Sepsis in mice stimulates muscle proteolysis in the absence of IL-6

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Williams, Arthur, Jing Jing Wang, Li Wang, Xiaoyan Sun, Josef E. Fischer, and Per-Olof Hasselgren. Sepsis in mice stimulates muscle proteolysis in the absence of IL-6. *Am. J. Physiol.* 275 (*Regulatory Integrative Comp. Physiol.* 44): R1983–R1991, 1998.—We tested the role of interleukin-6 (IL-6) in sepsis-induced muscle proteolysis by determining ubiquitin mRNA levels and protein breakdown rates in incubated extensor digitorum longus muscles from septic and sham-operated IL-6 knockout and wild-type mice. In addition, the effect of treatment of mice with human recombinant IL-6 on muscle protein breakdown rates was determined. Finally, protein breakdown rates were measured in myotubes treated for up to 48 h with different concentrations of IL-6. Sepsis in wild-type mice resulted in an approximately ninefold increase in plasma IL-6 levels, whereas IL-6 was not detectable in plasma of sham-operated or septic IL-6 knockout mice. Total and myofibrillar muscle protein breakdown rates were increased by ~30% and threefold, respectively, in septic IL-6 wild-type mice with an almost identical response noted in septic IL-6 knockout mice. Ubiquitin mRNA levels determined by dot blot analysis were increased during sepsis in muscles from both IL-6 knockout and wild-type mice, although the increase was less pronounced in IL-6 knockout than in wild-type mice. Treatment of normal mice or of cultured L6 myotubes with IL-6 did not influence protein breakdown rates. The present results suggest that IL-6 does not regulate muscle proteolysis during sepsis.

protein breakdown; ubiquitin; interleukin-6 knockout mice; cytokines; myofibrillar proteins; myotubes

SEPSIS IS ASSOCIATED WITH a pronounced catabolic response in skeletal muscle (3, 32). In severe and protracted sepsis, muscle breakdown results in muscle wasting and fatigue, impairing the recovery and ambulation in these patients with increased risk for pulmonary and thromboembolic complications. A better understanding of the mediators and mechanisms of sepsis-induced muscle catabolism, therefore, is of great clinical significance.

The catabolic response in skeletal muscle during sepsis is mainly caused by increased protein breakdown, in particular myofibrillar protein breakdown (17). Recent studies from our laboratory suggest that ubiquitin-proteasome-dependent protein degradation accounts for most of the sepsis-induced muscle proteolysis (18, 30–33). In other studies we found evidence that the catabolic response in skeletal muscle during sepsis is regulated by multiple mediators, including interleukin-1 (IL-1) (40), tumor necrosis factor (TNF) (39), and glucocorticoids (14, 30). Most of the catabolic effects of TNF are secondary to glucocorticoids, whereas IL-1 stimulates muscle protein breakdown through a glucocorticoid-independent mechanism (38).

In addition to IL-1 and TNF, IL-6 is another proinflammatory cytokine involved in the metabolic and endocrinological responses to sepsis (26). The role of IL-6 in the regulation of protein turnover in the liver is well established (9). In contrast, the role of IL-6 in the regulation of muscle proteolysis is controversial. Previous reports of muscle atrophy in IL-6 transgenic mice (34) and of IL-6-induced protein degradation in cultured myotubes (4) were interpreted as being consistent with a catabolic effect of IL-6. Reduced loss of carcass weight after treatment of tumor-bearing mice with anti-IL-6 antibody seemed to further support a role of IL-6 in the regulation of muscle protein breakdown (27). In contrast, in other studies, IL-6 did not stimulate muscle protein degradation in vitro (8) and did not induce a catabolic state in vivo (5). No experiments have been reported in which the role of IL-6 in sepsis-induced muscle proteolysis was determined.

In the present study, we tested the hypothesis that IL-6 participates in the regulation of muscle proteolysis during sepsis. This was done by measuring ubiquitin mRNA levels and protein breakdown rates in muscles from septic IL-6 knockout mice. The regulation of muscle protein breakdown by IL-6 was further examined by treating normal mice or cultured L6 myotubes with the cytokine. We found that sepsis increased the expression of ubiquitin mRNA and stimulated muscle proteolysis in IL-6 knockout mice in the absence of detectable plasma IL-6 levels and that treatment of normal mice or myotubes with IL-6 did not induce protein breakdown. Thus IL-6 does not seem to be a regulator of muscle protein degradation, at least not under these circumstances.

MATERIALS AND METHODS

Experimental animals. Four series of experiments were performed. In the first series of experiments, the influence of sepsis on muscle ubiquitin mRNA levels and protein breakdown rates in IL-6 knockout mice was examined. Male B6, 129 IL-6 knockout mice (IL-6 −/−) and corresponding wild-type mice (IL-6 +/+) were purchased from Jackson Laboratory (Bangor, ME). The mice were allowed to acclimatize in a room with a 12:12-h light-dark cycle and a temperature of 25°C for 5–7 days before experiments. With mice under pentobarbital sodium anesthesia (50 mg/kg ip), sepsis was induced by cecal ligation and puncture (CLP) as described previously (23). Control mice underwent sham operation, i.e.,

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laparotomy and manipulation, but no ligation or puncture, of the cecum. All mice were resuscitated with 100 ml/kg of normal saline administered subcutaneously on the back at the time of surgery. The mice had free access to water, but food was withheld after the surgical procedures to avoid any influence of differences in food intake between the groups of mice on muscle protein metabolism. Sixteen hours after CLP or the sham operation, mice were anesthetized with pentobarbital sodium (50 mg/kg ip) and the extensor digitorum longus (EDL) muscles were gently dissected with intact tendons for measurement of protein breakdown rates in vitro or for determination of ubiquitin mRNA levels. Blood was obtained by cardiac puncture for determination of plasma IL-6 levels by ELISA (R&D Systems, Minneapolis, MN). The time point for performing metabolic studies was based on results in previous studies in which muscle protein breakdown rates were consistently elevated 16 h after CLP in rats (17, 31). Protein breakdown rates and ubiquitin mRNA levels were measured in EDL muscles because in previous reports the catabolic response to sepsis was particularly pronounced in white, fast-twitch skeletal muscle (17, 33).

