Calpain Activation Contributes to Endotoxin-Induced Diaphragmatic Dysfunction

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Calpain activation occurs in skeletal muscle in response to infection, but it is unknown if calpain inhibition improves muscle functional capacity. We hypothesized that infection induces diaphragm calpain activation, that calpain activation results in cleavage of important diaphragm cytoskeletal proteins, and that inhibition of calpain attenuates infection-induced diaphragm dysfunction. Mice (n = 4–6/group) were given: (*1*) saline (intraperitoneal); (*2*) endotoxin (12 mg/kg intraperitoneal); (*3*) calpain inhibitor peptide III (12 mg/kg intraperitoneal); and (*4*) endotoxin (12 mg/kg) plus calpain inhibitor peptide III (12 mg/kg). At 24 hours, diaphragms were removed and the following determined: (*1*) calpain activity by fluorogenic assay; (*2*) calpain I and II protein levels; (*3*) talin protein levels; and (*4*) the force–frequency relationship. Endotoxin significantly increased diaphragm calpain activity ($P < 0.001$), active calpain I protein ($P < 0.001$), active calpain II protein ($P < 0.01$), levels of a calpain-specific cleavage talin degradation product ($P < 0.003$), and reduced diaphragm force ($P < 0.001$). Calpain inhibitor III administration prevented endotoxin-induced increases in calpain activity, reduced talin degradation, and attenuated reductions in diaphragm force. Diaphragm-specific force at 150 Hz stimulation was significantly higher in control, endotoxin plus calpain inhibitor III, and calpain inhibitor III alone groups (23 ± 1, 20 ± 1 and 23 ± 1 N/cm^2, respectively) than in the endotoxin alone group (15 ± 1 N/cm^2) ($P < 0.01$). This model of sepsis results in significant diaphragm calpain activation and calpain-dependent diaphragm cytoskeletal protein cleavage. Moreover, calpain inhibition attenuates endotoxin-induced diaphragm weakness, suggesting that such inhibitors may be a potential treatment to improve respiratory muscle function in infected patients.

Keywords: diaphragm; endotoxin; calpain; proteolysis

Recent studies using the magnetic twitch stimulation technique to assess diaphragm function in critically ill, mechanically ventilated patients found that these patients have severe weakness, averaging diaphragm twitch forces that are only 20–25% of those observed in normal subjects (1, 2). This severe weakness may contribute to the prolonged requirement for mechanical ventilation seen in a high percentage of critically ill patients, and may, in addition, predispose these patients to recurrent ventilatory failure after intensive care unit (ICU) discharge (3).

The precise mechanisms by which patients in the ICU acquire profound respiratory muscle weakness are incompletely understood, but many of these patients have infections, and infections have been shown to induce large reductions in respiratory muscle strength in both human and animal studies (4, 5). It has been postulated that infections may affect skeletal muscle function via activation of one or more members of the calpain family of proteases (6), but previous work examining this possibility has been limited to the study of limb skeletal musculature (6). Moreover, no previous study has determined if chemical inhibitors of the calpain family of proteases are capable of reducing either respiratory or limb skeletal muscle dysfunction in any animal model of infection or inflammation.

The purpose of the present experiment was to examine this important issue, and to test the specific hypotheses that: (*1*) calpain is activated in the diaphragm after endotoxin administration; (*2*) calpain activation is associated with proteolytic cleavage of diaphragm proteins; and (*3*) administration of a calpain inhibitor in addition to endotoxin prevents protein cleavage and preserves diaphragm force-generating capacity. Because previous work indicates that free radicals play a role in mediating inflammation-induced muscle dysfunction, we also performed experiments to test the hypothesis that free radicals play a role in modulating muscle calpain activation in response to inflammatory stimuli.

CLINICAL RELEVANCE

Most critically ill patients have significant respiratory muscle weakness. This report identifies a potential therapeutic target that could be exploited to prevent weakness and improve respiratory function in this patient population.

MATERIALS AND METHODS

Experimental Protocols

Experiments were performed using adult male mice of 20–35 g in weight (ICR strain). Approval for this work was granted by the Institutional Animal Care and Use Committee, University of Kentucky. Animals were given food and water ad libitum and housed in university facilities. Saline (60 mg/kg/d) was administered subcutaneously to all animals to maintain fluid volume status. All animals were sedated with pentobarbital (150 mg/kg intraperitoneally) before being killed.

