JCI The Journal of Clinical Investigation

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J Clin Invest. 1996;97(7):1610-1617. https://doi.org/10.1172/JCI118586.

Research Article

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Muscle Wasting in a Rat Model of Long-lasting Sepsis Results from the Activation of Lysosomal, Ca^{2+}-activated, and Ubiquitin-Proteasome Proteolytic Pathways

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Abstract

We studied the alterations in skeletal muscle protein breakdown in long lasting sepsis using a rat model that reproduces a sustained and reversible catabolic state, as observed in humans. Rats were injected intravenously with live *Escherichia coli*; control rats were pair-fed to the intake of infected rats. Rats were studied in an acute septic phase (day 2 postinfection), in a chronic septic phase (day 6), and in a late septic phase (day 10). The importance of the lysosomal, Ca^{2+}-dependent, and ubiquitin-proteasome proteolytic processes was investigated using proteolytic inhibitors in incubated epitrochlearis muscles and by measuring mRNA levels for critical components of these pathways. Protein breakdown was elevated during the acute and chronic septic phases (when significant muscle wasting occurred) and returned to control values in the late septic phase (when wasting was stopped). A nonlysosomal and Ca^{2+}-independent process accounted for the enhanced proteolysis, and only mRNA levels for ubiquitin and subunits of the 20 S proteasome, the proteolytic core of the 26 S proteasome that degrades ubiquitin conjugates, paralleled the increased and decreased rates of proteolysis throughout. However, increased mRNA levels for the 14-kD ubiquitin conjugating enzyme E2, involved in substrate ubiquitylation, and for cathepsin B and m-calpain were observed in chronic sepsis. These data clearly support a major role for the ubiquitin-proteasome dependent proteolytic process during sepsis but also suggest that the activation of lysosomal and Ca^{2+}-dependent proteolysis may be important in the chronic phase. (*J. Clin. Invest.* 1996. 97:1610–1617.) Key words: infection • protein breakdown • ubiquitin-dependent proteolysis • cathepsins • calpains

Introduction

It is difficult to estimate the true incidence of sepsis which is increasingly common. However, data from the Centers for Disease Control suggest that septicemia increased by 139% between 1979 and 1987 and was ranked in 1991 as the thirteenth leading cause of the death in the United States (1). Among other metabolic disturbances, sepsis causes a severe and persistent loss of body protein, much of which originates from skeletal muscle (2). In patients, these perturbations are lasting (3). Muscle wasting may be advantageous during the initial phase of sepsis, since, for example, protein mobilization provides substrates for acute phase protein synthesis. Because of the vital role of muscle in locomotion and respiratory effort, sustained loss of skeletal muscle may have a very negative impact upon rehabilitation or survival of the organism. A precise knowledge of the sequence of events as well as of the mechanisms responsible for the mobilization of muscle proteins is therefore needed to develop appropriate therapies.

Three models of sepsis are commonly used to investigate the alterations in skeletal muscle protein turnover, i.e., cecal ligation and puncture (4, 5), implantation of a sterilized fecal-agar pellet containing *Escherichia coli* and *Bacteroides fragilis* (6–8) and endotoxin administration (9, 10). None of these reproduces the lasting muscle wasting observed in septic patients. Indeed, the first two models are characterized by high mortality rates within a very short period of illness and are representative of an acute septic phase. Endotoxin administration has also very transient effects, due to resistance to endotoxin, even when multiple injections (9) or constant infusion (10) of LPS are given. This explains why nearly all experimental studies related to muscle atrophy have been performed in an early phase after induction of the stress.

In these different septic models, many studies have provided evidence that muscle wasting resulted mainly from increased protein breakdown (4, 5, 9–11), although depressed protein synthesis also contributed to the catabolic response (6, 7, 9, 10, 12, 13). However, the precise role of the different proteolytic processes is poorly defined, and contradictory results have been obtained during the acute septic phase. The most studied proteolytic process in skeletal muscle is the lysosomal pathway. It has been reported that cathepsin B (a major lysosomal proteinase in skeletal muscle with cathepsins L, H, and D) plays an important role in muscle wasting in septic rats (14, 15). By contrast, others studies have described no change in cathepsin B or L activities (8) or have reported little if any evidence for increased lysosomal proteolysis (5) in septic muscle. Skeletal muscle also contains Ca^{2+}-dependent proteinases, μ- and m-calpain, which differ in their affinities for Ca^{2+}. Benson et al. (16) and Bhattacharyya et al. (8) have provided evidence for a role of Ca^{2+}-dependent proteinases in sepsis, in contrast with Tiao et al. (5). Finally, recent evidence has indicated that the ATP-ubiquitin (Ub)[1]-dependent proteolytic

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Received for publication 21 August 1995 and accepted in revised form 19 January 1996.

J. Clin. Invest.

0021-9738/96/04/1610/08 $2.00
Volume 97, Number 7, April 1996, 1610–1617

1. *Abbreviations used in this paper:* EDL, extensor digitorum longus muscle; 14-kD E_2, 14-kD ubiquitin conjugating enzyme E2; Ub, ubiquitin.

pathway plays a major role in muscle wasting during various catabolic states (17–23). Degradation of a protein via this pathway involves two distinct steps: signaling of the protein by covalent attachment of multiple Ub molecules and degradation of the targeted protein by an ATP-dependent protease, the 26 S (1,500 kD) proteasome (24), with release of free and reutilizable Ub. Tiao et al. (5) have shown that sepsis stimulates an energy-dependent proteolytic system that accounted for the breakdown of myofibrillar proteins. Moreover, these authors reported increased Ub mRNA levels in the muscles from septic rats, suggesting that the ATP-dependent proteolytic pathway activated was the Ub system. However, Ub has many nonproteolytic functions (24). Furthermore, increased Ub mRNA levels are not necessarily correlated with enhanced protein breakdown in muscle wasting (25). Thus, additional experiments are clearly needed to elucidate the possible role of the Ub-proteasome proteolytic pathway in septic muscle.

