Calpain activity and muscle wasting in sepsis

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Submitted 11 February 2008; accepted in final form 13 May 2008

Smith IJ, Lecker SH, Hasselgren PO. Calpain activity and muscle wasting in sepsis. Am J Physiol Endocrinol Metab 295: E762–E771, 2008. First published May 20, 2008; doi:10.1152/ajpendo.90226.2008.—Muscle wasting in sepsis reflects activation of multiple proteolytic mechanisms, including lysosomal and ubiquitin-proteasome-dependent protein breakdown. Recent studies suggest that activation of the calpain system also plays an important role in sepsis-induced muscle wasting. Perhaps the most important consequence of calpain activation in skeletal muscle during sepsis is disruption of the sarcomere, allowing for the release of myofilaments (including actin and myosin) that are subsequently ubiquitinated and degraded by the 26S proteasome. Other important consequences of calpain activation that may contribute to muscle wasting during sepsis include degradation of certain transcription factors and nuclear cofactors, activation of the 26S proteasome, and inhibition of Akt activity, allowing for downstream activation of Foxo transcription factors and GSK-3β. The role of calpain activation in sepsis-induced muscle wasting suggests that the calpain system may be a therapeutic target in the prevention and treatment of muscle wasting during sepsis. Furthermore, because calpain activation may also be involved in muscle wasting caused by other conditions, including different muscular dystrophies and cancer, calpain inhibitors may be beneficial not only in the treatment of sepsis-induced muscle wasting but in other conditions causing muscle atrophy as well.

LOSS OF MUSCLE MASS IS COMMONLY seen in patients with sepsis (44, 55). Several other catabolic conditions, such as burn injury, cancer, uremia, and AIDS, are also associated with muscle wasting (20, 27, 29, 79). Muscle wasting has several significant clinical consequences. For example, loss of muscle mass results in weakness and fatigue, in turn resulting in delayed ambulation and prolonged rehabilitation. When patients are bedridden for long periods of time, the risks for thromboembolic events as well as for pneumonia and other pulmonary complications increase. Prolonged bed rest in itself causes loss of muscle mass, thus creating a vicious cycle (35). Patients treated in the intensive care unit may need ventilatory support for extended periods of time when respiratory muscles are atrophying (82).

Under normal conditions, muscle mass is maintained by a balance between protein synthesis and degradation, and muscle wasting can occur in any situation when this equilibrium is perturbed. There is evidence that loss of muscle mass during sepsis to a great extent reflects activated breakdown of muscle proteins, in particular the contractile proteins actin and myosin (48), but reduced protein synthesis may also contribute to sepsis-induced muscle wasting (62).

Although increased expression and activity of the ubiquitin-proteasome proteolytic pathway, including a dramatic upregulation of the muscle-specific ubiquitin ligases atrogin-1 and MuRF1 (16, 40), play an essential role in sepsis-induced muscle wasting (33, 113), other proteolytic mechanisms are also involved (50). For example, recent studies suggest that autophagy (67, 121) and lysosomal enzymes, in particular cathepsin L (26, 63), as well as certain peptidases, such as tripeptidyl peptidase II (115), are activated in skeletal muscle during sepsis.

The purpose of the present review is to discuss evidence that activation of the calpain system is an additional important mechanism of sepsis-induced muscle wasting. Although calpains may be activated secondary to cell injury and calcium leak, there is increasing evidence that calpain activity is also regulated by physiological and pathophysiological mechanisms in intact cells (39). Certain aspects of the role of calpains in muscle wasting were discussed recently by others (9, 22) and in a review from our laboratory (49). The present review adds to previous information by focusing mainly on the calpain system in sepsis-induced muscle wasting and by discussing some of the controversy that exists with regards to the relative importance of calpain- and caspase-dependent proteolysis in catabolic conditions. It also highlights pertinent recent reports on the role of calcium and calpains (4, 6, 15, 25, 33, 75, 83, 89, 92, 99, 104, 111) published after our previous review (49). Other aspects of the regulation of muscle mass during sepsis, including the role of transcription factors and nuclear cofactors and the influence of posttranslational modifications of these regulators, were reviewed recently (45, 46, 49). Various mechanisms by which calcium and calpain activity may regulate muscle mass and that are discussed in the present review are shown in Fig. 1. It should be noted that although the present review is focused on the role of calpain activation in skeletal muscle proteolysis; calcium; atrophy; calpastatin.
The Calpain System

Calpains are nonlysosomal, calcium-dependent cysteine proteases. The calpains constitute a family of at least 14 members that are ubiquitous enzymes, such as \( \mu \)- and m-calpain, or tissue specific, such as the muscle-specific calpain 3, also called CAPN3 or p94. The \( \mu \)- and m-calpains are heterodimers composed of two subunits of \( \sim 80 \) and \( 30 \) kDa, respectively. The larger subunit contains the catalytic domain, whereas the smaller unit has regulatory functions. The calpain system, its various members, as well as regulatory mechanisms were reviewed extensively elsewhere (39).