Three experiments were performed. In the first experiment, we determined if calpain was activated in the diaphragm in endotoxin-treated animals, and if administration of a calpain inhibitor (carbobenzoxy-valinyl-phenylalaninal, known as calpain inhibitor III) would blunt endotoxin-mediated diaphragm weakness. We compared (n = 4–5/group): (*1*) control, saline injected mice (0.3 ml intraperitoneally); (*2*) endotoxin injected mice (12 mg/kg of *Escherichia coli* LPS, 055:B5 [Sigma Chemicals, St. Louis, MO], administered intraperitoneally in 0.3 ml of saline); (*3*) mice given endotoxin (12 m/kg intraperitoneally in 0.3 ml of saline) and calpain inhibitor III (12 mg/kg via tail vein; Sigma Chemicals); and (*4*) mice given calpain inhibitor III alone (12 mg/kg via tail vein). Animals were killed 24 hours after injections and diaphragms removed. A portion of the left costal diaphragm was used for assessment of force generation. A small additional portion of freshly excised muscle was used for easily releasable myofilament (ERM) assays. The remaining diaphragm was frozen to −80°C, and later assayed for calpain activity, calpain I protein, calpain II protein, talin protein, and TNF-α levels (using an ELISA kit; Assay Designs, Ann Arbor, MI). TNF-α levels were assessed to determine if administration of calpain inhibitor III had systemic effects that could indirectly alter diaphragm muscle function (e.g., alterations in systemic production of

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TNF-α) rather than or in addition to direct effects on the diaphragm. If so, we would expect calpain inhibitor III administration to reduce diaphragm levels of TNF-α to control levels.

In a second experiment, we determined if polyethylene glycol–superoxide dismutase (PEG-SOD), a free radical scavenger, would prevent calpain activation in endotoxin-treated animals. We compared (n = 4–5/group): (*1*) control, saline-injected mice (0.3 ml, intraperitoneally); (*2*) endotoxin-injected mice (12 mg/kg in 0.3 ml given intraperitoneally); (*3*) mice given endotoxin (12 m/kg in 0.3 ml intraperitoneally) and PEG-SOD (2,000 U/kg intraperitoneally, obtained from Sigma Chemicals); and (*4*) mice given endotoxin (12 mg/kg intraperitoneally) plus heat-denatured PEG-SOD (2,000 U/kg intraperitoneally). Animals were killed 24 hours after injections. The left costal diaphragm was removed, and a portion used for determination of force generation. The remaining diaphragm was frozen to −80°C and later assayed for calpain I protein.

In a third experiment, we determined if administration of a mixture of cytokines (cytomix, a mixture of 20 ng/ml TNF-α, 50 U/ml IL-1β, 100 U/ml IFN-γ, and 10 μg/ml endotoxin) to isolated C2C12 myotubes evoked calpain activation, and if concomitant administration of a superoxide scavenger (i.e., PEG-SOD or tiron) blocked calpain activation by cytokines. For these studies: (*1*) saline (35 μl); (*2*) cytomix (volume, 35 μl); (*3*) cytomix plus PEG-SOD (2 U/ml); or (*4*) cytomix plus tiron (10 mM) were added to plates of differentiated myotubes (n = 4–5 plates/condition). Studies were performed by first seeding C2C12 myoblasts on culture plates (100 × 20 mm) containing Dulbecco's modified Eagle's medium media with antibiotics (penicillin and streptomycin) and 10% FBS (7 ml/plate). Cells were grown until plates were 70% confluent, then media switched to Dulbecco's modified Eagle's medium with antibiotics and 2% horse serum. Cells were differentiated for 9 days before exposure to experimental solutions. Cells were harvested 24 hours after exposures, and calpain levels determined on cell homogenates.