The aim of these experiments was to study the alterations in muscle protein turnover using a long lasting septic rat model that reproduces a sustained and reversible catabolic state, as observed in humans. These experiments were performed in an acute septic phase (day 2 postinfection), in a chronic septic phase (day 6 postinfection), and in a late septic phase (day 10 postinfection). The importance of the lysosomal, Ca^{2+}-dependent, and Ub-proteasome proteolytic processes was investigated during the three phases using proteolytic inhibitors in incubated epitrochlearis muscles, and by measuring mRNA levels for critical components of these pathways.

Methods

Animals. Male Sprague-Dawley rats (Iffa Credo, Saint-Germain sur l'Arbresle, France) were individually housed in wire-bottom cages in a temperature-controlled room (22–23°C) with a 12-h:12-h light-dark cycle. During a 6 d acclimatization period, all rats received a semisynthetic diet containing 12% protein distributed by an automatic device (13). During the 3 d preceding the experiments, the animals were weighed and food intake was measured.

Rats, with an initial body weight of 300 g, were divided into two groups. The infected group was injected with *E. coli* (serotype 0153: K^-:H^-; 7×10^8 CFU) into a lateral tail vein, as previously described (13). Control animals received an intravenous saline injection; since infection dramatically decreases food intake (13), they were pair-fed to the intake of infected rats. Usually pair-feeding is conducted on a daily basis, and the pair-fed animals would normally have been killed a day later than the infected animals. However, in vitro protein turnover measurements substantially varied from day to day. To avoid such temporal differences in protein turnover, the intake of controls was based on the intake of infected rats measured in previous experiments and corrected daily with the intake of the actual infected group. Animals were weighed every day until the completion of the studies (Fig. 1). A group of eight noninfected rats was killed at time 0. Thereafter, between five and eight infected rats were killed at 2, 6, and 10 d after infection. The number of pair-fed control animals was matched to those of the infected animals used. According to the variations in body weight throughout the study, three distinct phases were observed: an acute septic phase (day 1–2), a chronic septic phase (day 3–6), and a late septic phase (day 7–10) (see below). The mortality rate of the infected rats was 7±10% in these three groups. Each phase was studied separately. Animals were killed under anesthesia with sodium pentobarbital (6.0 mg/100 g body wt). Epitrochlearis muscles were dissected intact for incubation (see below) and extensor digitorum longus (EDL), soleus, and tibialis anterior muscles were frozen in liquid nitrogen and stored at −80°C until analysis.

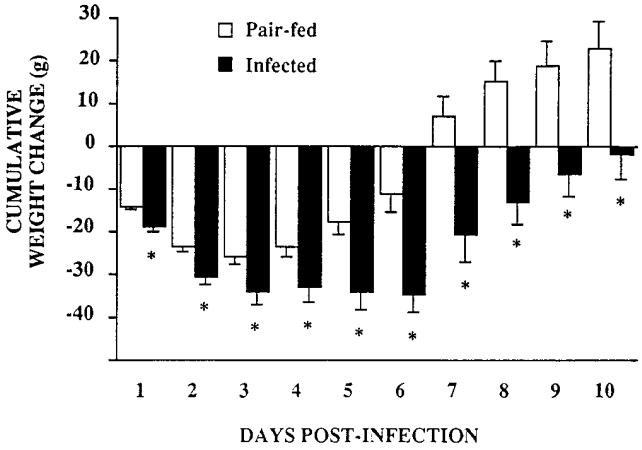

Figure 1. Time-course of cumulative weight changes for 10 d after infection in septic and pair-fed control rats. Changes in daily body weight for all animals studied are plotted. Values are means±SEM for 5–24 rats. $*P < 0.05$ vs. pair-fed.

Rates of protein turnover. Protein turnover was measured as previously described (26). Epitrochlearis muscles were preincubated for 30 min in Krebs Henseleit Buffer (KHB) (NaCl 120 mM, KCl 4.8 mM, $NaHCO_3$ 25 mM, $CaCl_2$ 2.5 mM, KH_2PO_4 1.2 mM, and $MgSO_4$ 1.2 mM, pH 7.4), supplemented with 5 mM glucose, 5 mM Hepes, 0.1% BSA, 0.17 mM leucine, 0.20 mM valine, 0.10 mM isoleucine, and 0.1 U/ml insulin. The medium was saturated with a 95% O_2–5% CO_2 gas mixture. Muscles were then transferred into fresh media of the same composition containing 0.5 mM [U-^{14}C]L-phenylalanine (0.15 μCi/ml) for 60 min. At the end of the incubation, muscles were blotted and homogenized in 10% TCA. TCA-insoluble material was washed three times with 10% TCA, and solubilized in 1 M NaOH at 37°C for determination of protein-bound radioactivity and protein. Tissue protein mass was determined using the bicinchoninic acid procedure (BCA; Pierce Chemical Co., Rockford, IL) (27). Rates of phenylalanine incorporation were multiplied by a value of 0.77, i.e., the molar ratio of tyrosine to phenylalanine in rat muscle proteins, to obtain tyrosine equivalents (28). Protein synthesis was expressed as nanomoles of tyrosine incorporated per mg protein per h.

