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 27–30% 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.

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.,

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 endocrinical 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 protein turnover 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 protein 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.
and intervals as described in RESULTS. Control mice were

Food was withheld after the surgical procedures to avoid any

The experimental protocols were approved by the National

Results and Discussion

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proteins were labeled with 1 µCi of L-[3,5-3H]tyrosine for 48 h for differentiation, cytosine arabinoside was removed and cells were cultured as described previously by Hong and Forsberg (19). After 10 µM cytosine arabinoside for 48 h to remove any remaining dividing myoblasts.

When the cells were approximately 90% confluent, they were removed by trypsinization 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 NaHCO₃, and 110 µg/ml of sodium pyruvate in a humidified atmosphere of 10% CO₂ and 90% air at 37°C. When the cells were almost identical (8%) in wild-type and IL-6 knockout mice. No detectable 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 and were 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 8–10 in each group.

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.

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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·h⁻¹, n = 6) and sham-operated (384 ± 49 nmol tyrosine·g⁻¹·h⁻¹, n = 6) mice and were increased to 577 ± 47 nmol tyrosine·g⁻¹·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.

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 2B). 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.

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·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·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 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 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
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·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·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).

**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 Ebisu 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 abscesses. The results did, however, provide further support for the concept that IL-6 is not a regulator of muscle proteolysis during sepsis, mediating increased 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 endotoxicemic IL-6-deficient mice (7).

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 endotoxicemic IL-6-deficient mice (7).

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