Hormone, cytokine, and nutritional regulation of sepsis-induced increases in atrogin-1 and MuRF1 in skeletal muscle


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Frost RA, Nystrom GJ, Jefferson LS, Lang CH. Hormone, cytokine, and nutritional regulation of sepsis-induced increases in atrogin-1 and MuRF1 in skeletal muscle. Am J Physiol Endocrinol Metab 292: E501–E512, 2007. First published September 26, 2006; doi:10.1152/ajpendo.00359.2006.—Various atrophic stimuli increase two muscle-specific E3 ligases, muscle RING finger 1 (MuRF1) and atrogin-1, and knockout mice for these “atrogenes” display resistance to denervation-induced atrophy. The present study determined whether increased atrogin-1 and MuRF1 mRNA are mediated by overproduction of endogenous glucocorticoids or inflammatory cytokines in adult rats and whether atrogene expression can be downregulated by anabolic agents such as insulin-like growth factor (IGF)-1 and the nutrient-signaling amino acid leucine. Both atrogin-1 and MuRF1 mRNA in gastrocnemius was upregulated dose and time dependently by endotoxin. Additionally, peritonsitis produced by cecal ligation and puncture increased atrogin-1 and MuRF1 mRNA in gastrocnemius (but not soleus or heart) by 8 h, which was sustained for 72 and 24 h, respectively. Whereas the sepsis-induced increase in atrogin-1 expression was completely prevented by IGF-1, the increased MuRF1 was not altered. In contrast to the IGF-1 effect, the sepsis-induced increased mRNA of both atrogens was unresponsive to either acute or repetitive administration of leucine. Whereas exogenous infusion of TNF-α increased atrogin-1 and MuRF1 in gastrocnemius, pretreatment of septic rats with the TNF antagonist TNF-binding protein did not prevent increased expression of either atrogene. Similarly, whereas dexamethasone increased atrogin expression, pretreatment with the glucocorticoid receptor antagonist RU-486 failed to ameliorate the sepsis-induced increase in atrogin-1 and MuRF1. Thus, under in vivo conditions in mature adult rats, the sepsis-induced increase in muscle atrogene expression was independent and unresponsive to leucine.

atrogin-1 and MuRF1 in skeletal muscle.

INFECTION IS A POTENT CATABOLIC INSULT that leads to a sustained hypermetabolic condition characterized by the erosion of lean body mass (47). When protracted, the depletion of both myofibrillar and sarcoplasmic protein leads to muscle weakness, which is associated with a greater risk for septic complications, longer hospital stays, and delayed rehabilitation (3, 59). In general, morbidity and mortality is adversely affected by the magnitude of the catabolic response (19). Tissue protein content in general and skeletal muscle protein in particular are maintained by the dynamic balance between rates of protein synthesis and degradation. Several catabolic insults, including sepsis, endotoxin, and excess inflammatory cytokines and glucocorticoids, produce an imbalance between protein synthesis and degradation (12, 22, 31–33, 40). The etiology of the sepsis-induced stimulation of protein degradation that exacerbates nitrogen loss is not completely understood.

Skeletal muscle contains at least three distinct pathways [e.g., lysosomal, calcium dependent, and ubiquitin (Ub)-proteasome dependent] by which proteolysis is regulated (24, 38). However, sepsis-induced changes in the Ub-proteasome proteolytic pathway are generally regarded as being the predominant atrophic mechanism, as evidenced by increased Ub-protein conjugates and mRNA content of ubiquitinating enzymes and proteasome subunits (24, 38, 55). Moreover, proteasome inhibition largely prevents sepsis-induced wasting (25). There are two functional facets to the Ub-proteasome system: the targeting of specific protein substrates for degradation and their ultimate destruction by the proteasome (49). The targeting requirement is fulfilled by the activity of a three-tiered hierarchical cascade of E1 (Ub-activating), E2 (Ub-conjugating), and E3 (Ub-ligating) enzymes in concert with their labeling cofactor Ub. Proteins degraded in this pathway are first sequentially linked to monomeric Ub forming a polyubiquitin chain. Subsequently, this chain is recognized by the 19S proteasome (e.g., regulatory cap) and leads to protein unfolding, insertion into the proteasome, and degradation of the protein into peptide fragments. Although the rate-limiting step in muscle proteolysis has not been identified, the coordinated upregulation of specific E2s and E3s and ubiquitin conjugation may be central to the atrophic response during catabolic injury. However, a sepsis-induced activation of calcium/calpain-dependent release of myofilaments from sarcomer may also be required to provide adequate substrate under conditions where ubiquitination is accelerated (13).

Historically, the ubiquitination of proteins in the N-end rule pathway by the Ub-conjugating enzyme E214k and the Ub ligase E3α was considered the primary regulatory pathway for muscle loss in wasting states, including sepsis (24, 38). However, a host of diverse atrophic stimuli, such as immobilization, denervation, diabetes, dexamethasone, and nutrient deprivation strongly increase the gene expression of two additional E3 ligases, muscle RING finger 1 (MuRF1) and muscle atrophy F-box (aka atrogin-1), that are muscle specific (2, 18, 37, 50). Furthermore, a reduced loss of muscle mass is evident in atrogin-1 and MuRF1 null mice after muscle denervation (2). In contrast, overexpression of atrogin-1 protein leads to a reduction in the size of cultured myocytes (2) and blunts cardiac hypertrophy (41). Hence, atrogin-1 and MuRF1 have collectively been referred to as atrophy-related genes or “atro-

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genes." Although initial studies reported elevated expression of these atrogene in response to sepsis (60) and endotoxemia (9), the purpose of the present study was to determine whether this increased expression is mediated by the overproduction of glucocorticoids or inflammatory cytokines in adult animals and whether atro gene expression can be downregulated by anabolic agents such as insulin-like growth factor (IGF)-1 and the nutrient-signaling amino acid leucine, which might be used therapeutically to minimize wasting.