Protein degradation was determined as described by Tischler et al. (28). Muscles were incubated as mentioned above. Since tyrosine is neither synthesized nor degraded by muscle, the release of this amino acid from muscle into the incubation medium reflects the net rate of protein breakdown. Tyrosine was assayed by the fluorometric method of Waalkes and Udenfriend (29). Total protein breakdown was then calculated by adding the rates of net protein breakdown and of protein synthesis. Additional experiments were performed to estimate the contribution of the lysosomal and Ca^{2+}-activated proteinases to overall protein breakdown, by following the rates of tyrosine release in the medium in the presence of 0.5 mM cycloheximide (28). One muscle was incubated without inhibitors. Nonlysosomal and Ca^{2+}-independent proteolysis was measured in the contralateral muscle in the presence of 10 mM methylamine and 50 μM E-64c in a Ca^{2+}-free medium (19–22). Protein degradation was expressed as nanomoles of tyrosine per mg protein per h.

Northern blot analysis. Total RNA was extracted from 0.5 g of tibialis anterior by the method of Chomczynsky and Sacchi (30). 20 μg of RNA was electrophoresed in formaldehyde agarose gels (1%) and transferred electrophoretically to nylon membranes (GeneScreen; NEN Research Products, Boston, MA). RNA was covalently bound to the membrane by UV crosslinking. Membranes were hybridized with cDNA probes encoding chicken polyUb (31), rat 14-kD Ub conjugating enzyme E2 (14-kD E2) (32), C8 and C9 rat proteasome subunits (33, 34), and human m-calpain (35). Hybridizations

Table I. Characterization of the Different Phases of Sepsis

	day 0	day 2	day 6	day 10
Food intake (%)	100±1*	25±3‡	80±5§	99±1*
Body weight (g)	299±2*	269±2‡	265±5‡	297±5*
Liver mass (g)	13.0±0.4*	12.4±0.5*	9.4±0.4‡	10.6±0.4*
Acute phase proteins				
Fibrinogen (mg/l)	3.47±0.03*	7.84±0.14‡	6.86±0.28§	6.24±0.26‖
Orosomucoid (mg/l)	19±1*	1,053±57‡	448±64§	372±43§
Bacterial counts				
Blood (bacteria/ml)	nd	$10^2 - 36 \times 10^2$	nd	nd
Spleen (bacteria/g)	nd	$10^6 - 6 \times 10^6$	$2 \times 10^3 - 15 \times 10^3$	$0 - 8 \times 10^3$
Liver (bacteria/g)	nd	$2 \times 10^4 - 17 \times 10^4$	$0 - 7 \times 10^3$	$0 - 5 \times 10^3$
Cytokines				
TNF-α (ng/ml)	nd	nd¶	nd	nd
IL-6 (ng/ml)	nd	< 25¶	< 7	< 4

Values for food intake, body weight, liver mass, and acute phase proteins are means±SEM for five to eight animals. *‡§‖ values within a line with different superscripts are significantly different ($P < 0.05$, one-way ANOVA). nd, not detectable. ¶TNF-α and IL-6 concentrations peaked at 1.5 and 3 h postinfection (30–40 and 2,000–4,000 ng/ml, respectively). Days 2, 6, and 10 postinfection are representative of acute, chronic, and late septic phases, respectively.

were conducted overnight at 65°C with [^{32}P]cDNA fragments labeled by random priming, as previously described (21). After washing at the same temperature, filters were autoradiographed at −80°C with intensifying screens on Hyperfilm-MP films (Amersham International, Little Chalfont, England). A cDNA probe encoding rat cathepsin B subcloned into EcoRI sites of pGem-blue was linearized with HindIII, as previously described (21). Membranes were hybridized at 60°C with a single-stranded antisense riboprobe synthetized using T7 RNA polymerase and digoxigenin-labeled UTP (Boehringer Mannheim Biochemicals, Indianapolis, IN) (25). After washing, specific hybridization was revealed autoradiographically using a chemiluminescence reaction as described by the manufacturer. After stripping of the different probes, the filters were reprobed with a mouse 18 S ribosomal probe (No. 63178; American Type Culture Collection, Rockville, MD). Autoradiographic signals were quantified by digital image processing and analysis (NIH Image 1.54) and normalized using the corresponding 18 S rRNA signals to correct for uneven loading.

TNF-α and IL-6 assays. Blood samples were collected in heparinized tubes, and plasma was stored at −80 and −20°C for TNF-α and IL-6 assays, respectively. TNF-α was measured by using an ELISA kit, according to the manufacturer's instructions (Genzyme Corp., Cambridge, MA). Biological activity of IL-6 was estimated in a bioassay using the B-9 hybridoma cell line (36). Briefly, B-9 cells (5,000/100 μl) were cultured in 96-well microtiter plates with serial dilutions of test samples. The IL-6 standard was human recombinant IL-6 (No. 89/548; National Institute for Biological Standards and Control, Hertfordshire, England), which was serially diluted. After 48 h of incubation at 37°C with 5% CO_2, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (2 mg/ml) was added to each well in the presence of phenazine methosulfate and incubated for an additional 2 h to determine cell proliferation (37). The water-soluble formazan product was quantitated at 490 nm in a MR 700 microplate reader (Dynatech Laboratories, Inc., Guernsey, England).

