, 25 μg was electrophoresed on 7.5 % (w/v) polyacrylamide gels and transferred on to a nitrocellulose membrane (0.45 μm; Schleicher & Schuell, Dassel, Germany). Membranes were blocked for 1 h in 5 % (v/v) non-fat dried milk in TTNS [25 mM Tris/HCl (pH 7.5)/0.1 % (v/v) Tween 20/0.9 % (w/v) NaCl] and incubated with a polyclonal rabbit anti-human antibody raised against Ub–protein conjugates (Affiniti) for 1 h. After washing three times in TTNS, the blots were incubated with a horseradish-peroxidase-conjugated goat anti-(rabbit IgG) for 45 min. The blots were washed extensively four times in TTNS for 20 min, incubated in ECL® reagent (Amersham Life Science), and exposed on radiographic film (Hyperfilm™ECL™; Amersham Life Science).

Effect of HWA 448 compared with other xanthine derivatives

To confirm that HWA 448 treatment effectively prevented muscle wasting in another catabolic model characterized by a hyperproduction of TNF, we studied septic rats. Rats were injected intravenously (i.v.) with live *E. coli*, as described previously [28], in order to reproduce a long-lasting catabolic state with significant muscle wasting. HWA 448 and other xanthine derivatives (A 2715, HWA 138, BL 194 and pentoxifylline) were administered i.p., 1 h before i.v. *E. coli* injection, at 100 mg/kg in 1 % CM-cellulose, as described above for HWA 448 treatment. Rats were killed 6 days after the infection and tibialis anterior muscles were excised and weighed. The effect of the various xanthine derivatives was also measured on the mRNA levels for the C2 subunit of the 20 S proteasome in these muscles as assessed by Northern blotting, as described above.

Statistical analysis

Each experiment was repeated twice. Data from a single experiment are presented as means$\pm$S.E.M. Statistical analysis was performed using the unpaired Student's t test or ANOVA, whichever was appropriate. Significance (P) was determined at the 0.05 level.

RESULTS

HWA 448 prevents muscle wasting in cancer rats

Table 1 shows that the Yoshida sarcoma-bearing rats lost only 8 % of their body mass in the space of 9 days after the implantation of the tumour, since the mass of some organs (i.e. spleen and lungs) increased dramatically [20]. This loss of body mass was totally prevented by HWA 448 treatment, and was not due to an inhibition of tumour growth, since neither the tumour mass (Table 1) nor the number of tumour cells (results not shown) was affected by the treatment. In fact, there was a tendency for tumour mass to increase following HWA 448 treatment ($P > 0.05$). Table 1 also shows that the changes in the protein content of the EDLs paralleled the body weight alterations. However, such changes were more marked because the protein content of the EDLs was reduced by 19 % in the tumour-bearing rats ($P < 0.05$) and by only 7 % in the HWA 448-treated animals ($P > 0.05$) compared with the controls. Similar observations were found in the tibialis anterior and gastrocnemius muscles (results not shown). Thus muscle wasting in the cancer rats was almost totally prevented by HWA 448. This beneficial effect is not due to changes in food intake, since control and HWA 448-treated tumour-bearing rats were pair-fed to the cancer animals (Table 1). Moreover, the drug itself did not exert any effect on animal growth or EDL protein mass in healthy control rats treated for 9 days with HWA 448 (Table 2).

HWA 448 prevents the LPS-induced increase in plasma TNF levels in cancer rats

Circulating levels of TNF were not detectable in healthy and tumour-bearing animals treated or not with HWA 448 (Figure 1). In contrast, when rats were challenged by LPS injection, TNF

Table 1   Effect of HWA 448 treatment on Yoshida sarcoma-bearing rats

For the calculation of the final body weight (g) (shown in the second row of data), the tumour mass was subtracted, if applicable. Values within a given row of data annotated with different symbols (* or †) are significantly different from each other as assessed by ANOVA ($P < 0.05$). Data are means$\pm$S.E.M. for six to ten animals. N.A., not applicable.

Parameter measured	Control	Tumour-bearing	Tumour-bearing HWA 448-treated
Initial body weight (g)	85 ± 1*	84 ± 2*	85 ± 1*
Final body weight (g)	157 ± 1*	144 ± 5†	155 ± 3*
Tumour mass (g)	N. A.	9.5 ± 1.3*	12.1 ± 1.3*
EDL protein content (mg/100 g initial body weight)	10.6 ± 2.0*	8.6 ± 0.6†	9.9 ± 0.5*†
Food intake (g/day)	11.3 ± 0.2*	11.1 ± 0.2*	11.1 ± 0.1*

Table 2   Effect of HWA 448 on growth, muscle mass and rate of proteolysis in healthy control rats

Data are means$\pm$S.E.M. for ten animals. Note that there are no statistical differences between the two groups within a given row of data, as assessed by the unpaired Student's t test ($P > 0.05$).

