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Sepsis Downregulates Myostatin mRNA Levels Without Altering Myostatin Protein Levels in Skeletal Muscle

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ABSTRACT

Myostatin is a negative regulator of muscle mass and has been reported to be upregulated in several conditions characterized by muscle atrophy. The influence of sepsis on myostatin expression and activity is poorly understood. Here, we tested the hypothesis that sepsis upregulates the expression and downstream signaling of myostatin in skeletal muscle. Because sepsis-induced muscle wasting is at least in part regulated by glucocorticoids, we also determined the influence of glucocorticoids on myostatin expression. Sepsis was induced in rats by cecal ligation and puncture and control rats were sham-operated. In other experiments, rats were injected intraperitoneally with dexamethasone (10 mg/kg) or corresponding volume of vehicle. Surprisingly, myostatin mRNA levels were reduced and myostatin protein levels were unchanged in muscles from septic rats. Muscle levels of activin A, follistatin, and total and phosphorylated Smad2 (p-Smad2) were not influenced by sepsis, suggesting that myostatin downstream signaling was not altered during sepsis. Interestingly, total and p-Smad3 levels were increased in septic muscle, possibly reflecting altered signaling through pathways other than myostatin. Similar to sepsis, treatment of rats with dexamethasone reduced myostatin mRNA levels and did not alter myostatin protein levels. Fasting, an additional condition characterized by muscle wasting, reduced myostatin mRNA and activin A protein levels, increased myostatin protein, and did not influence follistatin and p-Smad2 levels. Of note, total and p-Smad3 levels were reduced in muscle during fasting. The results suggest that sepsis and glucocorticoids do not upregulate the expression and activity of myostatin in skeletal muscle. The role of myostatin may vary between different conditions characterized by muscle wasting. Downstream signaling through Smad2 and 3 is probably regulated not only by myostatin but by other mechanisms as well. J. Cell. Biochem. 111: 1059–1073, 2010.

KEY WORDS: MYOSTATIN; ACTIVIN A; FOLLISTATIN; MUSCLE WASTING; SMAD; SEPSIS

Myostatin, also known as growth differentiation factor 8, is a member of the transforming growth factor-β (TGF-β) superfamily and is a negative regulator of muscle mass [McPherron et al., 1997]. There is evidence that myostatin may reduce muscle mass both by decreasing protein synthesis and by stimulating ubiquitin-proteasome-dependent protein breakdown [McFarlane et al., 2006; Amirouche et al., 2009], although inhibited activation and renewal of satellite cells may also be involved [McCroskery et al., 2003; Carnac et al., 2006]. The expression of myostatin has been reported to be upregulated in a number of conditions characterized by muscle atrophy, including cancer [Liu et al., 2007; Costelli et al., 2008], burn injury [Lang et al., 2001], HIV infection [Gonzalez-Cadavid et al., 1998], fasting [Jeanplong et al., 2003], denervation [Baumann et al., 2003], aging [Yarasheski et al., 2002], and disuse atrophy [Reardon et al., 2001]. In contrast, a recent study suggests that the expression of myostatin is not increased in skeletal muscle during sepsis [Lang et al., 2001]. This is a surprising observation since sepsis is associated with a substantial loss of muscle mass caused by inhibited protein synthesis [Lang et al., 2007] and stimulated ubiquitin-proteasome-dependent protein breakdown [Tiao et al., 1994; Hobler et al., 1999; Wray et al., 2003]. In addition, sepsis-induced muscle proteolysis is at least in part regulated by glucocorticoids [Hasselgren, 1999; Menconi et al., 2007] and glucocorticoids have been reported to increase the expression of myostatin [Lang et al., 2001; Ma et al., 2003; Gilson et al., 2007].

Of note, in previous experiments in which the influence of sepsis on myostatin expression was examined, muscle levels of myostatin

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mRNA levels were determined [Lang et al., 2001] and it is not known whether myostatin protein levels are affected by sepsis. This is significant because previous reports suggest that myostatin protein concentrations are regulated not only at the transcriptional level but by posttranscriptional mechanisms as well [Anderson et al., 2008; Lee, 2008]. Thus, unchanged myostatin mRNA levels do not rule out the possibility that myostatin protein levels may be increased. In addition, it is not known whether sepsis influences the expression of follistatin, an endogenous inhibitor of myostatin [Lee and McPherron, 2001; Haidet et al., 2008; Gilson et al., 2009]. This is important because reduced levels of follistatin (or other endogenous inhibitors) may increase myostatin-dependent signaling even in the absence of increased myostatin expression [Link and Nishi, 1997; McPherron et al., 1997; Gilson et al., 2009]. Activation of myostatin results in downstream phosphorylation (and activation) of Smad transcription factors secondary to ligand binding to the activin receptor type IIB [Tsuchida et al., 2008; Sartori et al., 2009]. The influence of sepsis on muscle levels of phosphorylated Smad (p-Smad) transcription factors is also unknown.

In the present study, we determined mRNA and protein levels for myostatin and protein levels for follistatin in skeletal muscle during sepsis in rats. We also measured muscle levels of p-Smad2 and p-Smad3 to test the influence of sepsis on downstream myostatin signaling. Surprisingly, sepsis resulted in a substantial down-regulation of myostatin mRNA levels in skeletal muscle, which was accompanied by unchanged myostatin protein levels and unchanged expression of follistatin and p-Smad2. Interestingly, p-Smad3 levels were increased in septic muscle, suggesting that Smad3 may be regulated by mechanism other than changes in myostatin levels during sepsis. The present results suggest that sepsis-induced muscle wasting does not reflect increased myostatin expression and activity.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Sepsis was induced in male Sprague–Dawley rats (55–65 g body weight) by cecal ligation and puncture (CLP) as described previously [Tiao et al., 1994; Hobler et al., 1999; Wray et al., 2003; Smith et al., 2010]. Control rats underwent sham-operation, that is, laparotomy and manipulation, but no ligation or puncture, of the cecum. Rats allocated to sham-operation or CLP were weight-matched in order to ensure identical initial body weights in the two groups of rats. To prevent hypovolemia and septic shock, rats were resuscitated with 10 ml/100 g body weight of saline administered subcutaneously on the back at the time of sham-operation or CLP. Food was withheld after the surgical procedures to avoid the influence of differences in food intake between septic and sham-operated rats on metabolic changes in muscle, but the animals had free access to water. In several previous experiments in which we studied sepsis-induced muscle wasting, rats weighing 50–60 g were used because rats of this size have lower extremity muscles that are thin enough to allow for in vitro incubation and measurement of protein degradation rates under physiological conditions [Tiao et al., 1994]. Rats of the same size were used here in order to make it possible to compare the present observations with previous studies. At different time points (4, 8, and 16 h) after sham-operation or CLP, extensor digitorum longus (EDL) muscles were harvested, weighed, immediately frozen in liquid nitrogen, and stored at −80°C. EDL muscles were studied here because in previous studies we found that white, fast-twitch skeletal muscles are particularly sensitive to the effects of sepsis [Tiao et al., 1994]. In a separate experiment, blood was collected in heparinized vials by heart puncture 16 h after sham-operation or CLP. The blood samples were centrifuged at 3,500*g* for 10 min at 4°C and the supernatant was stored at −80°C until used for determination of plasma myostatin levels as described below.

To test the role of glucocorticoids in the regulation of the myostatin pathway, two series of experiments were performed. First, rats were treated with the glucocorticoid receptor antagonist RU38486 (10 mg/kg) or corresponding volume of vehicle administered intraperitoneally 2 h before sham-operation or CLP. EDL muscles were removed 16 h after sham-operation or CLP for determination of myostatin mRNA and protein levels. We found in previous studies that treatment of rats with RU38486 prevented sepsis-induced muscle proteolysis and activation of the ubiquitin-proteasome pathway [Tiao et al., 1996; Wray et al., 2003] but the effect of RU38486 on the regulation of myostatin expression during sepsis is not known. In a second series of experiments, rats were treated with dexamethasone (10 mg/kg) or corresponding volume of vehicle administered intraperitoneally as described previously [Tiao et al., 1996; Yang et al., 2005]. Rats had free access to water but food was withheld after the injections. EDL muscles were harvested 16 h after administration of dexamethasone or vehicle for determination of atrogin-1, MuRF1, and myostatin mRNA levels and myostatin protein levels.

In a final experiment, the influence of fasting on myostatin expression and activity was examined. Food was withheld during 48 h in the fasted rats and control rats were provided regular chow. Both groups of rats had free access to drinking water during the experiment.

The animal experiments were approved by the Institutional Animal Care and Use Committee at the Beth Israel Deaconess Medical Center (Boston, MA).