Fibrinogen and orosomucoid assays. Fibrinogen and orosomucoid were measured by single radial diffusion, using anti–rat fibrinogen (Cappel, Turnhout, Belgium) and anti–rat orosomucoid produced in the laboratory, respectively.

Bacterial counts. Blood, liver, and spleen were collected under sterile conditions. The organs were homogenized with a Potter (Wheaton, Millville, NJ) in sterile saline. Viable bacteria were counted in duplicate in all tissues by using 10-fold serial dilutions plated on 0.05% desoxycholate lactose agar medium (Diagnostics Pasteur, Marnes La Coquette, France).

Statistics. All data are expressed as means±SEM and are representative of at least two different experiments. The significance of differences was analyzed by one-way ANOVA and by Student's *t* test where appropriate. Differences among means were considered significant when $P < 0.05$.

Results

Characterization of the septic model. The time-course for the body weight of infected rats fell into three distinct periods (Fig. 1); the first one was an acute septic phase that occurred between 0 and 3 d postinfection, during which body weight loss reached 35 g. The second phase was a chronic septic phase, in which rat body weights remained approximately stable until 6 d postinfection; the third and final stage was a late septic phase, between 7 and 10 d postinfection, in which their weight increased by 19 g. By contrast, pair-fed control rats only showed two periods of body weight change. Pair-fed rats lost 26 g within 3 d, and then rapidly recovered (Fig. 1). Despite the pair-feeding conditions, the body weight decrease was significantly ($P < 0.05$) higher in septic rats than in the pair-fed animals (Fig. 1). This difference in body weight increased progressively from the first day post-infection and remained elevated, even at day 10.

The acute septic phase was characterized by a very depressed food intake (the first and second days postinfection, rats ate, respectively, 24 and 25% of the preinfected food consumption), a high number of live bacteria in blood, spleen, and liver, increased levels of circulating acute phase proteins (fibrinogen and orosomucoid concentrations increased by 126 and 5,442%, $P < 0.001$) and of IL-6 (Table I). In contrast with IL-6, TNF-α concentration increased only very transiently at 1.5 h postinfection (30–40 ng/ml), but was no longer detectable 2 d postinfection (Table I; limit of detection of the ELISA: 15 pg/ml).

During the chronic septic phase, food intake gradually increased (at days 3 and 6 postinfection food intake represented

Table II. Effect of Sepsis on EDL, Soleus, Tibialis Anterior, and Epitrochlearis Muscle Masses from Infected and Pair-fed Control Rats 0, 2, 6, and 10 d Postinfection

	Days postinfection						
	0	2		6		10	
		PF	INF	PF	INF	PF	INF
EDL mass (mg)	132±5*	134±3*	112±3‡	140±3*	107±7‡	137±3*	115±5‡
Soleus mass (mg)	127±3*	120±1*	107±5‡	122±5*	93±6§	115±3*	87±4§
Tibialis anterior mass (mg)	542±13*	539±12*	444±18‡	563±13*	390±24§	584±16*	340±16‖
Epitrochlearis mass (mg)	52±3*	50±2*	42±2‡	52±2*	37±2§	58±4*	42±4‡§

Values are means±SEM for five to eight animals. *‡§‖ values within a line with different superscripts are significantly different ($P < 0.05$, one-way ANOVA). Days 2, 6, and 10 postinfection are representative of acute, chronic, and late septic phases, respectively. PF, pair-fed; INF, infected.

37 and 80% of preinfected intake, respectively), and no more live bacteria were detected in blood (Table I). By contrast, live bacteria were still detected in spleen and occasionally in liver. The circulating levels of fibrinogen, orosomucoid, and IL-6 remained high, but significantly less than in the acute septic phase (Table I).

Finally, during the late septic phase, food intake returned to normal, and live bacteria were only occasionally detected in spleen and liver. Acute phase proteins were still higher than in control animals, and circulating IL-6 was still detectable.

Effects of infection on skeletal muscle mass. Whatever the phase studied, there was no difference in skeletal muscle mass in pair-fed control rats compared to animals killed at time 0 (Table II). During the acute septic phase, a significant atrophy of EDL, soleus, tibialis anterior, and epitrochlearis muscles was observed in infected rats compared to pair-fed animals (−16%, $P < 0.001$; −11, −18, and −16%, $P < 0.005$, respectively) (Table II). During the chronic septic phase the mass of all muscles was further decreased (−24, −24, −31, and −29%, respectively; $P < 0.001$) with respect to pair-fed controls. During the late septic phase, the mass of all muscles studied remained lower than in the corresponding pair-fed control animals (−16, −24, −42 and −28% in the EDL, soleus, tibialis anterior, and epitrochlearis muscle, respectively, $P < 0.001$). Compared with the chronic septic phase, the mass of EDL, soleus, and epitrochlearis muscles remained stable ($P > 0.05$). Thus, the recovery in body weight was not yet associated with an increase in muscle mass and presumably largely reflected increases in gastrointestinal tract content and in the mass of nonmuscular tissues (e.g. liver, Table I).