MATERIALS AND METHODS

Animal preparation and experimental protocol. Adult specific pathogen-free male Sprague-Dawley rats (325–350 g; Charles River Breeding Laboratories, Cambridge, MA) were housed at a constant temperature, exposed to a 12:12-h light-dark cycle, and maintained on standard rodent chow (Harlan no. 2018, 18% protein rodent diet; Harland Teklad, Madison, WI) and water ad libitum for 1 wk before experiments were performed. All experiments were approved by the Animal Care and Use Committee at the Pennsylvania State University College of Medicine and adhered to the National Institutes of Health guidelines for the use of experimental animals.

The following animal protocols were used.

Protocol 1. Escherichia coli LPS (026:B6; Sigma-Aldrich, St. Louis, MO) or an equal volume of saline (0.5 ml/100 g body wt) was injected intraperitoneally at doses ranging between 10 and 1,000 μg/kg body wt and the gastrocnemius muscle sampled at various times thereafter (32).

Protocol 2. For the in vivo infusion of TNF-α, rats were anesthetized with an intramuscular injection of ketamine and xylazine (90 and 9 mg/kg, respectively), and sterile surgery was performed to implant a catheter in the jugular vein. This catheter was passed through a tightly coiled stainless steel spring and fixed to a freely rotating swivel (Instech, Plymouth, PA). Recombinant human TNF-α (Amgen, Thousand Oaks, CA) was diluted in 0.1% human serum albumin and infused intravenously for the next 24 h at a rate of 5 and 9 mg/kg, respectively, and sterile surgery was performed to implant a catheter in the jugular vein. This catheter was passed through a tightly coiled stainless steel spring and fixed to a freely rotating swivel (Instech, Plymouth, PA). Recombinant human TNF-α (Amgen, Thousand Oaks, CA) was diluted in 0.1% human serum albumin and infused intravenously for the next 24 h at a rate of 5 μg·kg⁻¹·h⁻¹ (0.35 ml/h) (33). Time-matched control animals were infused with an equal volume of vehicle.

Protocol 3. To determine whether an elevation in the plasma glucocorticoid concentration could alter muscle atrogin-1 and MuRF1 mRNA in control animals, a separate group of rats was injected subcutaneously with dexamethasone (100 μg/100 g body wt; Sigma) or an equal volume (0.5 ml/rat) of vehicle. This dose of dexamethasone decreases muscle protein synthesis, increases elements of the ubiquitin-proteasome system, and induces muscle wasting (5, 52). Gastrocnemius was sampled at 4 h and 24 h after injection of dexamethasone.

Protocol 4. Peritonitis was produced by cecal ligation and puncture (CLP) as previously described (30). Rats were anesthetized with a mixture of ketamine-xylazine, and a midline laparotomy was performed. The cecum was ligated at its base and punctured twice with a 20-gauge needle. The cecum was then returned to the peritoneal cavity, and the muscle and skin layers were closed. Rats were resuscitated with 10 ml of 0.9% sterile saline administered subcutaneously. Nonseptic control animals were subjected to a midline laparotomy with intestinal manipulation and then resuscitated with the same volume of saline. All septic rats and time-matched nonseptic control animals were injected subcutaneously with the analgesic morphine (0.2 mg/kg) immediately after surgery. In some studies, control and septic rats were treated with either TNF-binding protein (TNFBP), an antagonist of TNF-α, or placebo at various times after induction of sepsis. Muscle was sampled 12 h after the second injection of IGF-I/IGFBP-3. Other groups of control and septic rats were administered either saline (0.155 mol/l) or 1.35 g/kg body wt leucine (prepared as 54.0 g/l of l-amino acid in distilled water) by oral gavage. This dose of leucine was selected because it is equivalent to that consumed in a 24-h period, when rats of this age and strain are provided free access to food, and maximally stimulates muscle protein synthesis (8, 29). Moreover, based on the solubility of the amino acid and the volume of fluid that can be safely gavaged, this represents the near upper limit for the amount of leucine that can be administered by a single oral gavage. In this study, muscle was sampled 4 h after the final dose of leucine because the plasma leucine concentration is still elevated approximately twofold at this time point (data shown later). Finally, to assess the importance of endogenously produced glucocorticoids, control and septic rats were injected subcutaneously with the glucocorticoid receptor antagonist RU-486 (Mifepristone, 20 mg/kg body wt; Sigma) or an equal volume (0.3 ml) of vehicle 2 h before induction of peritonitis. RU-486 is an antiprogestin with antiglucocorticoid properties. RU-486 has a high affinity for cytosolic type II glucocorticoid receptors in various target tissues and exhibits little agonist activity. The dose of RU-486 used in the present study has been shown to attenuate glucocorticoid-induced increases in catabolism and ameliorate endotoxin- or cytokine-induced changes in the IGF system and muscle protein balance in adult rats (10, 34). Data for each of the catabolic insults was compared with data from appropriate time-matched control animals that were pair fed to match the food consumption of the catabolic group. In all studies, 24 h after induction of sepsis, a blood sample (1 ml) was collected from the abdominal aorta, and gastrocnemius, soleus, and heart (ventricle only) were excised.