REAL-TIME PCR

mRNA levels for myostatin, atrogin-1, and MuRF1 were determined by real-time PCR. Total RNA was extracted from EDL muscles as described by Chomczynski and Sacchi [1987]. Multiplex qRT-PCR with amplification of 18S RNA as endogenous control, TaqMan analysis and subsequent calculations were performed with an ABI Prism 7700 Sequence Detection System (Perkin-Elmer, Foster City, CA). For determination of myostatin mRNA levels, two separate TaqMan experiments were performed using the same RNA template but different myostatin primer sets. In one of the experiments we used an ABI Assay-on-Demand primer set (Applied Biosystems Assay ID: Rn00569683_m1) and in the other experiment, we used an in-house designed TaqMan primer set (forward, CCT CCA CTC CGG GAA CTG A; reverse, TCC AAA GAG CCG TCA CTG CT; and TaqMan probe FAM 5′-CGA TCA GTA CGA CGT CCA GAG GGA TGA-3′ TAMRA). The two primer sets used here comprise different areas of the gene; the ABI set spans the exon 1/exon 2 junction whereas the

in-house set lies entirely in exon 1. The sequences of the forward, reverse, and double-labeled oligonucleotides for atrogin-1 and MuRF1 used here were described recently [Menconi et al., 2008; Smith et al., 2010]. Amplification of 18S rRNA was performed in the same reaction wells as an internal standard with an alternatively labeled probe (VIC/TAMRA) to distinguish its message from the one derived for myostatin, atrogin-1, and MuRF1. mRNA concentrations were normalized to the 18S mRNA levels and were expressed as arbitrary units (AU). For the subsequent calculations, we used the comparative $\Delta\Delta C_t$ method.

WESTERN BLOTTING

Myostatin, follistatin, and total and p-Smad2 protein levels were determined by Western blotting of total muscle extracts. The muscle extracts were prepared by homogenizing muscles in ice-cold RIPA buffer (10 mM Tris–HCl, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, and 1% Nonidet P-40) containing protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN) followed by centrifugation at 10,000*g* for 10 min at 4°C. The supernatant was stored at −80°C until analysis. Aliquots (50 μg protein) of the muscle extracts or plasma samples were used for Western blotting performed as described in detail recently [Yang et al., 2005; Smith et al., 2010]. In short, protein extracts were subjected to SDS–PAGE using 10% or 4–20% gels, followed by transfer to PVDF membranes. The Western blot analyses were performed under reducing conditions which is important for the interpretation of the immunoprecipitated bands as demonstrated by Anderson et al. [2008]. The membranes were blocked with 5% non-fat milk in TTBS buffer (50 mM Tris–HCl, 150 mM NaCl, and 1% Tween-20, pH 7.4) and incubated with the following primary antibodies and the appropriate secondary antibodies: a rabbit polyclonal anti-human myostatin antibody recognizing the 52 kDa myostatin precursor protein (1:1,000, NB100-281, Novus Biologicals, Littleton, CO); a rabbit polyclonal anti-human follistatin (1:400, AB 58920, Abcam, Cambridge, MA); a rabbit polyclonal anti-human Smad2 antibody (1:1,000, #3103, Cell Signaling Technology, Danver, MA); a rabbit polyclonal anti-human phosphorylated Smad2 (Ser 465/467) antibody (1:500, #3101, Cell Signaling Technology); a rabbit monoclonal anti-human Smad3 antibody (1:1,000, #9523, Cell Signaling Technology); a rabbit monoclonal anti-human phosphorylated Smad3 (Ser 423/425) antibody (1:1,000, #9520, Cell Signaling Technology). The phosphorylation sites of Smad2 and Smad3 examined here were based on previous reports [Abdollah et al., 1997; Liu et al., 1997; Souchelnytskyi et al., 1997]. A mouse monoclonal anti-rat α-tubulin antibody (1:10,000, Sigma–Aldrich, St. Louis, MO) was used for loading control. Immunoreactive protein bands were detected by using the Western Lightning kit for enhanced chemiluminescence detection (Perkin-Elmer Life Sciences) and analyzed using the public domain Image J program (http://rsb.info.nih.gov/ij/index.html). The bands were quantified by densitometry and normalized to the appropriate loading controls. A preliminary Western blot was performed by using a blocking peptide (NB100281-PEP, Novus Biologicals) and a myostatin overexpression lysate (NBL1-13340, Novus Biologicals) to confirm the location of the myostatin protein band in the blots.

ELISA

Activin A protein levels were determined in total muscle extracts (prepared as described above) by using a commercially available ELISA assay kit (R&D Systems, Minneapolis, MN) following the manufacturer's instructions.

STATISTICS

Results are reported as means ± SEM. Statistical analysis was performed by using Student's *t*-test when the means from two groups were compared or ANOVA followed by Tukey's post hoc test when the means from more than two groups were compared. $P < 0.05$ was considered statistically significant.

RESULTS

Although we observed in several previous reports that muscle protein breakdown rates were increased after induction of sepsis by CLP in rats [Hasselgren et al., 1989; Tiao et al., 1994, 1996], changes in muscle weight in the present experimental model are less clear. In initial experiments, we determined the weight of EDL muscles 16 h after sham-operation or CLP and found that muscle weight was reduced by ~20% in septic rats (Fig. 1A). Because rats allocated to sham-operation or CLP were weight-matched, the initial body weight was almost identical in the two groups whereas the final body weight (16 h after sham-operation or CLP) was lower in septic than in control rats (58 ± 1.0 g and 61 ± 1.1 g, respectively; $P < 0.05$). The reduced body and muscle weights were accompanied by an ~20-fold increase in muscle atrogin-1 (Fig. 1B) and a 40-fold increase in MuRF1 mRNA levels (Fig. 1C) which is noteworthy because upregulated expression of the muscle-specific ubiquitin ligases atrogin-1 and MuRF1 is commonly used as "molecular markers" of muscle wasting. Taken together, the results in Figure 1 confirm that the present experimental model results in muscle wasting and molecular evidence of activation of a "muscle wasting program."

We next examined whether muscle wasting caused by CLP in rats is associated with increased expression of myostatin. Surprisingly, myostatin mRNA levels, determined by real-time PCR in the same muscles that were used for determination of atrogin-1 and MuRF1 mRNA levels, were reduced by almost 80% in septic rats (Fig. 2A). In this experiment, commercially available primer sets for rat myostatin were used for the real-time PCR. Because of the unexpected result, we wanted to make certain that the result did not reflect faulty primers and therefore performed an additional TaqMan experiment using an in-house-designed TaqMan primer set as described in the Materials and Methods Section and based on the published sequence of the rat myostatin gene [Yamanouchi et al., 2000]. Also when this primer set was used for real-time PCR, results suggested that myostatin mRNA levels were substantially reduced in muscles from septic rats (Fig. 2B).

Because changes in mRNA levels are not always accompanied by similar changes in protein levels, we next wanted to determine the influence of sepsis on myostatin protein levels. Myostatin is secreted as an ~50 kDa precursor protein (promyostatin), the processing of

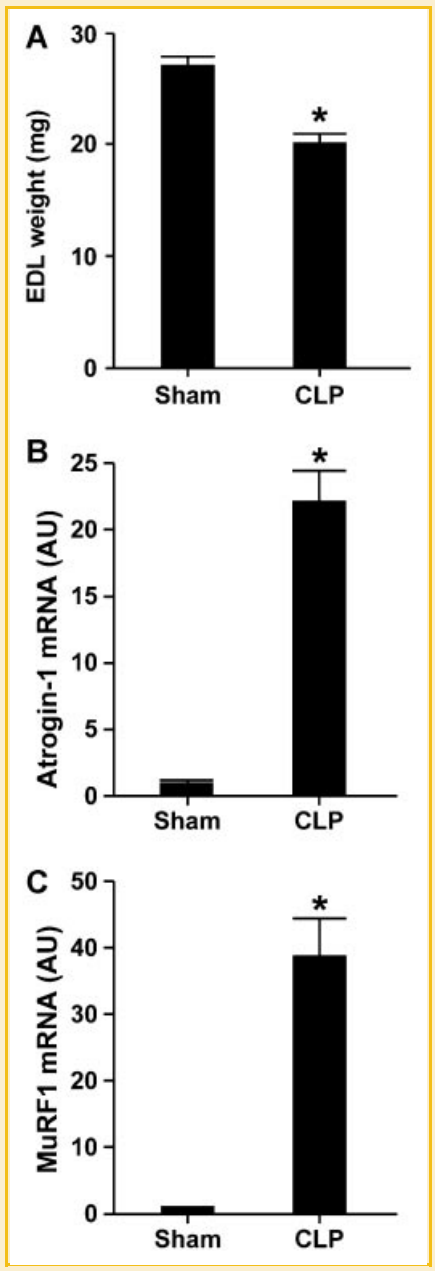

Fig. 1. The effects of sepsis on muscle weight and expression of atrogin-1 and MuRF1 in skeletal muscle. A: Muscle weight, (B) atrogin-1, and (C) MuRF1 mRNA levels were determined in EDL muscles 16 h after sham-operation or CLP in rats. For all panels, results are means ± SEM with n ≥ 6 per group. $^{*}P < 0.05$ versus sham by Student's *t*-test.

which yields an ~35 kDa inhibitory prodomain, also referred to as latency-associated peptide (LAP), and mature myostatin that can appear as a monomer (~15 kDa) or homodimer (~30 kDa) [Thomas et al., 2000; Anderson et al., 2008]. Because the 50 kDa precursor protein is the most abundant immunoreactive species in skeletal muscle [McFarlane et al., 2005; Anderson et al., 2008] with its protein level being inversely related to muscle mass [Baumann et al., 2003; Kinouchi et al., 2008; Welle et al., 2009], we used an antibody designed to recognize the ~50 kDa myostatin precursor peptide. To confirm the location of the myostatin protein band, we performed preliminary Western blot experiments using a myostatin overexpression lysate and a blocking peptide specific for the antibody used here. As shown in Figure 2C, a band was present at ~50 kDa in the myostatin overexpression lysate as well as in our samples and this band disappeared when the blocking peptide was used. The appearance of promyostatin as a 50 kDa band was expected since the Western blotting was performed under reducing conditions. This differs from the 105 kDa promyostatin dimer seen when the Western blotting is performed under non-reducing conditions [Anderson et al., 2008].