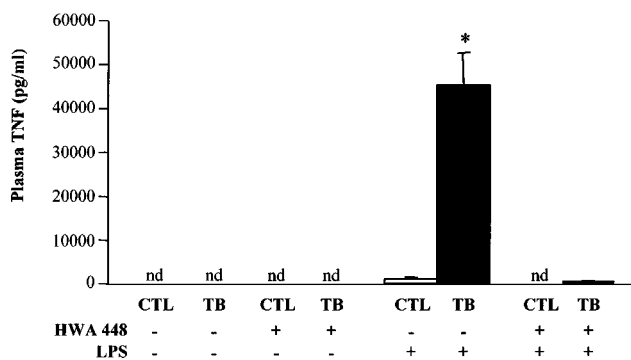|	Control	HWA 448-treated
Initial body weight (g)	74 ± 1	75 ± 1
Final body weight (g)	135 ± 3	134 ± 2
EDL protein content (mg/100 g initial body weight)	9.3 ± 0.2	9.1 ± 0.1
EDL total protein breakdown (nmol of tyrosine$\cdot$h$^{-1}\cdot$mg of muscle^{-1})	0.063 ± 0.005	0.062 ± 0.004

Figure 1   HWA 448 prevents the LPS-induced increase in plasma TNF levels in cancer rats

Control (CTL) and tumour-bearing (TB) animals were pretreated with HWA 448 or vehicle and were injected with LPS or physiological saline (see the Materials and methods section). After 3 h, blood was taken and the TNF concentration in the plasma was determined. Data are the means $\pm$ S.E.M. (vertical bars) for 4–5 animals. Statistical analysis (three-way ANOVA) indicated an effect of cancer, of HWA 448 treatment and of LPS injection, and an interaction between these factors for TNF production. nd, non-detectable ($<$ 12.5 pg/ml).
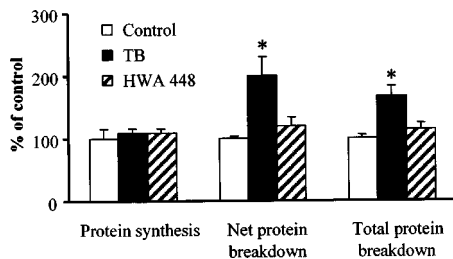

Figure 2   HWA 448 prevents increased proteolysis in EDLs from Yoshida sarcoma-bearing rats

Muscles were incubated in Krebs–Henselheit buffer. Values are means $\pm$ S.E.M. (vertical bars) for six to eight animals, and are expressed as the percentage of the value in control animals. Statistical differences were assessed using a one-way ANOVA test. *$P <$ 0.05 compared with control rats. TB, tumour-bearing rats; HWA 448, HWA 448-treated tumour-bearing rats.

plasma level increased by 45-fold in cancer animals compared with controls. Moreover, HWA 448 treatment totally blocked the increased TNF production in control animals and suppressed the LPS-induced hyperproduction of TNF in cancer animals (Figure 1).

HWA 448 suppresses the enhanced Ub–proteasome-dependent proteolysis in cancer rats

The muscle wasting observed in EDLs from the tumour-bearing rats was only attributable to enhanced rates of net ($+ 100\%$) and total ($+ 66\%$) proteolysis, without any alteration in protein synthesis (Figure 2). HWA 448 treatment almost completely suppressed the increased proteolysis observed in cancer animals. In addition, the basal rate of proteolysis was unaffected by HWA 448 treatment in healthy control rats (Table 2).

Using specific inhibitors of lysosomal/Ca²⁺-dependent proteases and of the proteasome, we then demonstrated that the increased rate of protein breakdown was exclusively accounted for by the stimulation of proteasome-dependent proteolysis (Figure 3). The large increases in the expression of Ub ($+ 470\%$; $P <$ 0.05), 14 kDa E2 ($+ 198\%$; $P <$ 0.001) and the C2 subunit of the 20 S proteasome ($+ 129\%$; $P <$ 0.05) in the atrophying
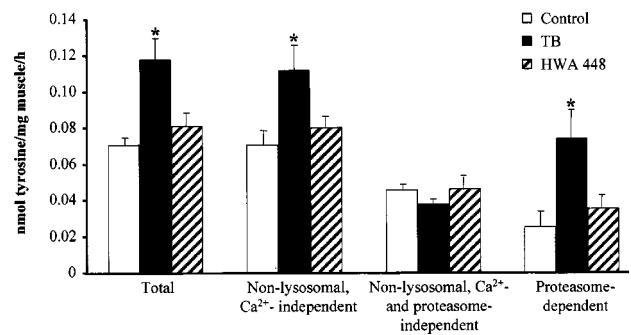

Figure 3   HWA 448 inhibits proteasome-dependent proteolysis in EDLs from Yoshida sarcoma-bearing rats

Mucles were incubated in Krebs–Henselheit buffer in the presence or absence of appropriate inhibitors. Non-lysosomal, Ca²⁺-independent proteolysis was measured in muscles incubated in the presence of methylamine and E-64 in a Ca²⁺-free medium. Non-lysosomal, Ca²⁺- and proteasome-independent proteolysis was measured using a medium containing methylamine and E-64 in a Ca²⁺-free medium, and the proteasome inhibitor, MG132. Proteasome-dependent proteolysis is the difference between non-lysosomal, Ca²⁺-independent proteolysis and non-lysosomal, Ca²⁺- and proteasome-independent proteolysis. Values are means $\pm$ S.E.M. (vertical bars) for six to ten animals. Statistical differences were assessed using a one-way ANOVA test. *$P <$ 0.05 compared with control rats. TB, tumour-bearing rats; HWA 448, HWA 448-treated tumour-bearing rats.

muscles from the tumour-bearing rats (Figure 4A) also supported a role for the Ub–proteasome pathway in muscle atrophy. Figure 4(B) shows that the mRNA levels for the ATPase subunit S7 and the non-ATPase subunit S5a of the 19 S complex also increased ($+ 129\%$; $P <$ 0.01, and $+ 245\%$; $P <$ 0.001 respectively) (Figure 4B) in the muscles from cancer rats. In contrast, the mRNA levels for the non-ATPase subunit S1 of the 19 S complex and the PA28α subunit of the 11 S regulatory complex (results not shown) were unaffected ($P >$ 0.05) by cancer cachexia.