Calpain Activity Levels

For this assay, muscle or cell homogenates containing 100 μm protein were added to assay buffer (total volume, 50 μl). Subsequently, a fluorogenic substrate that generates 7-Amino-4-methylcoumarin (AMC) when degraded by calpain was added (50 μl volume). Duplicate determinations were made for each sample in which a specific calpain inhibitor (0.1 mg/ml calpain inhibitor III) was added to the combined solution of muscle homogenate, assay buffer, and fluorogenic substrate. After the fluorogenic substrate was added, a baseline fluorescent measurement of AMC was performed using a spectrofluorophotometer (excitation frequency, 360 nm; emission frequency, 460 nm; temperature, 25°C; Molecular Devices, Sunnyvale, CA). This measurement was then repeated every 10 minutes for a total of 30 minutes. The fluorescence of a series of AMC standards was also determined, and a calculation made of the rate of AMC formation per minute for each experimental sample. The difference between AMC formation rates in the presence and absence of the calpain inhibitor was taken as an index of calpain activity (i.e., the rate of calpain-specific cleavage of the substrate).

Calpain I, Calpain II, and Talin Protein Levels

Western blotting was employed to measure diaphragm and myotube levels of calpain I, calpain II, and talin (a known target of calpain in cells) (7). We also measured levels of α-tubulin as a loading control. For these determinations, tissue samples were diluted with an equal volume of loading buffer (126 mM Tris-HCL, 20% glycerol, 4% SDS, 1.0% 2-mercaptoethanol, 0.005% bromphenol blue [pH 6.8]), loaded onto Tris glycine polyacrylamide gels, and sample mixtures separated by electrophoresis (Minicell II; Novex, Carlsbad, CA). Proteins were then transferred to polyvinylidene fluoride membranes and incubated overnight at 4°C with primary antibodies to targeted proteins (calpain I, calpain II [Biomol, Plymouth Meeting, PA] and talin [Sigma Chemicals]). Membranes were subsequently incubated with horseradish peroxidase–conjugated secondary antibodies, and antibody binding detected on film using enhanced chemiluminescence (Western Lighting, Perkin-Elmer, Boston, MA). Densitometry of filmed gels was performed using a scanner (Microtek, Carson, CA) and UN-SCAN-IT software (Silk Scientific, Orem, UT). After initial determinations, membranes were stripped and reprobed with primary antibodies to α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA) to verify equal loading across lanes. We chose α-tubulin for this normalization because this protein is not altered in skeletal muscle by sepsis (8). Densities of the α-tubulin blots were also determined using a Microtek scanner.

Assessment of ERM Levels

For this determination, myofibrillar proteins were first isolated by soaking a diaphragm sample in low-salt buffer (LSB; 0.1 M KCl, 2 mM $MgCl_2$, 2 mM EGTA, 0.5 mM DTT, 10 mM Tris-maleate [pH 7.0], containing 1% Triton X-100) for 90 minutes (9). Muscles were then homogenized at 4°C using a Polytron homogenizer (Brinkman, Westbury, NY), and this solution was centrifuged at 1,500 × g for 10 minutes. The pellet was resuspended with a Pasteur pipette in 10 ml LSB, filtered through gauze cloth, and recentrifuged. The pellet was then washed once in LSB containing Triton, and three times in LSB without Triton. ERMs were extracted from this fraction by repeated pipetting through a Pasteur pipette (10 passes using 1.5 ml of LSB containing 5 mM ATP). The suspension was layered over 0.75 ml LSB containing 20% glycerol in a conical tube, and centrifuged at 1,500 × g for 10 minutes. The supernatant was collected with a Pasteur pipette, and was centrifuged through 0.5 ml LSB containing 20% glycerol. The final supernatant contained the released myofilaments, and the pellet the residual myofibrillar fraction. Protein levels of both fractions were determined and ERMs were expressed as a percentage of the combined amount of protein in the two fractions.

