Sepsis is associated with increased ubiquitin-conjugating enzyme E2_{14k} mRNA in skeletal muscle

SCOTT C. HOBLER, JING JING WANG, ARTHUR B. WILLIAMS, FRANCESCO MELANDRI, XIAOYAN SUN, JOSEF E. FISCHER, AND PER-OLOF HASSELGREN. Sepsis is associated with increased ubiquitin-conjugating enzyme E2_{14k} mRNA in skeletal muscle. Am. J. Physiol. 276 (Regulatory Integrative Comp. Physiol. 45): R468-R473, 1999.—Previous studies provided evidence that sepsis is associated with increased ubiquitin-proteasome-dependent protein breakdown in skeletal muscle. The 14-kDa ubiquitin-conjugating enzyme (E2_{14k}) has been proposed to be a key regulator of the ubiquitin proteolytic pathway. We tested the hypothesis that E2_{14k} message and protein levels are increased in skeletal muscle during sepsis. Sepsis was induced in rats by cecal ligation and puncture (CLP). Control rats were sham operated. E2_{14k} mRNA and protein levels were quantitated after Northern and Western blot analysis, respectively, 16 h after CLP or sham operation. Sepsis resulted in a 70% increase in the 1.2-kb E2_{14k} transcript in the fast-twitch extensor digitorum longus muscle, whereas no changes were seen in the slow-twitch soleus muscle. E2_{14k} protein levels were not influenced by sepsis in any of the muscles studied. Although the changes in the expression of the E2_{14k} 1.2-kb transcript paralleled the differential effect of sepsis on protein breakdown in fast- and slow-twitch muscle, the potential role of E2_{14k} in the regulation of sepsis-induced muscle proteolysis needs to be interpreted with caution, because the results demonstrated that increased message levels were not associated with increased E2_{14k} protein levels.

proteolysis; cachexia; proteasome

ONE OF THE METABOLIC hallmarks of sepsis is the catabolic response in skeletal muscle characterized by increased protein breakdown, in particular myofibrillar protein breakdown (4, 9). This catabolic response results in release of amino acids from muscle tissue, providing the liver with substrates for acute phase protein synthesis and gluconeogenesis (16). Thus the catabolic response in skeletal muscle may be beneficial to the organism, at least during the early phase of sepsis. In severe and protracted sepsis, however, continued muscle protein breakdown results in muscle wasting and fatigue, which may impair recovery and lead to increased risk for thromboembolic and pulmonary complications if ambulation is delayed and respiratory muscles are affected. A better understanding of the mechanisms regulating muscle proteolysis during sepsis, therefore, is of great clinical significance and may be important for the development of future therapeutic modalities to inhibit the catabolic response in patients with sepsis.

Intracellular protein breakdown is regulated by different proteolytic pathways, including lysosomal and nonlysosomal mechanisms (6). In recent studies, we found evidence that the catabolic response in skeletal muscle during sepsis mainly reflects ubiquitin-dependent protein breakdown (19, 20). In this proteolytic pathway, the protein substrate is conjugated to ubiquitin, a small 76-amino acid polypeptide, before it is degraded by the large cytosolic protease complex, the 26S proteasome (for review, see Refs. 3, 8, and 10).

The regulation of the ubiquitin pathway in catabolic states is unclear. It has been postulated that the first irreversible reaction and the first reaction after a branch point in a metabolic pathway may be the rate-limiting and regulated step in that pathway (12). On the basis of such considerations, the 14-kDa ubiquitin-conjugating enzyme E2 (E2_{14k}) has been proposed to be a site of regulation in the ubiquitin-proteasome proteolytic pathway in skeletal muscle (22, 23).