Effects of infection on muscle protein turnover. To explain the variations in muscle mass, we measured protein turnover in incubated epitrochlearis muscles (Fig. 2). The rate of protein synthesis was not significantly altered in infected rats compared to their pair-fed controls during both the acute septic phase and the late septic phase. Protein synthesis was actually higher (27%; $P < 0.05$) in the infected rats than in their pair-fed controls in the chronic septic phase, i.e., when food intake had nearly returned to normal. By contrast, the rate of total protein breakdown increased by 49 and 66% in the acute and chronic septic phase, respectively ($P < 0.05$), but was not different during the late septic phase (Fig. 2). Thus muscle wasting resulted from enhanced protein breakdown in both acute and chronic septic phases. Furthermore, muscle mass stabilization during the late septic period did not result from increased protein synthesis. Because each phase of the infection was studied separately and because in vitro protein turnover measurements substantially varied from day to day, as previously reported by others (38), it was not possible to compare the measurements obtained throughout the time-course studied. However, the protein breakdown ratio, infected/pair-fed, which is close to 1 in basal conditions (i.e., before infection), was markedly elevated during the acute and chronic septic phases (1.50±0.30 and 1.66±0.06, respectively) and decreased to 1.12±0.05 in the late septic phase. We conclude from these experiments that increased protein breakdown was the major determinant of muscle wasting during the acute and chronic septic phases, and that the suppression of enhanced proteolysis characterized subsequent late septic phase.

Proteolytic systems responsible for muscle atrophy. To identify the proteolytic pathway(s) activated in the atrophying muscles from infected rats, we next measured rates of protein breakdown in the presence of inhibitors of both lysosomal and Ca^{2+}-dependent proteinases (19, 21, 22). Lysosomal protein breakdown was inhibited by adding to the media the weak-base methylamine, which raises intralysosomal pH; Ca^{2+}-dependent proteolysis was inhibited by using a Ca^{2+}-free medium containing E-64c, which inhibits cysteine proteinases (39), i.e., the calpains, as well as cathepsins B, H, and L. In

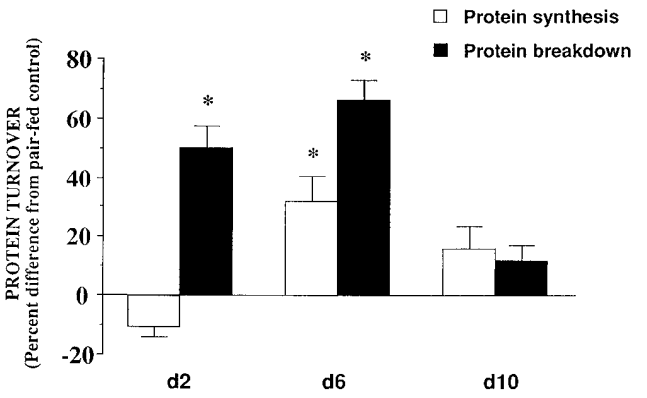

Figure 2. Effects of sepsis on in vitro protein turnover. Epitrochlearis muscles were incubated in a Krebs-Henseleit buffer. Protein synthesis and total protein breakdown (nmol tyrosine/mg protein per h) are expressed as percent differences from pair-fed controls. *d2*, 2 d postinfection (acute septic phase); *d6*, 6 d postinfection (chronic septic phase); *d10*, 10 d postinfection (late septic phase). Values are means±SEM for six to eight animals. *$P < 0.05$ vs. pair-fed.

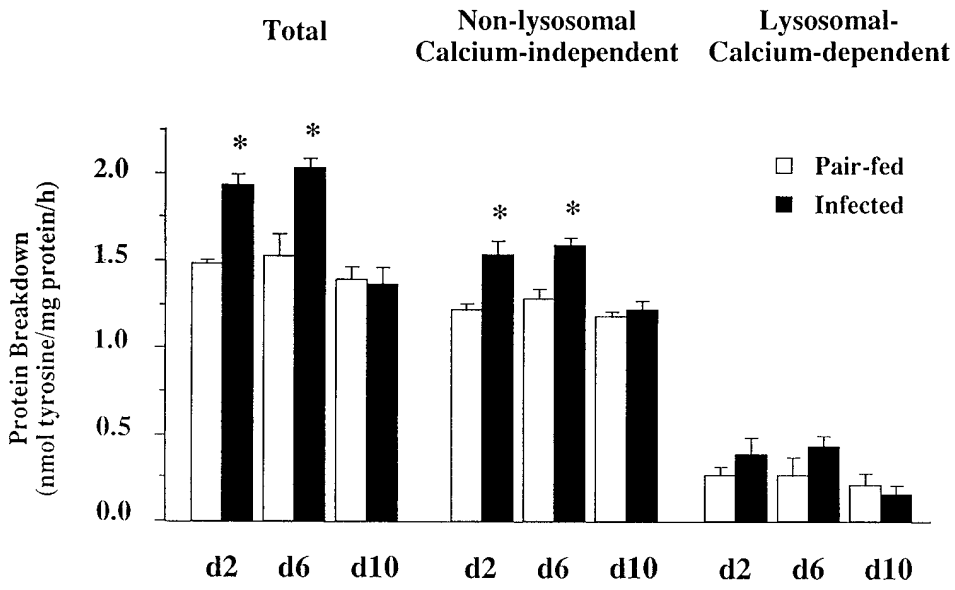

Figure 3. Effects of sepsis on total, nonlysosomal Ca^{2+}-independent, and lysosomal-Ca^{2+}-dependent protein breakdown. Proteolysis was estimated by the release of tyrosine into the incubation medium containing cycloheximide. Total protein breakdown was measured without proteolytic inhibitors. Nonlysosomal Ca^{2+}-independent proteolysis was measured in the presence of 10 mM methylamine and 50 μM E-64c in a Ca^{2+}-free medium. *d2*, 2 d postinfection (acute septic phase); *d6*, 6 d postinfection (chronic septic phase); *d10*, 10 d postinfection (late septic phase). Values are means±SEM for six to eight animals. $^*P < 0.05$ vs. pair-fed.