It should be noted that the role of p94 is probably unique and different (or even opposite) to that of the ubiquitous \( \mu \)- and m-calpains. For example, whereas the expression and activity of \( \mu \)- and m-calpain are increased in many muscle-wasting conditions, p94 is typically not affected or is even decreased. A striking example of the opposite role of p94 in muscle compared with the role of \( \mu \)- and m-calpain is the observation that certain types of muscular dystrophy are caused by p94 deficiency (57, 84, 102). Certain other muscle atrophy conditions (experimental cancer cachexia and muscle denervation) are also associated with the downregulation of p94 (17, 96). Of note, sepsis-induced muscle wasting may be unique with regards to the regulation of p94. For example, in previous studies (34, 112), we observed increased p94 mRNA levels in muscle from septic rats, although we have not found evidence of increased p94 activity in septic muscle. Interestingly, the activity of \( \mu \)- and m-calpain may be affected by p94 since p94 is able to cleave calpains (and calpastatin) (39). In the present review, we will mainly discuss the role of \( \mu \)- and m-calpain in sepsis-associated muscle wasting.

The regulation of calpain activity is complex. Calpains are typically in an inactive state under basal conditions. Calcium is the most important activator of calpains. Binding of calcium results in conformational changes, allowing for the catalytic site of the molecule to become activated. In addition to calcium, other factors may also influence calpain activity. For example, recent studies (39) suggest that \( \mu \)- and m-calpain activity can be influenced by phosphorylation. Other studies (86, 90, 120) suggest that certain phospholipids, in particular phosphatidylinositol, influence calpain activity by various mechanisms, including lowered calcium concentration needed for autolysis of \( \mu \)- and m-calpain. Additional molecules have also been found to regulate calpain activity by lowering the calcium requirements for activation, including isovaleryl-\( \Delta \)6ceramide (80) and a 40–45 kDa endogenous “activator” present in skeletal muscle (81).

An additional important regulator of calpain activity is the endogenous inhibitor calpastatin (39). Of note, calcium not only regulates the activity of calpains but also influences the binding of calpastatin to calpain, resulting in inhibited calpain activity. In addition, calpain is autocatalyzed, i.e., activated calpain degrades itself. Activated calpain can also degrade calpastatin, adding an additional level of complexity to the regulation of the calpain system.

**Muscle Wasting is Associated with Increased Calcium Uptake and Concentrations**

Multiple previous studies (36) provided evidence for a role of calcium-dependent mechanisms (probably at least in part reflecting calpain activation) in muscle wasting. In early studies (5), treatment of incubated muscles with calcium or a calcium ionophore increased protein degradation. In recent experiments (69) in our laboratory, treatment of cultured myotubes with the calcium ionophore A23187 stimulated proteasome activity and this effect was at least in part regulated by calpain activation. In other reports, muscle calcium uptake and intracellular concentrations were increased in sepsis (11, 34) as

![Fig. 1. Summary of the role of calpain activation in sepsis-induced muscle wasting.](image-url)
well as in other catabolic conditions, including burn injury (88), and cancer (21). Experiments using the “calcium antagonist” dantrolene, a drug that blocks the net release of calcium from the sarcoplasmic reticulum into the sarcoplasm (59), as well as recent (unpublished) observations in our laboratory using dexamethasone-treated myotubes, suggest that increased calcium levels in atrophying muscle reflects increased store operated calcium entry. Taken together, studies showing calcium-dependent regulation of muscle protein degradation provide important indirect evidence for a role of calpains in muscle wasting (although, of course, calcium may regulate protein degradation through other mechanisms as well).

Additional evidence for a role of calcium and calcium-regulated activation of calpains in muscle proteolysis was provided in a recent study by Smith and Dodd (92). In that study, incubated muscle preparations from rats were treated in vitro with 3.5 mM calcium in the absence or presence of the calpain inhibitor calpeptin. Exposing the muscles to calcium resulted in calpain activation, assessed by determining tissue levels of the 190 kDa talin cleavage fragment, and a 65% increase in protein degradation. Treatment of the muscles with calpeptin prevented calpain activation and the increase in protein degradation, providing evidence for a link between calcium-regulated calpain activation and muscle proteolysis.