Because other studies suggest that the role of IL-6 in the inflammatory response to sepsis-endotoxemia and to a sterile turpentine abscess may be different (7, 20), a second series of experiments was performed in which 200 μl of steam-distilled turpentine were injected subcutaneously on the back of wild-type and IL-6 knockout mice. Control mice were injected with corresponding volumes of sterile saline. Food was withheld, but the mice had free access to drinking water after injection of turpentine or saline. Sixteen hours after treatment, EDL muscles were harvested for measurement of protein breakdown rates in vitro and blood was collected for determination of plasma IL-6 levels by ELISA as previously described.

In a third series of experiments normal male A/J mice (20–25 g, Jackson Laboratory) were treated with human recombinant IL-6 (rIL-6, R&D Systems) injected with doses and intervals as described in RESULTS. Control mice were injected with corresponding volumes of solvent (phosphate-buffered saline, pH 7.4). All mice were fasted but had free access to water after the first IL-6 injection to avoid the influence on metabolic changes of reduced food intake caused by IL-6. EDL muscles were harvested as described previously for measurement of protein breakdown rates in vitro. Blood was obtained by cardiac puncture for determination of plasma IL-6 by ELISA as previously described. Because in a recent report from our laboratory treatment of mice with IL-6 resulted in increased protein synthesis in liver and intestinal mucosa (36), protein synthesis was measured in these tissues in separate groups of mice to provide a "positive control" for IL-6 injection.

All animals were cared for in accordance with the National Research Council *Guide for the Care and Use of Laboratory Animals*. The experimental protocols were approved by the University of Cincinnati Institutional Animal Care and Use Committee.

Ubiquitin mRNA levels. Tissue levels of ubiquitin mRNA were determined by dot blot analysis. Total RNA was extracted by the acid guanidinium thiocyanate-phenol-chloroform method (1), using an RNA Stat-60 kit (Tel-Test "B," Friendswood, TX). Because of the small size of the muscles, both EDL muscles from three or four mice were pooled for each experiment. For dot blot analysis, 20 μl (40 μg) of RNA solution were mixed with 80 μl of 37% formaldehyde and 140 μl of 20× saline-sodium citrate (SSC; 1× SSC, 0.15 M NaCl and 15 mM Na-citrate) and denatured at 60°C for 15 min. Aliquots containing 2.5, 5, and 10 μg RNA were then loaded onto magna charge nylon membrane (Micron Separations, Westborough, MA) in a dot blot filtration manifold apparatus, immobilized under ultraviolet light (302 nm for 30 s), and hybridized for 16 h at 42°C with a ^{32}P-labeled ubiquitin cDNA probe generated by polymerase chain reaction as described previously (31). Hybridization was performed in 5× saline-sodium phosphate EDTA buffer (SSPE; 1× SSPE, 0.15 M NaCl, 10 mM NaH_2PO_4, and 1 M EDTA), 1% SDS, 100 μg/ml denatured salmon sperm DNA, 50% deionized formamide, and 5× Denhardt's solution. After hybridization the membranes were washed twice with 2× SSPE-0.5% SDS at 44°C, three times with 1× SSPE-0.1% SDS at 50°C, and three times with 0.5× SSPE-0.1% SDS at 55°C and exposed to phosphor screen for quantification of signal intensity in a PhosphorImager SF (Molecular Dynamics, Sunnyvale, CA). The same membranes were then rehybridized with a mouse 18S ribosomal oligonucleotide probe and processed as above. Ubiquitin mRNA signals were normalized to 18S signals on the same blot.

Muscle incubations. Individual muscles were preincubated for 30 min and then incubated for 2 h in 1.5 ml oxygenated Krebs-Henseleit bicarbonate buffer (pH 7.4) containing 10 mM glucose and 0.5 mM cycloheximide. Cycloheximide was added to the incubation medium to prevent reincorporation of amino acids released during proteolysis. Total and myofibrillar protein breakdown rates were determined as net release during the 2-h incubation of tyrosine and 3-methylhistidine (3-MH), respectively, taking changes in tissue levels of free tyrosine and 3-MH into account as described in detail previously (17, 31). The in vitro method used here to measure total and myofibrillar protein breakdown rates in incubated muscles was characterized in a number of previous reports from our and other laboratories (10, 16, 17, 31). It should be noted that because protein breakdown rates were measured in vitro, results should be interpreted with caution. Previous studies have shown, however, that changes induced by various pathophysiological conditions, including sepsis, are adequately reflected by changes observed in incubated rodent muscles (13). One advantage of the present in vitro technique is the fact that muscle protein turnover rates are measured under standardized and well-controlled conditions with regard to concentrations of electrolytes and glucose, pH, temperature, and oxygenation. In addition, protein breakdown is measured directly in incubated muscles, whereas in vivo protein breakdown rates need to be calculated based on other measurements (such as protein synthesis and changes in muscle protein content over a certain period of time). The pros and cons of assessing sepsis-induced changes in muscle protein turnover in vitro or in vivo were reviewed previously (15).