Multi-probe template production for RNase protection assay. Primer selection for rat genes of interest was determined with the help of Genesis software (17). Amplified regions of various sizes were selected to allow distinct resolution during electrophoretic separation (Table 1). Primers were synthesized with 5' restriction sites for EcoRI or KpnI, with three extra bases at the extreme 5' end, which are indicated in italics (Table 2) (IDT, Coralville, IA). PCR was conducted using HotStarTag DNA Polymerase (Qiagen, Valencia, CA), and rat total RNA was reverse-transcribed with Superscript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA). PCR products were phenol-chloroform extracted, ethanol precipitated, and sequentially digested with KpnI and EcoRI (Promega, Madison, WI). Digested products were gel-purified, reextracted, and cloned into KpnI/EcoRI-digested pBluescript II SK+ plasmids (Stratagene, La Jolla, CA). Plasmid DNA was isolated with both QIAprep Spin Miniprep and Plasmid Maxi kits (Qiagen). Plasmids were verified by sequencing at the Molecular Genetics Core Facility at the Pennsylvania State College of Medicine. Final constructs were linearized with EcoRI, agarose gel-purified, and quantitated spectrophotometrically.

Table 1. Atro gene RPA template

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession No.</th>
<th>Amplified Region (inclusive)</th>
<th>Probe (bp)</th>
<th>Protected (bp)</th>
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<td>NM_133521</td>
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<tr>
<td>MuRF1</td>
<td>AYO59627</td>
<td>581–944</td>
<td>382</td>
<td>364</td>
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<tr>
<td>L32</td>
<td>NM_013226</td>
<td>19–149</td>
<td>152</td>
<td>131</td>
</tr>
<tr>
<td>GAPDH</td>
<td>BC059110</td>
<td>548–643</td>
<td>117</td>
<td>97</td>
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</table>

RPA, RNase protection assay; MuRF1, muscle RING finger-1.
Table 2. Primer sequences for RPA template

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences</th>
</tr>
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<tr>
<td>Atrogin-1</td>
<td>Forward 5'-GCA GAA TTC TGT GGG TGT ATC GAA TGG AGA CCA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-GCA GGT ACC GTG CAG TGC CCT TCG AAG AGA-3'</td>
</tr>
<tr>
<td>MuRF1</td>
<td>Forward 5'-GCA GAA TTC CAA GGA CAG AAG ACT GAA CTG AGC A-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-GCA GGT ACC TGA TGA GCG GCT TGG CAC TCA-3'</td>
</tr>
<tr>
<td>L32</td>
<td>Forward 5'-GCA GAA TTC CGG CCT CTG GTG AAG CCC AA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-GCA GGT ACC CTT CTC CGC ACC CTG TTG TCG-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward 5'-GCA GAA TTC CGC GCC AAG GTC ATC CAT GAC A-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-GCA GGT ACC GGG GCC ATC CAC AGT CTT CTG-3'</td>
</tr>
</tbody>
</table>

Primers were synthesized with 5' restriction sites for EcoRI or KpnI, with 3 extra bases at the extreme 5' end, which are indicated in italics.

The template was prepared so that a 2-μl aliquot contained 10 ng of each construct.

RNomease protection assay. A 2-μl aliquot of template was labeled using T7 Polynucleotide, RNasin and DName (Promega, Madison, WI), NTPs and rRNA (Sigma), and [32P]UTP (Amersham, Piscataway, NJ). Unless otherwise noted, the entire RNase protection assay (RPA) procedure, including labeling conditions, component concentrations, sample preparation, and gel electrophoresis, was performed according to the manufacturer’s protocol (BD Biosciences Pharmingen, San Diego, CA). Hybridization buffer was 80% formamide and 20% stock buffer (200 mM PIPES, pH 6.4, 2 M NaCl, 5 mM EDTA). Ten micrograms of total RNA were hybridized overnight at 56°C in a dry bath incubator (Fisher Scientific, Pittsburgh, PA) without the use of mineral oil. Samples were treated with RNase A + T1 (Sigma) in 1× RNomease buffer (10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 300 mM NaCl) followed by proteinase K (Fisher Scientific) in 1× proteinase K buffer (50 mM Tris, pH 8.0, 1 mM EDTA, 1% Tween 20). Following ethanol precipitation, samples were resuspended in loading buffer [98% formamide (vol/vol), 0.05% xylene cyanol (wt/vol), 0.05% bromphenol blue (wt/vol), 10 mM EDTA]; 34× 45 cm polyacrylamide gels were run at 75 W for 70 min in an S3S Sequencing System (Owl Separation Systems, Portsmouth, NH), transferred to chromatography paper, and dried for 10 min at 80°C (FG BD 45 Gel Dryer; Fisher Scientific). Gels were exposed to a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA), and the resultant data were quantified using Molecular Dynamics’ ImageQuant software (ImageQuant version 5.2). Although the data were normalized to L32, the same results were obtained if data were normalized to GAPDH (data not shown).