The nature of the lower bands seen in Figure 2C is unclear. Based on the molecular weight ladder used for the Western blots, the bands represented proteins with molecular weights of ~35 and 26 kDa. Although these molecular weights are close to the molecular weights for the prodomain and the mature myostatin dimer as reported by Anderson et al. [2008], we do not feel convinced that the bands represented myostatin-related proteins since the bands were not eliminated by the blocking peptide. We did not see a band corresponding to the mature myostatin monomer (15 kDa) on this or any other Western blots in the present study. In the remainder of the experiments, therefore, we used the immunoprecipitated band at ~50 kDa as a measure of myostatin protein expression. Although we realize that this band most likely represents promyostatin [Anderson et al., 2008], we have called it "myostatin" in most places in the manuscript because it is the most abundant myostatin species in skeletal muscle [McFarlane et al., 2005; Anderson et al., 2008] and because there is evidence that changes in the levels of this protein are directly related to changes in myostatin signaling and muscle mass [Baumann et al., 2003; Kinouchi et al., 2008; Welle et al., 2009]. The fact that we determined promyostatin protein expression (rather than the expression of mature myostatin) is a potential limitation of the present report. Previous studies strongly suggest, however, that changes in muscle promyostatin levels are directly related to the regulation of muscle mass [Baumann et al., 2003; Kinouchi et al., 2008; Welle et al., 2009] and determining promyostatin levels therefore should give accurate information about the potential role of myostatin signaling in most experimental conditions. Of note, the literature on myostatin protein expression is somewhat confusing and sometimes apparently contradictory with many reports not even defining the molecular weight of the immunoprecipitated bands on Western blots and with varying nomenclature used for the myostatin species being studied. Although different terminology has been used in the literature to describe the different myostatin species, in the present study we have mainly used the terminology adopted by Anderson et al. [2008]. The control experiment shown in Figure 2C using a (pro)myostatin-enriched lysate as a positive control and a blocking peptide as a negative control was important to ensure the identity of the band being examined in the present study.

We next determined myostatin protein levels in muscles from sham-operated and septic rats and found that in contrast to the reduced myostatin mRNA levels, myostatin protein levels were unchanged in EDL muscles 16 h after CLP (Fig. 2D).

Because in other studies, myostatin expression was increased in a time-dependent fashion during various catabolic conditions [Lang

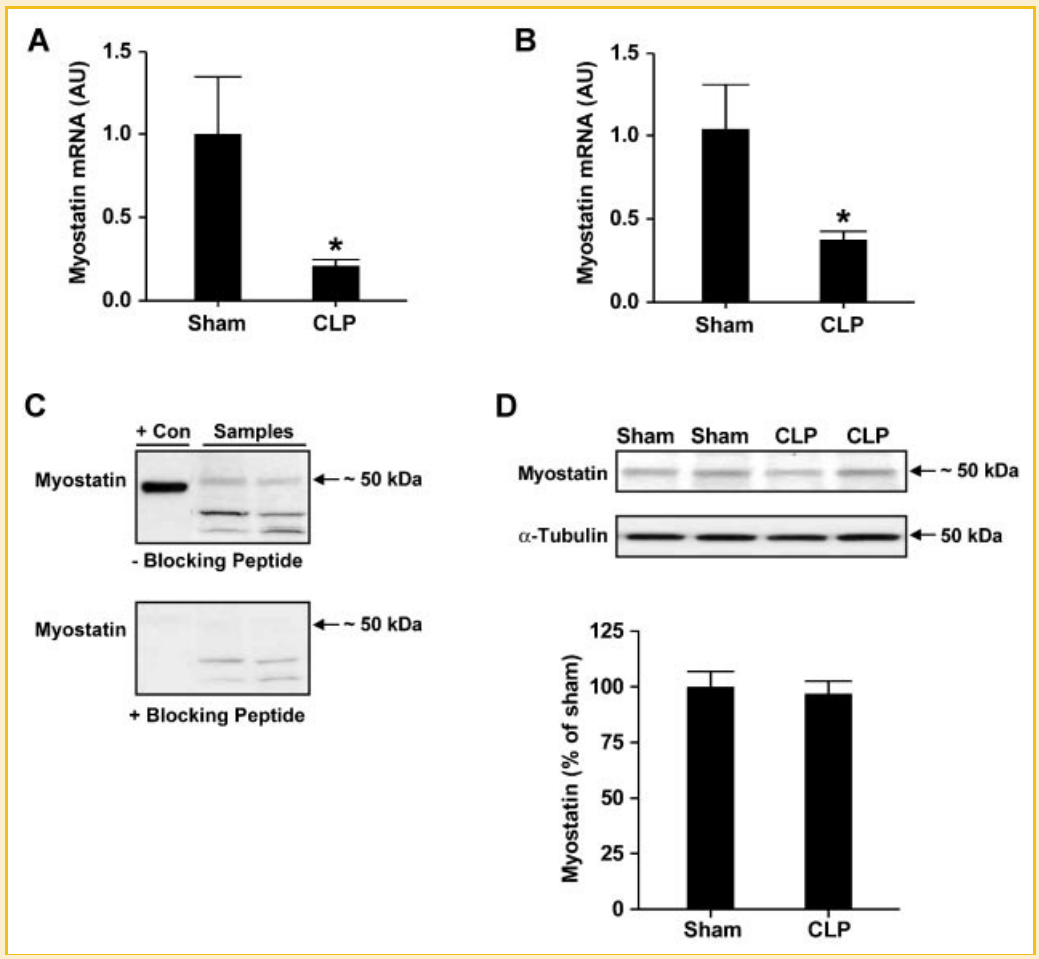

Fig. 2. The effect of sepsis on the expression of myostatin in skeletal muscle. Sepsis was induced by CLP in rats and control rats were sham-operated. EDL muscles were harvested 16 h after sham-operation or CLP and myostatin mRNA levels were determined by TaqMan real-time PCR using (A) a commercially available primer set or (B) an in-house-designed primer set based on the reported sequence of rat myostatin. C: Myostatin protein levels were determined by Western blotting of a myostatin overexpression cellular extract (Con) and of muscle extracts from non-septic rats (samples) in the absence or presence of a myostatin blocking peptide. D: Myostatin protein levels determined by Western blotting of muscle extracts 16 h after sham-operation of CLP. Representative blots are shown in the upper panel and densitometric quantifications of myostatin protein levels normalized to α-tubulin levels (used as loading controls) are shown in the lower panel. Results are means ± SEM with n = 7 or 8 in each group. $^{*}P < 0.05$ versus corresponding sham group by Student's *t*-test. AU, arbitrary units.

et al., 2001; Shao et al., 2007; Costelli et al., 2008], we next examined whether myostatin levels were increased at earlier time points after induction of sepsis in rats. When muscles were studied between 4 and 16 h after CLP, myostatin mRNA levels were unchanged at 4 and 8 h and were again significantly reduced 16 h after CLP (Fig. 3A). Myostatin protein levels were not altered at any of the time points studied here (Fig. 3B).

Although myostatin is produced in skeletal muscle and probably exerts most of its effects in an autocrine or a paracrine fashion, it is possible that circulating myostatin may also influence muscle mass [Gonzalez-Cadavid et al., 1998; Yarasheski et al., 2002; Zimmers et al., 2002]. In order to test whether sepsis increases circulating myostatin levels, we next measured plasma levels of myostatin 16 h after sham-operation or CLP and found that plasma myostatin levels were actually decreased in septic rats (Fig. 4). This result further supports the interpretation that sepsis-induced muscle wasting is not associated with increased myostatin expression. A discrepancy between changes in circulating and muscle myostatin protein levels (as observed here) has been reported by others as well [Costelli et al., 2008].

Because previous studies suggest that myostatin activity is inhibited by follistatin and that myostatin signaling can be increased by reduced levels of follistatin [Lee and McPherron, 2001; Haidet et al., 2008; Gilson et al., 2009] we next determined the influence of sepsis on follistatin protein levels in EDL muscles. Sepsis did not decrease follistatin protein levels in skeletal muscle but on the contrary resulted in a small (but statistically significant) increase in follistatin levels 16 h after CLP (Fig. 5A). Follistatin levels were unchanged 4 and 8 h after CLP (Fig. 5A).

