Sepsis Is Associated With Increased mRNAs of the Ubiquitin-Proteasome Proteolytic Pathway in Human Skeletal Muscle

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Abstract

Previous studies provided evidence that sepsis-induced muscle proteolysis in experimental animals is caused by increased ubiquitin-proteasome-dependent protein breakdown. It is not known if a similar mechanism accounts for muscle proteolysis in patients with sepsis. We determined mRNA levels for ubiquitin and the 20 S proteasome subunit HC3 by Northern blot analysis in muscle tissue from septic ($n = 7$) and non-septic ($n = 11$) patients. Plasma and muscle amino acid concentrations and concentrations in urine of 3-methylhistidine (3-MH), creatinine, and cortisol were measured at the time of surgery to assess the catabolic state of the patients. A three- to fourfold increase in mRNA levels for ubiquitin and HC3 was noted in muscle tissue from the septic patients concomitant with increased muscle levels of phenylalanine and 3-MH and reduced levels of glutamine. Total plasma amino acids were decreased by $\sim$ 30% in the septic patients. The 3-MH/creatinine ratio in urine was almost doubled in septic patients. The cortisol levels in urine were higher in septic than in control patients but this difference did not reach statistical significance. The results suggest that sepsis is associated with increased mRNAs of the ubiquitin-proteasome pathway in human skeletal muscle. (*J. Clin. Invest.* 1997. 99:163–168.) Key words: sepsis • ubiquitin • proteasome • muscle • proteolysis • human

Introduction

Muscle catabolism, resulting in muscle wasting and fatigue, is a characteristic metabolic response to sepsis. Sepsis-induced muscle catabolism is mainly caused by increased protein breakdown, in particular myofibrillar protein breakdown, although reduced protein synthesis and inhibited amino acid transport contribute to the metabolic response (1). Muscle breakdown may impair the recovery in septic patients and increase the risk for pulmonary and thrombo-embolic complications when respiratory muscles and ambulation are affected. An increased knowledge of the mechanisms involved in the regulation of muscle proteolysis during sepsis is therefore of great clinical significance and may be essential for the development of future treatment modalities in patients with sepsis.

Intracellular protein degradation is regulated by different proteolytic pathways, including the lysosomal pathway and the non-lysosomal, energy-ubiquitin-dependent proteolytic pathway (2). In recent studies, we found evidence that during sepsis in rats, muscle protein breakdown is mainly caused by upregulated activity in the energy-ubiquitin-dependent proteolytic pathway (3). In this mechanism, proteins that are to be degraded are first conjugated to multiple molecules of ubiquitin, a highly conserved 76–amino acid residue (4). Ubiquitin-conjugated proteins are recognized and degraded by the 20 S proteasome, the catalytic core of the larger 26 S proteolytic complex. Increased ubiquitin mRNA levels in muscles of septic rats suggest that this proteolytic mechanism is regulated at the transcriptional level during sepsis (3).

Previous studies implying a role of the ubiquitin-proteasome pathway in muscle proteolysis during sepsis (3), as well as several other catabolic conditions (5–8), were performed in experimental animals. It is not known if muscle protein breakdown is regulated by similar mechanisms in patients with sepsis. The present study, therefore, was undertaken to test the hypothesis that the ubiquitin- proteasome pathway is upregulated in skeletal muscle of septic patients. The expression of ubiquitin and one of the 20 S proteasome subunits, HC3, was examined in muscle tissue of non-septic and septic patients. There was a three- to fourfold increase in mRNA levels for ubiquitin and HC3, concomitant with increased tissue levels of phenylalanine and 3-methylhistidine (3-MH).[1] The results are consistent with the concept that muscle protein breakdown in patients with sepsis is associated with upregulated activity in the ubiquitin-proteasome pathway.