Protein synthesis in vivo. Protein synthesis in liver and jejunal mucosa was determined by measuring incorporation of [^{3}H]phenylalanine into protein after the intraperitoneal injection of a flooding dose of the amino acid (4 μCi [^{3}H]phenylalanine and 40 μmol of unlabeled phenylalanine dissolved in 0.4 ml of sterile saline per mouse) as described in detail previously (36). Exactly 15 min after injection of the isotope, a 10-cm segment of the jejunum was excised and immediately flushed with ice-cold saline to halt protein synthesis. The intestine was opened along the antimesenteric border, and mucosa was harvested with a microscope slide. The mucosa and a specimen from the left liver lobe were frozen in liquid nitrogen and stored at −70°C until analysis. The amount of radioactivity incorporated into TCA-precipitated proteins was determined as described previously (36). Because with the present method the specific radioactivity of the precursor amino acid equilibrates rapidly between the intra- and extracellular compartments and remains stable during the first

15–20 min after injection (36), differences in the amount of radioactivity incorporated into protein between different groups of mice reflect differences in protein synthesis rates rather than changes in specific activity of the precursor amino acid.

Cultured L6 myotubes. In a fourth series of experiments, cultured L6 myotubes were treated with different concentrations of human rIL-6. L6 rat skeletal muscle cells were purchased from the American Type Culture Collection (Rockville, MD). The cells were thawed and maintained by repeated subculturing at low density on 10-cm culture dishes and were used between *passages 2* and *8*. Cells were grown in DMEM (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS; Sigma Chemical, St. Louis, MO), 100 U/ml of penicillin, 100 μg/ml of streptomycin, 44 mM $NaHCO_3$, and 110 μg/ml of sodium pyruvate in a humidified atmosphere of 10% CO_2 and 90% air at 37°C. When the cells were ~80% confluent, they were removed by trypsinization [0.25% trypsin in calcium- and magnesium-free Hanks' balanced salt solution (HBSS)] and transferred to 12-well culture plates (2.5×10^4 cells/cm^2). The cells were grown in the presence of 10% FBS until they reached confluence, at which time the medium was replaced with DMEM containing 2% FBS for induction of differentiation. After 3 days, when ~90% of the cells had formed myotubes, the cells were treated with 10 μM cytosine arabinoside for 48 h to remove any remaining dividing myoblasts.

Protein breakdown was measured by determining the release of TCA-soluble radioactivity from proteins labeled with L-[3,5-^{3}H]tyrosine (New England Nuclear, Boston, MA) as described previously by Hong and Forsberg (19). After differentiation, cytosine arabinoside was removed and cell proteins were labeled with 1 μCi of L-[3,5-^{3}H]tyrosine for 48 h in DMEM containing 2% FBS. Cells were washed with HBSS and transferred to nonradioactive medium containing 2 mM tyrosine. The cells were treated with different concentrations (up to 2,000 ng/ml) of human rIL-6 for 6, 24, or 48 h. At the end of the experiment, culture medium was transferred to a microcentrifuge tube containing 100 μl of bovine serum albumin (10 mg/ml). TCA was added to a final concentration of 10%, and after incubation at 4°C for 1 h, samples were centrifuged for 5 min. The precipitates were dissolved with tissue solubilizer. The myotubes were washed with ice-cold phosphate-buffered saline and solubilized with 0.5 M NaOH containing 0.1% Triton X-100. Radioactivity in the myotubes and TCA-soluble and -insoluble radioactivity in the medium were measured in a Beckman scintillation counter. Protein degradation (%) was calculated as the TCA-soluble radioactivity in the medium divided by the TCA-soluble and -insoluble radioactivity in the medium and the myotube radioactivity times 100 (19).

Statistics. Results are reported as means ± SE. Student's *t*-test or analysis of variance followed by Tukey's test was used for statistical analysis.

RESULTS

In the first series of experiments, sepsis was induced in IL-6 knockout mice (IL-6 −/−) and corresponding wild-type mice (IL-6 +/+). Sixteen hours after CLP, plasma levels of IL-6 in wild-type mice were increased approximately ninefold above the levels in sham-operated mice (from 5.71 ± 2.49 to 52.5 ± 1.79 ng/ml, $n = 7$ in each group, $P < 0.01$), whereas IL-6 was not detectable in plasma of sham-operated or septic IL-6 knockout mice. Mortality rate 16 h after CLP was identical (8%) in wild-type and IL-6 knockout mice. No animals died after sham operation in either group of mice.

Total and myofibrillar protein breakdown rates were increased by ~30% and threefold, respectively, in septic IL-6 wild-type mice (Fig. 1), similar to the response to sepsis in rats (17, 18, 31, 40). The increase in protein breakdown rates in septic IL-6 knockout mice was almost identical to that seen in the wild-type mice (Fig. 1). No significant differences in total or myofibrillar protein breakdown rates were noted between corresponding groups of IL-6 knockout and wild-type mice.

Because the plasma IL-6 levels noted here in the sham-operated wild-type mice were relatively high, a control experiment was performed to test whether the sham procedure (i.e., laparotomy followed by 16 h of fasting) induced IL-6 release and a catabolic response in muscle. IL-6 levels and muscle protein breakdown rates were measured in untreated, fed, wild-type mice