Calpain Activity is Increased in Skeletal Muscle During Sepsis and May at Least in Part be Caused by Inhibition of Calpastatin Activity

In previous studies (34, 112), increased calcium levels and upregulated expression of calpains in skeletal muscle during sepsis provided indirect evidence of calpain activation. More direct evidence of increased calpain activity and expression in skeletal muscle during sepsis was reported by Bhattacharyya et al. (13) and Voisin et al. (105). In the study by Bhattacharyya et al. (13), calpain activity, determined by measuring the degradation of the calpain substrate azocasein in muscle extracts, was increased by ~70% in rats made septic by intraabdominal implantation of pellets containing Escherichia coli and Bacteroides fragilis bacteria. In the study by Voisin et al. (105), “chronic sepsis” 6 days after the intravenous injection of live E. coli bacteria was associated with an ~1.5-fold increase in mRNA levels for m-calpain (μ-calpain mRNA levels were not determined) and a tendency (although not statistically significant) for increased lysosomal and calcium-dependent protein breakdown determined by using the cysteine protease inhibitor E-64c. In a recent study (110) in our laboratory, the degradation of different calpain-specific substrates was increased in muscle extracts from septic rats, consistent with sepsis-induced increase of calpain activity. Because, in the same study, μ- and m-calpain activity was not increased in septic muscle when measured by zymography (a method in which calpains are separated from calpastatin), increased net calpain activity in muscle extracts (containing both calpains and calpastatin) may represent reduced calpastatin activity. This was indeed confirmed in the same study when calpastatin activity was measured separately and was reduced by 40–60% in muscles from septic rats. In more recent experiments (33) in our laboratory, transfection of cultured muscle cells with a plasmid expressing calpastatin cDNA resulted in elevated cellular calpastatin levels and reduced protein degradation in dexamethasone-treated muscle cells. The use of dexamethasone-treated muscle cells in those experiments was important because glucocorticoids are important mediators of muscle proteolysis during sepsis (43).

The observation that reduced calpastatin activity may be a mechanism of calpain activation in skeletal muscle during sepsis (33, 110) is in line with a recent report by Tidball and Spencer (103). In their study, transgenic overexpression of calpastatin in skeletal muscle of mice reduced muscle atrophy induced by unloading. Other reports as well support a role for changes in the balance between calpains and calpastatin in the regulation of muscle protein homeostasis. For example, Costelli et al. (23) reported that calcium-dependent muscle proteolysis in tumor-bearing rats reflected reduced calpastatin expression. In other studies (7), calpain-dependent protein degradation in differentiating myoblasts reflected reduced calpastatin expression and increased the calpain-to-calpastatin ratio.

Calpain Activation is Involved in Muscle Wasting During Other Catabolic Conditions

Although the present review is mainly focused on the role of calpains in sepsis-induced muscle wasting, it should be noted that other catabolic conditions as well have been associated with increased calpain expression and activity in studies from other laboratories. For example, evidence for a role of calpain-mediated proteolysis was reported in patients with acute quadriplegic myopathy (91) and in different muscular dystrophies (102). Activation of calcium-dependent proteolysis (23) and increased mRNA expression of m-calpain (17) were observed in skeletal muscle of tumor-bearing rats. In other experiments, transfection of cultured myocytes with a dominant negative m-calpain resulted in an ~30% inhibition of protein degradation and when calpastatin was overexpressed, protein degradation was reduced by ~60% (52). Interestingly, in the same study, inhibition of calpain activity stabilized nebulin, a protein that is essential for the integrity of the sarcomere. Other conditions that have been reported in more recent studies to be regulated by calcium- and calpain-dependent mechanisms include muscle unloading and immobilization, aging, and sarcopenia, as well as myoblast differentiation (4, 6, 25, 36, 75, 83, 89, 99).

Calpain Activation Disrupts the Sarcomere and Releases Myofilaments

It is possible that calpain activation regulates muscle mass by multiple mechanisms. One such mechanism is disruption of the sarcomere with release of actin and myosin (47, 52, 93, 94, 112) and probably other myofilaments as well, including desmin, α-actinin, titin, and nebulin (52, 65, 112).

