Energy-Ubiquitin–dependent Muscle Proteolysis during Sepsis in Rats Is Regulated by Glucocorticoids

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Abstract

Recent studies suggest that sepsis-induced increase in muscle proteolysis mainly reflects energy-ubiquitin–dependent protein breakdown. We tested the hypothesis that glucocorticoids activate the energy-ubiquitin–dependent proteolytic pathway in skeletal muscle during sepsis. Rats underwent induction of sepsis by cecal ligation and puncture or were sham-operated and muscle protein breakdown rates were measured 16 h later. The glucocorticoid receptor antagonist RU 38486 or vehicle was administered to groups of septic and sham-operated rats. In other experiments, dexamethasone (2.5 or 10 mg/kg) was injected subcutaneously in normal rats. Total and myofibrillar proteolysis was determined in incubated extensor digitorum longus muscles as release of tyrosine and 3-methylhistidine, respectively. Energy-dependent proteolysis was determined in incubated muscles depleted of energy with 2-deoxyglucose and 2,4-dinitrophenol. Levels of muscle ubiquitin mRNA and free and conjugated ubiquitin were determined by Northern and Western blot, respectively. RU 38486 inhibited the sepsis-induced increase in total and myofibrillar energy-dependent protein breakdown rates and blunted the increase in ubiquitin mRNA levels and free ubiquitin. Some, but not all, sepsis-induced changes in ubiquitin protein conjugates were inhibited by RU 38486. Injection of dexamethasone in normal rats increased energy-dependent proteolysis and ubiquitin mRNA levels. The results suggest that glucocorticoids regulate the energy-ubiquitin–dependent proteolytic pathway in skeletal muscle during sepsis. (*J. Clin. Invest.* 1996. 97:339–348.) Key words: ubiquitin • protein breakdown • myofibrillar proteins • muscle catabolism • 3-methylhistidine

Introduction

One of the most prominent metabolic consequences of sepsis and severe injury is muscle catabolism, resulting in muscle fatigue, muscle wasting, and whole body protein loss (1). The catabolic response in skeletal muscle during sepsis is caused mainly by increased protein breakdown, in particular myofibrillar protein breakdown (2), although reduced protein synthesis and inhibited amino acid uptake may contribute to muscle catabolism in this condition (3, 4).

Muscle catabolism during sepsis has several important clinical implications. Muscle breakdown results in release of amino acids which are taken up by the liver for gluconeogenesis and acute phase protein synthesis (5). Amino acids, in particular glutamine, are also utilized at an increased rate by cells in the immune system (6) and by enterocytes (7). Thus, the catabolic response in skeletal muscle may be beneficial to the organism, at least during the early phase of sepsis, by providing amino acids to essential tissues. In more severe and protracted sepsis, however, protein breakdown results in muscle wasting and fatigue which may significantly impair the recovery in septic patients. Of particular concern are the pulmonary complications that may result from breakdown of respiratory muscles. A better understanding of mediators and mechanisms involved in muscle protein breakdown is therefore important for the management of patients with sepsis and other catabolic conditions.

Intracellular proteins are degraded by different proteolytic mechanisms which can be divided into lysosomal and nonlysosomal pathways (for review see reference 1). Among the nonlysosomal proteolytic pathways, there are both energy-dependent and -independent mechanisms. Recent studies suggest that the energy-ubiquitin–dependent pathway plays an important role in the degradation of muscle proteins during various catabolic conditions. Ubiquitin is a 76–amino acid polypeptide that is conjugated to proteins targeted for degradation. The ubiquitin-protein conjugate is recognized by a 26S proteolytic complex which splits ubiquitin from the protein that is subsequently degraded. The ubiquitin mechanism of intracellular protein breakdown was reviewed elsewhere (8, 9).

We found recently that sepsis stimulates protein breakdown in skeletal muscle by a nonlysosomal energy-dependent proteolytic pathway, and because muscle levels of ubiquitin mRNA were also increased, the results were interpreted as indicating that sepsis-induced muscle protein breakdown is caused by upregulated activity of the energy-ubiquitin–dependent proteolytic pathway (10). The same proteolytic pathway has been implicated in muscle breakdown caused by denervation (11), fasting (12), acidosis (13), cancer (14), and burn injury (15).

Several previous reports suggest that glucocorticoids play an important role in the regulation of muscle proteolysis during sepsis. Plasma levels of glucocorticoids are elevated both in patients (16) and experimental animals with sepsis (17). Administration of cortisol in humans (18) or corticosterone or dexamethasone in rats (19, 20) resulted in increased muscle protein breakdown. Similar to sepsis, myofibrillar protein breakdown is particularly sensitive to glucocorticoids (20).

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Further evidence for a role of glucocorticoids in sepsis-induced muscle proteolysis was elicited in experiments in which treatment of septic rats with the glucocorticoid receptor antagonist RU 38486 significantly blunted the increase in muscle protein breakdown (21). Because in those studies the different intracellular proteolytic pathways were not individually examined, it is not known whether the effect of the glucocorticoid receptor antagonist reflected inhibited activity of the energy-ubiquitin–dependent proteolytic pathway.

The purpose of this study was to test the hypothesis that glucocorticoids are involved in the regulation of energy-ubiquitin–dependent muscle proteolysis during sepsis. This was done by determining energy-dependent total and myofibrillar proteolytic rates and tissue levels of ubiquitin mRNA and ubiquitin-conjugated proteins in muscle from septic rats treated with RU 38486. In other experiments, the same measurements were performed after treatment of normal rats with dexamethasone. We found that treatment with RU 38486 inhibited the sepsis-induced increase in energy-dependent muscle protein breakdown rates and ubiquitin mRNA levels as well as changes in free ubiquitin and ubiquitinated proteins. Conversely, treatment of normal rats with dexamethasone stimulated energy-dependent muscle protein breakdown and resulted in increased ubiquitin mRNA levels. These observations support the hypothesis that glucocorticoids regulate, at least in part, the energy-ubiquitin–dependent proteolytic pathway in skeletal muscle during sepsis.

Methods

Experimental design. Sepsis was induced in male Sprague-Dawley rats (40–60 grams body weight [bw])[1] by cecal ligation and puncture (CLP) as described previously (2–5, 10). With rats under pentobarbital anesthesia (50 mg/kg administered intraperitoneally [i.p.]), the abdomen was opened through a midline incision. The cecum was ligated proximal to the ileocecal valve with a 3-0 silk ligature and was punctured twice with an 18-gauge needle. The abdomen was closed with a running 2-0 silk suture. Other rats were sham-operated, i.e., they underwent laparotomy and manipulation but no ligation or puncture of the cecum. All animals were resuscitated with saline (10 ml/100 grams bw) administered subcutaneously on the back at the time of surgery. Metabolic studies were performed 16 h after CLP or sham-operation. Rats had free access to water after the operative procedures but were fasted to avoid any influence of different food intake between septic and nonseptic rats on metabolic changes.