Northern blot analysis of Ub. Total RNA was isolated using TRI Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer’s protocol. Samples (25 μg) of total RNA were run under denaturing conditions in 1.1% agarose/6% formaldehyde gels under denaturing conditions in 1.1% agarose/6% formaldehyde gels using 1× HEPES running buffer. Northern blotting occurred via capillary transfer to Nytran SuPerCharge membranes (Schleicher & Schuell, Keene, NH). A rat oligonucleotide for Ub (5’-GGATCTTG-GCAAGGACTTGGTCATTTGTCATGAGGTC-3’) was synthesized (IDT) and radioactively labeled using TdT (Promega). For normalization of RNA loading, an 18S oligonucleotide was labeled by the same method. Northern blots were hybridized using ULTRAhyb (Ambion, Austin, TX). All membranes were initially washed twice in 2× SSC/0.1% SDS for 5 min at 42°C and once in 0.2× SSC-0.1% SDS for 15 min at 42°C. All probes except 18S were additionally washed with 0.2× SSC-0.1% SDS for 10 min at 48°C. Finally, membranes were exposed to a PhosphorImager screen, visualized, and quantified as described above.

Plasma TNF-α, hormone, and amino acids concentrations. The plasma concentration of corticosterone and insulin was determined by radioimmunoassay (DSL, Webster, TX, and Linco Research, St. Charles, MO, respectively). Amino acid concentrations were determined by reverse-phase HPLC after precolumn derivatization of amino acids with phenylisothiocyanate.

Statistical analysis. Experimental data for each condition are summarized as mean ± SE, and the sample size is indicated in the figure legends. Statistical evaluation of the data from studies with four groups was performed with ANOVA followed by Student-Neuman-Keuls test to determine treatment effect (Instat, San Diego, CA). Differences between the groups were considered significant when P < 0.05.

RESULTS

Endotoxin- and sepsis-induced changes in atro gene mRNA. Low constitutive expression of atrogin-1 mRNA was observed in gastrocnemius of control rats by RPA. The injection of LPS first produced a detectable increase in atrogin-1 mRNA content of gastrocnemius at 8 h (Fig. 1, top and middle). The LPS-induced increase appeared to peak fourfold above time-matched control values at 12 h, and expression remained elevated for ≈24 h. Moreover, the LPS-induced increase in atrogin-1 mRNA was dose dependent (Fig. 1, bottom). A similar time and dose dependency for MuRF1 was also observed in gastrocnemius following LPS (Fig. 2), although the increase in MuRF1 mRNA had returned toward baseline by 24 h. The temporal progression of the sepsis-induced increase in both atrogin-1 and MuRF1 in gastrocnemius was comparable to that seen after LPS (Fig. 3). However, atrogin-1 mRNA content was still elevated in rats 72 h after CLP, whereas the sepsis-induced increase in MuRF1 mRNA was more transient and had declined back to control values between 24 and 72 h.

At the 24-h time point, soleus and cardiac muscles were also sampled from septic and time-matched control rats. Although both atrogin-1 and MuRF1 mRNA transcripts were detected by both RPA and Northern blot analysis in these striated muscles, there was no consistent change in either tissue in response to the septic insult (data not shown).

Sepsis-induced changes in TNF-α, corticosterone, insulin, and amino acids. The plasma concentration of corticosterone in septic rats was increased above values in time-matched control rats by the earliest time point (e.g., 4 h) (Table 3). The glucocorticoid concentration peaked at 24–48 h after CLP and was still elevated at the 72-h time point. In contrast to the sustained increase in glucocorticoids, the plasma TNF-α concentration was first statistically increased at 4 h post-CLP and was still marginally increased at 24 h but was not different from time-matched control values between 48 and 72 h (Table 3). The plasma insulin concentration in control rats gavaged with saline averaged 0.81 ± 0.10 ng/ml, and this value was not
statistically different from that detected in similarly-treated septic rats (0.91 ± 0.08 ng/ml). Moreover, although leucine is known to produce a transient hyperinsulinemia (8), there was no change in the plasma insulin concentration in control (0.79 ± 0.11 ng/ml) and septic (0.85 ± 0.09 ng/ml) rats 4 h after the last dose of leucine.

The plasma amino acid profiles from control and septic rats gavaged with leucine are presented in Table 4. Approximately 24 h after CLP, the plasma concentrations of arginine, asparagine, serine, and threonine were decreased 45–55%. Sepsis also increased tyrosine (25%) and tended to increase phenylalanine (25%), although the latter change failed to achieve statistical significance. There was no statistical change in the concentration of any other amino acid between control and septic rats under basal conditions. In general, these sepsis-induced changes are comparable to those previously reported (27, 53). In control rats gavaged with leucine (e.g., 6 PM and 12 AM on the day of CLP and 6 AM the following morning) and sampled 4 h thereafter, the plasma leucine concentrations were elevated twofold. Conversely, the plasma concentrations of phenylalanine and tyrosine were reduced compared with basal control values (35 and 51%, respectively). Leucine also tended to decrease the concentrations of the other branched-chain amino acids, isoleucine and valine, but these changes failed to achieve statistical significance. The ability of leucine and its keto analog, α-ketoisocaproate (α-KIC), to reduce...
isoleucine and valine has been previously reported in humans (46). There were no other statistically significant changes in control rats administered leucine. Although, in general, comparable changes were observed in septic rats treated with leucine, the leucine-induced decrease in phenylalanine and tyrosine was less in septic rats than in control animals.