The expression of Ub was normalized by HWA 448 administration to cancer rats, but the mRNA level for the C2 subunit of the 20 S proteasome was slightly decreased in the HWA 448-treated tumour-bearing animals so that C2 expression was not significantly different from either cancer or control rats (Figure 4A). In contrast, HWA 448 did not reduce the enhanced expression of 14 kDa E2 (Figure 4A) or of subunits S7 and S5a of the 19 S complex that prevailed in the atrophying muscles from the tumour-bearing animals (Figure 4B).

Most substrates targeted for degradation by the 26 S proteasome are polyubiquitinated proteins. Figure 5 shows that high-molecular-mass Ub-conjugates increased by 29% in the myofibrillar fraction of tumour-bearing rats ($P <$ 0.05), while the amount of conjugates was unaffected in the soluble sarcoplasmic fraction. HWA 448 treatment totally prevented the accumulation of Ub-conjugates in the myofibrillar fraction (Figure 5B).

HWA 448 down-regulates the elevated mRNA levels for the C2 subunit of the 20 S proteasome in septic muscles

To test whether the suppressive effect of HWA 448 on muscle wasting and activated proteasome-dependent proteolysis can be reproduced in another catabolic condition characterized by a hyperproduction of TNF, muscle mRNA levels for the C2 subunit of the 20 S proteasome were measured in septic rats treated or not with a variety of xanthine derivatives. These mRNA levels were strongly decreased in septic rats treated with HWA 448, A 2715 and HWA 138 (Figure 6A). In contrast,
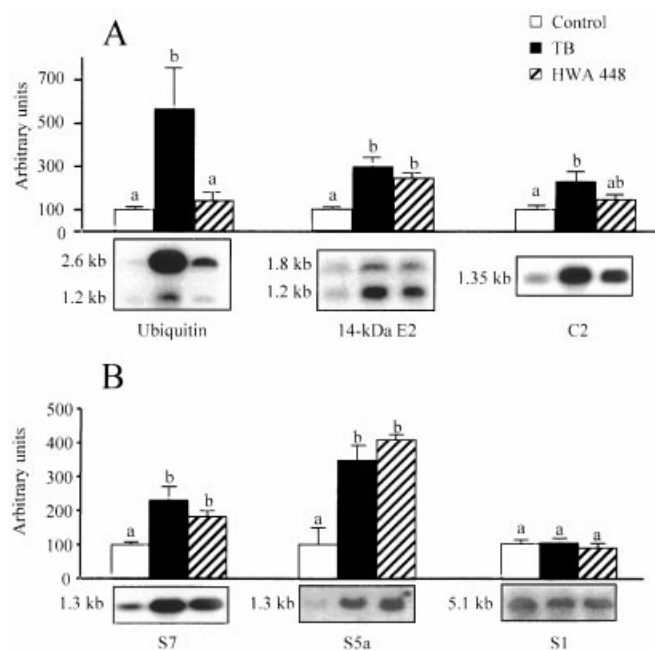
Figure 4   Effect of HWA 448 administration on mRNA levels for components of the Ub–proteasome-dependent proteolytic pathway in muscles from Yoshida sarcoma-bearing rats

RNA was extracted, electrophoresed, transferred on to a nylon membrane and hybridized (**A**) with ^{32}P-labelled cDNAs encoding ubiquitin, 14 kDa E2 and the C2 subunit of the 20 S proteasome or (**B**) with ^{32}P-labelled cDNAs encoding the S7, S5a and S1 subunits of the 19 S complex, as described in the Materials and methods section. After stripping of the probes, blots were re-hybridized with a ^{32}P-labelled cDNA encoding GAPDH. Densitometric signals were normalized by using the corresponding GAPDH mRNA values to take into account slight variations in RNA loading. Data are means $\pm$ S.E.M. (vertical bars) for four to six rats and are expressed as a percentage of the control values. Representative Northern blots are also shown. Statistical differences were assessed using a one-way ANOVA test. Bars labelled with different letters (a, b or c) are significantly different from each other ($P < 0.05$). TB, tumour-bearing rats; HWA 448, HWA 448-treated tumour-bearing rats.
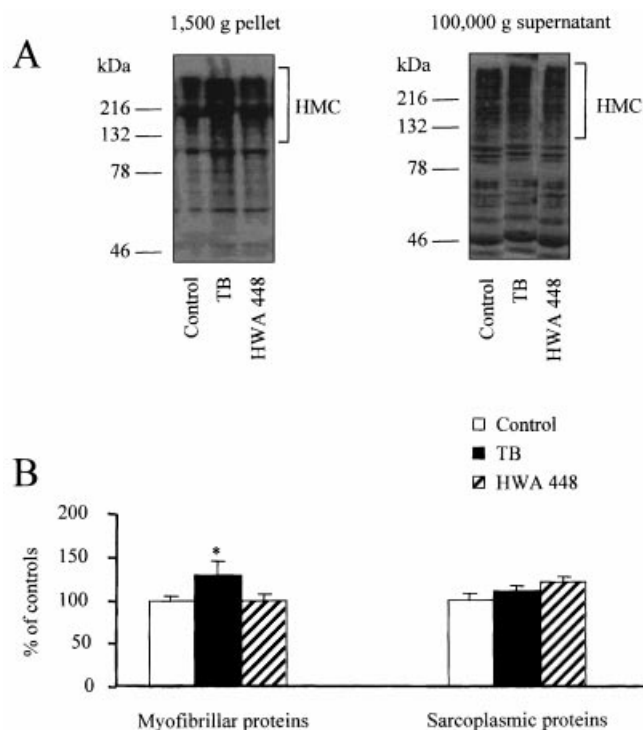