Measurement of Diaphragm Force Generation

Diaphragm force generation was assessed as we have previously reported (10). In brief, after diaphragms were excised and placed in a dissecting dish, muscle strips were dissected from the left midcostal portion. Strips were then mounted vertically in water jacketed glass organ baths containing Krebs-Henselheit solution (50 mg/L curare [pH 7.40], 135 mM NaCl, 5 mM KCl, 11.1 mM dextrose, 2.5 mM $CaCl_2$, 1 mM $MgSO_4$, 14.9 mM $NaHCO_3$, 1 mM $NaHPO_4$, 50 U/L insulin, 95% O_2/5% CO_2, 22°C). One end of each strip was tied to the base of the organ bath, and the other end to a force transducer (Scientific Instruments, Heidelberg, Germany). Platinum mesh field electrodes were used to deliver supramaximal currents using a biphasic constant current amplifier driven by a Grass S48 stimulator (Grass Tachnologies, West Warwick, RI). After a 15-minute equilibration period, muscle strip length was adjusted to the length at which strip force generation in response to a single stimulus was maximal. Strips were then sequentially stimulated with trains of 1-, 10-, 20-, 50-, 100-, and 150-Hz stimuli (train duration, 800 ms, 30 s between adjacent trains), and force recorded with a Gould 2,600 strip chart recorder (Gould, Cleveland, Ohio). Cross-sectional area was calculated as muscle strip weight divided by muscle density (1.06) and muscle length. Specific muscle force was calculated as raw force divided by cross-sectional area.

Statistical Analysis

Unpaired t tests (SigmaStat software, San Jose, CA) were used to compare parameters across experimental groups for the first group of experiments. ANOVA was used for comparison of calpain levels and diaphragm forces across experimental groups for the second and third group of experiments. Tukey's test was used to determine differences between individual groups following ANOVA. A P value of less than 0.05 was taken as indicating statistical significance. Data are presented as mean (±SEM).

RESULTS

Indices of Calpain Activation

Endotoxin administration resulted in increases in several indices of calpain activation in the diaphragm (Figures 1–4). First, diaphragm homogenates from endotoxin (LPS)-treated animals demonstrated significant increases in cleavage of an exogenous fluorogenic calpain substrate, as shown in Figure 1 ($P < 0.001$). Second, diaphragm levels of the cleaved form of microcalpain (calpain I) protein increased after endotoxin administration ($P < 0.001$, Figure 2). Third, endotoxin administration also elicited increases in diaphragm levels of the cleaved form

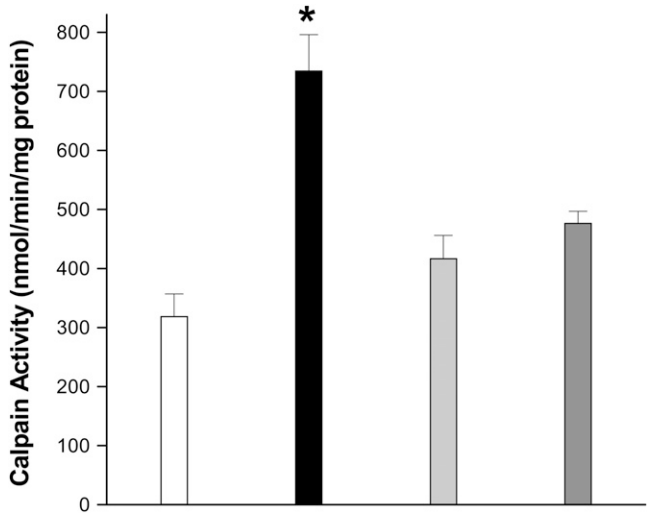

Figure 1. Calpain activity of diaphragm homogenates. Calpain activity measured as the cleavage of a fluorogenic substrate by diaphragm homogenates. Diaphragm samples from endotoxin (LPS)-treated animals (*closed bar*) had higher calpain activities than samples from control animals (*open bar*), animals given both endotoxin and calpain inhibitor (*light gray bar*), or animals given calpain inhibitor alone (*dark gray bar*). *Significant statistical difference.