The sarcomere is the smallest unit of the myofibril. The function and architecture of the sarcomere have been reviewed elsewhere (1, 98). The Z disks serve to anchor and organize the myofilaments and to mechanically link actin from one sarcomere to the next sarcomere along the myofibril. There is evidence that α-actinin is involved in the anchorage of actin to the Z disk and in the cross-linking of one actin molecule to the next actin molecule in the adjacent sarcomere. Actin and α-actinin make up a large proportion of the Z disk, but other...
Early studies provided evidence that release of myofilaments from the sarcomere occurs in various catabolic conditions, including fasting and treatment with glucocorticoids (24) and that calcium/calpain-dependent mechanisms may be involved (10, 30). Skeletal muscle contractile proteins, actin and myosin, are poor calpain substrates but other proteins that are important for the structural integrity of the sarcomere are excellent calpain substrates. For example, titin and nebulin, proteins that anchor myofilaments to the Z disk, are readily cleaved by calpain (52, 65). In a recent study (112), we found both biochemical and morphological evidence that disruption of the sarcomere, followed by release of actin and myosin, plays a role in sepsis-induced muscle wasting as well. Thus, sepsis in rats resulted in an increase in the fraction of “easily releasable myofilaments,” mainly accounted for by release of actin and myosin. Importantly, the sepsis-induced increase in “easily releasable myofilaments” was blocked in rats treated with dantrolene, suggesting that the effect of sepsis was at least in part regulated by a calcium-sensitive mechanism, possibly calpain activity.

In the same study (112), electron microscopy provided morphological evidence of sarcomere disruption. In particular, there was a loss of normal Z-band morphology and normal registry (alignment) of neighboring myofibrils (so called “streaming”). The sepsis-induced changes in the Z-bands were associated with increased amounts of released titin, α-actinin, actin, desmin, and myosin as determined by electrophoretic separation of proteins. Changes in Z disk morphology, similar to those observed in septic muscle, were reported in early studies on muscle atrophy caused by denervation and muscular dystrophy (76, 100). Thus, Z-disk disintegration and disruption of the anchorage of myofilaments to the Z disk may be a common response in different muscle wasting conditions. Importantly, the Z disk is a site of subcellular calpain and calpastatin localization (95, 119).

Recent studies (1) suggest that the M band is also essential for the integrity of the sarcomere. The M band, situated in the center of the sarcomere, links individual myosin molecules by a network of cross-linking filaments, including M-protein and myomesin (1, 12, 73). Titin is an additional important component of the M band. The influence of sepsis (and other muscle wasting conditions) on the integrity of the M band and its cross-linking filaments remains to be determined. Interestingly, tibial muscular dystrophy, an autosomal dominant late-onset distal myopathy, is associated with a defect of the M-line titin (41) and titin M-line deficient mice develop muscle atrophy (77). These observations suggest that M-line disruption may be involved in muscle wasting, probably by allowing for the release of myosin from the sarcomere.

**Calcium/Calpain-Dependent Release of Myofilaments from the Sarcomere is Upstream of the Ubiquitin-Proteasome System**

There are multiple levels of evidence supporting a model in which “upstream” release of myofilaments from the sarcomere is followed by ubiquitination of the myofilaments and degradation by the proteasome (27, 37, 47, 52, 56, 58, 93, 94, 112). Previous studies (52) suggest that the proteasome does not degrade intact myofibrils. For example, incubation of ovine skeletal muscle with proteasomes failed to induce morphological or biochemical evidence of myofibrillar protein breakdown. In other studies (93, 94), isolated muscle proteasomes degraded free actin and myosin but not intact myofibrils. These observations suggest that actin and myosin need to be released from the myofibrils before they can be ubiquitinated and degraded by the 26S proteasome and it is likely that mechanisms regulating the integrity of the sarcomere play an important role in maintaining muscle mass. We and others (37, 47, 52, 58, 93, 94) have proposed a model in which muscle wasting is associated with an early “upstream” dissociation of the myofibrils resulting in release of actin and myosin that are subsequently ubiquitinated and degraded by the 26S proteasome and reports both from our (47, 112) and other laboratories (9, 25, 37, 52, 58) suggest that calpain activation may be involved in the sarcomere disassembly during muscle wasting.

It should be noted that although several previous reports support a role of calcium-calpain-dependent release of myofilaments from the sarcomere as an integral part of protein degradation in atrophying muscle, apparently contradictory results have been reported, including a study from our laboratory (101). In that report, which provided the first evidence for a role of the ubiquitin-proteasome system in sepsis-induced muscle proteolysis, reducing the calcium concentration in the medium of incubated muscles in vitro inhibited total protein breakdown (assessed as net release of tyrosine). Because the same effect of calcium depletion on protein degradation was observed in muscles from sham-operated and septic rats and because protein breakdown rates remained higher in septic than in nonseptic muscles even in the absence of calcium in the incubation medium, we interpreted the results as indicating that calcium-dependent mechanisms are not involved in sepsis-induced muscle proteolysis. The findings in the same study (101) that changes in the calcium concentrations in the incubation medium and that treatment of the incubated muscles with the cysteine protease inhibitor E-64 did not alter myofibrillar protein degradation (assessed as net release of 3-methylhistidine) were interpreted as additional evidence that calcium-calpain-dependent mechanisms are not involved in sepsis-induced muscle proteolysis. It should be noted, however, that a lack of effect of changes in calcium concentrations in the incubation medium on myofibrillar protein breakdown rates does not rule out an involvement of calcium-dependent mechanisms in sepsis-induced myofibrillar protein breakdown in vivo. In the study by Tiao et al. (101), calcium concentration was increased from 0 to 2.5 mM (which is within normal range of serum concentrations) and it is possible that higher calcium concentrations would be needed to influence myofibrillar proteolysis. It is also possible that changes in cellular calcium levels caused by adding calcium to the medium of incubated muscles during a 2 h incubation period do not cause changes in calcium homeostasis identical to those occurring in vivo during the course of sepsis. In light of more recent experiments in which we found that treatment of rats in vivo with dantrolene prevented sepsis-induced muscle proteolysis (including myofibrillar protein breakdown) and release of myofilaments from the sarcomere (34, 112), it is likely that calcium-dependent mechanisms are indeed involved in sepsis-induced muscle wasting. That conclusion was also supported by a recent study in which we found that treatment of rats with the calpain inhibitors calpe-
tin and BN82270 prevented sepsis-induced muscle proteolysis (33). Therefore, some of our observations reported previously (101) probably need to be reinterpreted considering new information that has become available after that report was published.