Figure 5   HWA 448 suppresses the accumulation of high-molecular-mass (HMC) Ub–protein conjugates in the myofibrillar fraction of tibialis anterior muscles from cancer rats

(**A**) Muscle proteins were fractionated as described in the Materials and methods section and the 1500 **g** pellet and the 100 000 **g** supernatant containing myofibrillar and soluble proteins respectively were electrophoresed, transferred on to a nitrocellulose membrane and blotted with an antibody raised against polyUb chains. Representative immunoblots are shown. (**B**) Densitometric analysis of high-molecular-mass Ub conjugates shown in (**A**). Data are means $\pm$ S.E.M. (vertical bars) for four animals and are expressed as the percentage of control values; HWA 448, HWA 448-treated tumour-bearing rats. Statistical differences were assessed using a one-way ANOVA test. *$P < 0.05$ compared with controls and HWA 448-treated tumour-bearing rats.

pentoxifylline and BL 194 exercised only a small, if significant at all, effect on the increased mRNA levels of C2 that are characteristic of untreated septic rats (Figure 6A), with the effect of BL 194 being slightly more pronounced than that of pentoxifylline. In addition, Figure 6(B) clearly shows that the tibialis anterior muscle mass both from septic rats treated with the various xanthine derivatives and from untreated animals is inversely correlated with C2 mRNA levels ($P < 0.001$).

DISCUSSION

We report here the first direct evidence that HWA 448: (i) blocks the LPS-induced hyperproduction of TNF in cancer rats; (ii) has no effect in itself on muscle proteolysis in control healthy rats; (iii) prevents muscle wasting by suppressing the elevated proteasome-dependent proteolysis and accumulation of Ub-protein conjugates, both of which prevail in atrophying muscles from cancer rats; and (iv) is also effective in septic rats (which exhibit increased TNF production) and is much more potent than pentoxifylline or other xanthine derivatives.

The present study clearly demonstrates that HWA 448 reduced the muscle wasting in Yoshida sarcoma-bearing rats by inhibiting Ub–proteasome-dependent proteolysis. We [5,20] and others [3,6,36,37] previously reported that this pathway plays a major role in the muscle atrophy seen in rodents after tumour implantation. Such observations entirely support a crucial role for this degradative machinery in the muscle wasting observed in various animal models (for reviews, see [7–10]), as well as in head trauma [38], septic [39] and cancer [40] patients. Previous studies have shown that both an anti-TNF antibody [19] and pentoxifylline [20] prevent the increased expression of Ub and 20 S proteasome subunits in muscles from rats bearing the Yoshida hepatoma and the Yoshida sarcoma respectively. We show further that HWA 448 inhibited proteasome-dependent proteolysis (Figure 3) and prevented the accumulation of polyubiquitinated proteins in the myofibrillar fraction of muscles from cancer rats (Figure 5). These findings clearly support the concept that the Ub pathway degrades the major contractile proteins actin and myosins [7–10].