Effect of anabolic agents on atrogene expression. IGF-I has been shown to decrease the dexamethasone-induced increase in atrogene expression in C2C12 myocytes (50). To examine whether such a change could be mimicked in vivo, a sustained elevation in circulating IGF-I was produced by the injection of a binary complex consisting of equimolar amounts of IGF-I and IGFBP-3. Such a treatment modality fully reverses the sepsis-induced decrease in the plasma IGF-I concentration (53). In vivo elevation of IGF-I significantly decreased the constitutive expression of atrogin-1 by 55% in gastrocnemius from control rats (Fig. 4, top and middle). Moreover, IGF-I also decreased atrogin-1 mRNA content in septic rats to values not different from basal control values. In contrast, IGF-I did not significantly decrease MuRF1 mRNA in gastrocnemius from either control or septic rats at the time point examined (Fig. 4, bottom).

A separate group of rats was injected intravenously with native IGF-I (200 μg/kg body wt) 24 h after the induction of sepsis and muscle sampled 4 h thereafter (29). This study protocol differs from the previous one in that IGF-I was administered after the septic insult, at a time point where we have previously shown an increased atrogene expression. The 4-h time point was selected because IGF-I has been reported to achieve statistical significance (data not shown).

The branched-chain amino acid leucine also provides a potent anabolic stimulus, at least in part, by increasing muscle protein synthesis (1). However, there are conflicting data as to whether this nutrient-signaling molecule modulates proteolysis (45). Therefore, two studies were performed in which rats were orally gavaged with leucine. In the first study, the ability of leucine to acutely regulate atrogene expression was assessed in control and septic rats gavaged with leucine or vehicle and

Table 3. Plasma concentrations of corticosterone and TNF-α

<table>
<thead>
<tr>
<th>Time-Post Infection, h</th>
<th>Corticosterone, ng/ml</th>
<th>TNF-α, pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Septic</td>
</tr>
<tr>
<td>4</td>
<td>278±31</td>
<td>582±84*</td>
</tr>
<tr>
<td>8</td>
<td>215±23</td>
<td>431±32*</td>
</tr>
<tr>
<td>12</td>
<td>202±17</td>
<td>388±26*</td>
</tr>
<tr>
<td>24</td>
<td>167±21</td>
<td>431±32*</td>
</tr>
<tr>
<td>48</td>
<td>183±22</td>
<td>381±24*</td>
</tr>
<tr>
<td>72</td>
<td>202±18</td>
<td>341±29*</td>
</tr>
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Values are means ± SE; n = 7–8 rats/group at each time point. *P<0.05 compared to time-matched control value.

Table 4. Effect of sepsis and leucine on plasma amino acid concentrations

<table>
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<tr>
<th>Amino Acid</th>
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<th>Sepsis</th>
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<td>Ala</td>
<td>434±34</td>
<td>402±47</td>
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<td>Asn</td>
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<td>64±9*</td>
<td>34±3b</td>
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<tr>
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<td>Gln</td>
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<td>539±53</td>
<td>442±37</td>
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<td>Glu</td>
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<td>Met</td>
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</table>

Values are means ± SE; n = 7–8 rats/group, *P<0.05; the same amino acid values with different letters are significantly different from each other. Amino acid concentrations have units of μmol/L.
muscle sampled 4 h thereafter. This time point was selected on the basis of our previous results, demonstrating the ability of IGF-I to decrease atrogin-1 mRNA at this time. Using this experimental paradigm, we failed to detect a significant leucine effect on the expression of atrogin-1 or MuRF1 in gastrocnemius in either control or septic rats (data not shown). Subsequent experiments were performed in which the gastrocnemius was sampled at either 2 or 8 h after a single oral gavage of leucine \((n = 8 - 9\) rats/group). Leucine also failed to consistently alter the mRNA content for atrogin-1 or MuRF1 at these additional time points in either control or septic rats (data not shown). In a separate study, animals were gavaged at 6 PM on the day of surgery, again at 12 AM, and then for a third time at 6 AM the following morning to produce a sustained elevation in plasma leucine throughout the septic episode. Muscle was sampled 4 h after the final administration of leucine. Again, we were not able to detect a significant leucine effect in either control or septic rats on atrogin mRNA expression (Fig. 5).

We also assessed expression of Ub mRNA in muscle because this transcript reliably and sensitively reflects activation of Ub-proteasome-dependent proteolysis in atrophying skeletal muscle (50). Overall, the sepsis-induced increase in the 2.4-kb mRNA for Ub mRNA, as well as the differential ability of IGF-I and leucine to regulate its expression, was similar to that reported for atrogin-1 (Fig. 6). For example, sepsis increased Ub mRNA in skeletal muscle approximately threefold. Although this increase in muscle from septic rats was reversed by IGF-I, there was no detectable effect of IGF-I on Ub mRNA in muscle sampled 4 h thereafter. This time point was selected on the basis of our previous results, demonstrating the ability of IGF-I to decrease atrogin-1 mRNA at this time. Using this experimental paradigm, we failed to detect a significant leucine effect on the expression of atrogin-1 or MuRF1 in gastrocnemius in either control or septic rats (data not shown). Subsequent experiments were performed in which the gastrocnemius was sampled at either 2 or 8 h after a single oral gavage of leucine \((n = 5\) each). Leucine also failed to consistently alter the mRNA content for atrogin-1 or MuRF1 at these additional time points in either control or septic rats (data not shown). In a separate study, animals were gavaged at 6 PM on the day of surgery, again at 12 AM, and then for a third time at 6 AM the following morning to produce a sustained elevation in plasma leucine throughout the septic episode. Muscle was sampled 4 h after the final administration of leucine. Again, we were not able to detect a significant leucine effect in either control or septic rats on atrogin mRNA expression (Fig. 5).