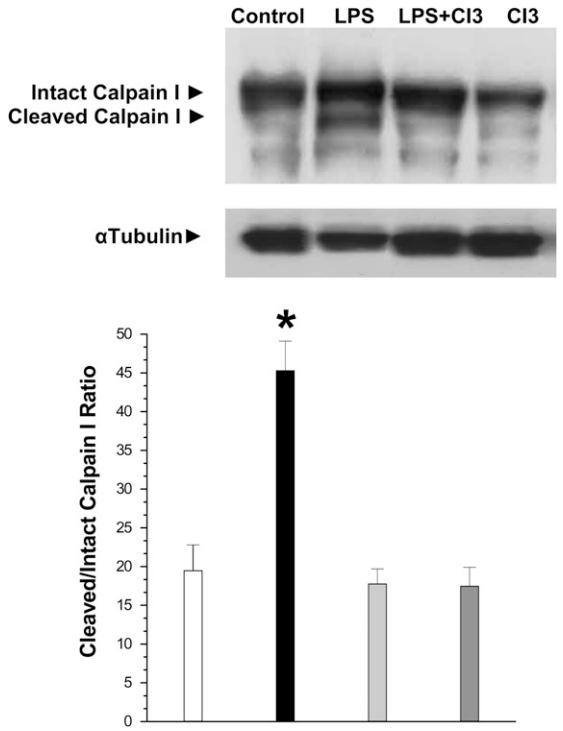

Figure 2. Calpain I protein levels for diaphragm samples. Western blots stained for calpain I protein, with *top panels* presenting blots for representative samples, and the *bottom graph* providing group mean densitometry data. Blots were reprobed with α-tubulin to verify equal loading of lanes. Levels of cleaved calpain I (78-kD protein) were higher for samples from endotoxin-treated animals (*closed bar*) than for samples from control animals (*open bar*), animals given both endotoxin and calpain inhibitor III (*light gray bar*), or animals given calpain inhibitor III alone (*dark gray bar*). *Significant statistical difference.

of macrocalpain (calpain II), as shown in Figure 3 ($P < 0.01$). In addition, diaphragms taken from endotoxin-treated animals contained fragments of talin (Figure 4), an important cytoskeletal protein known to be a target for calpain-mediated degradation ($P < 0.002$). All of these indices of calpain activation were diminished in animals given a combination of endotoxin and calpain inhibitor protein III when compared with animals given only endotoxin alone (Figures 1–4). Specifically, diaphragms from the endotoxin plus calpain inhibitor III–treated group had calpain fluorogenic activity, cleaved calpain I protein, active calpain II protein, and talin degradation product levels that were similar to values for saline-treated control animals, and markedly lower than levels for animals given endotoxin alone ($P < 0.001$ for comparison of calpain activity and cleaved calpain I between the endotoxin alone and the endotoxin plus calpain inhibitor protein III–treated groups; $P < 0.03$ for comparison of cleaved calpain II and talin degradation between these groups). Animals given only calpain inhibitor protein III had levels of diaphragm calpain activity, active calpain I protein, active calpain II protein, and talin degradation products that were similar to saline-treated control animals (Figures 1–4).

We also determined the effect of calpain inhibitor III administration on diaphragm TNF-α levels, assayed using an ELISA technique. TNF-α levels for control, endotoxin-, endotoxin plus calpain inhibitor protein III–, and calpain inhibitor protein III–treated groups of animals averaged 634 (±222), 1,912 (±341), 1,320 (±101), and 762 (±246) pg/g, respectively ($P <$ 0.01 for comparison of the control group to the endotoxin group). As a result, diaphragm TNF concentrations for the endotoxin and endotoxin plus calpain inhibitor III groups were similar.

Effect of Calpain Inhibitor Administration on ERM Levels and Diaphragm Force Generation

Measurement of muscle ERM levels has been used to examine the ease with which contractile proteins can be released from the myofilbrillar lattice of isolated skeletal muscles (9). It is thought that proteolytic enzymes, such as calpain and caspase, have the capacity to destabilize the contractile protein lattice by cleaving critical lattice elements, such as talin, myosin, and α-actinin, thereby making it possible for myofilaments to be released from the lattice (11). We found that endotoxin administration resulted in a significant increase in ERM levels for diaphragm homogenates, as shown in Figure 5 ($P < 0.002$). ERM levels for diaphragm homogenates taken from animals given both endotoxin and calpain inhibitor protein III were midway between levels for saline-treated control animals and the endotoxin treated group (Figure 5).