Rats that underwent either CLP or sham-operation were randomly divided into two different groups. One group was treated with 10 mg/kg of the glucocorticoid receptor antagonist RU 38486 (kindly provided by Roussel Uclaf, Romainville, France) administered by gavage 2 h before CLP or sham-operation. The drug was given as a suspension in an aqueous solution of 0.25% carboxymethylcellulose and 0.20% polysorbate (Sigma Chemical Co., St. Louis, MO). Control rats received a corresponding volume (0.5 ml/100 grams bw) of vehicle by gavage 2 h before CLP or sham-operation. RU 38486 is a potent glucocorticoid receptor antagonist with no agonist activity even at high concentrations (22). The drug has been used in previous studies to block sepsis- or dexamethasone-induced metabolic changes in skeletal muscle (21, 23).

Small rats were used in the present study because they possess lower extremity muscles that are thin enough to allow for in vitro incubation with maintained oxygenation and diffusion of substrates into the tissue (24). The septic model used here is clinically relevant since it resembles the situation in patients with sepsis caused by intraabdominal abscess and devitalized tissue. The model was characterized with respect to mortality rates and hemodynamic and metabolic changes in previous studies from our and other laboratories (25, 26). The time point of 16 h for metabolic studies was based on previous reports in which muscle proteolysis and ubiquitin mRNA levels were increased 16 h after CLP in rats (2, 10).

In a second series of experiments, male Sprague-Dawley rats (40–60 grams bw) were treated with dexamethasone dissolved in 1 ml/100 grams bw of PBS, pH 7.4, and administered subcutaneously at a dose of 2.5 or 10 mg/kg bw. Control rats received a corresponding volume of PBS subcutaneously. The rats were fasted but had free access to water after the treatment, and metabolic studies were performed after 16 h to make conditions similar to those in the septic rats. The doses of dexamethasone used here were chosen from previous studies in which they resulted in increased breakdown of muscle proteins (19, 23).

A third series of experiments was performed to test the specificity of the effects of RU 38486. Groups of rats were treated with 25 mg/kg of RU 38486 by gavage or a corresponding volume (0.5 ml/100 grams bw) of vehicle 2 h before the subcutaneous injection of 2.5 or 10 mg/kg bw of dexamethasone. Metabolic studies were performed 16 h after the injection of dexamethasone. A higher dose of RU 38486 was used in this series of experiments than in the first series of experiments because in pilot experiments the dose of 10 mg/kg (as used in the first series of experiments) did not affect the dexamethasone-induced metabolic changes. The higher dose of RU 38486 is consistent with the dosage used to block dexamethasone-induced metabolic changes reported in a previous study (23).

Total and myofibrillar protein breakdown in incubated muscles. 16 h after CLP or sham-operation (the first series of experiments) or 16 h after injection of dexamethasone or PBS (the second and third series of experiments), rats were anesthetized with pentobarbital (50 mg/kg i.p.), and the extensor digitorum longus muscles were gently dissected and excised with intact tendons. The muscles were mounted on stainless steel supports at resting length and immediately placed in 3 ml of oxygenated (O_2:CO_2 = 95:5) Krebs-Henseleit bicarbonate buffer (pH 7.4) with 5 mM glucose. The extensor digitorum longus muscle was used in this study because in previous reports this muscle, which is a white fast-twitch muscle, was particularly sensitive to the effects of sepsis (2). Muscles were incubated fixed at resting length, rather than flaccid, in order to better maintain their energy level and protein balance (27–29).

Muscles were preincubated in 3 ml of oxygenated Krebs-Henseleit bicarbonate buffer (pH 7.4) at 37°C in a shaking water bath for 30 min (except in the experiments in which muscles were energy depleted when preincubation was carried out for 90 min, see below). After preincubation, one muscle was homogenized in 0.4 M perchloric acid for determination of tissue levels of free tyrosine and 3-methylhistidine (3-MH) by HPLC as described previously (2, 28). The contralateral muscle was incubated for 2 h in fresh medium of the same composition as described above with the addition of 0.5 mM cycloheximide to prevent reincorporation of amino acids released during proteolysis. After incubation, muscle and medium concentrations of free tyrosine and 3-MH were measured. Total and myofibrillar protein breakdown rates were determined from the net release of tyrosine and 3-MH, respectively, taking changes in tissue levels of the amino acids during incubation into account as described in detail previously (2, 28). Because tyrosine is present in all proteins, its release reflects total protein breakdown. 3-MH is present only in actin and myosin, which is why its release reflects myofibrillar protein breakdown (30). This distinction is important because in previous studies we found that the myofibrillar proteins are particularly sensitive to the effect of sepsis (2). Total and myofibrillar protein breakdown rates are reported as nanomoles of tyrosine per gram of wet weight per 2 h and nanomoles of 3-MH per gram of wet weight per 2 h, respectively.

1. *Abbreviations used in this paper:* 2-DG, 2-deoxyglucose; 3-MH, 3-methylhistidine; bw, body weight; CLP, cecal ligation and puncture; GRE, glucocorticoid response element; LSB, low salt buffer; PRB, pyrophosphate relaxing buffer.

Energy-dependent proteolysis. To study the energy-dependent component of protein breakdown, muscles were depleted of intracellular ATP by 90 min of preincubation in medium containing 5 mM 2-deoxyglucose (2-DG) and 0.2 mM 2,4-dinitrophenol (DNP) (10, 31). Glucose was omitted from the incubation medium to which 2-DG and DNP were added. ATP-depleted muscles were then incubated for 2 h in the presence of 5 mM 2-DG and 0.2 mM DNP and compared with muscles preincubated and incubated in the presence of 5 mM glucose. All muscles were incubated in calcium-free medium containing insulin (1 mU/ml) and the branched-chain amino acids leucine, isoleucine, and valine present at concentrations five times those found in rat plasma. Insulin and the branched-chain amino acids were added to the incubation medium to block lysosomal protein breakdown. Therefore, in these experiments, changes induced by incubating muscles in the presence of 2-DG and DNP reflected nonlysosomal, calcium-independent, energy-dependent proteolysis. Total and myofibrillar protein breakdown rates were determined as described above. In previous studies, tissue levels of ATP were almost completely abolished when muscles were incubated with 2-DG and DNP as described here (31). In the same studies, muscles generated ATP and metabolized glucose after recovery in medium without inhibitors of energy metabolism, suggesting that the ATP-depleted muscles were not irreversibly damaged but were still viable.