basal conditions (i.e., before infection) the inhibition of both lysosomal and Ca^{2+}-dependent proteinases reduced total protein breakdown by ~ 20% in epitrochlearis muscle (data not shown). During either phase of sepsis, blocking lysosomal and Ca^{2+}-dependent proteinases inhibited total protein breakdown by 15–20% ($P < 0.05$) (Fig. 3). There was a tendency for increased lysosomal and Ca^{2+}-dependent proteolysis during both the acute and chronic septic phases, which corresponded to increased mRNA levels for cathepsin B and m-calpain (+134 and +144%, respectively; $P < 0.05$) in only the chronic period (Fig. 4). These data suggest a possible activation of both lysosomal and Ca^{2+}-dependent proteolytic pathways during that phase. By contrast, mRNA levels for cathepsin B and m-calpain returned to control pair-fed values in the late septic phase (Fig. 4).

The increased rate of proteolysis seen in acute and chronic septic phases was, however, not suppressed by the inhibitors of lysosomal and Ca^{2+}-dependent proteinases (Fig. 3). Thus, a nonlysosomal and Ca^{2+}-independent proteolytic process was mainly responsible for enhanced protein breakdown. Since the Ub-proteasome pathway has been suggested to be stimulated in an acute septic rat model (5), we measured mRNA levels for Ub, 14-kD E2, and proteasome subunits. Increased mRNA levels for Ub (+483%, $P < 0.05$), C8 (+116%, $P < 0.05$), and C9 (+86%, $P < 0.05$; data not shown) subunits of the 20 S proteasome, which is the proteolytic core of the 26 S proteasome that degrades Ub-conjugates (24), were observed in the atrophying muscles during the acute septic phase (Fig. 5). However, the mRNA levels for the 14-kD E2, the only mammalian E2 so far reported to be up-regulated in various catabolic conditions in muscle (21, 25, 32, 40) remained unchanged during that period in the muscles from infected rats (Fig. 5). By contrast, the abundance of mRNAs for Ub, the lowest 1.2-kb transcript of the 14-kD E2, and C8 and C9 proteasome subunits was higher (+84, +205, +51, and +62%, respectively; $P < 0.05$) in infected rats than in pair-fed animals during the chronic septic phase. In the late septic phase, mRNA levels for Ub, C8 and C9 proteasome subunits, and 14-kD E2 were not significantly different in the muscles from infected and pair-fed rats.

Discussion

The present study clearly demonstrated for the first time, to our knowledge, that sustained increased protein breakdown is the major determinant of muscle atrophy in a long-lasting rat septic model that reproduces the catabolic state seen in humans. The data suggest that the Ub-proteasome pathway was presumably essential for the bulk of increased skeletal muscle protein breakdown, but that a coordinate activation of the lysosomal, Ca^{2+}-dependent and ATP-Ub-dependent proteolytic pathways prevailed during a chronic septic phase.

In septic patients, muscle wasting occurs progressively (3), and increased skeletal muscle proteolysis contributes to this loss (41–43). In animal models of sepsis, it is well documented

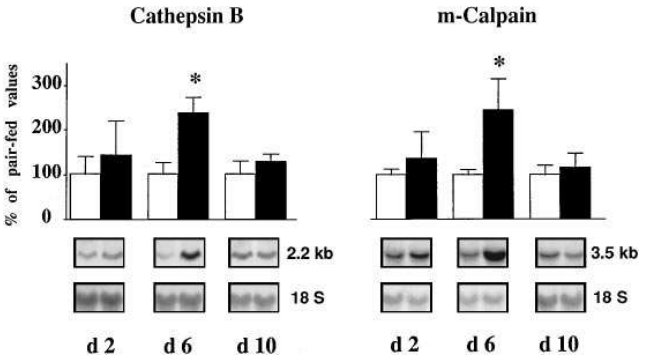

Figure 4. Effects of sepsis on mRNA levels for cathepsin B and m-calpain. RNA was extracted from tibialis anterior muscles of septic and pair-fed rats. Samples (20 μg) were electrophoresed, transferred to nylon membranes, and hybridized with ^{32}P-labeled cDNAs encoding cathepsin B and m-calpain. After stripping of the probes, blots were rehybridized with an 18 S ribosomal oligonucleotide. Data are expressed as a percentage of pair-fed controls that were corrected for 18 S rRNA abundance, to take into account variations in RNA loading. Values are means±SEM for four rats. Representative northern blots are also shown. *d2*, 2 d postinfection (acute septic phase); *d6*, 6 d postinfection (chronic septic phase); *d10*, 10 d postinfection (late septic phase). $^*P < 0.05$ vs. pair-fed.