Methods

Two groups of consecutive patients undergoing abdominal surgery were included in the study. One group ($n = 7$) consisted of patients with sepsis based on the following criteria: (*a*) clinical evidence of infection; (*b*) fever (body temperature > 38°C) or hypothermia (body temperature ≤ 36°C); (*c*) tachycardia (heart rate > 90/min); (*d*) tachypnea (respiration rate > 20/min) or patient on ventilator; (*e*) positive blood culture was not required. These criteria were based on a report by Bone (9). The other group ($n = 11$) consisted of patients undergoing elective laparotomy for various non-septic conditions, such as peptic ulcer disease, ventral hernia, diverticulosis, and vascu-

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Received for publication 1 July 1996 and accepted in revised form 7 November 1996.

J. Clin. Invest.

0021-9738/97/01/163/06 $2.00
Volume 99, Number 2, January 1997, 163–168

1. *Abbreviation used in this paper*: 3-MH, 3-methylhistidine.

lar disease. Patients with cancer were not included since malignancy is associated with muscle catabolism (6). None of the patients were treated with sympathomimetic drugs, glucocorticoids, or any other drugs known to induce muscle catabolism at the time of surgery. A written consent was obtained from the patient or a family member. The study was approved by the Institutional Review Board at the University of Cincinnati.

Protocol. A blood sample was obtained from an antecubital vein immediately before surgery for determination of plasma amino acids. Plasma amino acids were measured to assess the overall metabolic response in the septic patients and to get an indirect measure of the catabolic state in muscle. Previous studies have shown that typical changes in plasma amino acids occur in septic patients and that some of these changes may reflect muscle catabolism (10–13).

To further assess the clinical condition, APACHE II score was calculated for each patient immediately before surgery. The APACHE II score is a commonly used grading system to assess the severity of the disease (14).

Immediately before surgery was started, a 10-ml urine sample was obtained through the Foley catheter that was routinely placed before operation. The urine sample was immediately frozen at −70°C for subsequent determination of 3-MH, creatinine, and cortisol. Because most of the septic patients were admitted to the hospital and taken to the operating room on an emergency basis and because all control patients were admitted to the hospital on the day of surgery, it was not possible to collect urine during the 24 h preceding surgery in most patients. We therefore choose to make the measurements described above on urine samples obtained at the time of surgery. The 3-MH/creatinine ratio in urine was calculated because this ratio has been suggested to be a useful index of the fractional rate of muscle protein breakdown in injured and septic patients (15). Cortisol concentration in urine was determined because sepsis is associated with high levels of cortisol and because there is evidence that glucocorticoids regulate ubiquitin-dependent muscle proteolysis during sepsis (16).

A biopsy specimen was obtained from the rectus abdominis muscle during the initial phase of the operation. After skin incision and dissection through the subcutaneous fat, the anterior sheet of the rectus abdominis muscle was opened with scissors and a muscle biopsy specimen weighing ~ 1 g was obtained. The biopsy specimen was divided into two portions that were immediately frozen in liquid nitrogen and then stored at −70°C until analysis. One portion of the biopsy was used for determination of tissue amino acid levels and the other portion for Northern blot analysis (see below). After the muscle biopsy had been obtained, small bleeding vessels were carefully controlled with ligatures and cautery, whereafter the operation continued in a routine fashion. No complications occurred from the biopsy procedure.

Plasma and tissue amino acids. Plasma was deproteinized with 5% (wt/vol) sulfosalicylic acid containing 200 nmol/ml thienylalanine as internal standard and amino acid concentrations were measured in an automated amino acid analyzer (Beckman Amino Acid Analyzer; Beckman Instruments, Inc., Fullerton, CA). To measure tissue amino acids, the muscle biopsy specimens were homogenized in three volumes of 5% sulfosalicylic acid and after centrifugation at 5,000 *g* for 30 min, amino acids in the supernatant were measured by high performance liquid chromatography using a modification of the method of Graser et al. (17) as described recently in a report from our laboratory (18).

Metabolites and cortisol in urine. 3-MH concentration in urine was measured by HPLC using a technique described previously (3). Urine was not hydrolyzed before analysis because 3-MH is excreted in nonacetylated form in man (19). Urinary creatinine was determined using an automated analyzer, Hitachi 717 (Boehringer Mannheim, Indianapolis, IN). Cortisol in urine was measured by HPLC according to the method of Canalis et al. (20).