Muscle ubiquitin mRNA levels. Muscle ubiquitin mRNA levels were determined by Northern blot analysis 16 h after CLP or sham-operation (in the first series of experiments) or injection of dexamethasone or PBS (in the second and third series of experiments). RNA was extracted from individual muscles as described previously (32). A rat ubiquitin cDNA probe was generated by PCR as described recently in a report from our laboratory (10). Muscle RNA (10 μg) was denatured in glyoxal and fractionated on a 1% agarose gel in 10 mM sodium phosphate, pH 7.0. The RNA was transferred to nylon membranes (Micron Separation Inc., Westboro, MA) by capillary action in 25 mM sodium phosphate (pH 6.4) overnight. RNA was covalently attached to the nylon membrane by ultraviolet light. The blot was prehybridized for 4 h in 50% formamide, 5× SSC (1× SSC = 0.15 M NaCl, 15 mM Na-citrate, pH 7.0), 50 mM sodium phosphate, pH 7.0, 1 mM EDTA, 2% SDS, 10× Denhardt's solution, and 10 μg/ml salmon sperm at 42°C. The probe was labeled by random priming (Stratagene, La Jolla, CA) using [α-^{32}P]ATP (New England Nuclear, Boston, MA). The blots were hybridized with 1×10^8 cpm of labeled probe overnight at 42°C in the same buffer that was used for prehybridization, except that sodium phosphate concentration was decreased to 20 mM and Denhardt's solution to normal concentration. The blots were washed three times in 1× SSC, 0.1% SDS at room temperature, once with 0.2× SSC, 0.1% SDS at 65°C, and autoradiographed for 24 h at −70°C. An 18S rat ribosomal oligonucleotide probe (GACAAGCATATGCTACTGGC) was used to control for equal loading of RNA. Autoradiographs of the blots were quantitated on a PhosphorImager using the Image Quant Program (Molecular Dynamics Inc., Sunnyvale, CA).

Free and conjugated ubiquitin in muscle tissue. Free and conjugated ubiquitin was determined both in the sarcoplasmic and myofibrillar protein pools. Sarcoplasmic and myofibrillar protein fractions were prepared from muscles of sham-operated and septic rats as described previously (33), with minor modifications. Muscles were homogenized in 20 vol of pyrophosphate relaxing buffer (PRB) consisting of 4 mM $Na_4P_2O_7$, 10 mM Tris maleate (pH 7.4), 100 mM KCl, 2 mM $MgCl_2$, 2 mM EGTA, 10 mM *N*-ethylmaleimide, 0.02% sodium azide, and 1 mM dithiothreitol. After centrifugation (800 *g* for 10 min at 4°C) of the homogenate, the supernatant was centrifuged at 100,000 *g* for 1 h at 4°C. The resulting supernatant constituted the sarcoplasmic proteins. The 800 *g* pellet was washed six times in 20 vol of low salt buffer (LSB) (pH 7.4) containing the same components as PRB but without 4 mM $Na_4P_2O_7$, 10 mM *N*-ethylmaleimide, and 0.02% sodium azide. The pellet was then washed once in LSB with 0.5% Triton X-100, once in LSB with 0.1% Na-deoxycholate, and four times in LSB. The resulting pellet, which constituted the myofibrillar proteins, was suspended in 20 vol of PRB. The amount of protein in the sarcoplasmic and myofibrillar fractions was determined according to Lowry et al. (34) using BSA as standard.

Western blots for the detection of free and conjugated ubiquitin were generated using aliquots of sarcoplasmic and myofibrillar protein fractions (10 μg/lane) electrophoresed (20 mA) on a 12.5% polyacrylamide gel (1.5 × 80 × 60 mm) in the presence of SDS. After SDS-PAGE, the gels were equilibrated in transfer buffer (20 mM Tris base, 144 mM glycine, 10% [vol/vol] methanol) for 30 min. Proteins were then transferred from the slab gel onto an Immobilon polyvinylidenedifluoride (PVDF) transfer membrane (Millipore, Bedford, MA) by electroelution (480 Vh) using a Trans blot cell (Bio-Rad, Hercules, CA). After heat fixing the membrane at 75°C for 30 min, the membrane was blocked using 5% milk in buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) for 2 h with constant shaking at room temperature. The membranes were incubated for 1.5 h in buffer A containing 1% milk, 0.05% Tween 20, and monospecific antibody against heat- and SDS-denatured ubiquitin raised in rabbits. The monospecific antibody to ubiquitin was purified by affinity chromatography on a column with ubiquitin covalently bound to Sepharose. Membranes were then washed in buffer A for 10 min followed by two 10-min washes in buffer A plus 0.05% Triton X-100 and again in buffer A for 10 min. The bound antibody was detected by a 1-h incubation in buffer A containing 1% milk, 0.05% Tween 20, and peroxidase-conjugated anti–rabbit IgG. The washes were repeated as described above. The membrane was then incubated for 1 min at room temperature in the presence of Renaissance chemiluminescence reagents (New England Nuclear). The membrane was blotted dry and exposed to Kodak X-OMAT AR film (Eastman Kodak, Rochester, NY). The intensities of the bands on the film were quantitated using an Ultrascan XL enhanced laser densitometer (Pharmacia Biotech Inc., Piscataway, NJ). A ubiquitin standard was loaded onto each gel and its signal was shown to be linear over the amounts tested (0–100 ng).

It should be noted that the procedure used here to measure free and conjugated ubiquitin differed somewhat from the method described earlier (10). By using a 12.5% polyacrylamide gel for the Western blots in the present experiments, rather than a 10% gel as in our previous report (10), it was possible to detect free ubiquitin on the gel. In our previous study, free ubiquitin was determined using dot blots that used a relatively narrow range of sensitivity. In addition, the ubiquitin antibody used in the present study was purified by affinity chromatography and the bound antibody was detected by using chemiluminescence reagents, rather than ^{125}I-protein A. These modifications further increased the sensitivity in our system. These technical modifications may explain some of the differences in the results that were noticed between the present and our previous (10) study (see below).

Statistics. Results are presented as means±SEM. ANOVA followed by Scheffe's test was used for statistical analysis and $P < 0.05$ was considered significant.

Results

The effect of RU 38486 on energy-dependent muscle proteolysis and ubiquitin mRNA levels in septic rats. In a previous study in our laboratory, pretreatment of septic rats with 5 mg/kg of RU 38486 significantly blunted, but did not normalize, muscle protein breakdown rates (21). In an effort to block sepsis-induced muscle proteolysis even further, a higher dose (10 mg/kg) of RU 38486 was used in this study. Administration of 10 mg/kg of RU 38486 2 h before induction of sepsis almost completely abolished the sepsis-induced increase in total (tyrosine release) and myofibrillar (3-MH release) protein breakdown (Fig. 1). This dose of the glucocorticoid receptor blocker was therefore used in subsequent experiments in this report. The almost doubled total protein breakdown rate and the fourfold