The E2 enzymes are a large family of proteins consisting of at least 30 different members with different functions (11). Among the E2 enzymes, E2_{14k} supports ubiquitin-dependent proteolysis (22). Several recent studies have examined the expression of E2_{14k} mRNA in catabolic muscle (5, 17, 22). Parallel changes in the levels of the 1.2-kb E2_{14k} transcript and protein breakdown rates were seen in muscles of starved (22), dexamethasone-treated (5), and tumor-bearing rats (17). However, there is not an absolute correlation between E2_{14k} mRNA expression and muscle proteolysis. For example, the catabolic response in skeletal muscle caused by metabolic acidosis was not associated with increased levels of E2_{14k}-encoding transcripts despite stimulated ubiquitin-proteasome-dependent proteolysis (15). Thus the expression of E2_{14k} mRNA needs to be examined in different catabolic conditions before its role in the regulation of muscle proteolysis can be determined.

The effect of sepsis on the expression of E2_{14k} mRNA has not been reported. In addition, the correlation between E2_{14k} mRNA and E2_{14k} protein levels in catabolic muscle is not known. We determined the influence of sepsis on E2_{14k} mRNA and protein levels in different types of skeletal muscle. To test the specificity of changes in muscle E2_{14k}, E2_{14k} mRNA and protein levels were examined in liver and kidney as well. We found that sepsis upregulated the expression of the 1.2-kb E2_{14k} transcript in fast-twitch muscle, liver, and kidney but not in slow-twitch muscle. E2_{14k} protein...
levels did not change during sepsis in any of the tissues studied.

MATERIALS AND METHODS

Animals and experimental model. Sepsis was induced by cecal ligation and puncture (CLP) in male Sprague-Dawley rats weighing 40–60 g as described previously (9, 19). Control rats underwent sham operation, i.e., laparotomy and manipulation but no ligation or puncture of the cecum. All rats were resuscitated with 10 mL saline/100 g body wt administered subcutaneously on the back at the time of surgery. The rats had free access to water, but food was withheld after surgery to avoid any influence on metabolic changes of different food intake between the two groups of rats. This experimental model of sepsis is clinically relevant because it resembles the situation in patients with sepsis caused by intra-abdominal abscess and devitalized tissue. The model was characterized with respect to hemodynamic and metabolic changes and survival rates in previous studies from our (14) and other laboratories (1). Rats weighing 40–60 g were used in the present experiments because rats of this size were used in previous reports from our laboratory in which protein breakdown rates were measured in incubated skeletal muscles (9, 19). Results from the present experiments could therefore be compared with results in our previous experiments. It should be noted that although protein turnover rates are higher in young growing rats than in adult rats, in a previous study we found that the relative changes in protein breakdown rates induced by CLP were similar in young growing and adult rats (24), suggesting that the catabolic response to sepsis is not age dependent, at least not in rats using the present septic model. Sixteen hours after CLP or sham operation, the extensor digitorum longus (EDL) and soleus muscles, liver, and kidney were harvested and tissue was frozen at −70°C for subsequent study. In some experiments, EDL muscles were incubated in vitro for determination of E214k mRNA stability (see mRNA stability). The time point for metabolic studies was based on previous reports in which total and myofibrillar ubiquitin-dependent muscle protein breakdown rates were increased, and rats were in a hyperdynamic state of sepsis 16 h after CLP (1, 9, 14, 18, 19). All experiments were conducted and animals cared for in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved by the Institutional Animal Care and Use Committee at the University of Cincinnati.