Northern blot analysis. RNA was extracted from muscle tissue using guanidinium isothiocyanate followed by purification in phenol: chloroform (1:1). For Northern blot analysis, 10 μg of RNA was denatured in glyoxal and fractionated on 1% agarose gel in 10 mM sodium phosphate, pH 7.0. The RNA was transferred to nylon membranes (Micron Separation, Inc., Westborough, 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× sodium chloride-sodium citrate solution (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% sodium dodecyl sulfate (SDS), 10× Denhardt's solution, and 10 μg/ml salmon sperm DNA at 42°C. A ubiquitin cDNA probe was generated by PCR as described previously (3). A rat C3 (RC3) cDNA probe was kindly provided by Dr. K. Tanaka (University of Tokushima, Japan). Homology between the human C3 (HC3) subunit and RC3 has been reported (21). The blots were hybridized with 1×10^8 cpm [α^{32}P]-dATP labeled ubiquitin or RC3 cDNA probe overnight at 42°C in the same buffer that was used for Prehybridization except the sodium phosphate concentration was decreased to 20 mM and Denhardt's solution to normal concentration. The blots were washed twice in 1× SSC, 0.1% SDS at room temperature and auto-radiographed for 24 h at −70°C. An 18 S ribosomal oligonucleotide probe (GACAAGCATATGCTACTGGC) and a GAPDH cDNA probe were used to control for equal loading of RNA. Autoradiographs were quantitated on a Phosphoimager using the Image Quant Program (Molecular Dynamics Inc., Sunnyvale, CA).

Statistics. Results are presented as means±SEM. Student's *t* test was used for statistical analysis.

Results

Seven septic and eleven control patients were included in the study. The septic group consisted of five men and two women with a mean age of 71±2 years (range 63–77 yr). All but one of the septic patients had fecal peritonitis caused by perforated colon secondary to diverticulitis or ischemic colitis. One patient in the septic group had strangulated small bowel with perforation secondary to incarcerated inguinal hernia. The control group consisted of seven men and four women with a mean age of 48±5 yr (range 22–72 yr). The mean age was significantly lower ($P < 0.05$) in the control group than in the group of septic patients. Patients in the control group underwent elective laparotomy for ventral hernia (one), afferent loop syndrome (one), chronic pancreatitis (one), diverticulosis (one), gastric outlet obstruction (one), peptic ulcer (two), ulcerative colitis in a non-active phase (two) and aortic aneurysm (two). The severity of the disease among the septic pa-

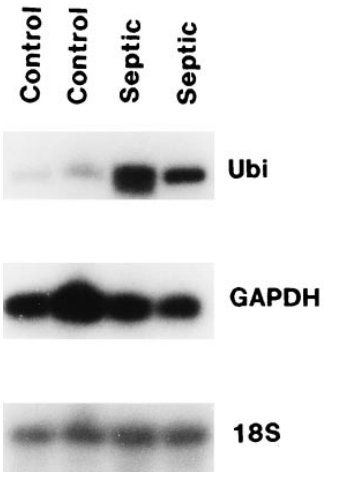

Figure 1. Ubiquitin mRNA levels in muscle tissues from two control and two septic patients determined by Northern blotting. Similar results were observed in the remaining 9 control and 5 septic patients.

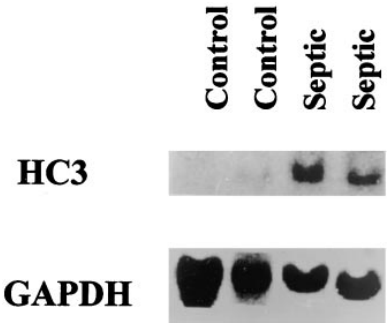

Figure 2. HC3 mRNA levels in muscle tissue from two control and two septic patients determined by Northern blotting. Similar results were observed in the remaining 9 control and 5 septic patients.

tients was confirmed by an APACHE II score of 14.9±2.6 as compared to 2.5±0.8 for the control patients ($P < 0.01$).

Northern blot analysis revealed higher mRNA levels for ubiquitin (Fig. 1) and the 20 S proteasome subunit HC3 (Fig. 2) in muscle from septic than in muscle from control patients. Quantitation of the ubiquitin and RC3 mRNA levels showed a three- to fourfold increase in muscle from septic patients (Fig. 3).