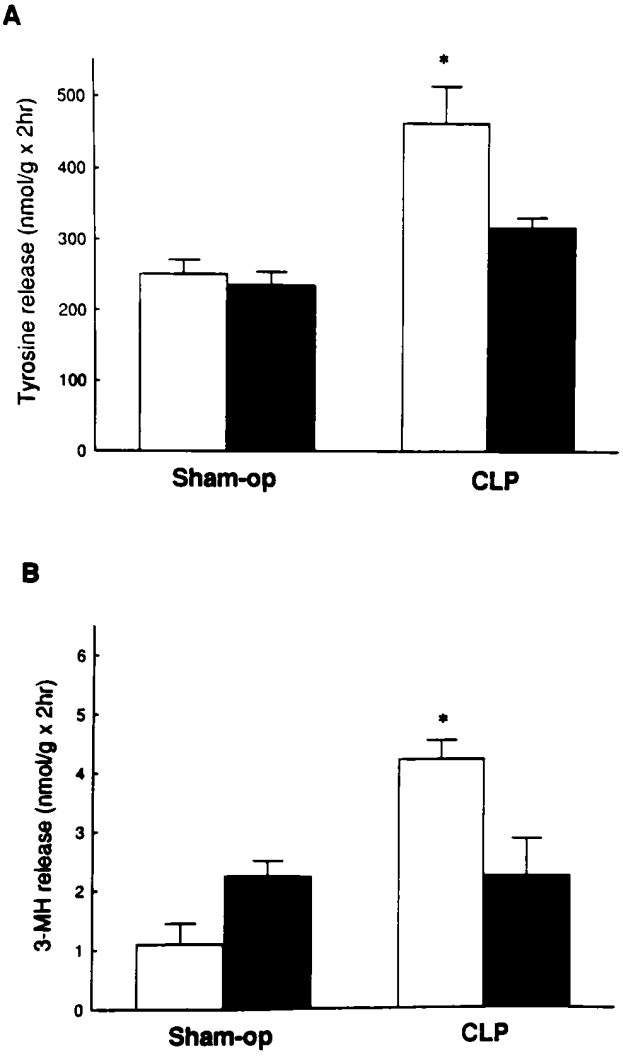

Figure 1. Total (*A*) and myofibrillar (*B*) protein breakdown rates in incubated extensor digitorum longus muscles from sham-operated and septic (CLP) rats. Groups of rats were pretreated by gavage with 10 mg/kg of RU 38486 (*filled bars*) or corresponding volume of vehicle (*open bars*) 2 h before sham-operation or CLP. $n = 6$ or 7 in each group. $^*P < 0.05$ vs. all other groups by ANOVA.

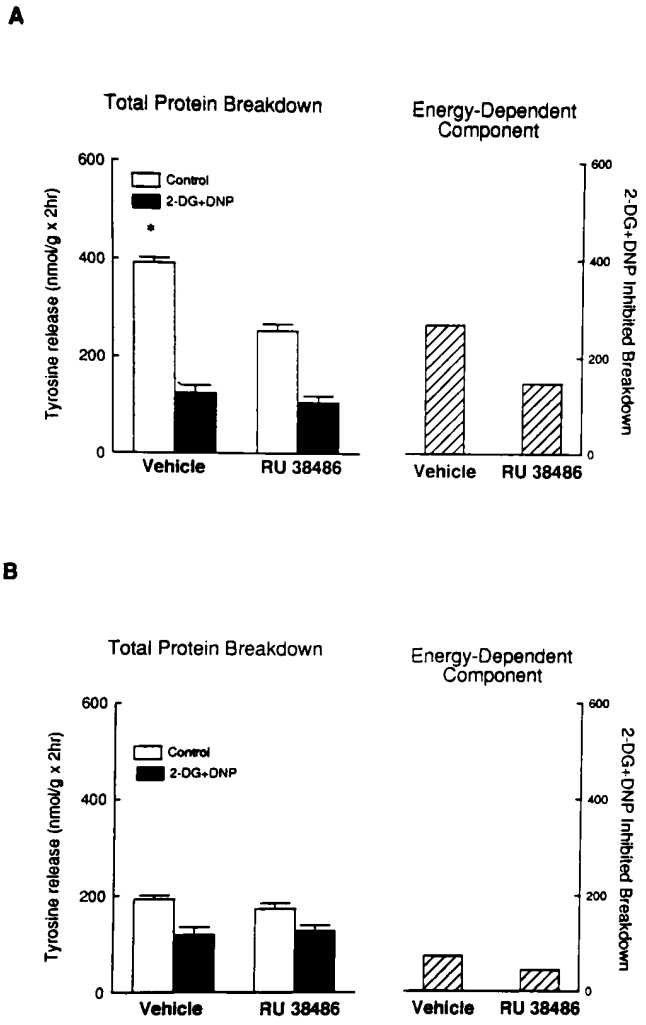

Figure 2. Total protein breakdown rates in incubated muscles from septic (*A*) and sham-operated (*B*) rats. Muscles from both groups of rats were incubated in control medium containing 5 mM glucose (*open bars*) or in medium containing 5 mM 2-DG (substituting for glucose) and 0.2 mM DNP (*filled bars*). The difference in protein breakdown rates between muscles incubated in control medium and medium containing 2-DG and DNP was calculated and represented the energy-dependent component of total protein breakdown (*hatched bars*). The energy-dependent component of total muscle protein breakdown was increased in septic rats (compare *A* and *B*) and was reduced by $\sim 50\%$ in RU 38486–treated septic rats (*A*). $n =$ 6 or 7 in each group. $^*P < 0.05$ vs. muscles incubated in control medium from RU 38486–treated septic rats.

increase in myofibrillar protein breakdown rate noted in vehicle-treated septic rats compared with vehicle-treated sham-operated rats (Fig. 1) are in line with several previous reports from our laboratory (2, 10, 21).

We next examined the effect of RU 38486 on the sepsis-induced increase in energy-dependent muscle proteolysis. Muscles were incubated under energy-providing or -depleting conditions (as described in Methods), and any difference in protein breakdown rates between these conditions was interpreted as being reflective of energy-dependent proteolysis. Calculated in this way, sepsis resulted in a substantial increase in energy-dependent total protein breakdown; compare the energy-dependent component in vehicle-treated septic (*A*) and sham-operated (*B*) rats in Fig. 2. An even more pronounced increase in the energy-dependent component of myofibrillar protein breakdown was noted in septic rats (Fig. 3). It should be emphasized that the energy-dependent component of total and myofibrillar protein breakdown was calculated as the difference in protein breakdown rates between muscles incubated in control medium and in medium containing 2-DG and DNP. Muscles from different groups of rats (rather than paired muscles) were used for the different incubation conditions since both extensor digitorum longus muscles from each rat were required to make it possible to measure changes in tissue levels of tyrosine and 3-MH during incubation (see Methods). This explains why no standard errors are given for the energy-dependent component of total and myofibrillar protein breakdown in Figs. 2 and 3 since the calculated energy-dependent component was the difference between two mean values. The increase in energy-dependent protein breakdown noted here confirms a recent study from our laboratory in which we