The changes in mRNA levels for Ub, 14 kDa E2 and the C2 subunit of the 20 S proteasome paralleled the enhanced rate of proteolysis in the muscles from Yoshida sarcoma-bearing rats, as reported previously in this cancer model [5,20]. The 20 S proteasome associates with either the 19 S complex (to form the 26 S proteasome that degrades polyubiquitinated proteins) or PA28
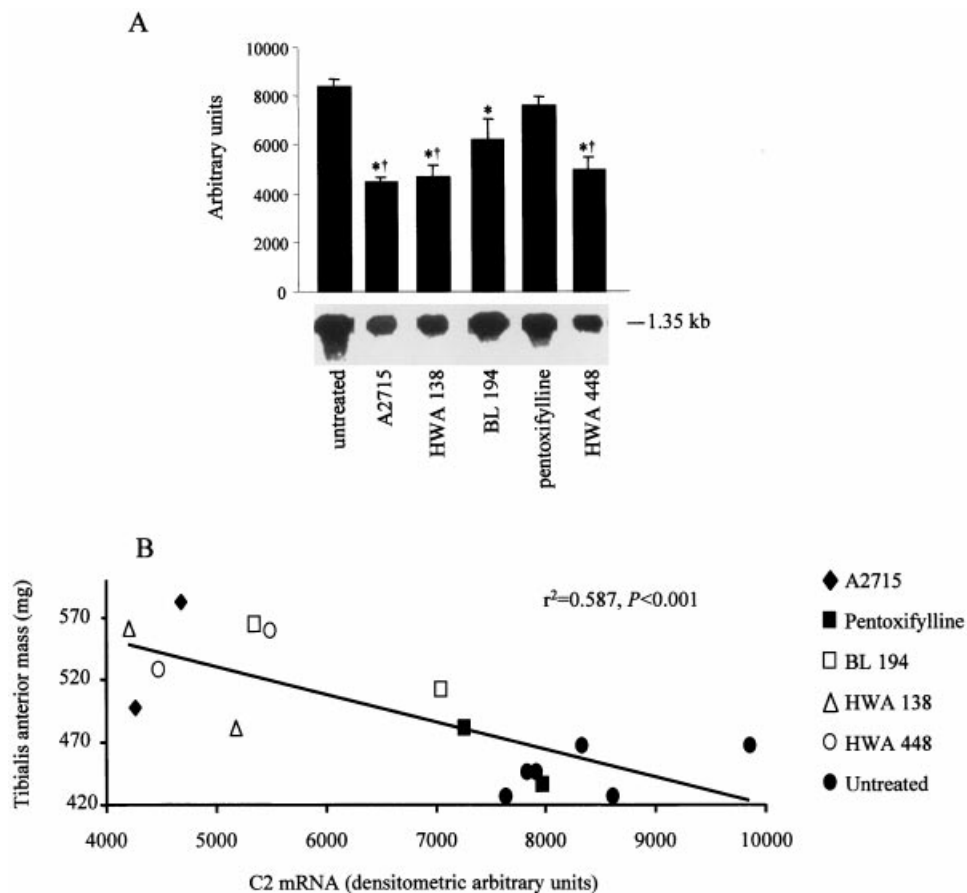
Figure 6   HWA 448 is a potent suppressor of the elevated expression of the C2 subunit of the 20 S proteasome in septic muscles

Total RNA from septic rat muscles was extracted 6 days after infection, electrophoresed, transferred on to a nylon membrane and hybridized with ^{32}P-labelled cDNA encoding the C2 subunit of the 20 S proteasome, as described in the Materials and methods section. (**A**) Quantification of representative Northern blots. Data are means ± S.E.M. (vertical bars) for two to six animals and are expressed in arbitrary units. Statistical differences were assessed using a one-way ANOVA test. *$P < 0.05$ compared with untreated rats; †$P < 0.05$ compared with pentoxifylline-treated rats. (**B**) Relationship between the tibialis anterior muscle mass and the densitometric signals for C2 mRNA levels.

to form either proteasome PA28 and/or hybrid proteasomes (see [12] for a recent review). These regulatory components modulated the peptidase activities of the 20 S proteasome in insect [41] and lobster [42] muscles. However, the muscle mRNA levels for PA28α did not change in cancerous rats ([20], and results not shown), suggesting that both the proteasome PA28 and/or the hybrid proteasome played little if any significant role in muscle wasting [2]. mRNA levels for one ATPase and two non-ATPase subunits of the 19 S complex did not increase in concert in the atrophying muscles from cancer rats (Figure 4B). The ATPase subunits of the 19 S complex provide energy for the formation of the 26 S proteasome and the breakdown of polyubiquitinated proteins, for the gating of the 20 S proteasome channel and, presumably, for the denaturation and injection of the substrates into the proteolytic chamber [13]. Accordingly, S7 expression increased in the muscles from tumour-bearing rats, as reported previously [20]. Changes in the mRNA levels for the non-ATPase subunit S5a also paralleled rates of proteasome-dependent proteolysis in cancer rats, while mRNA levels for S1 did not change (Figure 4B). Thus there is no co-ordinate up-regulation of the expression of all subunits of the 19 S complex in this model of muscle wasting. S5a is the only 26 S proteasome subunit that has been clearly identified as a polyUb chain receptor [43]. The

increased mRNA levels for S5a reported in the present work is in good agreement with results from Western blotting experiments, which showed an increased content of this particular subunit in the atrophying muscles from the tobacco hornworm *Manduca sexta* [44]. However, a major observation in that study was that the HWA 448-induced down-regulation of the Ub pathway did not correlate with a suppression of the elevated expression of all components of the pathway. Indeed, only Ub and, to a lesser extent, C2 mRNA levels returned to almost-basal levels in the muscles from HWA 448-treated rats (Figure 3) and paralleled the suppression both of the accumulation of high-molecular-mass Ub-conjugates (Figure 5) and of increased proteasome-dependent proteolysis (Figure 2). In contrast, the expression of S7, S5a and 14 kDa E2 remained elevated when proteolysis decreased, suggesting that the level of expression of these proteins is not crucial in the overall activity of the pathway. There are several simple explanations for such observations. For example, S5a cannot be the only polyUb receptor, because deletion of its gene homologue in yeast does not prevent Ub-dependent protein breakdown [45]. Similarly, 14 kDa E2 has been reported to play a major role in the ubiquitination of soluble muscle proteins in various instances of muscle atrophy [46,47]. However, our data clearly show that high-molecular-mass Ub–protein conjugates increased predomi-

nantly in the myofibrillar, but not in the soluble, fraction (Figure 5), in accordance with previously published data [48].