Northern blot analysis. For determination of E214k mRNA levels, total RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction as described by Chomczynski and Sacchi (2). RNA was denatured in glyoxal and separated by electrophoresis on 1% agarose gel in 10 mM sodium phosphate (pH 7.0). The RNA was transferred from the gel to nylon membranes (Micron Separations, Westboro, MA) by capillary action in 25 mM sodium phosphate (pH 6.4) overnight. RNA was immobilized by ultraviolet cross-linking. The blots were prehybridized at 42°C for 4 h in 50% formamide, 6× sodium chloride-sodium phosphate-EDTA (SSPE) (1× SSPE = 0.15 M NaCl, 10 mM NaH2PO4, 1 M EDTA), 5× Denhardt’s solution, 0.5% SDS, and 100 µg/mL salmon sperm DNA. A cDNA probe for E214k was labeled by random priming with [32P]dATP (Stratagene, La Jolla, CA). The blots were hybridized with the [32P]-labeled E214k cDNA probe in the same buffer as described above at 42°C overnight. The blots were then washed twice in 1× 0.1 M NaCl, 15 mM Na-citrate, pH 7.0 (SSC) and 0.1% SDS at room temperature, washed once in 0.1× SSC and 0.1% SDS at 65°C, and autoradiographed for 12–24 h at −70°C. The blots were stripped and rehybridized with an 18S oligonucleotide probe to control for equal loading of RNA. The blots were quantitated on a phosphorimager using the Image Quant Program (Molecular Dynamics, Sunnyvale, CA), and the relative mRNA abundance was expressed as the ratio of E214k mRNA to 18S. The E214k cDNA probe was generated as described previously from our laboratory (19) on the basis of the reported structure of the rat E214k gene (22).

Because the relative abundance of E214k mRNA was expressed as the ratio of E214k mRNA to 18S, it was important to make certain that an increase in this ratio did not merely reflect a reduced amount of RNA in catabolic muscle. A separate experiment was therefore performed in which RNA was extracted (2) from EDL muscles of sham-operated and septic rats, and the different lanes of the gel were loaded either with identical amounts of RNA (20 µg) or with the amount of RNA adjusted to reflect the same amount of tissue (5 mg muscle).

mRNA stability. The stability of E214k mRNA was assessed by determining the degradation of mRNA during incubation of muscles. Sixteen hours after sham operation or CLP, the EDL muscle from one side of the rat was immediately frozen in liquid nitrogen and stored at −70°C until further analysis. The contralateral muscle was incubated at resting length in 3 mL of oxygenated (O2:CO2 = 95:5) Krebs-Henseleit bicarbonate buffer (pH 7.4) containing 10 mM glucose and 5 µg/mL actinomycin D. The incubations were carried out at 37°C in a shaking water bath for up to 4 h, after which the muscle was frozen in liquid nitrogen and stored at −70°C until further analysis. The medium was supplemented with actinomycin D to block synthesis of mRNA. The decline in mRNA levels during incubation, therefore, reflected degradation of mRNA. The amount of E214k mRNA present in the muscle at the end of incubation was expressed as percent of E214k mRNA present in the contralateral muscle that was immediately frozen after harvesting from the rat. E214k mRNA levels were quantitated after Northern blot analysis as described above. Muscles were incubated for 4 h because in a recent study, using an identical experimental approach to assess mRNA stability, we found that there was only a minimal decline in mRNA levels in the first 2 h of incubation (21).

Western blot analysis. Soluble (sarcomplasmic) proteins were extracted from EDL muscles of sham-operated and septic rats as described previously (19). Proteins (10 µg) were fractionated by electrophoresis on a 15% acrylamide gel using 200 V for 1.5 h at room temperature and were then transferred to an Immobilon polyvinylidene difluoride membrane (Millipore, Bedford, MA) at 33 V at 4°C overnight. The membranes were incubated with rabbit anti-rat E214k polyclonal antibody [diluted 1:8,000 in 5% milk/Tris-buffered saline (TBS-T; 25 mM Tris, pH 7.4; 137 mM NaCl; 0.1% Tween 20)] for 1 h at room temperature. After washing with TBS-T 3× 10 min, the membranes were incubated with fluorescein-linked anti-rabbit antibody (diluted 1:2,000 in 5% milk/TBS-T) for 1 h at room temperature. After washing with TBS-T 3× 10 min, the membranes were incubated with anti-fluorescein alkaline phosphate conjugate antibody (diluted 1:5,000 in 5% milk/TBS-T) for 1 h at room temperature. The bound antibodies were detected with a Vistra Atto Phos and were quantitated with a Storm 840 System (Molecular Dynamics, Sunnyvale, CA) by comparing to a standard curve established by using known amounts of recombinant E214k protein.