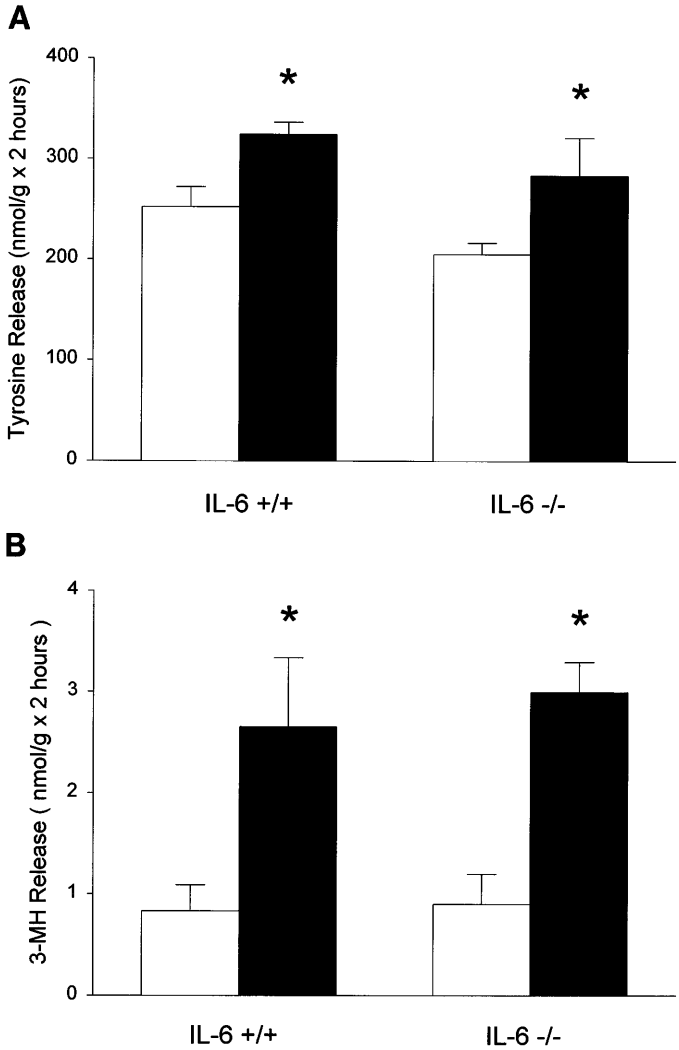

Fig. 1. Total (*A*) and myofibrillar (*B*) protein breakdown rates in incubated extensor digitorum longus (EDL) muscles of wild-type interleukin-6 (IL-6 +/+) and IL-6 knockout (IL-6 −/−) mice. Groups of mice underwent sham operation (open bars) or cecal ligation and puncture (CLP; filled bars) 16 h before muscle incubations. 3-MH, 3-methylhistidine. *$P < 0.05$ vs. sham operation by ANOVA; n = 8–10 in each group.

and in wild-type mice 16 h after sham operation or CLP. Plasma IL-6 levels were 0.3 ± 0.12 ng/ml in fed, untreated mice (n = 7), 9.7 ± 0.5 ng/ml in sham-operated mice (n = 6, $P < 0.05$ vs. fed mice), and 55.4 ± 4.0 ng/ml in septic mice (n = 6, $P < 0.05$ vs. fed and sham-operated mice). Total protein breakdown rates were almost identical in muscles from fed (407 ± 35 nmol tyrosine·g^{-1}·2 h^{-1}, n = 6) and sham-operated (384 ± 49 nmol tyrosine·g^{-1}·2 h^{-1}, n = 6) mice and were increased to 577 ± 47 nmol tyrosine·g^{-1}·2 h^{-1} (n = 6, $P < 0.05$) in muscles from septic mice. Thus, although the sham-procedure resulted in increased plasma IL-6 levels, it did not induce increased muscle proteolysis.

Representative dot blots illustrating the expression of ubiquitin mRNA in EDL muscles from sham-operated and septic wild-type and IL-6 knockout mice are shown in Fig. 2*A*. Densities of ubiquitin mRNA and 18S rRNA hybridization signals were linearly related to total RNA applied, and the mean of ubiquitin-18S signal density ratios measured for 2.5, 5, and 10 µg total RNA blots was used as the normalized value of ubiquitin mRNA for each sample.

Muscle ubiquitin mRNA levels were increased approximately fivefold in septic wild-type mice compared with sham-operated wild-type mice, and were almost doubled in septic IL-6 knockout mice (Fig 2*B*). The ubiquitin mRNA levels were significantly lower in muscles from septic IL-6 knockout mice than in muscles from septic wild-type mice. There was no significant difference in ubiquitin mRNA levels between sham-operated wild-type and sham-operated knockout mice.

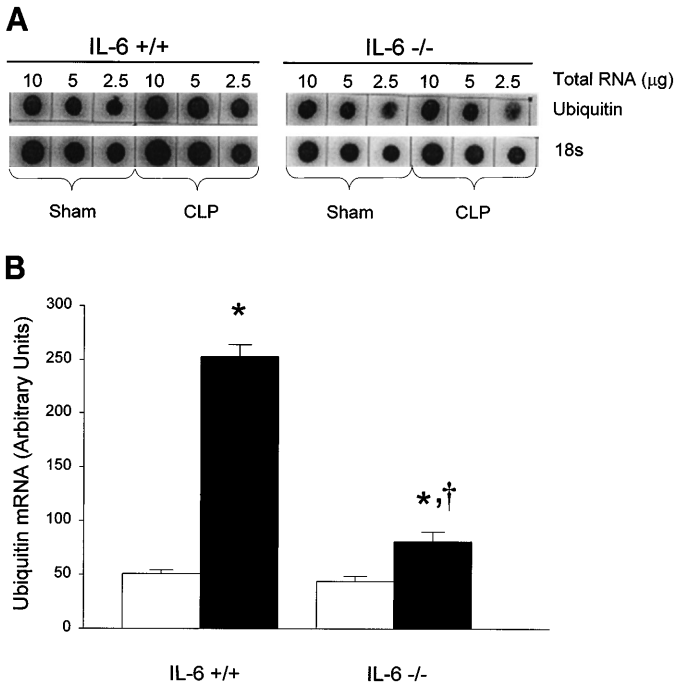

Fig. 2. *A*: representative dot blots, illustrating expression of ubiquitin mRNA in EDL muscles from sham-operated and septic wild-type (IL-6 +/+) and IL-6 knockout (IL-6 −/−) mice. *B*: densities of ubiquitin mRNA and 18S rRNA were linearly related to total RNA applied and mean of ubiquitin-18S signal density ratios measured for 2.5, 5, and 10 µg total RNA blots was used as normalized value of ubiquitin mRNA for each sample. Results are means ± SE; n = 3 in each group and both EDL muscles from 3 or 4 mice were pooled for each sample. Open bars, sham-operated mice; filled bars, septic mice 16 h after CLP. *$P < 0.05$ vs. corresponding sham group; †$P < 0.05$ vs. septic IL-6 +/+.