A second major observation in these studies is that HWA 448 was effective in preventing both the muscle wasting and the activation of the Ub–proteasome-dependent pathway in another catabolic model. To investigate this possibility, we used a septic rat model in which it is evident that TNF is involved in the catabolic response [28,49]. The improvement in muscle mass in the septic rats that were treated with HWA 448 was approx. 22%, clearly indicating that the drug is able to promote muscle recovery even when the degree of wasting is quite severe. In addition, Figure 6 shows that HWA 448 was more potent than pentoxifylline in reducing the enhanced muscle expression of proteasome subunit C2, and that the suppressive effect of HWA 448 on C2 mRNA levels was correlated with the preservation of muscle mass.

The precise mechanisms by which HWA 448 inhibits skeletal-muscle Ub–proteasome-dependent proteolysis in cancer animals remain to be elucidated. This study indicates that the drug itself did not affect body weight, food intake, muscle mass or protein breakdown in healthy rats (Table 2). HWA 448 inhibits the LPS-induced production of TNF in cancer rats ([21] and Figure 1), suggesting that the inhibition of increased proteolysis in these animals may reflect a suppression of the hypersensitivity of tumour-bearing animals to the stress-induced hyperproduction of TNF. Although further experiments are clearly needed to identify whether the modulation of a TNF-signalling pathway is involved in the inhibition of the catabolic response, several lines of evidence support this interpretation. First, HWA 448 was totally unable to prevent muscle wasting in atrophying non-weight-bearing muscles [50]. As in cancer cachexia [1,2], muscle wasting results mainly from an activation of Ub–proteasome-dependent proteolysis in non-weight-bearing animals [51]. However, simulated weightlessness is a non-pathological model of muscle disuse, which is not associated with increased TNF production. Secondly, enhanced skeletal-muscle proteolysis has been reported repeatedly to be mediated by TNF in some, but not all, tumour-bearing animals [1,2,18–20]. For example, treatment of Yoshida hepatoma-bearing animals with an anti-TNF antibody suppressed both muscle wasting and the increased expression of Ub and a subunit of the 20 S proteasome in skeletal muscle [19], as did HWA 448 in the present experiments. However, it cannot be discounted that HWA 448 may be acting at some other point(s) in the Ub–proteasome-dependent pathway.

In conclusion, our data show that HWA 448 limited muscle wasting by suppressing the activation of Ub–proteasome-dependent proteolysis, which plays a prominent role in the breakdown of muscle proteins, and was much more potent than pentoxifylline. In addition, HWA 448 did not alter peripheral blood mononuclear cell viability, and has a longer serum half-life and presumably lower toxicity than pentoxifylline, which is commonly used in humans [52]. Taken together, the data suggest that the administration of HWA 448 may contribute to the prevention of the muscle wasting seen in cancer and other catabolic states characterized by a hyperproduction of TNF, such as sepsis or trauma.