Similarly, our observation that treatment of incubated muscles with E-64 did not regulate protein breakdown rates (101) may need to be reinterpreted as well. In those experiments, E-64 was added to incubated muscles together with the lysosomal inhibitor methylamine and considering the potential interaction between different proteolytic mechanisms and the fact that E-64 is not a specific calpain inhibitor but also blocks the lysosomal cysteine proteases cathepsin B, H, and L (8), the results from our previous experiments probably do not allow for the interpretation that calpain activity is not involved in sepsis-induced muscle proteolysis. Indeed, in subsequent experiments, using more specific calpain inhibitors added to incubated muscles in the absence of other inhibitors, results strongly supported a role of calpain-dependent mechanisms in sepsis-induced muscle proteolysis (33).

Calpains may regulate the ubiquitin-proteasome system at multiple levels. For example, calpains may activate the ubiquitin-proteasome pathway by increasing substrate flux through the system. Indeed, evidence suggesting that the ubiquitin-proteasome system is regulated by the availability of substrates was reported by Li et al. (64). In that study, E3-ligase activity was increased by substrate-regulated stabilization of one component of an E3-ligase complex. Previous work from our laboratory also supports a model in which calpain cleavage products become substrates for, and thereby drive, the ubiquitin-proteasome system. For instance, we recently found that treating cultured myotubes with the calcium ionophore A23187 or thapsigargin resulted in a dose- and time-dependent increase in proteasome activity and that this effect was, at least in part, regulated by calpain activity, although other mechanisms were also involved including calmodulin- and calcium-calmodulin kinase II-dependent mechanisms (the role of caspases was difficult to assess because caspase inhibitors reduced basal proteasome activity and the effect of A23187 was similar in the absence and presence of caspase inhibitors) (69). Additional support for this model was provided in a recent study by Smith and Dodd (92) in which calcium/calpain-mediated protein degradation in incubated rat diaphragm muscle preparations was blocked by the proteasome inhibitor epoxomicin. In the same study, calpain activation resulted in increased proteasome-dependent protein degradation. Also in the cardiomyocyte, there is evidence that upstream calpain-dependent mechanisms regulate ubiquitination of proteins and activation of the proteasome (37).

It should be noted that apparently contradictory results have been reported with regard to the influence of calcium and calpain activity on muscle ubiquitin-proteasome activity. In the study by Smith and Dodd (92), treatment of incubated preparations from rat diaphragms muscles with calcium did not alter proteasome activity despite calpain activation. Additionally, in experiments in our laboratory, treatment of incubated extensor digitorum longus muscles from septic rats with the calpain inhibitor BN82270 did not influence overall proteasome-dependent protein degradation and treatment of rats in vivo with calpain inhibitor did not prevent sepsis-induced increase in muscle atrogin-1 and MuRF1 expression (33). Thus, additional studies are needed to better define the role of calcium and calpains in activation of the ubiquitin-proteasome system in catabolic muscle.