In addition to myostatin, other molecules as well can induce signaling via the activin receptor type IIB. One such molecule is activin A, which was shown in previous studies to be involved in the regulation of signaling pathways commonly activated by myostatin [Kingsley, 1994; Link and Nishi, 1997; Gilson et al., 2009]. In order

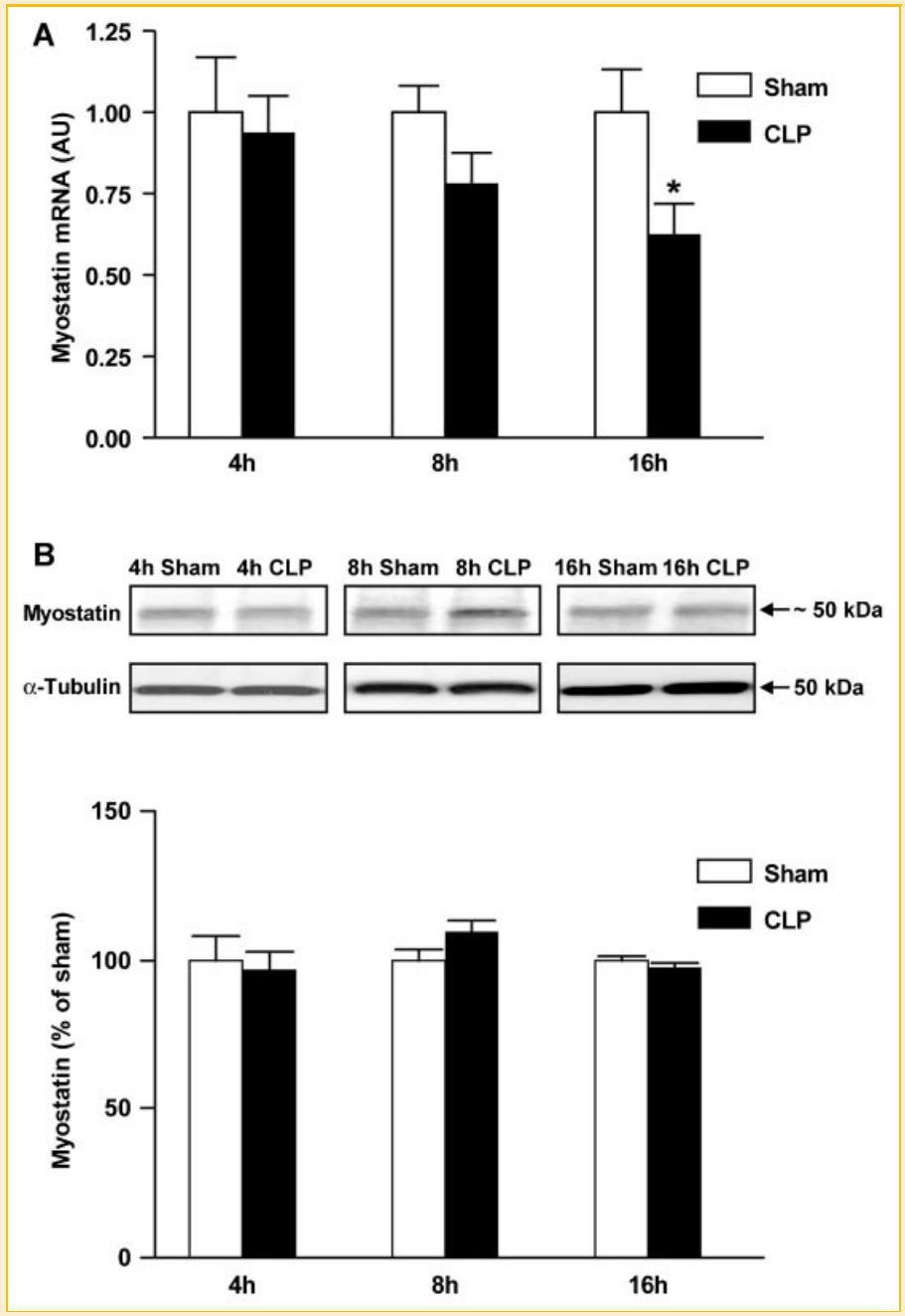

Fig. 3. Myostatin mRNA and protein levels at different time points after sham-operation or CLP in rats. A: Myostatin mRNA levels in EDL muscles determined by real-time PCR 4, 8, and 16 h after sham-operation or CLP. B: Myostatin protein levels in EDL muscles determined by Western blotting 4, 8, and 16 h after sham-operation or CLP. Representative Western blots are shown in the upper panel and densitometric quantifications of myostatin protein levels normalized to α-tubulin are shown in the lower panel. Results are means ± SEM with n = 6 in each group. $^{*}P < 0.05$ versus corresponding sham group by ANOVA. AU, arbitrary units.

to test the potential role of activin A in sepsis-induced muscle wasting, we determined activin A protein levels in EDL muscles from sham-operated and septic rats. Similar to myostatin protein levels, activin A protein levels were not influenced by sepsis under the present experimental conditions at any of the time points studied (Fig. 5B).

In addition to myostatin and activin A, several other members of the TGF-β superfamily may also signal through the myostatin-like pathway [Lee et al., 2005]. A common downstream effect of signaling through the activin receptor type IIB is phosphorylation (activation) of Smad transcription factors [Lee et al., 2005; Tsuchida et al., 2008; Sartori et al., 2009]. Although the levels of p-Smad2 [Ohsawa et al., 2006; Shao et al., 2007; Trendelenburg et al., 2009] and p-Smad3 [Sartori et al., 2009] are elevated during muscle wasting caused by other conditions, the influence of sepsis on Smad2 and Smad3 is not known. In order to further examine the

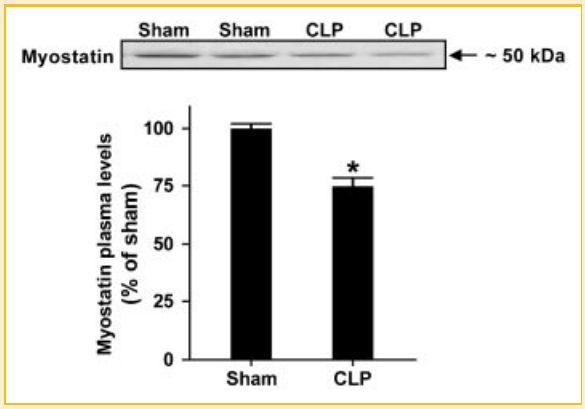

Fig. 4. The effect of sepsis on plasma myostatin levels. Blood was drawn by heart puncture 16 h after sham-operation or CLP in rats. Plasma was prepared and myostatin levels were determined by Western blotting. The upper panel shows representative Western blots and the lower panel shows quantification densitometry of Western blots with n = 6 in each group. Results are means ± SEM with n = 8 per group. $^{*}P < 0.05$ versus sham by Student's *t*-test.

potential role of TGF-β signaling in sepsis, we next measured muscle levels of total and phosphorylated Smad2 and Smad3 in sham-operated and septic rats. Sepsis did not influence total or p-Smad2 levels in skeletal muscle (Fig. 6) whereas both total and p-Smad3 levels were increased in muscle from septic rats (Fig. 7). In light of unchanged myostatin levels, the increased levels of p-Smad3 in septic muscle was unexpected but may reflect regulation of Smad3 by mechanisms other than changes in myostatin levels.

In previous studies, treatment of rats with the glucocorticoid receptor antagonist RU38486 blocked the sepsis-induced increase in ubiquitin-proteasome-dependent muscle proteolysis suggesting that sepsis-induced muscle wasting is at least in part regulated by glucocorticoids [Tiao et al., 1996; Wray et al., 2003]. We next wanted to test the potential involvement of glucocorticoids in the sepsis-induced changes in myostatin mRNA expression. Although it may seem counterintuitive that glucocorticoids should be involved in the sepsis-induced decrease in myostatin expression, it was important to examine the potential role of glucocorticoids in the reduced myostatin mRNA levels observed here, in particularly since previous reports on the effects of glucocorticoids on myostatin expression have not been consistent [Ma et al., 2003; Rodgers et al., 2003; Carraro et al., 2009]. When rats were treated with RU38486, the sepsis-induced decrease in myostatin mRNA levels was not significantly affected by RU38486 (Fig. 8A). Myostatin protein levels remained unaffected by sepsis and were also not affected by RU38486 (Fig. 8B). A lack of significant involvement of glucocorticoids in sepsis-induced downregulation of myostatin mRNA expression does not rule out the possibility that glucocorticoids can regulate myostatin. Indeed, previous reports suggest that glucocorticoids can upregulate myostatin expression in skeletal muscle [Lang et al., 2001; Ma et al., 2003]. In order to further test the role of glucocorticoids in the regulation of myostatin expression, we next treated rats with 10 mg/kg of dexamethasone, a dose that increased ubiquitin-proteasome-dependent muscle proteolysis and the expression and activity of muscle wasting-related transcription factors in previous experiments [Tiao et al., 1996; Yang et al., 2005]. Treatment of rats with dexamethasone resulted in sepsis-like changes in myostatin expression, that is, a substantial down-regulation of myostatin mRNA levels accompanied by unchanged myostatin protein levels (Fig. 8C,D). These results differ from a study by Ma et al. [2003] in which treatment of rats with dexamethasone resulted in increased gene and protein expression of myostatin. Although the reason for these apparently contradictory results is not known at present, the differences may at least in part reflect longer duration of dexamethasone treatment (5–10 days) in older rats (weighing 250–420 g) in the study by Ma et al. [2003]. Another difference between the study by Ma et al. [2003] and the present report is that we used a higher dose of dexamethasone (10 mg/kg) than Ma et al. [2003] who used doses ranging from 0.06 to 1.2 mg/kg. However, because those doses were repeated daily for 5 days, the total amount of dexamethasone administered by Ma et al. [2003], that is, a maximum of 6 mg/kg, was not dramatically different from the amount of dexamethasone administered in the present study. It should be noted that previous reports on the effects of glucocorticoids on myostatin have been inconsistent with evidence of both glucocorticoid-induced increase [Ma et al., 2003; Carraro et al., 2009] and decrease in myostatin expression [Rodgers et al., 2003].