REFERENCES

1   Tisdale, M. J. (2000) Protein loss in cancer cachexia. Science **289**, 2293–2294
2   Attaix, D., Combaret, L., Tilignac, T. and Taillandier, D. (1999) Adaptation of the ubiquitin-proteasome proteolytic pathway in cancer cachexia. Mol. Biol. Rep. **26**, 77–82
3   Baracos, V. E., DeVivo, C., Hoyle, D. H. R. and Goldberg, A. L. (1995) Activation of the ATP-ubiquitin-proteasome pathway in skeletal muscle of cachectic rats bearing a hepatoma. Am. J. Physiol. **268**, E996–E1006
4   Lundholm, K., Bylund, A. C., Holm, J. and Schersten, T. (1976) Skeletal muscle metabolism in patients with malignant tumour. Eur. J. Cancer. **12**, 465–473
5   Temparis, S., Asensi, M., Taillandier, D., Aurousseau, E., Larbaud, D., Obled, A., Béchet, D., Ferrara, M., Estrela, J. M. and Attaix, D. (1994) Increased ATP-ubiquitin-dependent proteolysis in skeletal muscles of tumour-bearing rats. Cancer Res. **54**, 5568–5573
6   Llovera, M., Garcia-Martinez, C., Agell, N., Marzabal, M., Lopez-Soriano, F. J. and Argiles, J. M. (1994) Ubiquitin gene expression is increased in skeletal muscle of tumour-bearing rats. FEBS Lett. **338**, 311–318
7   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
8   Attaix, D. and Taillandier, D. (1998) The critical role of the ubiquitin-proteasome pathway in muscle wasting in comparison to lysosomal and Ca^{2+}-dependent systems. In Advances in Molecular and Cell Biology, Intracellular protein degradation (Bittar, E. E. and Rivett, A. J., eds), vol. 27, pp. 235–266, JAI Press, Inc., Greenwich, CT
9   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 (suppl. 1S)**, 227S–237S
10 Hasselgren, P. O. and Fischer, J. E. (2001) Muscle cachexia: current concepts of intracellular mechanisms and molecular regulation. Ann. Surg. **233**, 9–17
11 Hershko, A., Ciechanover, A. and Varshavsky, A. (2000) The ubiquitin system. Nat. Med. **6**, 1073–1081
12 Attaix, D., Combaret, L., Pouch, M.-N. and Taillandier, D. (2001) Regulation of proteolysis. Curr. Opin. Clin. Nutr. Metab. Care **4**, 45–49
13 Voges, D., Zwickl, P. and Baumeister, W. (1999) The 26S proteasome: a molecular machine designed for controlled proteolysis. Annu. Rev. Biochem. **68**, 1015–1068
14 Todorov, P., Cariuk, P., McDevitt, T., Coles, B., Fearon, K. and Tisdale, M. (1996) Characterization of a cancer cachectic factor. Nature (London) **379**, 739–742
15 Tessitore, L., Costelli, P., Bonetti, G. and Baccino, F. M. (1993) Cancer cachexia, malnutrition and tissue protein turnover in experimental animals. Arch. Biochem. Biophys. **306**, 52–58
16 Oliff, A., Defeo-Jones, D., Boyer, M., Martinez, D., Kiefer, D., Vuocolo, G., Wolfe, A. and Socher, S. H. (1987) Tumours secreting human TNF/cachectin induce cachexia in mice. Cell **50**, 555–563
17 Tracey, K. J., Wei, H., Manogue, K. R., Fong, Y., Hesse, D. G., Nguyen, H. T., Kuo, G. C., Beutler, B., Cotran, R. S., Cerami, A. and Lowry, S. F. (1988) Cachectin/tumour necrosis factor induces cachexia, anemia, and inflammation. J. Exp. Med. **167**, 1211–1227
18 Costelli, P., Carbo, N., Tessitore, L., Bagby, G. J., Lopez-Soriano, F. J., Argiles, J. M. and Baccino, F. M. (1993) Tumour necrosis factor-alpha mediates changes in tissue protein turnover in a rat cancer cachexia model. J. Clin. Invest. **92**, 2783–2789
19 Llovera, M., Carbo, N., Garcia-Martinez, C., Costelli, P., Tessitore, L., Baccino, F. M., Agell, N., Bagby, G. J., Lopez-Soriano, F. and Argiles, J. M. (1996) Anti-TNF treatment reverts increased muscle ubiquitin gene expression in tumour-bearing rats. Biochem. Biophys. Res. Commun. **221**, 653–655
20 Combaret, L., Rallière, C., Taillandier, D., Tanaka, K. and Attaix, D. (1999) Manipulation of the ubiquitin-proteasome pathway in cachexia: pentoxifylline suppresses the activation of 20S and 26S proteasomes in muscles from tumour-bearing rats. Mol. Biol. Rep. **26**, 95–101
21 Semmler, J., Gebert, U., Eisenhut, T., Moeller, J., Schönharting, M. M., Alléra, A. and Endres, S. (1993) Xanthine derivatives: comparison between suppression of tumour necrosis factor-alpha production and inhibition of cAMP phosphodiesterase activity. Immunology **78**, 520–525
22 Vary, T., Dardevet, D., Grizard, J., Voisin, L., Buffière, C., Denis, P., Breuillé, D. and Obled, C. (1999) Pentoxifylline improves insulin action limiting skeletal muscle catabolism after infection. J. Endocrinol. **163**, 15–24
23 Dezube, B. J., Sherman, M. L., Fridovich-Keil, J. L., Allen-Ryan, J. and Pardee, A. B. (1993) Down-regulation of tumour necrosis factor expression by pentoxifylline in cancer patients: a pilot study. Cancer Immunol. Immunother. **36**, 57–60
24 Goldberg, R. M., Loprinzi, C. L., Mailliard, J. A., O'Fallon, J. R., Krook, J. E., Ghosh, C., Hestorff, R. D., Chong, S. F., Reuter, N. F. and Shanahan, T. G. (1995) Pentoxifylline for treatment of cancer anorexia and cachexia? A randomized, double-blind, placebo-controlled trial. J. Clin. Oncol. **13**, 2856–2859
25 Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. (1985) Measurement of protein using bicinchoninic acid. Anal. Biochem. **150**, 76–85

26 Tischler, M., 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

27 Waalkes, T. P. and Udenfriend, S. J. (1957) A fluorometric method for the estimation of tyrosine in plasma and tissues. Lab. Clin. Invest. **50**, 733–736

28 Voisin, L., Breuillé, D., Combaret, L., Pouyet, C., Taillandier, D., Aurousseau, E., Obled, C. and Attaix, D. (1996) Muscle wasting in a rat model of long lasting sepsis results from the activation of lysosomal, Ca^{2+}-activated and ubiquitin-proteasome proteolytic pathways. J. Clin. Invest. **97**, 1610–1617

29 Rock, K. L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D. and Goldberg, A. L. (1994) Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. Cell **78**, 761–771

30 Chomczynski, P. and Sacchi, N. (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162**, 156–159

31 Shibuya, H., Irie, K., Ninomiya-Tsuji, J., Goebl, M., Taniguchi, T. and Matsumoto, K. (1992) New human gene encoding a positive modulator of HIV Tat-mediated transactivation. Nature (London) **357**, 700–702