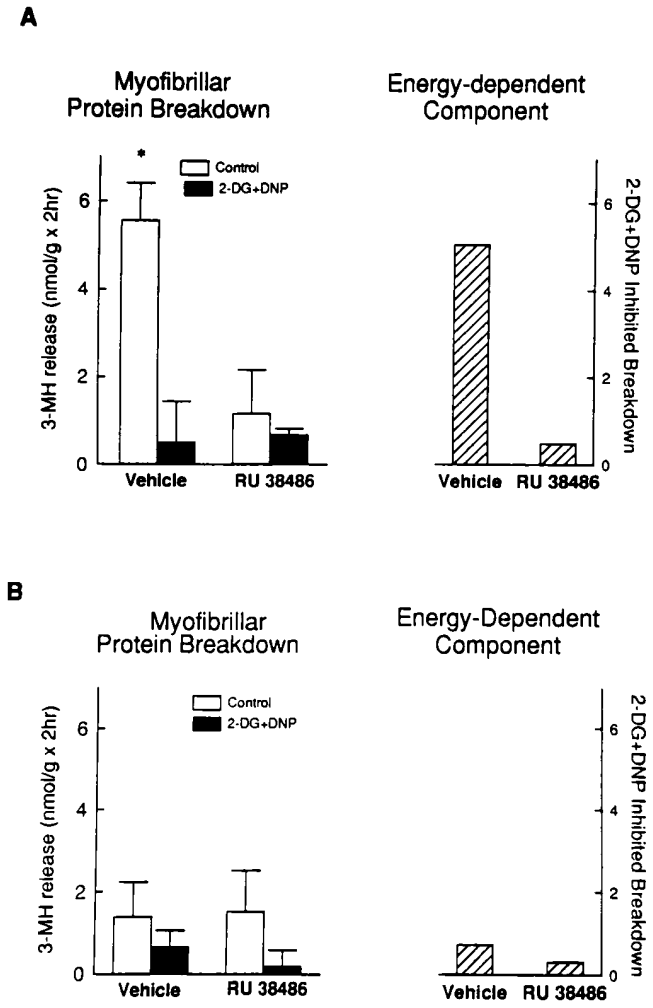

Figure 3. Myofibrillar protein breakdown rates in incubated muscles from septic (*A*) and sham-operated (*B*) rats. Experimental conditions and symbols were identical to those in Fig. 2. Note the substantial increase in the energy-dependent component of myofibrillar protein breakdown in muscles from septic rats (compare *A* and *B*) and the inhibition of the energy-dependent myofibrillar protein breakdown in RU 38486–treated septic rats (*A*).

found that sepsis specifically stimulated energy-dependent total and myofibrillar protein breakdown without increasing lysosomal or calcium-dependent muscle proteolysis (10). When rats were pretreated with 10 mg/kg of the glucocorticoid receptor antagonist RU 38486, the sepsis-induced increase in energy-dependent total protein breakdown was reduced by $\sim$ 50% (Fig. 2) and the increase in the energy-dependent myofibrillar protein breakdown was almost completely abolished (Fig. 3). These results support the concept that glucocorticoids regulate energy-dependent muscle proteolysis during sepsis. RU 38486 did not influence energy-dependent muscle proteolysis in sham-operated rats.

The most important energy-dependent proteolytic pathway in skeletal muscle is the ubiquitin-dependent mechanism (8, 9). There is evidence that ubiquitin is encoded by a multigene family. In humans, the genes have been labeled ubiquitin A, ubiquitin B, and ubiquitin C with mRNA lengths of $\sim$ 0.6, 1.1, and 2.5 kb, respectively (35). The rat cDNA homologues of ubiquitin B and ubiquitin C were reported recently (36). Two different sizes of ubiquitin C mRNA were identified, a 2.8- and a 3.2-kb signal, and individual rats expressed different patterns of the two signals by Northern blot analysis (36). The different patterns of expression were attributed to mRNA polymorphism which was reported previously in the human ubiquitin C gene family (37).

In this study, muscle levels of the ubiquitin C mRNA were increased 16 h after induction of sepsis (Fig. 4), confirming a recent study from our laboratory (10). The ubiquitin C mRNA levels were quantitated by phospho-imaging analysis in three consecutive experiments and showed an approximately ninefold increase in muscles from septic rats compared with muscles from sham-operated rats. Ubiquitin B mRNA levels were not affected by sepsis (data not shown). No ubiquitin A message was detected in muscles from sham-operated or septic rats. These results suggest that the different ubiquitin genes are individually regulated during sepsis.

It should be noted that at the time of our previous study (10), the sizes of the rat ubiquitin B and C genes had not been reported. A marker of 2.4 kb was used to identify the size of the signal in those experiments, and the Northern blot indicated ubiquitin mRNA to be $\sim$ 2.4 kb. In retrospect, however, the signal in those experiments most likely represented a size of 2.8 kb. Indeed, the Northern blots in the present study show that the ubiquitin C mRNA we detected was bigger than the 2.4 kb marker and corresponded to the recently reported size of 2.8 kb.

Pretreatment of rats with RU 38486 abolished the sepsis-induced increase in ubiquitin C mRNA levels and also reduced ubiquitin C mRNA levels in muscle from sham-operated rats in some of the experiments (Fig. 4). The reduction of ubiquitin C mRNA in sham-operated rats pretreated with RU 38486 probably reflects a certain degree of stimulation of the ubiquitin-dependent proteolytic pathway induced by the laparotomy and the 16-h period of fasting.

Free and conjugated muscle ubiquitin during sepsis and after treatment with RU 38486. The sarcoplasmic and myofibrillar proteins were fractionated based on their solubility from muscles 16 h after sham-operation or CLP in groups of rats pretreated with vehicle or RU 38486. As expected, free ubiquitin (molecular mass $\sim$ 8.5 kD) was present only in the sarcoplasmic fraction (Fig. 5). Densitometry of the autoradiograms revealed a 48% increase in free ubiquitin in muscle from septic rats pretreated with vehicle compared with vehicle-treated sham-operated rats. Pretreatment of rats with RU 38486 decreased the sepsis-induced increase in free ubiquitin by 21% but had little effect on ubiquitin levels in muscles from sham-operated rats. Of note was the 2.2-fold increase in ubiquitination of the highest molecular mass (> 200 kD) sarcoplasmic protein observed in muscles from septic rats compared with controls. Pretreatment of the septic rats with RU 38486 decreased the ubiquitination of this protein by 29%.

Several proteins in the myofibrillar fraction were conjugated to ubiquitin (Fig. 5). A comparison between sham-operated and septic rats showed that two proteins in particular, a 21- and a 200-kD protein, had different levels of ubiquitination. The 200-kD protein contained 55% more ubiquitin in muscles from septic rats than in muscles from sham-operated rats. Pretreatment of septic rats with RU 38486 decreased by 19% the amount of ubiquitin conjugated to the 200-kD myofibrillar protein. In contrast, RU 38486 appeared to have no ef-