Because unchanged or decreased myostatin expression and activity in skeletal muscle during sepsis (despite evidence of muscle wasting with loss of muscle mass and a substantial upregulation of atrogin-1 and MuRF1 expression) and after treatment with dexamethasone were surprising observations, we wanted to examine the influence of an additional condition characterized by muscle wasting, that is, fasting. This is relevant because sepsis (like several other muscle wasting conditions) is associated with reduced food intake. Fasting is associated with activation of muscle atrophy-related genes and loss of muscle mass [Dehoux et al., 2004; Lecker et al., 2004]. Of note, previous studies on the influence of food deprivation and fasting on the expression of myostatin have been inconsistent with both increased, decreased, and unchanged myostatin levels being reported [Jeanplong et al., 2003; Rodgers et al., 2003; Guernec et al., 2004; Larsen et al., 2006; Terova et al., 2006; Yamaguchi et al., 2006]. The differences between previous studies may in part reflect different species and different lengths of fasting being studied.

In the present study, fasting of rats for 48 h resulted in reduced myostatin mRNA levels (Fig. 9A) and increased myostatin protein levels in skeletal muscle (Fig. 9B). Muscle levels of the endogenous myostatin inhibitor follistatin were not influenced by fasting (Fig. 10). Because increased myostatin and unchanged follistatin levels may result in stimulated myostatin signaling, we next tested whether fasting increased myostatin signaling by determining muscle levels of total and p-Smad2. Fasting resulted in a small (but statistically significant) reduction of total Smad2 levels (Fig. 11A) but did not significantly alter p-Smad2 levels (Fig. 11B), suggesting that activin receptor type IIB-dependent signaling was not upregulated, despite increased myostatin protein levels. This was further supported by reduced levels of both total Smad3 and p-Smad3 levels in muscles from fasted rats (Fig. 12).

Because unchanged p-Smad2 and reduced p-Smad3 levels, despite increased myostatin protein expression, may reflect reduced

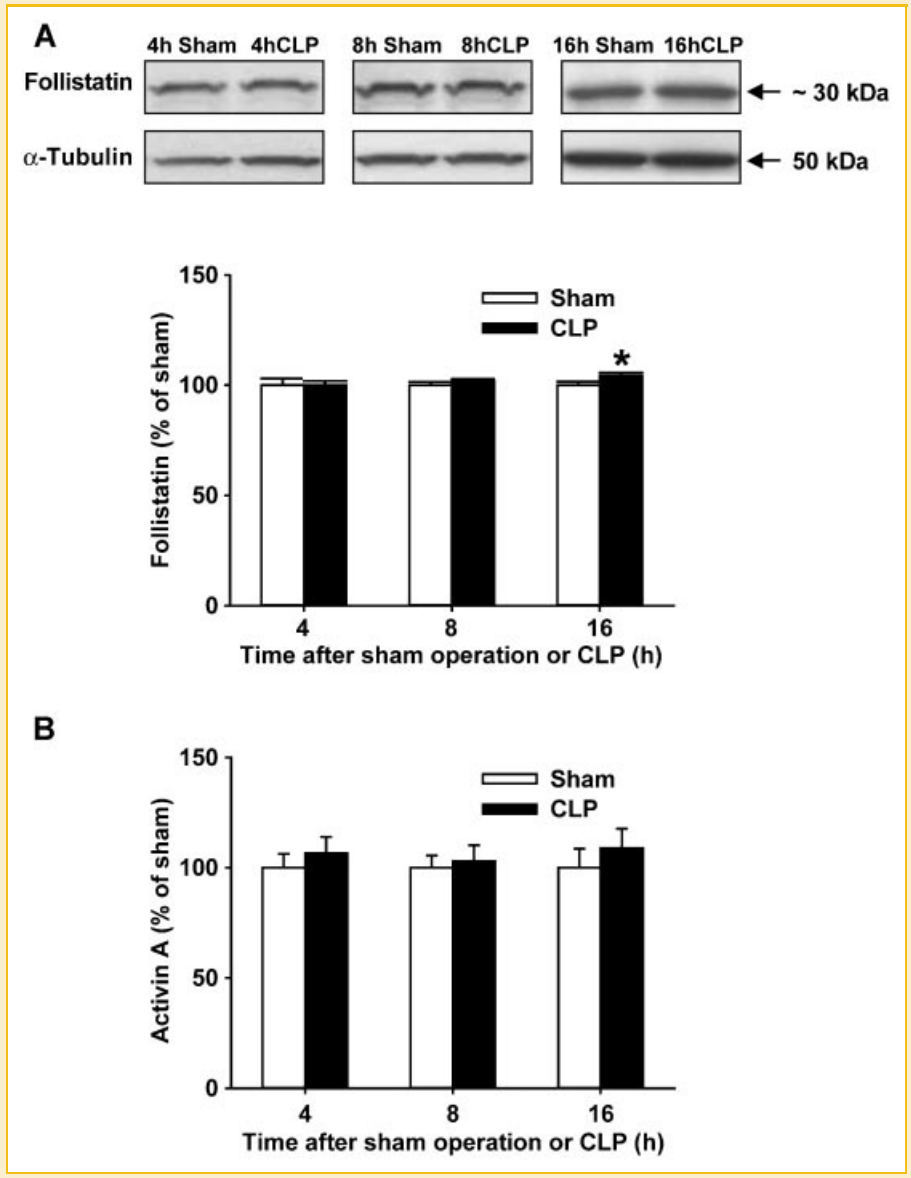

Fig. 5. The effect of sepsis on the expression of follistatin and activin A in skeletal muscle. A: Follistatin protein levels were determined in EDL muscles 4, 8, and 16 h after sham-operation or CLP by Western blotting. Representative Western blots for follistatin and α-tubulin (loading control) are shown in the upper panel and densitometric quantifications of myostatin protein levels normalized to α-tubulin levels are shown in the lower panel. B: Activin A protein levels were determined in EDL muscles 4, 8, and 16 h after sham-operation or CLP by ELISA. Results are means ± SEM with n = 6–8 in each group. $^{*}P < 0.05$ versus corresponding sham group by ANOVA.

levels of other molecule(s) signaling through the activin receptor type IIB, we next determined activin A levels in muscles from fasted rats. Fasting resulted in an almost 50% reduction of activin A protein levels (Fig. 13) providing a potential explanation why p-Smad2 and p-Smad3 levels were not increased despite increased myostatin protein levels in muscle from fasted rats.

DISCUSSION

Previous studies provided convincing evidence that myostatin is a negative regulator of muscle mass. For instance, animals [McPherron and Lee, 1997; McPherron et al., 1997] and humans [Schuelke et al., 2004] lacking the myostatin gene develop substantial muscle hypertrophy and overexpression of myostatin results in muscle atrophy [Zimmers et al., 2002; Reisz-Porszasz et al., 2003]. In addition, previous studies suggest that various conditions associated with muscle atrophy, including cancer [Liu et al., 2007; Costelli et al., 2008], HIV infection [Gonzalez-Cadavid et al., 1998], burn injury [Lang et al., 2001], fasting [Jeanplong et al., 2003], denervation [Baumann et al., 2003], aging [Yarasheski et al., 2002], and muscle disuse [Reardon et al., 2001] are associated with upregulated expression of myostatin. From those observations it has been suggested that muscle wasting conditions may be associated with increased expression of myostatin and that