32 Yokota, K., Kagawa, S., Shimizu, Y., Akioka, H., Tsurumi, C., Noda, C., Fujimuro, M., Yokosawa, H., Fujiwara, T., Takahashi, E. et al. (1996) cDNA cloning of p112, the largest regulatory subunit of the human 26S proteasome, and functional analysis of its yeast homologue, sen3p. Mol. Biol. Cell **7**, 853–870

33 Ferrell, K., Deveraux, Q., van Nocker, S. and Rechsteiner, M. (1996) Molecular cloning and expression of a multiubiquitin chain binding subunit of the human 26S protease. FEBS Lett. **381**, 143–148

34 Ahn, J. Y., Tanahashi, N., Akiyama, K., Hisamatsu, H., Noda, C., Tanaka, K., Chung, C. H., Shibmara, N., Willy, P. J., Mott, J. D. et al. (1995) Primary structures of two homologous subunits of PA28, a gamma-interferon-inducible protein activator of the 20S proteasome. FEBS Lett. **366**, 37–42

35 Fort, P., Marty, L., Piechaczyk, M., El Sabrouty, S., Dani, C., Jeanteur, P. and Blanchard, J. M. (1985) Various rat adult tissues express only one major mRNA species from the glyceraldehyde-3-phosphate dehydrogenase multigenic family. Nucleic Acids Res. **13**, 1431–1442

36 Lorite, M. J., Thompson, M. G., Drake, J. L., Carling, G. and Tisdale, M. J. (1998) Mechanism of muscle protein degradation induced by a cancer cachectic factor. Br. J. Cancer **78**, 850–856

37 Fujita, J., Tsujinaka, T., Yano, M., Ebisui, C., Saito, H., Katsume, A., Akamatsu, K., Ohsugi, Y., Shiozaki, H. and Monden, M. (1996) Anti-interleukin-6 receptor antibody prevents muscle atrophy in colon-26 adenocarcinoma-bearing mice with modulation of lysosomal and ATP-ubiquitin-dependent proteolytic pathways. Int. J. Cancer. **68**, 637–643

38 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

39 Tiao, G., Hobler, S., Wang, J. J., Meyer, T. A., Luchette, F. A., Fischer, J. E. and Hasselgren, P. O. (1997) Sepsis is associated with increased mRNAs of the ubiquitin-proteasome proteolytic pathway in human skeletal muscle. J. Clin. Invest. **99**, 163–168

40 Williams, A., Sun, X., Fischer, J. E. and Hasselgren, P. O. (1999) The expression of genes in the ubiquitin-proteasome proteolytic pathway is increased in skeletal muscle from patients with cancer. Surgery **126**, 744–749

41 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, 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

42 Mykles, D. L. (1996) Differential effects of bovine PA28 on six peptidase activities of the lobster muscle proteasome (multicatalytic proteinase). Arch. Biochem. Biophys. **325**, 77–81

43 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

44 Takayanagi, K., Dawson, S., Reynolds, S. E. and Mayer, R. J. (1996) Specific developmental changes in the regulatory subunits of the 26 S proteasome in intersegmental muscles preceding eclosion in *Manduca sexta*. Biochem. Biophys. Res. Commun. **228**, 517–523

45 van Nocker, S., Sadis, S., Rubin, D. M., Glickman, M., Fu, H., Coux, O., Wefes, I., Finley, D. and Vierstra, R. D. (1996) The multiubiquitin-chain-binding protein Mcb1 is a component of the 26S proteasome in *Saccharomyces cerevisiae* and plays a nonessential, substrate-specific role in protein turnover. Mol. Cell. Biol. **16**, 6020–6028

46 Solomon, V., Baracos, V., Sarraf, P. and Goldberg, A. L. (1998) Rates of ubiquitin conjugation increase when muscles atrophy, largely through activation of the N-end rule pathway. Proc. Natl. Acad. Sci. U.S.A. **95**, 12602–12607

47 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

48 Wing, S. S., Haas, A. L. and Goldberg, A. L. (1995) Increase in ubiquitin-protein conjugates concomitant with the increase in proteolysis in rat skeletal muscle during starvation and atrophy denervation. Biochem. J. **307**, 639–645

49 Dinarello, C. A. (2000) Proinflammatory cytokines. Chest **118**, 503–508

50 Aboudrar, S., Desplanches, D., Graber-von-Bergen, F., Favier, R., Okyayuz-Baklouti, I. and Hoppeler, H. (1992) Effects of torbafylline on muscle atrophy: prevention and recovery. Can. J. Physiol. Pharmacol. **70**, 814–820

51 Taillandier, D., Aurousseau, E., Meynial-Denis, D., Béchet, D., Ferrara, M., Cottin, P., Ducastaing, A., Bigard, A. X., Guezennec, C. Y., Schmid, H.-P. and Attaix, D. (1996) Coordinate activation of lysosomal, Ca^{2+}-activated and ATP-ubiquitin-dependent proteinases in the unweighted rat soleus muscle. Biochem. J. **316**, 65–72

52 Jackobsen, P. H., Koch, C. and Brendtzen, K. (1997) Inhibition of LPS and *Plasmodium falciparum* induced cytokine secretion by pentoxifylline and two analogs. Scand. J. Immunol. **45**, 546–550