Statistics. Results are presented as means ± SE. Student’s t-test was used for statistical comparisons.
RESULTS

Two E2\textsubscript{14k} mRNA transcripts were observed in the different tissues examined here, \( \text{1.2} \) and \( \text{1.8} \) kb in size, respectively, similar to previous reports (22, 23). Changes in E2\textsubscript{14k} mRNA levels in muscle, liver, and kidney are shown in Fig. 1. Sepsis resulted in a 73% increase in the expression of the 1.2-kb transcript but did not influence the levels of the 1.8-kb transcript in the fast-twitch EDL muscle. A selective increase in the 1.2-kb E2\textsubscript{14k} mRNA transcript was noted in other catabolic conditions as well (5, 17, 22).

In recent studies in our laboratory, sepsis in rats resulted in a pronounced increase in energy-ubiquitin-dependent proteolysis in EDL muscle (a white, fast-twitch muscle), with no or only minimal changes noted in the red, slow-twitch soleus muscle (21). To test whether sepsis resulted in a similar differential regulation of E2\textsubscript{14k}, we next determined E2\textsubscript{14k} mRNA levels in different tissues from sham-operated and septic rats. Sepsis resulted in small but statistically significant increases in E2\textsubscript{14k} mRNA levels in both tissues (Fig. 1). Interestingly, in renal tissue the levels of both E2\textsubscript{14k} mRNA transcripts were increased, whereas in liver tissue only the 1.2-kb transcript was upregulated.

Because the relative abundance of E2\textsubscript{14k} mRNA shown in Fig. 1 was expressed as the ratio of E2\textsubscript{14k} mRNA to 18S after loading of the lanes with identical amounts of RNA, it was important to test whether the apparent increase in E2\textsubscript{14k} mRNA was a true increase or merely reflected a decrease in muscle total RNA. Indeed, total RNA in EDL muscle was reduced by 19% in septic rats compared with sham-operated rats (from \( 89 \pm 4.5 \) to \( 72 \pm 2.1 \) \( \mu \)g RNA/muscle; \( P < 0.05 \), \( n = 7 \) in each group). The corresponding muscle weights were \( 26.8 \pm 0.95 \) and \( 27.2 \pm 1.9 \) mg (not significant). In a separate experiment, Northern blot analysis was performed after loading of the lanes with identical amounts of RNA (20 \( \mu \)g) or with the amount of RNA adjusted to reflect the same amount of tissue (5 mg muscle). In this experiment, the relative abundance of the 1.2-kb E2\textsubscript{14k} mRNA was increased by 89% when measured in identical amounts of RNA and by 70% when measured in RNA adjusted to reflect the same amount of tissue (Table 1). The somewhat less pronounced increase in mRNA when expressed per 5 mg muscle probably reflected the reduced amount of RNA in septic muscle (see above). Importantly, however, sepsis was associated with a significant increase in 1.2-kb E2\textsubscript{14k} mRNA in the EDL muscle regardless of whether the gels were loaded with identical amounts of RNA or RNA reflecting the same amount of tissue. Thus the results in Fig. 1 reflected a true increase in 1.2-kb E2\textsubscript{14k} mRNA in septic EDL muscle. This interpretation was further supported by the selective increase in the 1.2-kb transcript. If the results only reflected a decreased amount of total RNA in muscle, a parallel apparent increase for both the 1.2- and 1.8-kb transcripts would have been expected. The present results are similar to those reported by Wing and Banville (22), who found that 1.2-kb E2\textsubscript{14k} mRNA levels were significantly increased in EDL muscles of fasted rats, both when expressed per microgram of RNA and per milligram of muscle.