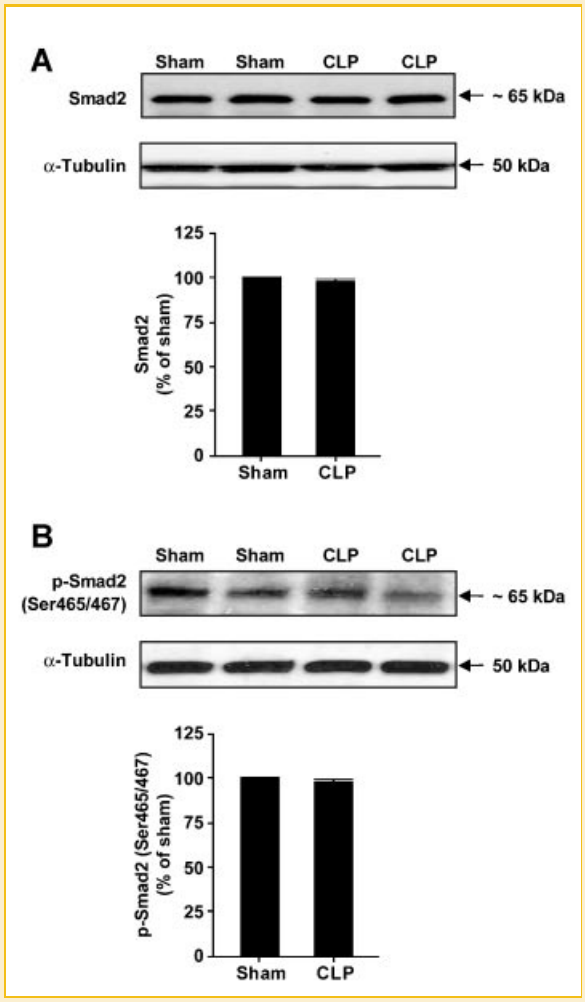

Fig. 6. The effect of sepsis in rats on (A) total Smad2 and (B) p-Smad2 (Ser 465/467) protein levels determined by Western blotting. EDL muscles were studied 16 h after sham-operation or CLP. Representative Western blots are shown in the upper panels and densitometric quantifications normalized to α-tubulin levels (loading control) are shown in the lower panels. Results are means ± SEM with n = 8 in each group.

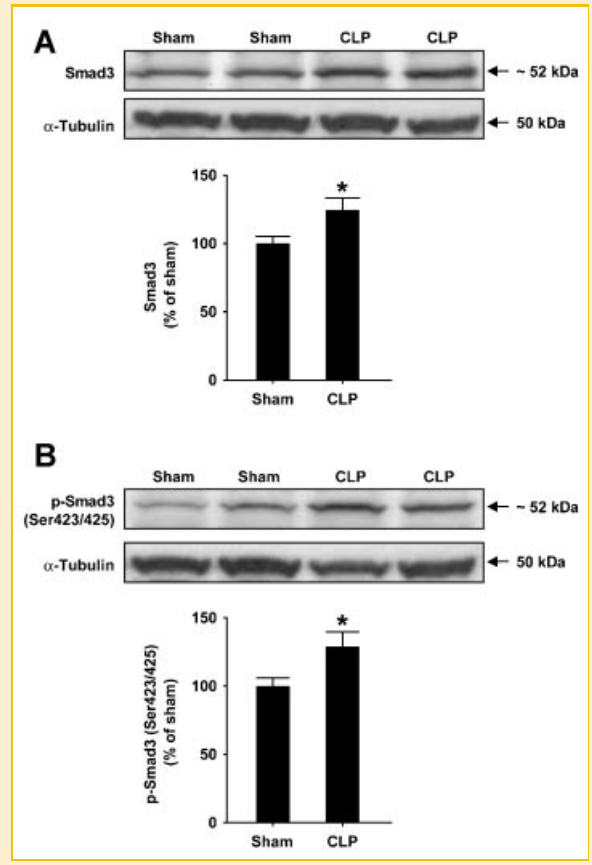

Fig. 7. The effect of sepsis in rats on (A) total Smad3 and (B) p-Smad23 (Ser 423/425) protein levels determined by Western blotting. EDL muscles were studied 16 h after sham-operation or CLP. Representative Western blots are shown in the upper panels and densitometric quantifications normalized to α-tubulin levels (loading control) are shown in the lower panels. Results are means ± SEM with n = 8 in each group. $^{*}P < 0.05$ versus sham by Student's *t*-test.

inhibition of myostatin expression and activity may be useful in the prevention and treatment of muscle wasting. Other studies supported the concept that blocking myostatin may prevent or reduce muscle wasting [Bogdanovich et al., 2002; Wagner, 2005; Carnac et al., 2006; Tsuchida et al., 2008].

It should be noted that although increased expression and activity of myostatin were observed previously in several muscle wasting conditions [Gonzalez-Cadavid et al., 1998; Lang et al., 2001; Baumann et al., 2003; Jeanplong et al., 2003; Liu et al., 2007; Costelli et al., 2008], conflicting results have also been reported. For example, in a study by Carlson et al. [1999], 7 days of hind leg unloading in mice resulted in a more than 40% atrophy of the soleus muscle without detectable changes in myostatin mRNA levels. In another study, Sakuma et al. [2000] examined the relationship between muscle mass and myostatin protein levels in rats and found that mechanical overloading resulted in a significant hypertrophy of the plantaris muscle accompanied by increased (rather than decreased) myostatin protein levels. In the same study, denervation-induced atrophy of the soleus muscle was accompanied by reduced (rather than increased) myostatin protein levels.

Additional evidence for a lack of a close correlation between the regulation of muscle mass and myostatin expression was found in a report by Baumann et al. [2003]. In that study, age- and denervation-induced muscle atrophy was examined in rats. Although gastrocnemius muscle weight was reduced by ~50% between 12 and 27 months, myostatin mRNA levels were substantially reduced (rather than increased) during the same time interval. In contrast, myostatin protein levels were increased in gastrocnemius muscle of old rats providing an additional example of a discrepancy between changes in myostatin mRNA and protein levels. In the same study [Baumann et al., 2003], denervation of the gastrocnemius muscle resulted in a significant loss of muscle mass detectable after 4 days and amounting to an ~50% loss of muscle mass after 8 days. At both these time points, myostatin mRNA and protein levels were not significantly altered.

Also, in cancer cachexia, evidence suggests that there is not a close correlation between muscle wasting and myostatin expression.

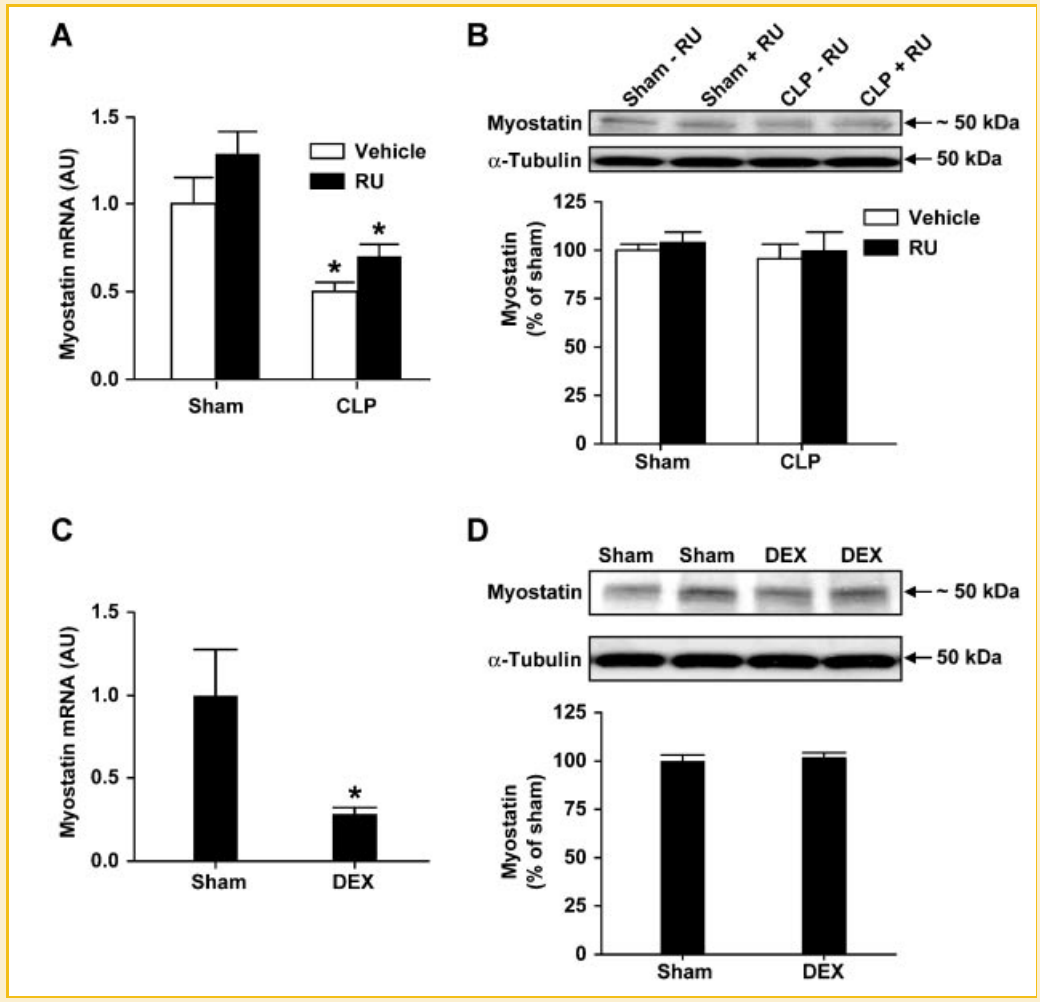

Fig. 8. The effects of sepsis, RU38486, and dexamethasone on myostatin expression in skeletal muscle. Rats were treated with RU38486 (RU, 10 mg/kg) or vehicle administered intraperitoneally 2 h before sham-operation or CLP and (A) myostatin mRNA levels were determined by real-time PCR and (B) myostatin protein levels were measured by Western blotting in EDL muscles 16 h after sham-operation or CLP. Results are means ± SEM with n = 7 or 8 in each group. In other experiments, rats were treated with dexamethasone (10 mg/kg) or corresponding volume of vehicle administered intraperitoneally and EDL muscles were harvested after 16 h for determination of (C) myostatin mRNA levels and (D) myostatin protein levels. Results are means ± SEM with n = 7 or 8 in each group. $^{*}P < 0.05$ versus corresponding sham group by ANOVA (panel A) or Student's *t*-test.