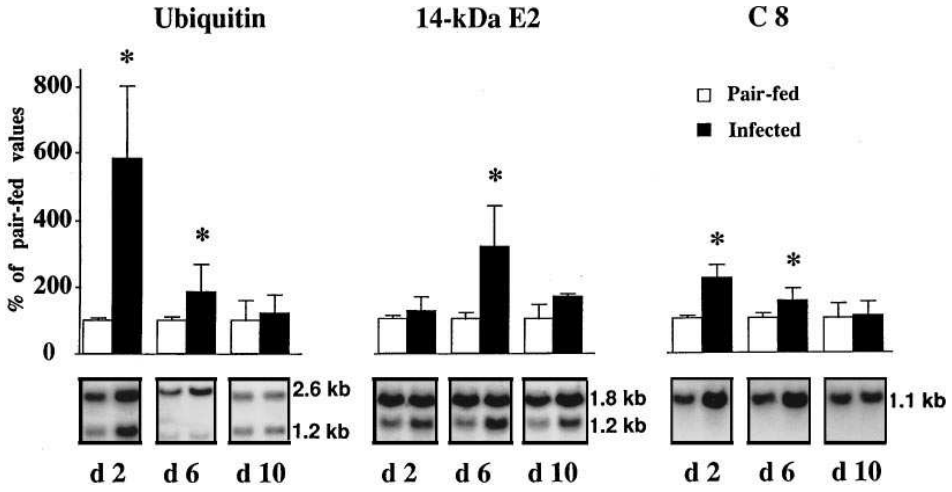

Figure 5. Effects of sepsis on mRNA levels for ubiquitin, 14-kD E2, and C8 proteasome subunit. RNA was extracted from tibialis anterior muscles of septic and pair-fed rats. Samples (20 μg) were electrophoresed, transferred to nylon membranes, and hybridized with ^{32}P-labeled cDNAs encoding ubiquitin, 14-kD E2, and C8 proteasome subunit. After stripping of the probes, blots were rehybridized with an 18 S ribosomal oligonucleotide. Data are expressed as a percentage of pair-fed controls that were corrected for 18 S rRNA abundance, to take into account variations in RNA loading. Values are means±SEM for four rats. Data for ubiquitin and 14-kD E2 refer to both transcripts and to the lowest 1.2-kb transcript, respectively. Representative northern blots are also shown. *d2*, 2 d postinfection (acute septic phase); *d6*, 6 d postinfection (chronic septic phase); *d10*, 10 d postinfection (late septic phase). *$P < 0.05$ vs. pair-fed.

that the early acute catabolic phase is characterized by enhanced skeletal muscle proteolysis (4, 5, 9–11). However, the most commonly used models of sepsis present at least two major drawbacks. First, they result in a brief catabolic state, with little if any loss of muscle proteins (5, 9). Second, the animals either quickly recover (in particular with endotoxin administration) or exhibit a very high mortality rate (4, 5, 8). By contrast, the early acute catabolic period reproduced in our septic model is followed by a severe chronic catabolic state. Indeed, body weight recovery is not observed before 7 d postinfection (Fig. 1), and muscle atrophy is progressive in all muscles between days 2 and 6 postinfection. This muscle atrophy reflects protein loss, since protein concentration was not modified by the infection (12.0±0.5, 12.8±0.5, 11.9±0.6, and 12.0±0.5 in controls rats, and in the acute, chronic, and late septic phases, respectively). It is noteworthy that the muscle wasting is particularly long lasting, because it was stopped, but not reversed, 10 d postinfection (Table II). Therefore, the catabolic state induced by our model is very similar to that observed in patients. Furthermore, the different phases of sepsis can be characterized by several parameters (Table I), and a low mortality rate is seen in this model.

Since infection induces an acute anorexia, and because food deprivation is well known to affect protein turnover in skeletal muscle (44), the data obtained in infected rats were systematically compared to pair-fed animals. Not surprisingly, we found an increased rate of protein breakdown in skeletal muscle during the acute septic phase. This observation is consistent with in vitro (4, 5, 10) and in vivo (9) data reported by others, using various septic models. These observations prevailed in the different skeletal muscles studied, especially pale fast-twitch muscles such as the EDL (5, 45), which has a fiber composition close to the epitrochlearis muscle used here. To our knowledge, there is no information on skeletal muscle protein turnover disturbances during both a chronic septic phase and the subsequent late septic phase. The present in vitro measurements clearly demonstrated that increased protein breakdown was the major determinant of muscle wasting during chronic sepsis and that the suppression of enhanced proteolysis contributed to the cessation of muscle wasting observed 10 d postinfection (Table II and Fig. 2). However, we did not observe an inhibition of protein breakdown in the late septic phase, as recently reported by Samuels and Baracos (46) in catch-up growth after *E. coli* infection in weanling rats. These discrepancies may result from differences in the recovery process between the two experiments (since neither catch-up growth [Fig. 1] nor an improvement in muscle mass [Table II] was detectable 10 d postinfection in our experiments) or alternatively from age-related differences of the animals.

All available data concerning the proteolytic pathways responsible for increased proteolysis have been obtained in the early phase after the induction of the stress. These reports are conflicting. Indeed, studies by several groups supported a role for lysosomal (14, 15) or Ca^{2+}-dependent proteinases (8, 16). In striking contrast, no evidence for a role of either cathepsins (8) or both lysosomal and Ca^{2+}-activated proteinases (5) has also been reported. In accordance with Tiao et al. (5), we found that the inhibition of both the lysosomal and Ca^{2+}-dependent proteolytic pathways did not suppress increased protein breakdown (Fig. 2). In agreement with the in vitro experiments, the mRNA levels for cathepsin B or m-calpain did not change during the acute septic phase in the atrophying muscles (Fig. 3). Thus, a nonlysosomal Ca^{2+}-independent proteolytic process is presumably responsible for enhanced protein breakdown in these conditions. It has already been suggested that the ATP-Ub-dependent proteolytic pathway may play an important role in acute sepsis, since ATP depletion suppressed increased protein breakdown and because increased Ub mRNA levels were observed in septic muscle (5). The present study confirms this observation (Fig. 5). Since Ub may serve also nonproteolytic functions (24, 47), we also measured the mRNA levels for the 14-kD E2 and the C8 and C9 proteasome subunits. The 14-kD E2 species is one of the mammalian E2s that best supports E3-dependent conjugate formation and protein breakdown (32, 47). The abundance of the lowest 1.2-kb transcript of the 14-kD E2 has previously been reported to increase in muscles from fasted (32), tumor-bearing (21), dexamethasone-treated (25), and hindlimb-suspended rats (20), but not in acidotic animals (22) or in the first phase of our model (Fig. 5). By contrast, as shown here, in-