Increased mRNA levels may reflect increased gene transcription, increased mRNA stability, or a combination of these changes. Because initial attempts to isolate nuclei from the muscles studied in the present report were unsuccessful, mainly reflecting the small size of the muscles and the relatively tough tissue, we were not able to determine transcription rates for the E2\textsubscript{14k} gene. Instead, we assessed E2\textsubscript{14k} mRNA stability by incubating EDL muscles from sham-operated and septic rats for 4 h in the presence of actinomycin D. At the end of the incubation period, the amount of both the 1.8- and 1.2-kb transcripts, expressed as percent of
mRNA levels at the start of incubation, was almost identical in muscles from sham-operated and septic rats, suggesting that sepsis did not influence the stability of E214k mRNA (Fig. 2).

Increased levels of E214k mRNA do not necessarily mean that E214k protein levels are increased. The influence of sepsis (and other catabolic conditions) on the expression of E214k protein is not known. Indeed, previous attempts to measure E214k in skeletal muscle were reported to be unsuccessful due to the low concentrations of the protein (22). In the present study, a sensitive Western blot assay was established allowing for determination of E214k at low concentrations (Fig. 3). Sepsis did not influence E214k protein levels in any of the tissues studied (Fig. 4). It should be noted that the same amount of soluble proteins (10 µg) were used from both sham-operated and septic rats for Western blotting in this experiment. In a previous study, we found that the amount of soluble proteins was not different in control and septic EDL muscles 16 h after sham operation or CLP in rats (19). Thus the results shown in Fig. 4, at least those for EDL muscles, were probably not influenced by different concentrations of soluble proteins in control and septic muscles.

**DISCUSSION**

In the present study, sepsis in rats was associated with increased levels of the 1.2-kb E214k transcript in the fast-twitch EDL muscle, whereas no changes were observed in transcript levels in the slow-twitch soleus muscle.
muscle. This differential effect of sepsis in the different types of skeletal muscle parallels the sepsis-induced changes in ubiquitin-proteasome-dependent protein breakdown in EDL and soleus muscles (21). The results support the concept (but do not prove) that E214k is a regulating site of the ubiquitin proteolytic pathway in skeletal muscle during sepsis.

Upregulated expression of E214k mRNA in skeletal muscle was reported in other catabolic conditions as well, including starvation (22), cancer (17), and treatment with dexamethasone (5). Also, in those studies, it was mainly the 1.2-kb E214k mRNA transcript that was increased with no or only minimal changes observed in the 1.8-kb transcript. Increased mRNA levels for E214k may reflect upregulated transcription of the E214k gene and/or increased stability of the mRNA. Results in the present study suggest that mRNA stability was not influenced by sepsis, at least not in incubated EDL muscles. Thus it is possible that sepsis stimulated the transcription of the E214k gene in the EDL muscle. Because the two E214k transcripts probably originate from two different polyadenylation sites of the E214k gene (22), the present result of a selective increase in the 1.2-kb transcript suggests that the transcription of different sites of the E214k gene is differentially regulated. The contribution of gene transcription vs. mRNA stability to changes in E214k mRNA levels in skeletal muscle during other catabolic conditions was not addressed in previous reports (5, 17, 22).

Although the correlation between E214k expression and protein breakdown rates noted here and in other studies (5, 17, 22) is consistent with the concept that E214k is a site of regulation of the ubiquitin proteolytic pathway in skeletal muscle, it should be noted that increased ubiquitin-proteasome-dependent muscle proteolysis in acidotic rats was not associated with increased expression of E214k mRNA (15). Thus it is possible that E214k is involved in the regulation of the ubiquitin-proteasome pathway in skeletal muscle in some, but not all, catabolic conditions.