Thus, in a report by Costelli et al. [2008], an experimental model of cancer cachexia in rats resulted in an ~15% and 20% reduction of gastrocnemius muscle weight 4 and 7 days, respectively, after tumor implantation. In those experiments, muscle levels of myostatin mRNA and protein were unchanged in tumor-bearing rats on day 4 but were increased on day 7. Interestingly, Smad DNA binding activity was increased 4 days after tumor implantation (despite unchanged myostatin expression) but was unchanged on day 7 (despite increased myostatin expression) providing further support for the concept that the relationship between the regulation of muscle mass and the expression and activity of myostatin and myostatin-dependent signaling is complex. Interestingly, results suggesting that muscle levels of myostatin protein levels are not universally increased in patients with cancer were reported recently by the same group of investigators [Muscaritoli et al., 2009]. In that study, myostatin protein expression was increased in muscle tissue from patients with gastric carcinoma but not in muscle from patients with lung cancer. Interestingly, muscle levels of p-Smad2/3 were not increased in muscle from gastric cancer patients (despite increased myostatin protein levels) but were increased in patients with lung cancer (despite unchanged myostatin levels), further suggesting that the regulation of myostatin expression and activity in cancer cachexia is complex.

The present study adds sepsis to the list of muscle wasting conditions that are not associated with increased expression and activity of myostatin in skeletal muscle. Although the influence of sepsis on the expression of myostatin in skeletal muscle was examined in a previous report [Lang et al., 2001], the present study adds important novel information about the influence of sepsis on the myostatin pathway. Thus, in the present study, protein levels of myostatin, follistatin, and activin A as well as p-Smad2 and p-Smad3 levels were determined in addition to measurement of myostatin mRNA levels. This is important because previous reports suggest that there is not an absolute correlation between myostatin

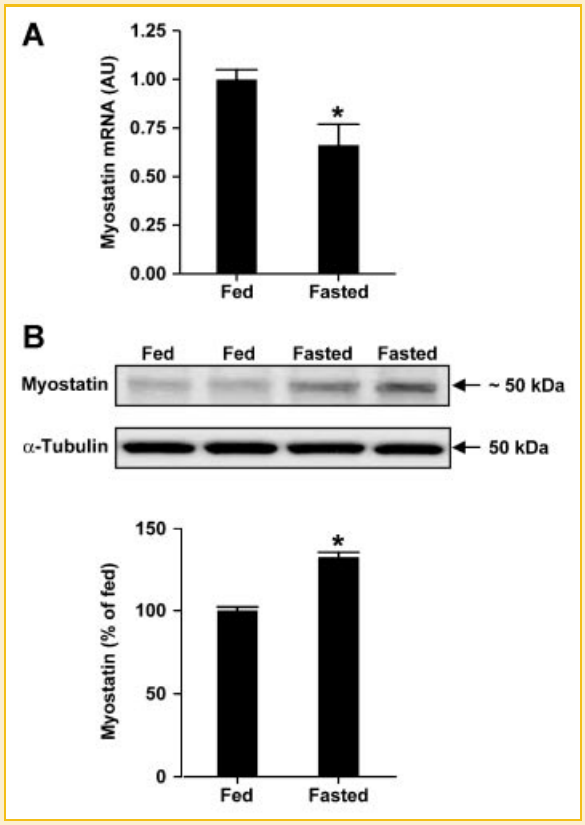

Fig. 9. The effects of fasting (48 h) on myostatin expression in skeletal muscle. A: Myostatin mRNA levels were determined in EDL muscles by real-time PCR and (B) myostatin protein levels were determined by Western blotting in EDL muscles of fed and fasted rats. Representative Western blots from EDL muscles for myostatin and α-tubulin (loading control) are shown in the upper panel and densitometric quantifications are shown in the lower panel of (B). Results are means ± SEM with n = 8 in each group. $^{*}P < 0.05$ versus corresponding fed group by Student's *t*-test.

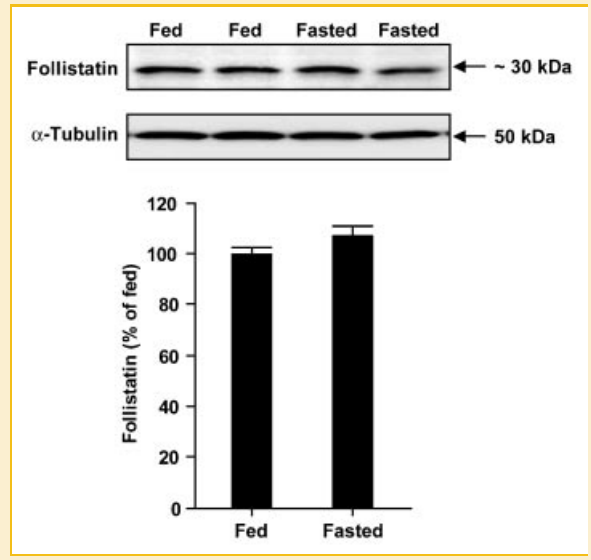

Fig. 10. The effect of fasting (48 h) on follistatin expression in skeletal muscle. Representative Western blots from EDL muscles are shown in the upper panel and densitometric quantifications of follistatin protein normalized to α-tubulin levels are shown in the lower panel. Results are means ± SEM with n = 8 in each group. The difference between the groups was not statistically significant by Student's *t*-test.

mRNA and protein levels in atrophying muscle [Baumann et al., 2003] and that activin A receptor type IIB signaling (assessed here by measuring p-Smad2 and p-Smad3 levels) can be influenced by changes in follistatin and activin A [Gilson et al., 2009].

An interesting observation in the present study was the discrepancy between changes in myostatin mRNA and protein levels. Of note, all three models of muscle wasting used in the present study (sepsis, glucocorticoid treatment, and fasting) were associated with reduced myostatin mRNA levels (rather than increased myostatin mRNA levels). In contrast, myostatin protein levels were differentially regulated in the three conditions with unchanged levels noticed in sepsis and after treatment with dexamethasone and increased myostatin protein levels in muscles from fasted rats. Unchanged or increased myostatin protein levels despite reduced myostatin mRNA levels suggest that myostatin protein levels were regulated by posttranscriptional mechanisms in the present experiments. Such mechanisms may include increased myostatin synthesis secondary to increased translational efficiency or reduced degradation of myostatin. A discrepancy between changes in myostatin mRNA and protein levels was reported in other conditions characterized by muscle atrophy as well [Baumann et al., 2003].

A noteworthy difference between the present study and the previous report by Lang et al. [2001] was the finding of a substantial reduction of myostatin mRNA levels in the present experiments whereas myostatin mRNA levels were unaltered in muscle from septic rats in the report by Lang et al. [2001]. One reason for this difference may be the different septic models used in the present and the previous experiments (CLP and intraperitoneal implantation of a fecal-agar pellet inoculated with *E. coli* and *Bacteroides fragilis*, respectively). Another reason may be the fact that rats of different ages were used in the two studies. Thus, mature rats weighing 250–300 g were used by Lang et al. [2001] whereas in the present study, young growing rats weighing 50–60 g were used. Despite these differences, the important conclusion of the present and previous studies [Lang et al., 2001] is that sepsis does not seem to increase the expression of myostatin in skeletal muscle.