Calpain Activation Downregulates Akt Activity in Skeletal Muscle

In a recent study (92), the increased calpain activity and calpain-dependent protein degradation noted in calcium-treated incubated rat muscles was associated with the novel observation of reduced levels of phosphorylated (Ser473) Akt, consistent with reduced Akt activity. Interestingly, in the same study, the abundance of total Akt was not influenced by treatment of the muscles with calcium, suggesting that post-translational modifications of Akt (reduced phosphorylation) account for the majority of inhibited Akt activity. Of note, the antibody used for detection of Akt in those experiments reacts with all three isoforms of Akt and it is not known if any of the Akt isoforms was differentially regulated in calcium-treated muscles. The reduced Akt activity in calcium-treated muscles was accompanied by reduced phosphorylation of various downstream signaling molecules, including mammalian target of rapamycin (mTOR) and GSK-3β. Although the mechanisms of calpain-regulated inhibition of Akt phosphorylation and activity are not fully understood, it is possible that degradation of kinase(s) at least in part can explain the reduced expression of phosphorylated Akt after calpain activation. Additional studies will be important to better understand the mechanisms of calpain-dependent regulation of Akt.

Regardless of the mechanisms, the observation that calpain activity may downregulate Akt signaling is important considering previous evidence that Akt signaling provides an essential anabolic influence in skeletal muscle (38, 70). Multiple downstream mechanisms may account for the anabolic effects of Akt signaling (and catabolic effects of inhibited Akt signaling; Ref. 68). For example, inhibition of Akt activity results in reduced phosphorylation (activation) of GSK-3β. Recent observations support a role of activated GSK-3β in muscle wasting caused by sepsis (31) and burn injury (32). Thus, in a recent study (31) we found that muscle levels of phosphorylated (Ser9) GSK-3β were reduced in muscles from septic rats, consistent with activation of the kinase, and in the same study, treatment of septic muscles with GSK-3β inhibitors reduced protein breakdown. In another study (32), burn-induced muscle proteolysis was associated with activation of GSK-3β determined as reduced p(Ser9)-GSK-3β levels and increased GSK-3β kinase activity. Reduced phosphorylation and activity of mTOR, downstream of inhibited Akt activity, are important for the development of muscle wasting mainly by inhibition of protein synthesis (61). Recent studies (107) suggest that inhibited mTOR signaling in dexamethasone-treated rats and in cultured muscle cells treated with dexamethasone (models that are both associated with muscle atrophy) was at least in part mediated by increased expression of the mTOR repressors REDD 1 and Tuberin. Reduced Tuberin phosphorylation after inhibition of Akt activity may be an additional mechanism activating Tuberin and reducing mTOR activity (106).

An additional mechanism by which reduced Akt activity regulates muscle mass is by inhibiting phosphorylation of Foxo transcription factors (18). Reduced phosphorylation is an important mechanism of nuclear translocation and transcriptional...
activation of FoxO transcription factors (53). Recent studies (87, 97) have provided strong evidence for a role of activation of different members of the family of Foxo transcription factors in muscle wasting, in particular their role in regulating the expression and activity of atrogin-1 and MuRF1. It will be important in future experiments to determine the role of calpain activity in the activation of Foxo transcription factors (as well as in the activation of GSK-3β and the inhibition of mTOR) for the loss of muscle mass during sepsis and other catabolic conditions.

**Calpains Regulate the Expression of Transcription Factors**

An additional mechanism by which calpains may influence muscle mass is by regulating the turnover of transcription factors and other regulatory proteins involved in muscle wasting. Previous studies provided evidence that CEBP-β DNA binding activity was increased in cultured pituitary cells treated with the calcium ionophore A23187 (109) consistent with possible calpain-dependent upregulation of CEBP-β activity. This observation was particularly interesting considering our previous observation of increased CEBP-β activity in muscle from septic and dexamethasone-treated rats (78, 116). Surprisingly, however, we found in recent experiments that treatment of cultured myotubes with different calpain inhibitors or transfection of myoblasts with a plasmid expressing calpastatin resulted in increased CEBP-β levels secondary to reduced degradation of the transcription factor (111). This effect of calpain inhibition was associated with upregulated CEBP-β DNA binding activity and transcriptional activity. When μ- and m-calpain was overexpressed in myoblasts, CEBP-β expression was reduced, further supporting the concept that CEBP-β degradation in muscle cells is calpain dependent. In additional experiments, coimmunoprecipitation provided evidence for protein-protein interaction between CEBP-β and μ- and m-calpain, suggesting that CEBP-β is a calpain substrate. Interestingly, other members of the CEBP family were not influenced by calpain activation or inhibition, suggesting that the role of calpains was specific for CEBP-β, at least in skeletal muscle cells. Of note, even though the expression of CEBP-β in skeletal muscle is influenced by sepsis (78) and calpain activation (111), more studies are needed to determine if calpain-dependent regulation of CEBP-β expression and activity actually influences the transcription of genes involved in muscle wasting.