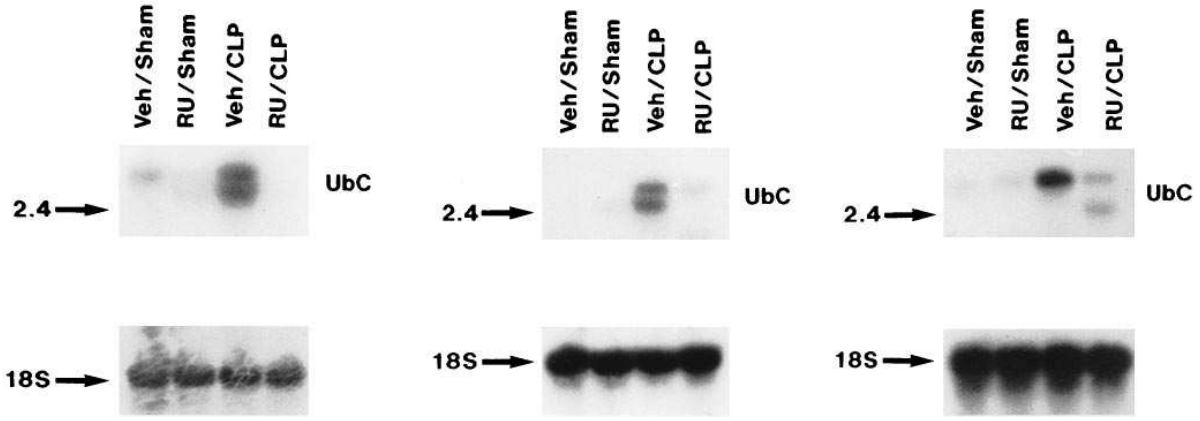

Figure 4. Ubiquitin C mRNA levels in extensor digitorum longus muscles from sham-operated and septic (CLP) rats pretreated with 10 mg/kg of RU 38486 or corresponding volume of vehicle. The Northern blots were generated with a rat ubiquitin cDNA probe. The blots were stripped and rehybridized with a rat 18S oligonucleotide probe to control for equal loading of RNA between the lanes. The three different blots represent three separate experiments. The increase in ubiquitin mRNA levels noted in muscles from vehicle-treated septic rats was blocked after treatment with RU 38486 (*RU/CLP*).

fect on the 21-kD protein, which contained 46% more ubiquitin in sham-operated than in septic rats.

It should be noted that in addition to a different degree of ubiquitination, changes in the intensity of the different bands shown in Fig. 5 may also be interpreted as indicating changes in breakdown of individual proteins. For example, a pronounced reduction of a band, as noted in septic muscle for the 21-kD myofibrillar protein fraction, may reflect enhanced proteolysis of that particular protein.

The effect of dexamethasone on energy-dependent muscle proteolysis and ubiquitin mRNA levels. To further test the role of glucocorticoids in the regulation of energy-ubiquitin–dependent muscle proteolysis, normal rats were treated with dexamethasone. In initial experiments, rats were treated with 2.5 or 10 mg/kg of dexamethasone, doses which have been shown in previous studies to stimulate muscle proteolysis (19, 23). Because the higher dose resulted in both increased total and myofibrillar protein breakdown (Fig. 6), this dose was used to assess the role of dexamethasone in energy-dependent muscle proteolysis. Injection of 10 mg/kg of dexamethasone resulted 16 h later in a doubling of the energy-dependent component of total protein breakdown and an approximately fourfold increase in energy-dependent myofibrillar protein breakdown (Fig. 7).

We next examined whether the dexamethasone-induced increase in energy-dependent muscle proteolysis was associated with increased ubiquitin mRNA levels. Muscle levels of ubiquitin C mRNA were increased after treatment with dexamethasone (Fig. 8). Finally, we tested the specificity of RU 38486 by administering the drug to rats treated with dexamethasone. RU 38486 pretreatment completely abolished the 2.5 mg/kg and reduced the 10 mg/kg dexamethasone-induced

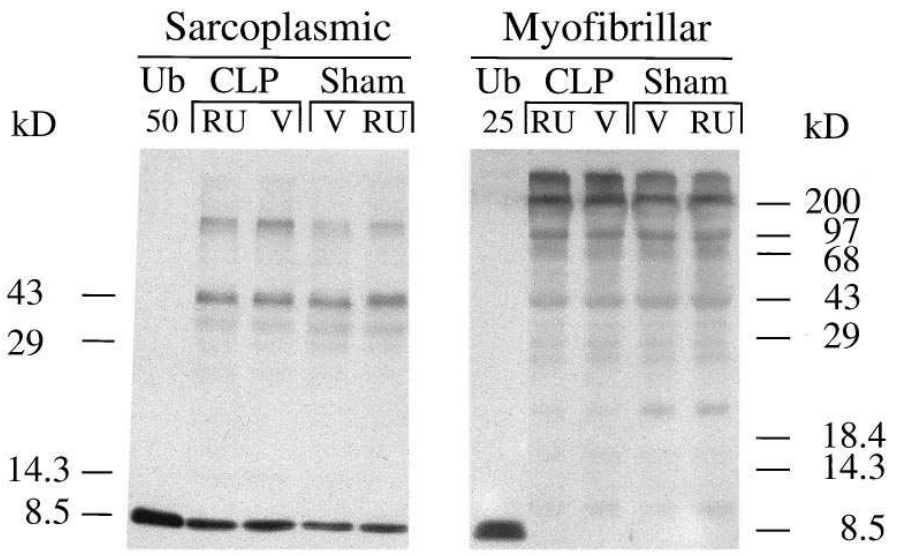

Figure 5. Western blot analysis of free and conjugated ubiquitin in the sarcoplasmic and myofibrillar protein pools of muscles from sham-operated and septic (CLP) rats. Rats were pretreated by gavage with 10 mg/kg of RU 38486 (*RU*) or corresponding volume of vehicle (*V*). 10-μg aliquots of either sarcoplasmic or myofibrillar proteins were loaded per lane on 12.5% SDS-polyacrylamide gels. Purified ubiquitin (50 and 25 ng, respectively) was loaded onto the sarcoplasmic and myofibrillar gels to make it possible to identify the position of free ubiquitin. Proteins were transferred to PVDF Immobilon membranes. The ubiquitinated proteins were detected by using affinity-purified ubiquitin antibody and chemiluminescence reagents and visualized by autoradiography. The intensity of the individual bands was quantitated with a laser densitometer. The positions of different proteins with known molecular weights are indicated on the left and right sides of the gels. The blots represent one muscle per lane. Note that the order between V and RU is not the same under CLP and sham. The results shown in the figure reflect the actual sequence of loading onto the gels shown in this figure.