Plasma amino acids in control and septic patients are shown in Table I. The marked depletion of the nonessential amino acids glutamate, serine, glutamine, glycine, arginine, and ornithine as well as the reduction in total plasma amino acids are similar to previous reports in patients with sepsis or severe trauma (12, 13). In addition to the nonessential amino acids, the concentrations of isoleucine and lysine were also reduced in the septic patients. The ratio between the branched chain amino acids (valine, leucine, and isoleucine) and the aromatic amino acids phenylalanine and tyrosine was significantly lower in the septic than in the control patients, similar to previous reports in septic patients and consistent with muscle catabolism (11). The ratio between phenylalanine and tyrosine, another indicator of muscle breakdown (22), was higher in the septic than in the control patients although this difference did not reach statistical significance. It should be noted that the phenylalanine/tyrosine ratios noted both in our control and septic patients were somewhat above the limits reported previously for normal subjects (22). Thus, the present result of a slightly higher phenylalanine/tyrosine ratio in septic than in control patients needs to be interpreted with caution. Changes in concentrations of phenylalanine and tyrosine in plasma and muscle (see below) suggest that the increased phenylalanine/tyrosine ratio during sepsis mainly reflected higher phenylalanine levels.

Table I. Plasma Amino Acids in Control and Septic Patients

	Control ($n = 11$)	Sepsis ($n = 7$)
Aspartic acid	17±3	10±3
Glutamate	125±13	78±3*
Aspargine	34±2	29±6
Serine	83±4	51±9*
Glutamine	527±44	397±36*
Glycine	224±22	130±16*
Histidine	57±4	58±11
Threonine	79±5	49±10*
Citrulline	20±4	13±2
Arginine	71±4	50±5*
Alanine	356±49	237±33
Taurine	57±20	42±12
Tyrosine	43±5	44±8
Valine	192±38	159±22
Methionine	20±6	12±3
Cysteine	49±16	36±7
Tryptophan	22±2	15±4
Phenylalanine	55±5	68±7
Isoleucine	45±5	26±9*
Leucine	111±24	85±13
Ornithine	46±19	8±4*
Lysine	148±31	72±13*
Total	2,398±117	1,692±162*
Phe/Tyr	1.30±0.12	1.73±0.28
BCAA/Aromatic	3.46±0.30	2.43±0.25*

Results are expressed as μmol/liter. *$P < 0.05$ vs. control.

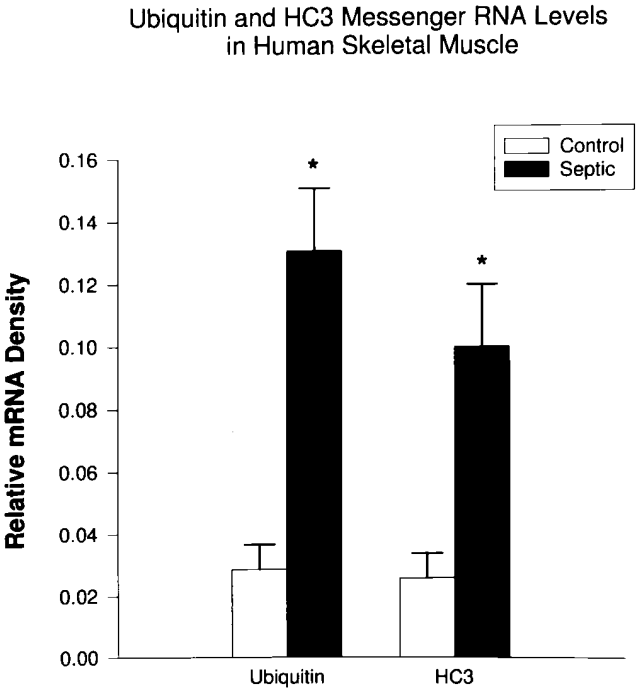

Figure 3. Relative abundance of mRNA for ubiquitin and the 20 S proteasome subunit HC3. Results represent the means±SEM from 11 control and 7 septic patients. *$P < 0.05$ vs. control.