The influence of starvation in rats on E214k mRNA levels in different types of skeletal muscle and other tissues was reported by Wing and Banville (22). When different types of muscle were examined, they found the most pronounced changes in the fast-twitch EDL muscle, similar to the current study, with about a fivefold increase in the 1.2-kb transcript after 2 days of starvation. In contrast to the present report, Wing and Banville (22) noted increased expression of E214k in the slow-twitch soleus muscle as well, although this increase was much less pronounced than in the EDL muscle. Interestingly, in the soleus muscle, the levels of both the 1.2- and 1.8-kb transcripts were increased. Thus it is possible that various catabolic conditions regulate the E214k gene in slow-twitch muscle differentially. Alternatively, slow-twitch muscle may not become catabolic until after a more severe and prolonged stress. We did not observe any substantial changes in ubiquitin-dependent protein breakdown rates (21) or E214k mRNA levels (present study) in the soleus muscle 16 h after induction of sepsis, whereas the increased expression of E214k in soleus muscle was noted after 2 days of starvation (22).

The mediator(s) of increased E214k mRNA levels in the EDL muscle during sepsis are not known from the present study. Although the catabolic response in muscle is regulated by multiple factors, including the proinflammatory cytokines interleukin-1 and tumor necrosis factor, glucocorticoids are probably the most important regulator of sepsis-induced muscle catabolism (18). Glucocorticoid levels were not measured in the present experiments, but in a previous report from our laboratory, plasma levels of corticosterone (the predominant glucocorticoid in rats) were increased after CLP in rats (7).

Similar to the present study, E214k mRNA levels were increased in liver and kidney during starvation, with the increases being statistically significant only for the smaller transcript (22). The results were interpreted as supporting the role of E214k in the regulation of proteolysis because liver and kidney lose weight upon fasting. It should be noted, however, that increased expression of E214k in liver and kidney does not necessarily reflect a catabolic state, because during sepsis liver weight and protein content are typically increased, and protein breakdown is not stimulated (13). The present finding of upregulated expression of both E214k mRNA transcripts in kidney during sepsis, as opposed to the selective increase in the 1.2-kb transcript during starvation (22), suggests that the E214k gene is differentially regulated by different catabolic conditions in renal tissue. The biological role of increased E214k mRNA levels in liver and kidney during sepsis and other catabolic conditions remains to be determined.

The influence of catabolic conditions on E214k protein levels in skeletal muscle or other tissues has not been previously reported. Results in the present study suggest that sepsis-induced changes in E214k mRNA levels are not accompanied by changes in E214k protein levels. This observation may have several explanations. The results could be consistent with reduced translational efficiency of the E214k mRNA or increased production of E214k with a concomitant increase in the breakdown of E214k protein. It is also possible that the increased E214k mRNA protein levels noted here 16 h after CLP may be followed by increased E214k protein levels later in the septic process. Further studies are needed to determine the reason for the apparent discrepancy between E214k mRNA and protein levels in muscle and other tissues during sepsis.

The interpretation of the present results that E214k may be a regulator or activator of ubiquitin-dependent muscle proteolysis during sepsis needs to be done with caution for several reasons. First, increased E214k mRNA levels do not necessarily mean that the amount and, more importantly, the activity of the enzyme are increased. Indeed, results in the present study suggest that E214k protein levels are not altered in septic muscle. This was an important finding considering several previous reports in which increased E214k mRNA levels alone were interpreted as indicating a role of E214k as a regulating site of muscle proteolysis in
various catabolic conditions (5, 17, 22). First, the influence of sepsis and other catabolic conditions on the activity of the E214k enzyme is not known at present. Second, the lack of upregulated E214k expression in catabolic muscles of acidic rats (15) suggests that E214k is not the only site for regulation of the ubiquitin-proteasome proteolytic pathway. Third, whereas the influence of sepsis on ubiquitin-dependent proteolysis seems to be specific for fast-twitch skeletal muscle with no changes in other tissues (21), in the present study E214k mRNA levels were increased in liver and kidney as well. It is obvious, then, that more studies are needed to define the role of the E214k enzyme in the regulation of muscle proteolysis during sepsis. The present study is important because it provides the first information on the expression of E214k mRNA and protein in skeletal muscle during sepsis.

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REFERENCES