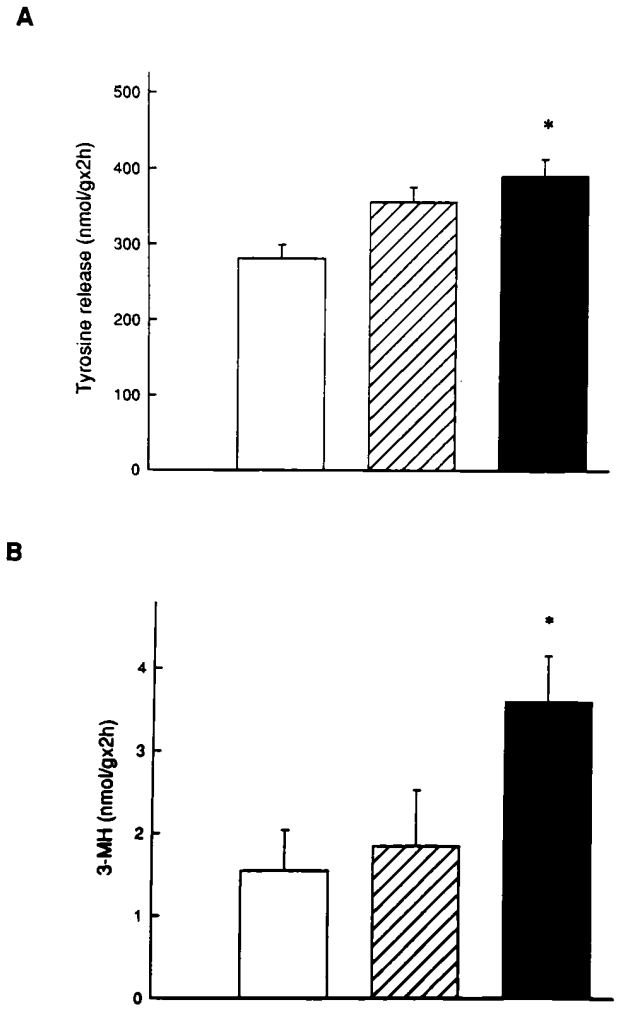

Figure 6. Total (*A*) and myofibrillar (*B*) protein breakdown rates in incubated extensor digitorum longus muscles harvested from rats 16 h after the subcutaneous injection of PBS (control, *open bars*) or dexamethasone at a dose of 2.5 (*hatched bars*) or 10 mg/kg (*filled bars*). $n = 6$ or 7 per group. $^*P < 0.05$ vs. control by ANOVA.

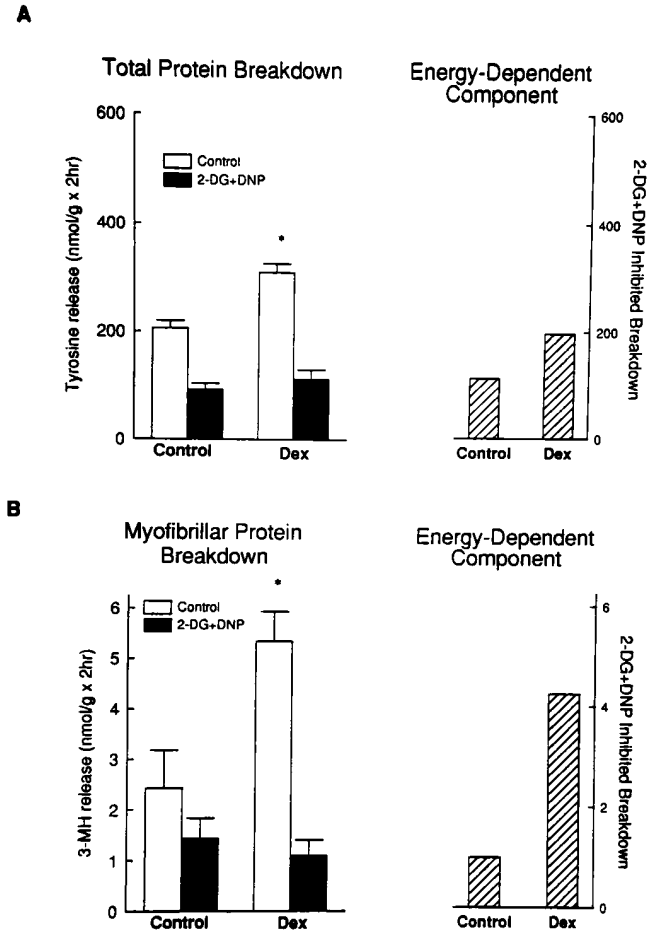

Figure 7. Total (*A*) and myofibrillar (*B*) protein breakdown rates in incubated extensor digitorum longus muscles harvested from rats 16 h after the subcutaneous injection of PBS (*Control*) or 10 mg/kg of dexamethasone (*Dex*). The in vitro conditions and symbols were identical to those in Fig. 2. The energy-dependent component (*hatched bars*) of both total and myofibrillar protein breakdown was increased in muscles from dexamethasone-treated rats. $n = 6$ or 7 in each group. $^*P < 0.05$ vs. muscles from control injected rats.

increase in ubiquitin C mRNA (Fig. 9), suggesting that the effect of RU 38486 on the energy-ubiquitin–dependent proteolytic pathway is glucocorticoid receptor mediated.

Discussion

In this study, treatment of rats with the glucocorticoid receptor antagonist RU 38486 blocked the sepsis-induced increase in energy-dependent muscle proteolysis and ubiquitin C mRNA levels and reduced the concentration of free ubiquitin and certain ubiquitin-protein conjugates. The data strongly support the hypothesis that glucocorticoids are involved in the regulation of the energy-ubiquitin–dependent muscle proteolysis during sepsis. The results are in line with previous reports suggesting that glucocorticoids may regulate ubiquitin-dependent muscle proteolysis during fasting and acidosis (12, 38). Further support for the concept that glucocorticoids regulate the ubiquitin mechanism of muscle proteolysis was obtained in this study when treatment of normal rats with dexamethasone resulted in increased energy-dependent total and myofibrillar protein breakdown rates as well as increased muscle levels of ubiquitin C mRNA.

Increased muscle protein breakdown after treatment of rats with dexamethasone, as observed here, is similar to previous reports in which glucocorticoid treatment of both humans (18) and experimental animals (19, 20, 23) stimulated muscle proteolysis. Similar to a report by Kayali et al. (20), our results suggest that myofibrillar proteins are particularly sensitive to the effect of glucocorticoids. The present study added to previous observations by providing evidence that the increased muscle protein breakdown after treatment with glucocorticoids may reflect stimulated activity in the energy-ubiquitin–dependent pathway.

In this study, skeletal muscle ubiquitin C mRNA levels increased in normal rats after the administration of dexamethasone. In two recent reports, the effect of dexamethasone on muscle ubiquitin mRNA levels was studied in adrenalectomized rats during starvation (12) and metabolic acidosis (38). In one of the studies (12), administration of dexamethasone increased muscle levels of ubiquitin mRNA, whereas in the

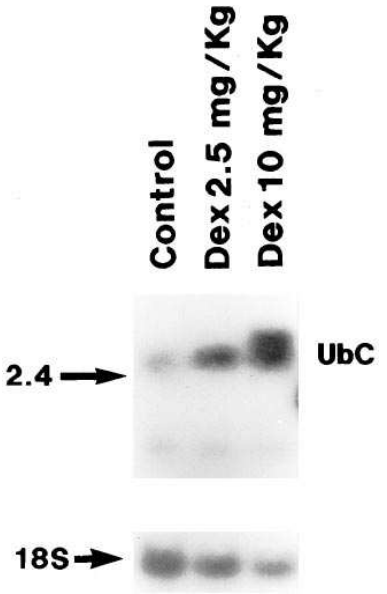

Figure 8. Ubiquitin C mRNA levels in muscles harvested from rats 16 h after the subcutaneous injection of PBS (*Control*) or dexamethasone (*Dex*) at a dose of 2.5 or 10 mg/kg. Northern blots were generated by using a rat ubiquitin cDNA probe. The blots were stripped and rehybridized with an 18S oligonucleotide probe as a loading control. The 18S bands indicate that the Dex 10 mg/kg lane was probably loaded with less RNA than the other lanes. Thus, the apparent increase in ubiquitin C mRNA in this lane was probably underestimated. A similar dose-dependent increase in muscle ubiquitin C mRNA levels as observed in this figure was noted in three separate experiments.