Some of the muscle amino acid levels in the control and septic patients are shown in Fig 4. Changes in muscle concentrations of glutamine, glycine, alanine, 3-MH, tyrosine and phenylalanine were shown here because they have been found previously to be particularly affected by sepsis and other catabolic conditions (12, 18). Muscle glutamine concentration was approximately 40% lower in septic than in control patients, similar to previous reports in patients and experimental animals with muscle catabolism (12, 18). Glycine and alanine levels were unchanged which is similar to a previous report in septic patients (12) but contrasts to reduced concentrations of these amino acids during severe sepsis in rats (18). Muscle concentration of phenylalanine was approximately twofold higher and that of 3-MH ~ 60% higher in septic than in control patients. Similar differences have been reported previously in

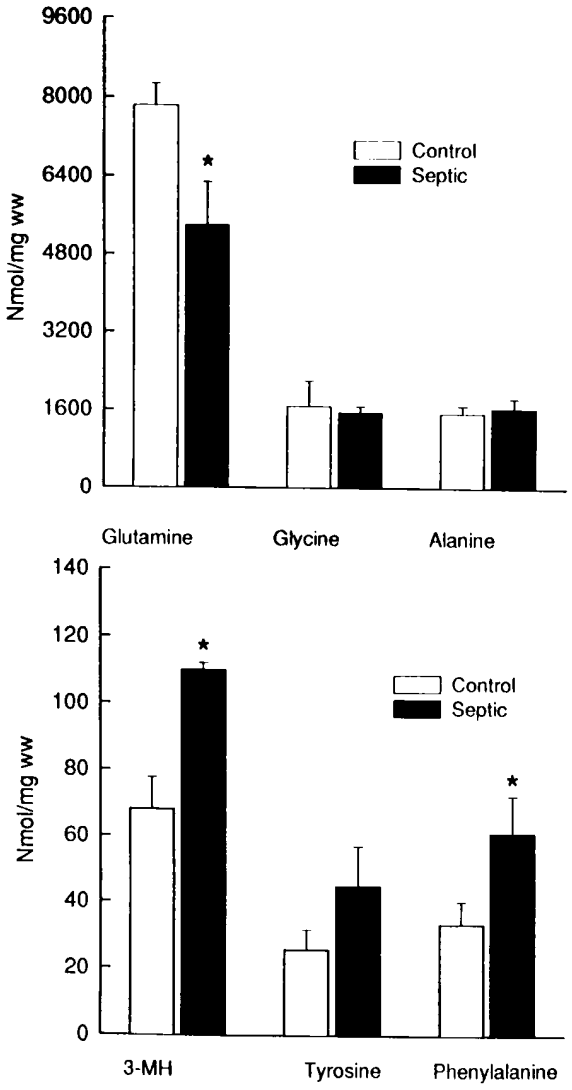

Figure 4. Concentrations of selected amino acids in muscle tissue from 11 control and 7 septic patients. *$P < 0.05$ vs. control.

muscle tissue of both patients and animals during sepsis and other conditions characterized by increased muscle protein breakdown (12, 18).

The 3-MH/creatinine ratio in urine was 167±9.8 and 308±54 nmol 3-MH/mg creatinine in control and septic patients, respectively ($P < 0.05$). Urinary cortisol concentration was 179±120 and 882±569 μg/liter in the two groups of patients. There was a large variation in cortisol concentrations both in the control (range < 5 to 1,122 μg/liter) and septic patients (range 41 to 3,678 μg/liter) and the difference in urinary cortisol between the two groups of patients was not statistically significant.

Discussion

Recent studies from our laboratory provided evidence that the increase in muscle proteolysis during sepsis in rats was caused by stimulated activity of the ubiquitin-dependent proteolytic pathway (3, 16). The present study is the first report of upregulated expression of the ubiquitin gene in human skeletal muscle during sepsis and suggests that similar intracellular mechanisms are operational in muscle tissue of septic patients as previously reported in experimental animals. A recent study in patients with head trauma supports the notion that muscle protein breakdown is regulated by the ubiquitin pathway in human muscle tissue (23).