Endotoxin administration evoked a large reduction in diaphragm force-generating capacity, as shown in Figure 6, decreasing the force generated by isolated muscle samples in response to a wide range of stimulation frequencies (1–150 Hz, $P < 0.01$ for comparison for force between control and endotoxin-treated groups for stimulation frequencies of 10–150 Hz). Administration of calpain inhibitor peptide III attenuated the effects of endotoxin administration, with significantly greater forces generated for stimulation frequencies between 50 and 150 Hz for diaphragms from the endotoxin plus calpain inhibitor III group as compared with the forces generated for muscles taken from animals given endotoxin alone ($P < 0.02$ for comparison of force for stimulation frequencies between 50 and 150 Hz for these two groups).

Effect of SOD Administration on Calpain Activation

Previous work has demonstrated that administration of PEG-SOD, a potent superoxide scavenger, also attenuates endotoxin-induced diaphragm dysfunction (12). To determine if a potential link exists between superoxide generation and calpain activation, we examined the effect of administration of PEG-SOD on

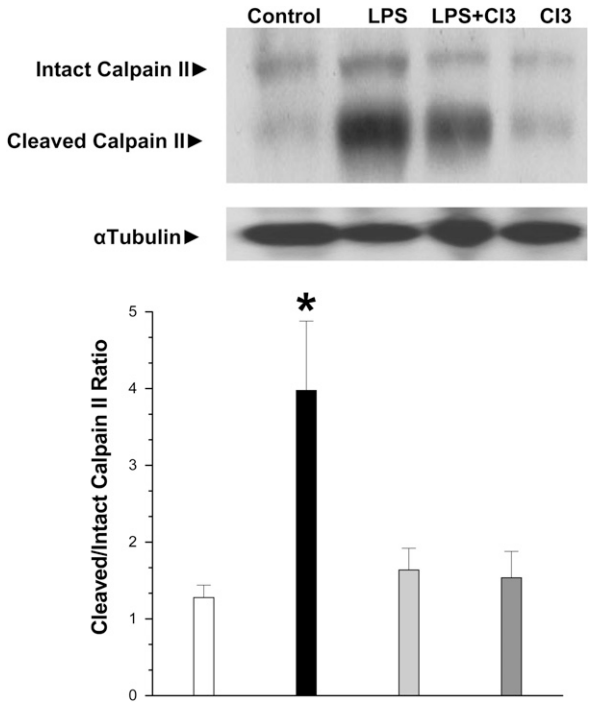

Figure 3. Calpain II protein levels for diaphragm samples. Western blots stained for calpain II protein, with *top panels* presenting blots for representative samples, and the *bottom graph* providing group mean densitometry data. Blots were reprobed with α-tubulin to verify equal loading of lanes. Levels of cleaved calpain II (58-kD protein) were higher for samples from endotoxin-treated animals (*closed bar*) than for samples from control animals (*open bar*), animals given both endotoxin and calpain inhibitor III (*light gray bar*), or animals given calpain inhibitor III alone (*dark gray bar*). *Significant statistical difference.

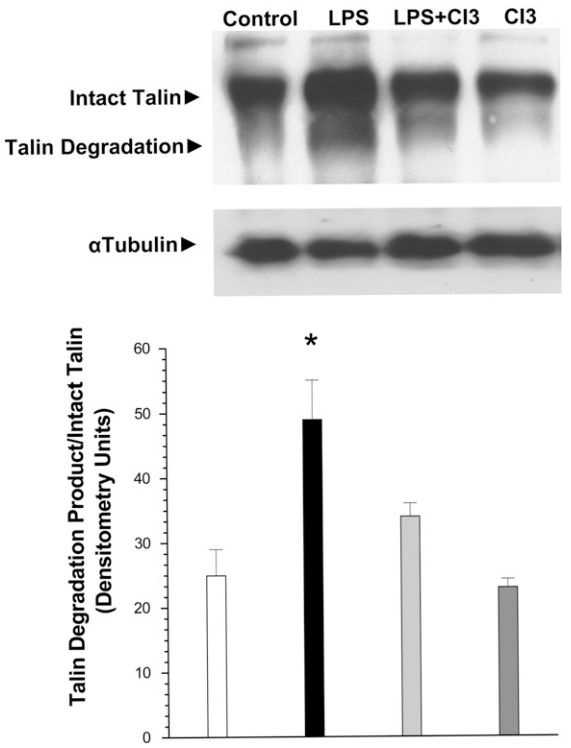

Figure 4. Talin protein levels for diaphragm samples. Western blots stained for talin protein, with *top panels* presenting blots for representative samples, and the *bottom graph* providing group mean densitometry data. Blots were reprobed with α-tubulin to verify equal loading of lanes. Levels of cleaved talin were higher for samples from endotoxin-treated animals (*closed bar*) than for samples from control animals (*open bars*), animals given both endotoxin and calpain inhibitor III (*light gray bar*), or animals given calpain inhibitor III alone (*dark gray bar*). *Significant statistical difference.