In a second series of experiments mice were injected subcutaneously with sterile turpentine or saline, and plasma IL-6 levels and muscle protein breakdown rates were measured 16 h later. In wild-type mice turpentine injection resulted in increased plasma IL-6 levels (from 5.76 ± 2.62 to 599 ± 138 pg/ml, $P < 0.05$) but did not influence total muscle protein breakdown rates (212 ± 14 and 214 ± 17 nmol tyrosine·g^{-1}·2 h^{-1} in saline- and turpentine-injected mice, respectively). IL-6 was not detectable in plasma from saline- or turpentine-injected IL-6 knockout mice, and total muscle protein breakdown rates were unaffected by turpentine injection in these mice as well (296 ± 41 and 303 ± 15 nmol tyrosine·g^{-1}·2 h^{-1} in saline- and turpentine-injected IL-6 knockout mice, respectively). Thus the second series of experiments could not resolve the question of whether the role of IL-6 in the catabolic response to a sterile turpentine abscess is different from the role of IL-6 in sepsis-induced muscle breakdown. The results showed, however, that the increased plasma IL-6 levels in turpentine-injected wild-type mice were not associated with increased muscle protein breakdown rates.

Although the results described strongly suggest that sepsis-induced increase in muscle proteolysis can occur in the absence of IL-6 and that increased plasma IL-6 levels are not always associated with increased muscle protein breakdown, the results do not rule out the possibility that IL-6 can stimulate muscle proteolysis. To further test the role of IL-6 in the regulation of muscle protein breakdown, a third series of experiments was performed in which mice were treated with three intraperitoneal injections of rIL-6. Each injection, given in 8-h intervals, provided 100 µg/kg of the cytokine. Metabolic studies were performed 24 h after the first cytokine injection. This protocol was identical to that used in a recent study from our laboratory in which protein synthesis was increased in liver and jejunal mucosa 24 h after the first IL-6 injection (36). Treatment of mice with IL-6 did not influence total or myofibrillar protein breakdown rates determined as net release of tyrosine and 3-MH, respectively, in incubated EDL muscles (Fig. 3). The higher myofibrillar protein breakdown rates in control mice noted in this experiment compared with the first series of experiments (compare with Fig. 1) may reflect the different strains of mice used in the two experiments. In addition, the difference may reflect the longer period of fasting that animals were subjected to in the third series (24 h) than in the first series (16 h) of experiments. The difference may also reflect the day-to-day variation in protein breakdown rates that is typically seen with the present in vitro technique and which makes it necessary to include different groups of mice on the same day when comparisons between groups are

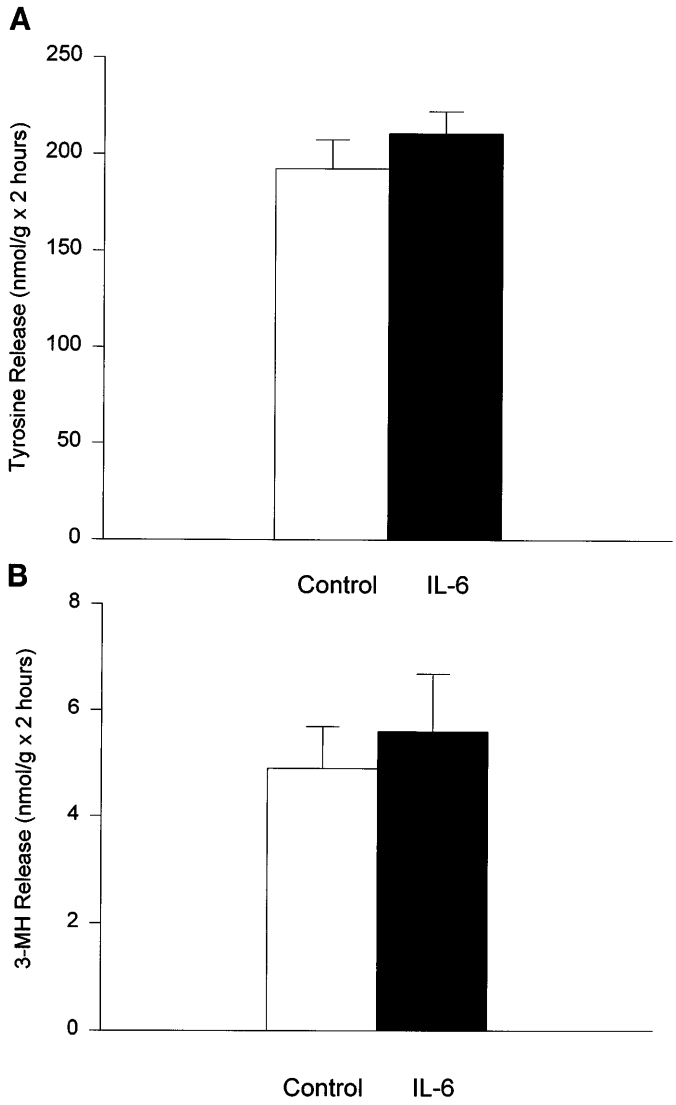

Fig. 3. Total (*A*) and myofibrillar (*B*) protein breakdown rates in incubated EDL muscles from control (open bars) and IL-6-injected mice (filled bars). Mice were treated with 3 doses of human recombinant (r) IL-6 (each dose 100 μg/kg ip) with 8-h intervals; $n = 6$ for each group. Control mice received corresponding injections with solvent. Metabolic studies were performed 24 h after the first control or IL-6 injection.

made. In the present study the different groups of mice within each experiment were always studied on the same day.