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Fig. 5. Leucine does not alter the sepsis-induced increase in atrogin-1 and MuRF1 mRNA in skeletal muscle. Atrogin-1 (middle) and MuRF1 (bottom) were determined by RPA in gastrocnemius 24 h after CLP or in time-matched control animals. Leucine was administered by oral gavage at 6 PM on the day of surgery, again at 12 AM, and for a third time at 6 AM the following morning. Muscle was sampled 4 h after the last administration of leucine. Values are expressed as means ± SE; \(n = 7 - 8\) rats/group. Groups with different letters are significantly different from each other \((P < 0.05)\). For all data, mRNA abundance was expressed in AU as determined by densitometry and normalized to L32 mRNA. Top: representative RPA for atrogin-1, MuRF1, and L32 as indicated (note: all 4 experimental groups were run on the same gel, with the clear area of the blot, indicating a blank lane).
gastrocnemius from control rats (Fig. 6, middle). In contrast to
the IGF-I effect, there was no detectable effect of leucine on
Ub mRNA expression in skeletal muscle at the time point
examined (Fig. 6, bottom). No sepsis-induced increase in Ub
mRNA was detected in either heart or soleus muscle (data not
shown).

**Potential role of TNF in regulating E3 ligases.** In the third
experimental protocol rats were infused overnight with a non-
lethal dose of TNF-α, for which the metabolic and hormonal
alterations have been previously reported (33). Gastrocnemius
sampled 24 h after initiating the TNF-α infusion demonstrated
a marked increase in mRNA content for atrogin-1 and MuRF1
(Fig. 7, top and upper middle). However, despite the stimulat-
ing effect of exogenous TNF-α, pretreating rats with TNF$_{bp}$
before CLP to neutralize endogenously secreted TNF-α did not
prevent the sepsis-induced increase in atrogin-1 expression
(Fig. 7, bottom). TNF$_{bp}$ also did not alter the increase in
muscle MuRF1 mRNA produced by sepsis (Fig. 7, lower
middle). The relative ineffectiveness of TNF$_{bp}$ at reversing
the sepsis-induced increase in atrogene expression could not be
accounted for by the inability of this agent to inhibit TNF-
mediated effects (Fig. 7, top; compare TNF-α alone vs.
TNF$_{bp}$ + TNF-α groups).

**Potential role of glucocorticoids in regulating E3 ligases.** In
the final in vivo study, naïve rats were first treated with the
synthetic glucocorticoid dexamethasone. Atrogin-1 mRNA
was modestly increased by 4 h and more substantially elevated
24 h after a single subcutaneous injection of dexamethasone
(Fig. 8, top and upper middle). This response appears largely
mediated via the type II glucocorticoid receptor because it was
agonized by pretreatment of rats with RU-486. In contrast,
treatment of septic rats with RU-486 prior to CLP failed to
attenuate the increase in atrogin-1 mRNA (Fig. 8, bottom).
Comparable findings were observed in muscle from septic
rats ± RU-486 when MuRF1 mRNA content was determined
(data not shown).

**DISCUSSION**

Negative nitrogen balance is a common complication of
sepsis and results from both a decreased rate of protein syn-
thesis and accelerated rate of protein breakdown in skeletal
muscle. The purpose of the present study was to investigate the
in vivo control of potential mechanisms capable of modulating
the latter process by focusing on the regulation of two muscle-
specific E3 ligases believed to be causally linked to muscle
wasting induced by other atrophic stimuli (2, 18, 37, 39, 50).
The present study indicates that the mRNA content for both
atrogin-1 and MuRF1 was upregulated in skeletal muscle by
inflammatory insults in mature rats. When LPS was used as a
model of infection, atrogene expression was upregulated be-
tween 4 and 8 h and the activation was dose-dependent. A
time-dependent effect of LPS on muscle atrogin-1 and MuRF1
mRNA has been previously reported for a 12-h period (9).
Likewise, hypermetabolic sepsis produced by CLP-induced
peritonitis also increased atrogene expression. However, it is
noteworthy that the temporal progression of the sepsis-induced
increase in atrogin-1 and MuRF1 diverged over time. That is,
the increase in atrogin-1 mRNA was maintained for at least
72 h after infection, whereas MuRF1 mRNA content returned
to basal values between 24 and 48 h. These data confirm the
observations of Wray et al. (60), which showed increased
atrogin-1 and MuRF1 mRNA in fast-twitch extensor digitorum
longus muscle 16 h after CLP in studies using juvenile (e.g.,
50–60 g) rats. However, our data extend these original find-
ings in two important ways: 1) our data highlight that the
sepsis-induced increase in atrogene expression was not re-
stricted to muscle of rapidly growing animals; and 2) because of
the transient nature of the change, the muscle wasting
observed in response to a more sustained septic or inflamma-
tory insult is unlikely to be mediated by increased MuRF1. Finally, our data indicate that, at later time points, sepsis produces a discordant regulation of the so-called atrophy-related transcription program (2).