In addition to sepsis, other catabolic conditions as well are associated with increased expression of the ubiquitin-proteasome pathway, including burn injury (8), fasting, denervation (5), acidosis (7), and cancer (6). Because those studies were performed in experimental animals, the role of the ubiquitin-dependent proteolytic pathway in patients with these catabolic conditions remains to be determined.

In addition to ubiquitin, the expression of one of the 20 S proteasome subunits (HC3) was examined in the present study. The 20 S proteasome, a barrel-shaped particle consisting of four stacked, seven-membered rings, is the catalytic core of the ubiquitin pathway (21, 24). The subunits of the outer rings are called subunits and those of the inner rings β subunits. The functions of the subunits include substrate recognition and unfolding whereas the β subunits contain the catalytic sites (25).

The present result of upregulated expression of mRNA for HC3, an α subunit of the 20 S proteasome, is similar to a recent report in septic rats (26) and suggests that at least some of the genes encoding the 20 S proteasome subunits may be transcribed at an increased rate during sepsis in humans. The effect of sepsis on the expression of other α subunits and on β subunits of the 20 S proteasome is not known at present.

It should be noted that although increased mRNA levels for ubiquitin and HC3, as reported here, may reflect upregulated gene transcription during sepsis, the results need to be interpreted with caution for several reasons. First, increased mRNA steady-state levels do not necessarily reflect increased gene transcription but may also reflect increased mRNA stability. Several observations, however, support the interpretation of the present results as indicating upregulated gene transcription. For example, in recent experiments we found evidence that ubiquitin mRNA levels were increased in rat skeletal muscle during sepsis but mRNA stability was not affected (unpublished observation). In a recent report by Mitch et al. (27), the transcription rate of the ubiquitin gene, measured by nuclear run-on assay, was increased in muscle of rats with metabolic acidosis, concomitant with increased ubiquitin mRNA levels. Thus, it is likely that increased ubiquitin mRNA in catabolic muscle reflects increased transcription of the ubiquitin gene. Second, increased mRNA levels do not necessarily translate into increased protein levels. In fact, in our previous studies in septic rats, the changes in free and conjugated ubiquitin concentrations were less pronounced than the changes in message levels (3, 16) most likely because the ubiquitin protein levels are regulated both by production and breakdown of the protein. Third, increased ubiquitin and RC3 mRNA levels do not prove that the activity in the ubiquitin-proteasome pathway is stimulated. However, several lines of evidence suggest that increased ubiquitin and C3 mRNA concentrations in skeletal muscles during sepsis may reflect upregulated activity of the ubiquitin-proteasome pathway. In a recent study we found that the sepsis-induced increase in muscle proteolysis in rats was blocked by a specific proteasome inhibitor (26). In other experiments, we found evidence of increased activity of the 20 S proteasome isolated from muscle of septic rats (unpublished observations). Thus, it is reasonable to assume that the present

results of increased ubiquitin and HC3 mRNA levels reflect upregulated gene transcription and stimulated activity in the ubiquitin-proteasome proteolytic pathway.

The mediators of increased expression of the ubiquitin-proteasome pathway in muscle of septic patients are not known from the present study. In a recent study we found evidence that glucocorticoids play an important role in upregulating ubiquitin-dependent proteolysis in muscle of septic rats (16). A similar role of glucocorticoids for the activation of the ubiquitin-proteasome pathway was reported in catabolic muscle from rats with metabolic acidosis (28). Although the difference in urinary cortisol levels between control and septic patients was not statistically significant in the present report, it is still possible that circulatory cortisol levels were increased in the septic patients and that cortisol may have participated in the upregulation of the ubiquitin-proteasome pathway. Catecholamines and cytokines are other factors that may influence the expression of the ubiquitin pathway (29). Further studies will be necessary to define the mediators of sepsis-induced muscle proteolysis in patients with sepsis.