To make certain that the human IL-6 preparation used here was active in mice, we measured protein synthesis in liver and jejunal mucosa. Protein synthesis was increased in liver and jejunal mucosa of IL-6-treated mice (Fig. 4). Thus it is not likely that the lack of effect of IL-6 on muscle protein breakdown was caused by an inactive rIL-6 preparation or by the fact that a human cytokine was used in mice.

Another reason why treatment of the mice with IL-6 did not stimulate muscle proteolysis could be that the plasma levels of IL-6 were too low and did not reach plasma levels seen during sepsis. As seen in Fig. 5, plasma levels of IL-6 reached levels similar to those

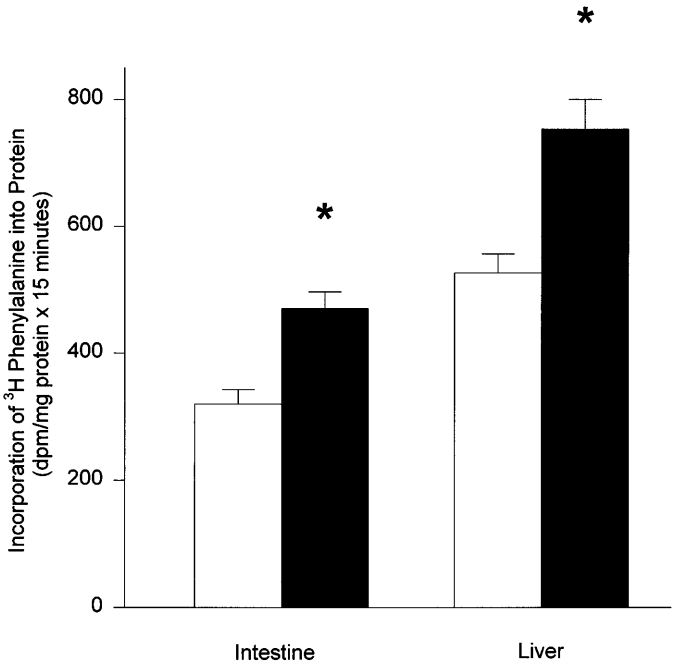

Fig. 4. Protein synthesis in jejunal mucosa and liver of control (open bars) and IL-6-injected (filled bars) mice. Experimental protocol was identical to that in Fig. 3. $*P < 0.05$ vs. control; $n = 6$ for each group.

observed in this study 16 h after CLP (see above) and to those in another study from our laboratory in which IL-6 levels were measured at multiple time points after CLP in mice (23). However, these high levels of IL-6 were seen only early (1 h) after each IL-6 injection, and

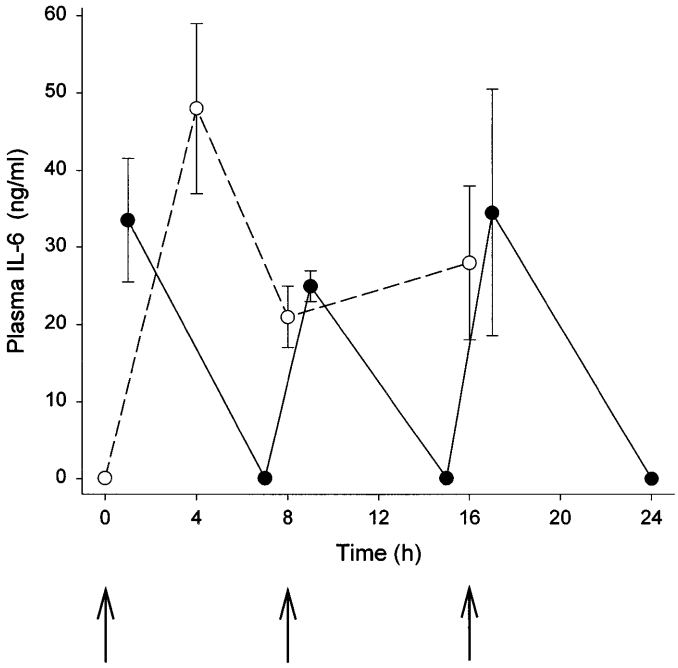

Fig. 5. Plasma IL-6 levels in mice injected with 3 intraperitoneal doses of 100 μg/kg of human rIL-6 (solid line; time points of IL-6 injection indicated by arrows). For comparison, plasma IL-6 levels in septic mice (sepsis induced by CLP) reported in previous study from this laboratory (23) have been plotted (dashed lines); $n \geq 3$ for each data point.

IL-6 levels decreased to control levels 1 h before each subsequent cytokine injection.

It should be noted that plasma IL-6 levels in the present experimental protocol were reduced to basal levels when muscles were harvested for in vitro incubation, i.e., 8 h after the last cytokine injection (Fig. 5). Thus an additional reason why muscle protein breakdown rates were not increased in the present experiments may be that the interval between the last IL-6 injection and muscle incubation was too long. Indeed, in a study by Goodman (12), the time interval between IL-6 injection and measurement of muscle protein degradation was found to be critical. Thus, in that study, the intraperitoneal injection in rats of IL-6 did not result in increased muscle protein degradation when the time interval between the cytokine injection and muscle incubation was 12 h. However, a small but significant increase was noted in total and myofibrillar protein breakdown rates (15 and 40%, respectively) in incubated EDL muscles 3 h after the last of two IL-6 injections of 125 μg/kg each (12). When we used an experimental protocol identical to that reported by Goodman (12), i.e., mice were injected twice with 125 μg/kg of IL-6 intraperitoneally and protein breakdown rates were measured in incubated EDL muscles 3 h after the last cytokine injection, IL-6 did not stimulate total or myofibrillar muscle protein breakdown; total protein breakdown rates were 332 ± 17 and 364 ± 20 nmol tyrosine·g^{-1}·2 h^{-1} in saline- and IL-6-injected mice, respectively (not significant, NS). The corresponding myofibrillar protein breakdown rates were 3.5 ± 0.5 and 3.9 ± 0.7 nmol 3-MH·g^{-1}·2 h^{-1} (NS, $n = 7$ in each group).