An important question in light of our observations of unchanged or even decreased expression and activity of myostatin in septic muscle is whether the present experimental model resulted in muscle wasting. Without activation of a "muscle wasting program," the expectation of increased myostatin expression and activity may not be justified. Results in the present study suggest that the muscles in septic rats were indeed displaying evidence of wasting. Thus, muscle weight was lower in septic than in sham-operated rats and the mRNA levels for atrogin-1 and MuRF1 were dramatically upregulated. An additional reason why the expectation of increased myostatin expression and activity was justified is a recent study in which we found that FOXO1 expression and activity, as well as atrogin-1 and MuRF1 expression, were upregulated 8 and 16 h after

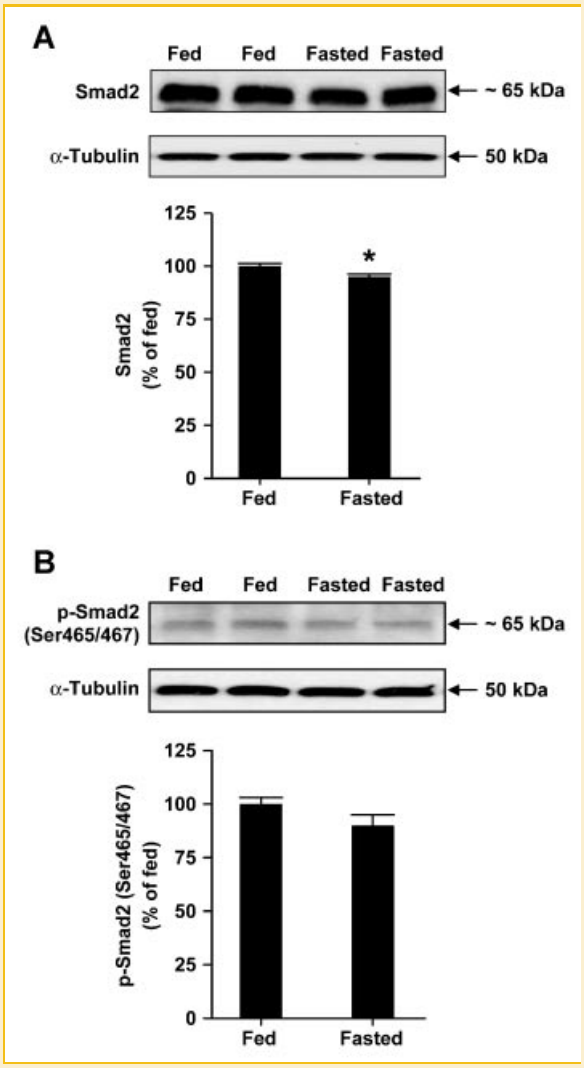

Fig. 11. The effects of fasting (48 h) on (A) total Smad2 and (B) p-Smad2 (Ser 465/467) protein levels in skeletal muscle. Representative Western blots are shown in the upper panels and densitometric quantifications are shown in the lower panels. Results are means ± SEM with n = 8 in each group. $^{*}P < 0.05$ versus corresponding fed group by Student's *t*-test.

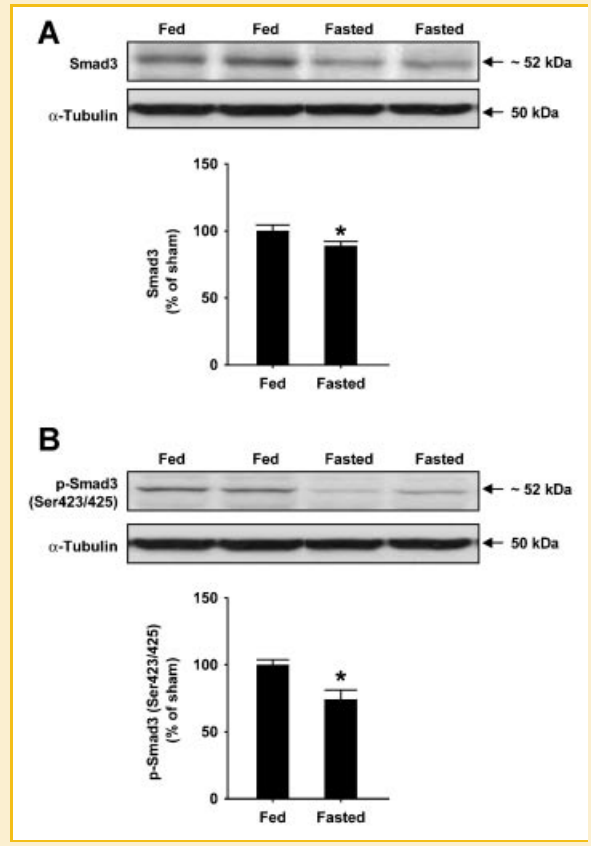

Fig. 12. The effects of fasting (48 h) on (A) total Smad3 and (B) p-Smad3 (Ser 423/425) protein levels in skeletal muscle. Representative Western blots are shown in the upper panels and densitometric quantifications are shown in the lower panels. Results are means ± SEM with n = 8 in each group. $^{*}P < 0.05$ versus corresponding fed group by Student's *t*-test.

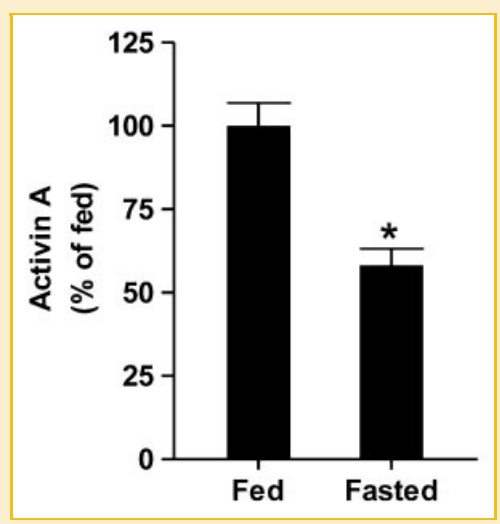

Fig. 13. The effect of fasting (48 h) on activin A expression in skeletal muscle. Activin A protein levels were determined by ELISA as described in the Materials and Methods Section. Results are means ± SEM with n = 8 in each group. $^{*}P < 0.05$ versus fed animals by Student's *t*-test.

CLP in rats [Smith et al., 2010]. In the same study, FOXO1 regulated atrogin-1 and MuRF1 expression in atrophying cultured muscle cells. In a recent report from another laboratory, evidence was found that high levels of myostatin induce muscle atrophy by a FOXO1-dependent upregulation of components of the ubiquitin-proteasome pathway, including atrogin-1 and MuRF1 [McFarlane et al., 2006]. Therefore, since both FOXO1 expression and activity and atrogin-1 and MuRF1 expression were upregulated 8 and 16 h after CLP in rats [Wray et al., 2003; Smith et al., 2010], it would be justified to expect myostatin expression and activity to be increased if myostatin plays a role in muscle wasting during sepsis. We therefore believe it is reasonable to interpret the results in the present study as indicating that sepsis-induced muscle wasting does not reflect increased myostatin expression and activity, at least not when sepsis is induced by CLP in young growing rats.

Although the present study suggests that sepsis does not increase the expression and activity of myostatin in skeletal muscle, the results should be interpreted with caution for several reasons. First, results in septic rats may not necessarily reflect the situation in patients with sepsis and although a previous study suggests that the catabolic response to sepsis may be similar in humans and rats [Tiao et al., 1997], it will be important in future studies to determine myostatin expression and activity in muscle from patients with sepsis. Second, the results observed here do not rule out the possibility that myostatin is involved in the regulation of sepsis-induced muscle wasting. For example, myostatin signaling may be influenced not only by changes in the expression of myostatin and follistatin but also by changes in the activity of proteases and other proteins that regulate the processing of promyostatin, such as members of the furin family of proprotein convertases or proteins that can interact with and influence the stability of promyostatin, such as latent TGF-β-binding protein-3 (LTBP-3) [Anderson et al., 2008]. It is also possible that maintained (basal) myostatin protein levels are required for the induction of ubiquitin-proteasome-dependent protein degradation in septic muscle. If that is the case, inhibition of the myostatin pathway, as has been suggested in other catabolic conditions [Bogdanovich et al., 2002; Wagner, 2005; Carnac et al., 2006; Haidet et al., 2008; Tsuchida et al., 2008], may prove beneficial in sepsis despite unchanged myostatin protein levels in septic muscle. Third, other members of the TGF-β superfamily than those examined here (myostatin and activin A) may be upregulated in septic muscle.

Finally, even though unchanged p-Smad2 levels suggest that activin receptor type IIB signaling was not stimulated under the present experimental conditions, the results do not rule out that TGF-β superfamily members activated other signaling pathways, such as the p38 MAPK signaling pathway [Philip et al., 2005; Carnac et al., 2006; Roffe et al., 2010]. Our present observation of increased p-Smad3 levels in septic muscle (consistent with activation of Smad3) in the presence of unchanged p-Smad2 levels suggests that mechanisms other than changes in myostatin and activin A levels may mediate muscle wasting by selectively activating Smad3. Interestingly, recent studies suggest that Smad3 may be activated by reduced PI3K/Akt activity [Conery et al., 2004; Remy et al., 2004]. Reduced IGF-I expression in septic muscle [Lang et al., 2006] may be consistent with activation of Smad3 secondary to reduced PI3K/Akt signaling during sepsis. Although the reduced p-Smad3 levels noticed here in muscle from fasting rats may reflect the reduced activin A levels noticed in the same muscles, it is also possible that increased IGF-I-induced PI3K/Akt signaling may have contributed to the inhibition of Smad3 [Conery et al., 2004; Remy et al., 2004] as suggested by increased IGF-I expression in skeletal muscle during fasting [Vendelbo et al., 2010]. Further evidence for the complex roles of myostatin, Smad2, and Smad3 in the regulation of muscle mass was provided in a recent study in which activation of both Smad2 and Smad3 by myostatin signaling activated the atrogin-1 promoter but not the MuRF1 promoter [Sartori et al., 2009]. It is obvious that more studies will be needed to provide a better understanding of the roles of myostatin, Smad2, and Smad3 in the regulation of muscle mass during sepsis and other catabolic conditions.

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