The increased CEBP-β levels in muscle cells after inhibition of calpain activity (111) may seem contradictory to previous reports in which we found that CEBP-β levels and calpain activity were increased in muscle during sepsis (78, 110). It should be noted, however, that increased expression of CEBP-β in catabolic muscle is probably caused by transcriptionally regulated production of CEBP-β, mainly regulated by glucocorticoids (78, 116). Calpain-dependent degradation of CEBP-β in the same muscle may serve the purpose of “fine-tuning” cellular levels of the transcription factor or may reflect compartmentalization of CEBP-β synthesis and degradation. Regardless, the observations suggest that CEBP-β expression may be regulated by both increased synthesis and calpain-dependent degradation in skeletal muscle during sepsis.

In addition to CEBP-β, the expression of other transcription factors involved in muscle wasting may also be influenced by calpain activity. For example, studies suggest that the expression of members of the STAT family of transcription factors (74), NF-κB (66), and AP-1 (51) may also be degraded by calpain-dependent mechanisms. Importantly, studies suggest that calpains may activate NF-κB by regulating the degradation of the inhibitory protein IKKα in parallel to ubiquitin-proteasome-dependent degradation of IKKα (42). Similarly, the degradation of p300, a nuclear cofactor involved in muscle wasting (117, 118), is at least in part regulated by calpain activity (85).

**Treatment with Calpain Inhibitors Reduces Sepsis-Induced Muscle Proteolysis, a Clinically Important Observation**

In previous studies in our laboratory, treatment of rats with the “calcium antagonist” dantrolene prevented sepsis-induced muscle proteolysis (34, 112, 114). This response was associated with the prevention of the sepsis-induced increase in muscle calcium levels (34), suggesting that the inhibition of protein degradation at least in part reflected calpain inhibition. This was supported by reduced expression of μ- and m-calpain in the same experiments. It should be noted, however, that the interpretation of the results in that study was complicated by the fact that treatment with dantrolene also resulted in the prevention of the sepsis-induced increase in circulating TNF-α and corticosterone levels (34) and it is therefore possible that some of the muscle-sparing effects were caused by reduced TNFα and corticosterone levels in addition to reduced muscle calpain activity.

More direct evidence that inhibition of calpain activity can reduce protein breakdown was found in experiments in which treatment in vitro of incubated muscles from septic rats with the calpain inhibitor calpeptin resulted in reduced calpain activity and protein degradation (33). Importantly, in the same study, treatment of rats with calpain inhibitors in vivo also prevented the sepsis-induced muscle proteolysis. Thus treatment with calpeptin resulted in a dose-dependent inhibition of muscle protein degradation in septic rats with an ~25% inhibition of proteolytic rates noticed after administration of a total dose of calpeptin of 12 mg/kg. Because calpeptin, like most pharmacological inhibitors, is not completely specific, we also examined the effects of a novel calpain inhibitor, BN82270 (3). This drug as well inhibited sepsis-induced muscle proteolysis, both when septic rats were treated in vivo (total dose of 180 mg/kg) and when incubated muscles from septic rats were treated in vitro (100 μM), providing further support for the concept that treatment with calpain inhibitors may prevent sepsis-induced muscle proteolysis. Because, in those experiments, administration of the calpain inhibitors in vivo was commenced before the onset of sepsis, it is not known if calpain inhibitors can be used as treatment, rather than prevention, of sepsis-induced muscle wasting. However, the fact that treatment of incubated muscles from septic rats with calpeptin or BN82270 reduced protein degradation (33, 110) suggests that calpain inhibitors may reduce the catabolic response in skeletal muscle in which protein breakdown has already been activated, an observation that has clinical implications by suggesting that treatment of sepsis-induced muscle proteolysis with calpain inhibitors may actually be possible. Further studies will be needed, however, to test that notion by administering calpain inhibitors in vivo after the onset of sepsis.
The Role of Calpains in Muscle Wasting Remains Controversial

Although multiple previous studies support a role of calpains in muscle atrophy, it should be noted that apparently contradictory results have also been reported, sometimes even in the same paper. For example, in the study by Voisin et al. (105), sepsis induced by injection of live E. coli bacteria in rats increased m-calpain mRNA levels in skeletal muscle (providing support for a role of calpains) but did not result in statistically significant changes in calcium-lysosome-dependent proteolysis (although there was a trend towards increased calcium-lysosome-dependent proteolysis). When the influence of different muscle wasting conditions on the gene expression in skeletal muscle was examined using micro array hybridization, mRNA levels for calpains were not altered (63). Although that observation may be interpreted as indicating that muscle wasting is not caused by calpain-dependent mechanisms, the results do not rule out a role of calpain activation since calpain protein levels and activity were not determined and the expression and activity of calpastatin were also not examined in that study (63).