In a recent study by Ebisui et al. (4), treatment of cultured C2C12 myotubes with IL-6 was reported to stimulate protein degradation. Because the increase in protein degradation was small (6%) and because in other reports conflicting results were observed with no evidence for a direct effect of IL-6 on muscle protein breakdown (8, 12), we examined the effect of different concentrations (up to 2,000 ng/ml) of IL-6 on protein degradation in cultured L6 myotubes. Protein degradation in the myotubes was not affected by IL-6 at any of the concentrations tested and when treated with the cytokine for up to 48 h (Fig. 6). It should be noted that the highest IL-6 concentration tested here was 40–50 times higher than the plasma concentrations that were seen in septic mice (present study and Ref. 23) and in mice treated with intraperitoneal injections of IL-6 (see Fig. 5) and approximately 10 times higher than the concentration used in the study by Ebisui et al. (4).

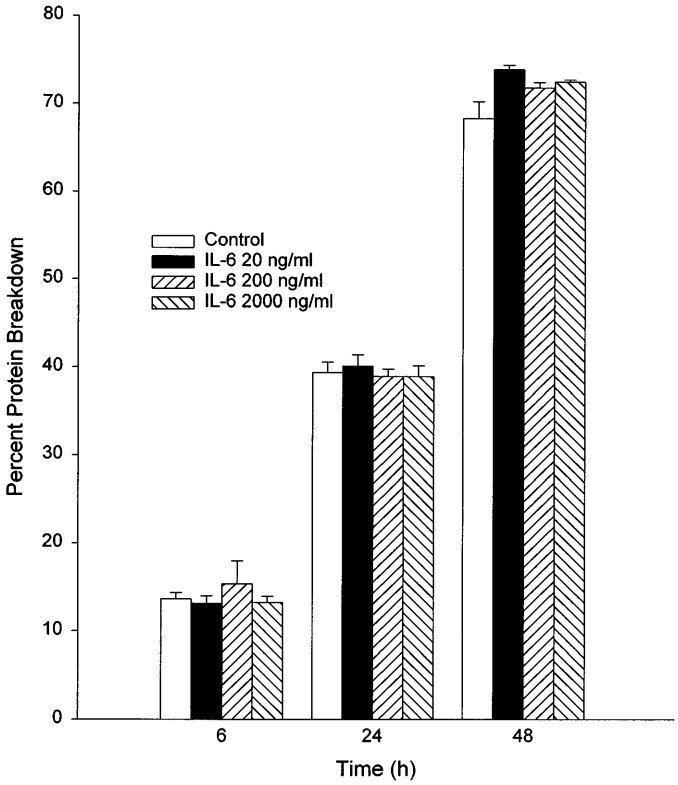

Fig. 6. Protein degradation in cultured L6 myotubes after treatment with different concentrations of human rIL-6 for 6, 24, or 48 h. Results are means ± SE from 3 experiments for each IL-6 concentration at each time point. Each experiment was performed in triplicate, and the result from each experiment was calculated as mean from 3 wells.

DISCUSSION

The results in the present study of stimulated total and myofibrillar protein breakdown in septic IL-6 knockout mice suggest that IL-6 is not a mediator of muscle proteolysis during sepsis, at least under these experimental conditions. An alternative interpretation of the results is that in IL-6 knockout mice, other mediators of muscle proteolysis were substantially increased, substituting for IL-6. For example, in a recent study by Fattori et al. (7), endotoxin injection in IL-6-deficient mice produced three times more TNF than in wild-type mice. Thus the present results do not rule out that IL-6 regulates muscle proteolysis in normal septic mice. The data show, however, that muscle proteolysis can be increased during sepsis in the absence of IL-6. The results from the additional experiments in which mice were treated with high doses of IL-6 or cultured L6 myotubes were treated with IL-6 in vitro further support the concept that IL-6 is not a major regulator of muscle protein degradation. The present results are important from a clinical standpoint because blockade of IL-6 has been proposed as a treatment modality to prevent the catabolic response in skeletal muscle during sepsis (35). In light of the results in the present study, such an approach would probably not be successful.

In previous studies from this laboratory the increase in muscle proteolysis seen during sepsis was associated with upregulated expression of ubiquitin mRNA in muscle tissue (30–33), supporting the concept that sepsis-induced muscle proteolysis reflects ubiquitin-proteasome-dependent protein degradation. In recent studies, specific proteasome blockers inhibited the sepsis-induced increase in muscle protein breakdown (18, 28), further supporting the role of the ubiquitin-proteasome pathway in the catabolic response to sepsis. A close correlation between protein breakdown

rates and ubiquitin mRNA expression in skeletal muscle has been observed in other catabolic conditions as well, including fasting (37), muscle denervation (22), cancer (29), metabolic acidosis (24), burn injury (6), and glucocorticoid treatment (30).

Although the several-fold increase in ubiquitin mRNA levels noted here in muscles of septic wild-type mice lends further support to the involvement of the ubiquitin-proteasome pathway in the regulation of muscle protein breakdown during sepsis, the significantly less pronounced increase in ubiquitin mRNA levels in muscles from septic IL-6 knockout mice (despite a similar increase in muscle protein breakdown rates) suggests that there is not an absolute correlation between ubiquitin mRNA levels and protein breakdown rates. This in turn may reflect the involvement of the ubiquitin pathway in intracellular metabolic events other than protein degradation (2).