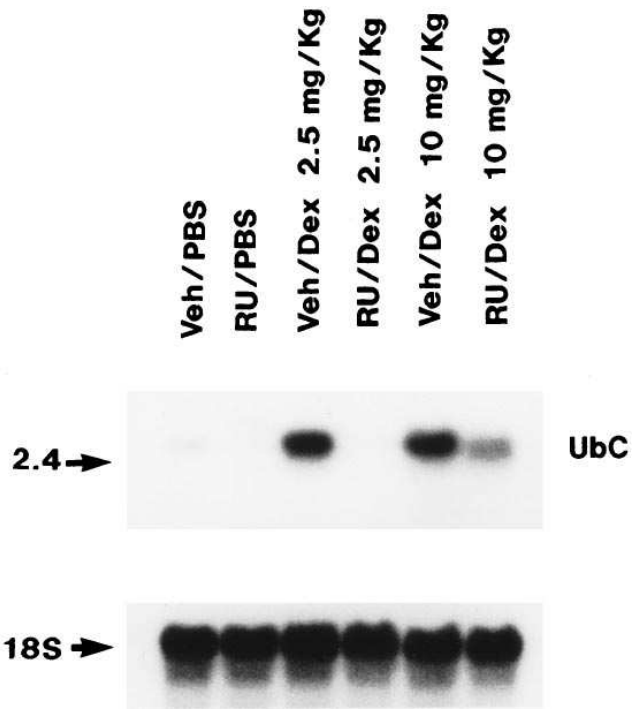

Figure 9. Ubiquitin C mRNA levels in muscles harvested from rats 16 h after the subcutaneous injection of PBS or dexamethasone (*Dex*) at a dose of 2.5 or 10 mg/kg. Rats were treated by gavage with 25 mg/kg of RU 38486 (*RU*) or vehicle (*Veh*) 2 h before the injection of PBS or dexamethasone. RU 38486 abolished the increase in ubiquitin C mRNA induced by 2.5 mg/kg of dexamethasone and substantially reduced the increase induced by 10 mg/kg of dexamethasone.

other study (38), dexamethasone alone did not upregulate the message levels. However, dexamethasone was necessary in that study in order for metabolic acidosis to stimulate an increase in ubiquitin mRNA levels. In neither of those studies was the effect of glucocorticoids tested in normal rats. Despite the different experimental models in the two previous studies (12, 38) and in our current report, the results strongly indicate that glucocorticoids play an important role in mediating changes in ubiquitin mRNA expression.

In this study, a 48% increase in free ubiquitin was noted in muscle from septic rats, whereas in our previous report (10) the concentration of free ubiquitin was not different between septic and nonseptic muscles. One reason for this difference may be varying metabolic response to sepsis in individual rats. However, a more important explanation is probably the differences in techniques between the two studies. The technical modifications of the methods used in this study to detect free and protein-conjugated ubiquitin (described in Methods) increased the sensitivity of the assay. The technical modifications may also explain why ubiquitination of individual proteins in the sarcoplasmic and myofibrillar fractions was apparently different between the two studies. It should be noted, however, that although the effect of sepsis on the ubiquitination of individual proteins was not identical between the two reports, both studies showed that sepsis affects individual proteins differently, with some proteins being ubiquitinated at an increased rate and other proteins at a decreased rate. Thus, the regulation of the ubiquitin pathway is probably complex, not merely involving an upregulation of the overall activity and expression of the pathway, but targeting specific proteins for degradation during sepsis.

The changes in muscle levels of free ubiquitin during sepsis and after treatment with RU 38486 were less dramatic than the changes in ubiquitin C mRNA levels. Unchanged free ubiquitin levels, as observed in our recent study (10), or only small changes, as observed here, do not rule out an increased activity of the energy-ubiquitin–dependent pathway and do not necessarily contradict the finding of increased ubiquitin C mRNA levels. It is possible, for example, that only small changes in free ubiquitin levels, despite increased production of ubiquitin (as suggested by increased mRNA levels), reflect a stimulated turnover of ubiquitin during sepsis with accelerated synthesis and degradation of ubiquitin. Increased conjugation of ubiquitin to proteins may also explain why free ubiquitin levels were not increased to a greater extent. Another possible explanation for the finding may be that the translation of ubiquitin mRNA is not increased during sepsis.

It should be noted that although RU 38486 is a potent glucocorticoid receptor antagonist, the drug is not completely specific. Thus, in addition to blocking the glucocorticoid receptor, it also blocks the progesterone receptor (22). Because previous studies suggest that skeletal muscle does not contain progesterone receptors (23), it is not likely that the effects of RU 38486 noted in the current study were caused by blockade of progesterone receptors. In addition, recent studies suggest that RU 38486 may act as an antioxidant (39). This may be of particular importance for interpretation of the present results since oxygen free radicals are generated during sepsis (40) and oxidatively damaged proteins are degraded at an increased rate (41). However, the results in the present study of increased energy-ubiquitin–dependent protein breakdown after treatment of rats with dexamethasone and inhibition of this effect by RU 38486 support the interpretation that the effects of RU 38486 in septic rats were caused, at least in part, by blockade of the glucocorticoid receptor.

The mechanism by which glucocorticoids activate the ubiquitin pathway is not known from the present study. It is well known that glucocorticoids can activate gene transcription by binding to cytosolic glucocorticoid receptors and forming a complex which translocates to the nucleus. In the nucleus, this complex acts as a transcriptional activator by binding to a glucocorticoid response element (GRE) in the promoter of a target gene. Although the region directly upstream to the promoter of ubiquitin C in humans does not contain a GRE (35), enhancers can function from great distances. It is possible that further upstream sequencing of the human promoter will demonstrate the presence of a GRE. Promoters also vary from species to species. The ubiquitin promoter region of the nematode *Caenorhabditis elegans* was shown to contain a steroid response element (42). Currently, no information is available regarding the rat ubiquitin C promoter.

The results in this study are important from a clinical standpoint because muscle catabolism is a significant metabolic response in septic patients (1, 43). Although most previous reports of increased energy-ubiquitin–dependent muscle proteolysis during various catabolic conditions were based on animal experiments, we recently found evidence of increased expression of the ubiquitin gene in muscle from septic patients (our unpublished observation), suggesting that this proteolytic pathway is important for muscle catabolism in humans as well. A better understanding of the regulation of energy-ubiquitin–dependent muscle proteolysis may become important in the future for the metabolic management of patients with sepsis and perhaps other catabolic conditions as well.

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