In our study, the upregulation of atrogin-1 and MuRF1 occurred in the gastrocnemius, a muscle with a predominance of fast-twitch type II fibers, but not the soleus, a muscle composed primarily of slow-twitch type I fibers. Similarly, we confirmed that sepsis increases gene expression of Ub in the...
gastrocnemius, and we extend these observations by demonstrating no such increase in soleus muscle. Importantly, the changes in E3 ligases and Ub were associated with the preferential sepsis-induced muscle atrophy seen in the gastrocnemius (20, 58). These results are consistent with previous studies (23, 25, 56) reporting the sepsis-induced increase of protein degradation, Ub mRNA, and other biomarkers of proteolysis that were preferentially or exclusively detected in fast-twitch, not slow-twitch, skeletal muscle. Moreover, the expression of the E3 ligases and Ub in cardiac muscle was not increased by sepsis. Hence, these data indicate a tissue-specific regulation of atrogin-1, MuRF1, and Ub after sepsis and suggest an upregulation of Ub-proteasome-dependent protein degradation. Collectively, these data are consistent with previous studies (16, 26) showing sepsis-induced increases in expression and activity of the 14-kDa Ub-conjugating enzyme E2_L4k and the Ub ligase E3α.

Many of the hormonal and metabolic responses to sepsis and endotoxemia are mediated directly or indirectly via enhanced synthesis and secretion of various inflammatory cytokines, chief among these being TNF-α. In the present study, plasma TNF-α concentrations were elevated 4–24 h after induction of peritonitis, but not at latter time points (e.g., 48–72 h). The production of this particular cytokine can lead to muscle wasting, which is mediated by a fall in protein synthesis (33) and stimulation of protein degradation (15). Previous work reported exogenous TNF-α increases Ub gene expression and Ub-conjugating activity in skeletal muscle under both in vivo and in vitro conditions (16, 42, 43). Our present data revealed that exogenous TNF-α also markedly increases the mRNA content of both atrogin-1 and MuRF1 under in vivo conditions. Although these results clearly indicate the capability of TNF-α to elevate atrogin expression, they do not provide direct evidence for its role in mediating the sepsis-induced increase in either atrogin-1 or MuRF1. To address this caveat we injected rats with TNFα to antagonize endogenously secreted TNF-α. The dose of TNFα used was shown to prevent increased atrogin expression in rats infused with recombinant TNF-α. Moreover, this dose of TNFα has been previously demonstrated to be efficacious in preventing a portion of the sepsis-induced decrease in muscle mass in adult rats (7). However, in the present study, TNFα failed to attenuate the sepsis-induced increase in atrogin expression. These data suggest that the increment in atrogin-1 and MuRF1 mRNA produced acutely by CLP was mediated by a TNF-independent mechanism. Our data do not exclude the possibility that atrogin expression may be driven by TNF-α in other catabolic conditions where the circulating concentration of this cytokine is more dramatically elevated (6).

Sepsis, endotoxin, and inflammatory cytokines also produce a rapid and sustained elevation in the circulating glucocorticoid concentration (31–33), and such an elevation was corroborated in the present study. The catabolic effect of exogenously administered glucocorticoid (e.g., dexamethasone) is well established in skeletal muscle and is in part mediated by an increased rate of degradation (6). Although multiple proteolytic pathways are stimulated, the dexamethasone-induced increase in proteolytic activity is largely prevented by pretreatment with proteasome inhibitors (40). Furthermore, proteasome inhibition also prevents the increased release of 3-methylhistidine, a marker of myofibrillar degradation, in dexamethasone-treated myocytes (54). Moreover, pretreatment of septic rats with the glucocorticoid receptor antagonist RU-486 largely prevents the increased muscle proteolysis and the upregulation of the Ub ligase E3α (14, 55). However, the results of the present study indicate that the sepsis-induced increase in muscle atrogin-1 and MuRF1 is not mediated by increased secretion of endogenous glucocorticoids and suggests that the increased rate of muscle proteolysis observed in adult septic rats is glucocorticoid independent. Since indexes of muscle wasting (e.g., mass or protein content) were not directly assessed in the present study, we cannot speculate on the overall effectiveness of RU-486 in preventing sepsis-induced muscle catabolism in adult rats. Although the exact reason for this apparent discrepancy is not known, studies showing a glucocorticoid-dependent increase in atrogin expression after sepsis used smaller, immature rats (40–60 g) compared with the present study, which used adult rats (>325 g), and demonstrated that the sepsis-induced increase in atrogin-1 and MuRF1 was glucocorticoid independent. In this regard, the rate of myofibrillar breakdown has been reported to be threefold greater in immature rats compared with rates in mature animals (62). Alternatively, it has been posited that elevations in endogenous glucocorticoids may require a second signal, such as low insulin, to activate muscle proteolysis (38).

Although the circulating insulin concentration was not different between our control and septic rats, we cannot exclude the possibility that such a permissive effect might be mediated by the presence of muscle insulin resistance, which is produced by a relative as opposed to an absolute insulinopenic condition (28). Finally, our data are consistent with the glucocorticoid-independent activation of the Ub system observed in response to cancer cachexia (44).