In addition, a recent study by Du et al. (28) questioned the importance of calpains in the development of muscle wasting, at least as seen in diabetic and uremic rats. Results in that study suggested that increased caspase-3 activity, rather than increased calpain activity, may be a mechanism of sarcomere disruption and release of myofilaments. The conclusions in that study were based on experiments in which incubation of purified actomyosin or muscle lysates with recombinant caspase-3 resulted in the generation of a 14-kDa cleaved actin fragment. In additional experiments in the same study, the 14-kDa actin fragment was used as a marker of actin degradation in serum-deprived cultured L6 myotubes and in muscles from rats with acute diabetes or chronic uremia. When muscles from these rats were incubated with a caspase-3 inhibitor, the 14-kDa actin fragment levels decreased consistent with the concept that caspase-3 regulated the cleavage of actomyosin and actin.

Of note, calpain activity was not determined in the experiments reported by Du et al. (28) and the results therefore do not rule out the possibility that calpain activity as well was increased in the catabolic muscle preparations studied in those experiments. This is particularly important considering the fact that various inhibitors, including caspase-3 inhibitors, are not completely specific. In addition, the authors reported that tyrosine release from incubated epitrichelaris muscles from diabetic rats was reduced by ~13% by the caspase-3 inhibitor Ac-DEVD-CHO. This was a relatively modest reduction of protein degradation that did not prevent the diabetes-induced increase in muscle proteolysis. Thus protein degradation remained increased by ~35% compared with muscles from nondiabetic rats even in the presence of Ac-DEVD-CHO. It would have been interesting to see the effects of calpain inhibition in those experiments.

It should be noted that in our recent study, in which we found evidence for a role of calpain activity in sepsis-induced muscle proteolysis, we found no evidence for a role of caspase-3 activity (110). Thus in those experiments, caspase-3 expression and activity were not altered in muscles from septic rats and treatment of the muscles with Ac-DEVD-CHO did not influence the sepsis-induced activation of protein degradation. Similarly, treatment of dexamethasone-treated cultured L6 myotubes with various caspase inhibitors did not prevent the dexamethasone-induced increase in protein degradation whereas treatment of the myotubes with calpeptin blocked the dexamethasone-activated proteolysis. In more recent experiments, we found that the levels of the 14-kDa actin cleavage fragment were not different in muscles from sham-operated and septic rats (unpublished observations). Thus, at this point, we lack evidence for a role of caspase-3 activity in sepsis-induced and glucocorticoid-regulated muscle proteolysis, at least in rats with septic peritonitis and in dexamethasone-treated myotubes.

The interpretation of previous observations regarding the roles of calpain and caspase activities in muscle wasting is complicated by the fact that the calpain and caspase systems can interact with each other (72). For example, truncation of caspase-3 by calpains may activate caspase-3 (14) and calpain-mediated cleavage of caspase-9 may result in loss of the ability to activate caspase-3 (19). Furthermore, caspases may increase calpain activity by cleaving calpastatin (108). It is possible that muscle wasting in various catabolic conditions is regulated by cross-talk between calpains and caspases. It is also possible, and perhaps even likely, that the relative importance of the two systems (and other proteolytic mechanisms as well) may vary in different conditions. The existence of apparently contradictory reports with regards to the role of calpains and caspases in muscle wasting makes a review of this field particularly important, not only to lend a broader perspective to a controversial topic, but also to provide further insight into potential reasons behind conflicting experimental results.

Concluding Remarks

This review argues for the concept that calcium-regulated calpain activation is an important mechanism of sepsis-induced muscle wasting. Although disruption of the sarcomere with release of actin and myosin may be the most critical consequence of calpain activation, other effects of calpain activation probably contribute significantly to the development of muscle wasting during sepsis. Of note, several of the effects of calpain activity are interrelated and may influence each other, adding further complexity to calpain-dependent regulation of muscle proteolysis. Considering the pivotal role of the calpain system, future studies aimed at determining the effects of calpain inhibitors in the prevention and treatment of muscle wasting will be important. Before such studies are performed, however, several areas of potential controversy with regards to calpain activation and muscle wasting need to be resolved. Among such areas, the interaction between calpains and other proteolytic mechanisms, in particular caspase activation, as well as the differential involvement of calpains in various disease states, need to be addressed. In addition, the potential role of calpain activity in some of the novel mechanisms regulating muscle mass, such as AMP-activated protein kinase-mediated expression of atrogin-1 and MuRF1 and myofibrillar protein degradation (60, 71) should be explored.

ACKNOWLEDGMENTS

We thank Marleen Marino, Beth Israel Deaconess Medical Center Media Services, for skillful assistance in the preparation of Fig. 1.
GRANTS

This work was supported in part by National Institutes of Health Grants R01-DK-73908 (F. O. Hasselgren), R01-NR-08545 (F. O. Hasselgren), and R01-D-62307 (S. H. Lecker).

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