endotoxin-induced diaphragmatic calpain activation in mice. PEG-SOD administration reduced calpain activity, as assessed by measuring cleavage of a fluorogenic calpain substrate (Figure 7; $P < 0.02$ for comparison of calpain activity between the endotoxin-treated group and the group receiving endotoxin plus PEG-SOD). PEG-SOD administration also reduced the formation of active calpain I protein in the diaphragm, as shown in Figure 8 ($P < 0.001$ for comparison of endotoxin to endotoxin plus PEG-SOD groups). In keeping with previous reports, PEG-SOD also prevented endotoxin-induced reductions in diaphragm force generation in these studies (Figure 7; $P < 0.01$ for comparison of force between endotoxin and endotoxin plus PEG-SOD groups for stimulation frequencies of 1–150 Hz).

We also examined the effect of superoxide scavenger administration on calpain activity in a muscle cell line (C2C12 cells) exposed to cytokines (Figure 8). We found that exposure of C2C12 cells to cytomix (a mixture of LPS, TNF-α, IL-1β, and IFN-γ) resulted in a significant increase of cell calpain activity, measured using a fluorogenic assay ($P < 0.02$). Administration of either PEG-SOD or tiron (another superoxide scavenger) prevented cytomix-induced increases in C2C12 cell calpain activity ($P < 0.02$ for comparison of cytomix to either cytomix plus PEG-SOD or cytomix plus tiron groups).

DISCUSSION

Calpain Activation in Sepsis

Although several previous studies have suggested that calpain may become activated in skeletal muscle in animal models of sepsis (13, 14), the present work represents the first: (*1*) evidence of sepsis-induced calpain activation in the diaphragm; (*2*) demonstration that a relatively specific calpain inhibitor prevents sepsis-induced reductions in muscle force generation; and (*3*) utilization of calpain I cleavage, calpain II cleavage, and talin cleavage as indices of sepsis-induced skeletal muscle calpain activation. We used these newer indices to assess calpain activation, because the traditional approaches for assessing activation of this enzyme in intact tissues have limitations. Specifically, traditional activity assays used to assess calpain activity in muscle homogenates are performed using homogenized samples, and homogenization, *per se*, will release calcium from the sarcoplasmic reticulum, altering calpain activation from that present under *in vivo* conditions. This limitation is not a factor, however, when using evidence of calpain I cleavage, calpain II cleavage, and talin cleavage to assess calpain activity, because our method of harvesting tissues results in inhibition of calpain activity as soon as these tissues leave the body (i.e., a cell-permeant calpain inhibitor, leupeptin [2 μg/ml], is added to the buffer in which tissues are homogenized for preparation of tissue samples for Western blotting; this inhibitor is not added to portions of muscle prepared for fluorogenic activity assays). As a result, the presence of these specific cleavage products in diaphragm homogenates could only have occurred from *in vivo* calpain activation in the diaphragm.

Our finding, that sepsis increases both calpain I and calpain II autocatalytic cleavage product formation, implies that both of these enzymes are activated in the diaphragm in this particular