The conclusion reached in the present study that IL-6 does not regulate muscle protein breakdown is similar to that in a report by Garcia-Martinez et al. (8). In that study, incubated EDL and soleus muscles from rats were treated in vitro with 2,000 U/ml of human rIL-6. This treatment did not stimulate protein breakdown, measured as release of tyrosine. The present experiments expanded the observations of Garcia-Martinez et al. (8) by specifically assessing the role of IL-6 during sepsis and by including measurements of myofibrillar protein breakdown rates, which is particularly important considering the pronounced effect of sepsis on myofibrillar protein degradation (17, 31, 39, 40). The present results are also in line with a recent study in which treatment of mice with IL-6 over 7 days did not induce a catabolic response (5).

Although results in some previous studies have been interpreted as indicating that IL-6 may regulate muscle protein breakdown, close inspection of the data and experimental design in several of those reports suggests that IL-6 may not be an important regulator of muscle proteolysis. For example, in a study by Goodman (12), only one of three treatment protocols with IL-6 resulted in increased protein breakdown rates in incubated EDL muscles. The increase noticed in both total and myofibrillar protein breakdown rates was small compared with the increase commonly seen in muscles from septic rats. In the same study, treatment of muscles in vitro with IL-6 for up to 6 h did not influence total or myofibrillar protein breakdown rates.

Tsujinaka et al. (34) reported that muscle underwent atrophy in IL-6 transgenic mice (overexpressing IL-6) and that the catabolic response in skeletal muscle was associated with increased expression of lysosomal cathepsins. It is possible, however, that the results in those studies did not reflect the effect of IL-6 per se but may have been caused by other mediators induced by IL-6. It is well known, for example, that IL-6 can induce the release of glucocorticoids (25) [although IL-6 is not required for the release of glucocorticoids (7)] and that glucocorticoids are an important mediator of muscle protein breakdown (14, 19, 30, 37). In addition, the finding in the report by Tsujinaka et al. (34) of increased expression of lysosomal proteases makes those results less relevant with regard to sepsis-induced muscle proteolysis because muscle protein breakdown during sepsis is mainly caused by upregulated ubiquitin-proteasome-dependent protein breakdown with no increase in lysosomal protein breakdown (31).

In another study, treatment of C2C12 myotubes with IL-6 was reported to stimulate protein breakdown, and it was concluded that IL-6 may play an important role in muscle degradation during sepsis or after severe injury (4). It should be noted, however, that the reported increase in protein breakdown in that study was small (6% increase in myotubes treated with 20 ng/ml of IL-6). Somewhat surprisingly, in the same study, TNF reduced protein degradation in the cultured C2C12 myotubes. This finding complicates the interpretation of the results in the study by Ebisui et al. (4) considering the well-known catabolic effect of TNF (11, 38, 39).

In a recent report, administration of IL-6 receptor antibody prevented muscle atrophy seen in the IL-6 transgenic mice and it was concluded that IL-6 receptor antibody may be effective against muscle wasting induced by sepsis or cancer (35). Interpretation of the results in that report, however, is difficult for several reasons. First, it is well known from previous studies that treatment with anti-IL-6 antibody results in a paradoxical increase in circulating IL-6 levels and that biological effects may be exerted by the increased IL-6 concentrations (21, 36). Because IL-6 levels were not measured in the study by Tsujinaka et al. (35), it is not known if the treatment with IL-6 receptor antibody resulted in a similar increase in IL-6 levels as reported previously after treatment with anti-IL-6 antibody. Second, if the catabolic response in skeletal muscle in the IL-6 transgenic mice was caused by glucocorticoids or other mediators released by IL-6 (as discussed previously), it is possible that the results observed after treatment with IL-6 receptor antibody reflected inhibited release of other regulators of muscle catabolism rather than an effect of IL-6 itself. Finally, because protein breakdown rates were not measured by Tsujinaka et al. (35), it is difficult to assess the role of IL-6 in the regulation of muscle proteolysis from the data in that report. Further exploration of the experimental model using IL-6 transgenic mice, including actual measurements of muscle protein breakdown rates, IL-6, and glucocorticoids, would be important to determine the role of IL-6 and other putative mediators of muscle proteolysis.

It should be noted that the septic model used in the present study results in peritonitis with growth of intestinal bacteria in peritoneal fluid and blood and that metabolic changes in this model are probably at least in part caused by endotoxemia. This is an important aspect because previous studies suggest that the role of IL-6 may be different in different types of inflammation. For example, reports by Fattori et al. (7) and Kopf et al. (20) provided evidence that the inflammatory response to tissue injury caused by sterile turpentine injection was severely compromised in IL-6-deficient mice, whereas the systemic inflammation

caused by endotoxin was not influenced by the absence of IL-6. In the present study, we injected an identical volume of sterile turpentine that was described in previous reports (7, 20), but because this treatment did not result in increased muscle proteolysis, we could not test the role of IL-6 in the catabolic response to turpentine abscess. The results did, however, provide further support for the concept that IL-6 is not a regulator of muscle proteolysis because circulating IL-6 levels were increased in turpentine-injected mice without noticeable changes in muscle protein breakdown.

In summary, the present results strongly argue against a role of IL-6 as a regulator of muscle proteolysis. IL-6 may be more important as an anabolic signal during sepsis and other critical illness, mediating increased protein synthesis in the liver and intestinal mucosa (9, 26, 36). Previous reports suggest that other mediators than IL-6 may be more important for the stimulation of muscle proteolysis during sepsis, including IL-1, TNF, and glucocorticoids (14, 30, 38–40). These mediators may have been involved in the regulation of muscle proteolysis in both wild-type and IL-6 knockout mice in the present study because both TNF and corticosterone are produced in endotoxemic IL-6-deficient mice (7).

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