The anabolic hormone IGF-I is important for the normal accretion of muscle protein and mass. The circulating concentration, as well as the muscle content of IGF-I, is markedly decreased by sepsis, LPS, and inflammatory cytokines (31–33). Such a reduction in the bioavailable endogenous IGF-I would increase muscle proteolysis, whereas exogenous administration should decrease proteolysis (35). Specifically, exogenous administration of IGF-I inhibits breakdown of myofibrillar proteins under both in vitro and in vivo conditions (11, 12). Moreover, IGF-I impairs protein degradation by inhibiting multiple proteolytic pathways, including the Ub-proteasome system (40, 50). Our data indicate that, in control rats, the in vivo administration of IGF-I, either as part of a binary complex with IGFBP-3 or as the native peptide, preferentially decreased the mRNA content of atrogin-1 but not MuRF1. A similar effect has been reported in C2C12 myocytes cultured with IGF-I, which activates the PI3K/Akt pathway, thereby resulting in the phosphorylation and inhibition of the forkhead transcription factor (FOXO) family of transcription factors as well as a FOXO-independent Akt/mTOR-dependent pathway (36, 51). It is also noteworthy that a sustained elevation in plasma IGF-I produced by repeated injection of IGF-I/IGFBP-3 complex differentially affected atrogin expression. In this regard, the binary complex prevented the sepsis-induced increase in atrogin-1, but in contrast, MuRF1 mRNA remained increased. The molecular mechanism by which IGF-I differentially regulates atrogin-1 and MuRF1 expression remains to be elucidated. We also showed that native IGF-I (e.g., IGF-I not complexed with IGFBP-3) was administered 24 h after
CLP acutely (e.g., within 4 h) and dominantly downregulates the elevated atrogin-1 mRNA content. These data do not support the observed IGF-I unresponsive state reported in immature septic rats (11). Together, our data suggest that the therapeutic efficacy of IGF-I was maintained in adult septic rats regardless of the duration between the onset of infection and the time of treatment.

Leucine is a central nutritional regulator of muscle protein balance specifically stimulating translational control of protein synthesis (45). Moreover, leucine and IGF-I mediate their metabolic effects by sharing many of the same distal signaling elements (1). Although there are exceptions in the literature, the consensus is that branched-chain amino acids in general and leucine in particular decrease muscle proteolysis under both in vivo and in vitro conditions (4, 45, 48). Moreover, leucine also dose-dependently inhibits chymotrypsin-like activity of the proteasome (21). Hence, we anticipated that leucine, similar to IGF-I, would downregulate the expression of atrogin-1 and possibly MuRF1. However, using two experimental paradigms where the plasma leucine concentration was either acutely or repetitively elevated, we were unable to detect a consistent leucine-induced decrease in either constitutive or sepsis-induced atrogene expression. The oral gavage of leucine produced a relatively prolonged increase in the plasma leucine concentration that ranged between ~10-fold above control values at 20 min and twofold above control at 4 h (8, 30, and present data). Although we cannot exclude the possibility of a transient leucine effect on atrogene expression, this possibility seems unlikely because muscle sampled at 2 and 8 h after leucine administration also failed to show a detectable change in either atrogin-1 or MuRF1 mRNA. Hence, the ability of leucine to inhibit muscle proteolysis in rats was not associated with a sustained downregulation of atrogin-1 or MuRF1 mRNA. Although muscle proteolysis per se was not directly assessed in the present study, the ability of leucine to ameliorate a portion of the sepsis-induced increase in the plasma concentration of both phenylalanine and tyrosine implies that leucine did inhibit whole-body proteolysis. Previous studies (57) have shown that the transamination of leucine to its keto acid is essential for the inhibitory effect of this amino acid. However, the intracellular α-KIC content was not determined in the present study. Finally, our data also suggest that the mechanism by which IGF-I decreases atrogin-1 mRNA is mediated at or before the level of PI3K and PKB, which are both activated by IGF-I but not by leucine under in vivo conditions (1, 29). This conclusion is consistent with findings from in vitro studies showing that inhibition of PI3K prevents the ability of IGF-I to decrease atrogin-1 (50) and that adenoviral infection of myocytes with a dominant-negative PI3K suppresses the ability of insulin to inhibit protein degradation (39).

Overall, the present investigation advances our understanding by providing novel data pertaining to the in vivo regulation of atrogene expression by catabolic and anabolic stimuli under basal and septic conditions in mature rats. In this regard, sepsis rapidly increases both atrogin-1 and MuRF1 mRNA, but only the expression of the former transcript is sustained for several days, suggesting its relative importance and contribution toward muscle proteolysis. Moreover, the sepsis-induced increase in atrogene mRNA was not observed in soleus or heart, striated muscles that show no or limited changes in protein content during sepsis. Our data indicate that exogenous IGF-I markedly downregulates atrogin-1 mRNA and, in conjunction with its known ability to increase protein synthesis and control stress-induced hyperglycemia, supports the therapeutic use of this peptide. In contrast, leucine does not appear to modulate muscle proteolysis via alterations in either atrogin-1 or MuRF1. Finally, it is recognized that one limitation of the present studies is that muscle proteolysis was not directly quantitated. Therefore, although the physiological relevance of the sepsis-induced increase in muscle atrogene expression remains to be assessed, our data suggest that this response in adult rats is independent of endogenous increases in both TNF-α and glucocorticoids functioning as extracellular signals (61).

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