creased expression of proteasome subunits was systematically observed in atrophying muscles where nonlysosomal Ca^{2+}-independent proteolysis rose (18, 20–23, 48). Taken together these data suggest that the proteolytic activities of the proteasome, rather than the rate of substrate ubiquitylation, could be a potential rate-limiting step in the ATP-Ub-dependent pathway. This is in accordance with our previous observations in tumor-bearing rats (21). The accumulation of Ub-protein conjugates that has been reported in fasting, denervation atrophy (38), and cancer (23, 49) also support this hypothesis.

During the chronic septic phase, we observed that most of the increased proteolysis appeared to be nonlysosomal Ca^{2+} independent. We are the first to make such observation in the chronic septic phase. This was similar to what we observed during the acute septic phase. In accordance with this observation, we found elevated mRNA levels for Ub and proteasome subunits, suggesting that the ATP-Ub-dependent proteolytic pathway played a major role in the chronic septic phase. By contrast with the acute period, however, 14-kD E2 mRNA levels were significantly enhanced in septic muscle. This suggests that the rate of substrate ubiquitylation may become rate limiting during prolonged muscle wasting. We also observed an increase in mRNA levels for cathepsin B and m-calpain during the chronic septic phase, suggesting that both the lysosomal and the Ca^{2+}-dependent proteolytic pathways may be activated. However, the increase in mRNA levels for cathepsin B and m-calpain that we observed may not necessarily imply an increase in lysosomal and/or Ca^{2+}-dependent proteolysis. The different proteolytic pathways can be regulated by several mechanisms, including inhibitors, compartmentalization of autophagy, or posttranslational modifications; further experiments should test these possibilities, since there are no data in the literature with which to compare.

Our data clearly support a minor role for lysosomal and Ca^{2+}-dependent proteinases in the chronic septic phase, since the in vitro inhibition of these pathways did not suppress the overall enhanced protein breakdown (Fig. 3), as in acute sepsis (5). Only the ATP-Ub-dependent proteolytic pathway accounts for a large part of the overall increase in muscle protein breakdown in many catabolic states (17–23) and is responsible for the degradation of long-lived proteins (50), including actin and myosin (5, 17, 18). A concomitant stimulation of the Ub-proteasome pathway with either the Ca^{2+}-dependent (21) or the lysosomal process (19, 23) or both (17, 20, 51, 52) has been observed in many muscle-wasting conditions. These lysosomal and Ca^{2+}-dependent proteinases may be qualitatively important for the degradation of specific but quantitatively minor muscle proteins. For example, calpains are involved in rapid removal of Z-disks (53, 54) and N2 lines (54), and degrade nebulin, titin, troponin, tropomyosin, but not myosin and actin (53), which are quantitatively important.

Finally, in the late septic phase, we found normalized mRNA levels for cathepsin B, m-calpain, Ub, 14-kD E2, and proteasome subunits. These changes clearly correlated with the in vitro suppression of enhanced protein breakdown. Similarly, upon refeeding, total proteolysis and mRNA levels for Ub and proteasome subunits (48) and the 14-kD E2 (32) returned to control levels in skeletal muscles from fasted rats.

In summary, in a long-lasting rat septic model, muscle wasting mainly resulted from a sustained increase in protein breakdown, despite the recovery in food intake. Only the rise and fall of mRNA levels for Ub and proteasome subunits paralleled the measured rates of protein breakdown throughout the different phases of sepsis (Fig. 2). These data clearly support a major role for the Ub-proteasome proteolytic pathway in muscle wasting in sepsis, as recently demonstrated in various catabolic states (17–23, 25, 38, 48, 49, 52). However, a concomitant activation of the lysosomal, Ca^{2+}-dependent and ATP-Ub-dependent proteolytic systems seems to occur during the chronic septic phase, and all proteolytic processes were inactivated during the subsequent late septic phase. It is therefore clear that a primary goal in sepsis should be to try to reduce muscle wasting by inhibiting the ATP-Ub-dependent proteolytic process. One possible strategy would be to limit the deleterious effects of cytokines. Indeed, TNF and IL-1, which may signal increased protein breakdown in septic skeletal muscle (55, 56), were reported to alter skeletal muscle ATP-Ub-dependent protein breakdown when administered in vivo (40, 57). Similarly, IL-6, another candidate for enhanced muscle protein breakdown (58), was recently shown to increase the expression of proteasome subunits (C2, C8, S4) and of cathepsins B and L in cultured myotubes (59). Thus, further experiments should test whether or not manipulating cytokine levels with antibodies or receptor antagonist may limit increased ATP-Ub-dependent proteolysis in sepsis and reduce muscle wasting.

Acknowledgments

We would like to thank Dr. Keiji Tanaka (Institute for Enzyme Research, Tokushima, Japan) for the gift of the plasmids encoding the rat proteasome subunits, Dr. Simon S. Wing (McGill University, Montréal, Canada) for providing us with the cDNA of the rat 14-kD E2, and Dr. Susan S. Samuels for helpful discussions.

This study was supported by Clintec Technologies, the French Ministère de l'Enseignement Supérieur et de la Recherche, and the Institut National de la Recherche Agronomique.

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