One limitation of the present study is that actual muscle protein breakdown rates were not measured. In previous reports, total and myofibrillar proteolytic rates were determined by measuring the release of tyrosine and 3-MH, respectively, from incubated intact muscles of septic rats (3, 16). Although protein breakdown rates have been assessed in incubated human muscle tissue in previous studies (30–32), the fact the muscle fibers have to be cut before incubation makes measurement of protein breakdown rates in incubated human muscle tissue less than optimal. In the present study, therefore, we relied on indirect evidence of accelerated muscle proteolysis. The changes in muscle amino acids noted here, i.e., increased tissue concentrations of tyrosine, phenylalanine and 3-MH and reduced levels of glutamine, together with increased urinary 3-MH/creatinine ratio, support the concept that the septic patients were in a catabolic state. In several previous reports, increased muscle protein breakdown rates were associated with, and perhaps caused by reduced intracellular glutamine levels (33, 34). Tyrosine and phenylalanine are not synthesized or metabolized in skeletal muscle. Increased tissue levels of these amino acids, therefore, are consistent with increased protein breakdown, although reduced protein synthesis and changes in transmembrane transport of the amino acids may give rise to similar results. Because 3-MH is present only in actin and myosin and is not reutilized for protein synthesis after its release during proteolysis (35), increased 3-MH levels in muscle and increased 3-MH/creatinine ratio in urine of septic patients, as reported here, are consistent with stimulated myofibrillar protein breakdown. It should be noted that because urinary excretion of 3-MH is influenced by the diet as well as by myofibrillar protein breakdown in the gastrointestinal tract, interpretation of the higher 3-MH/creatinine ratio in urine noticed here in septic patients must be done with great caution. The results, however, are consistent with previous studies in septic rats (3, 16) and patients (15) in which evidence was found that sepsis results in increased myofibrillar protein breakdown in skeletal muscle. In addition to muscle amino acid concentrations, several changes in plasma amino acids noticed here were similar to changes reported previously in patients with sepsis or trauma (12, 13), further supporting the interpretation that our septic patients were in a catabolic state.

Another limitation of the present study is that the septic patients were older than the control patients. The reason for this difference was the design of the study, entering consecutive patients undergoing laparotomy for sepsis or non-septic condition, rather than matching the control patients by age. Although it is possible that the higher ubiquitin and HC3 mRNA levels in the septic patients reflected the age difference rather than (or in addition to) the septic condition, several observations support the interpretation that the results were not merely caused by the higher age of the septic patients: (*a*) there was no correlation between age and ubiquitin or HC3 mRNA levels within each group of patients (data not shown), despite the fact that the age range was 22–72 yr among the control patients and 63–77 yr among the septic patients; (*b*) in previous studies we found evidence that the response to sepsis of muscle protein breakdown was not age-dependent in rats (36); (*c*) in a recent study by Dardevet et al. (37), the increase in muscle ubiquitin mRNA levels after treatment with dexamethasone was almost identical in adult (7-mo-old) and old (22-mo-old) rats.

Furthermore, differences in nutritional status were not controlled for in the present study. This is an additional limitation of our report because fasting may increase ubiquitin-dependent protein breakdown in skeletal muscle (38). It should be noted, however, that most of our septic patients were operated on emergently and although food intake was diminished they had not been fasting before surgery. In contrast, the control patients underwent elective surgery and had been fasting for at least 12 h before operation. Thus, it is likely that the difference in muscle levels of ubiquitin and HC3 mRNA between the septic and control patients did not reflect differences in food intake.

Despite the limitations described above, the present results are important because they suggest that the catabolic response to sepsis may be regulated at the gene level in human muscle tissue. The results provide further support for the role of the ubiquitin-proteasome pathway in the regulation of muscle proteolysis during sepsis. An important implication of the findings in this report is that changes in the ubiquitin-proteasome proteolytic pathway noted previously during sepsis in rats (3, 16) reflect changes that occur in patients with sepsis. Thus, the experimental model used in our previous studies (3, 16) should be valid to explore mediators and mechanisms of the sepsis-induced activation of the ubiquitin-proteasome system and, more importantly, to test different therapeutic interventions aimed at reducing the catabolic response to sepsis.

Acknowledgments

This work was supported in part by National Institutes of Health grant DK-37908 and grants 8580 and 8610 from the Shriners of North America. G. Tiao and S. Hobler were also supported by NIH Training Grant 1T32GM08478.

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