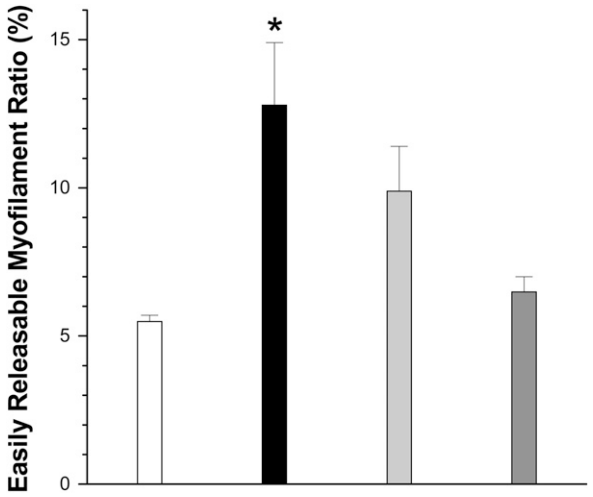

Figure 5. Easily releasable myofilament (ERM) levels for diaphragms. ERM level as a ratio to total myofilament concentration for diaphragm homogenates. Diaphragm samples from endotoxin (LPS)-treated animals (*closed bar*) had higher levels of ERM than samples from control animals (*open bar*), animals given both endotoxin and calpain inhibitor (*light gray bar*), or animals given calpain inhibitor alone (*dark gray bar*). *Significant statistical difference.

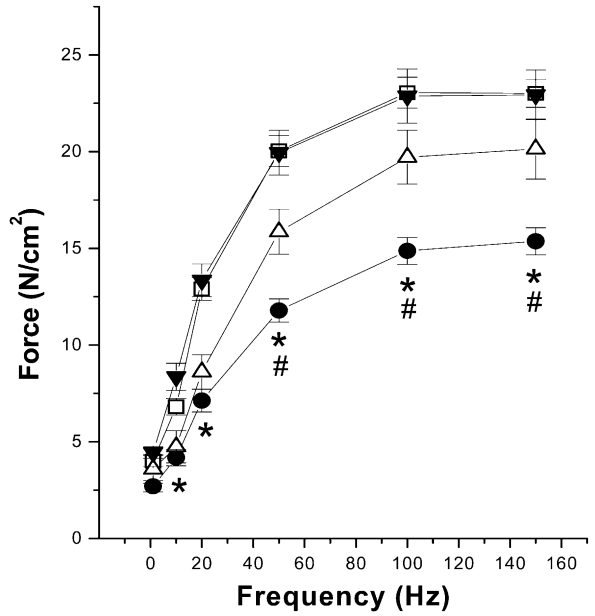

Figure 6. Diaphragm force–frequency curves. Force generation was significantly lower at stimulation frequencies from 10–150 Hz for diaphragms from LPS-treated animals (*closed circles*) than for control animals (*open squares*). Diaphragms from animals given both calpain inhibitor III and LPS (*open triangles*) generated forces significantly higher than diaphragms from animals given endotoxin alone for frequencies from 50 to 150 Hz. Force generation for muscles taken from animals given calpain inhibitor III alone (*closed triangles*) were similar to levels for control animals. *Significant statistical difference between control and endotoxin; #statistical significance between endotoxin and endotoxin plus calpain inhibitor III groups.

model of sepsis (15). These enzymes are known to be present in both type I and type II muscle fibers, and to have high concentrations at z-disks (16, 17). Activation of these enzymes in skeletal muscle is known to occur normally during growth and development, and may be required for remodeling of muscle cells during myotube formation. Activation of these enzymes is also thought to occur in some forms of muscular dystrophy, and, as in the current study, activation of these enzymes in dystrophic muscle is thought to contribute to the development of muscle damage and dysfunction (18). The relative importance of activation of these two in inducing alterations in skeletal muscle structure and function is not known.

The mechanism of activation of these enzymes in both physiological and pathological conditions is generally thought to be related to regional increases in calcium concentrations in the microenvironment surrounding the enzymes, although other mechanisms of activation (e.g., alterations in phosphorylation related to ERK) have been proposed (19, 20). Several previous studies examining animal models of sepsis have reported increases in resting skeletal muscle cytosolic calcium concentrations in response to this stress (21, 22), and this increase in calcium represents one possible mechanism by which endotoxin administration may have induced activation of diaphragmatic calpain in the present experiments.

Relationship of Calpain Activation to Force Reductions

Many recent publications have emphasized the importance of the proteasomal proteolytic system as the major mechanism by which skeletal muscle proteins are degraded in pathological conditions (23). Certainly, a number of publications have confirmed the key role played by the proteasome in muscle wasting due to cancer, diabetes, cirrhosis, and several other diseases (23, 24). It is also commonly held that the proteasome plays a key role in mediating the development of muscle dysfunction in sepsis, but careful consideration of existing data suggests that this enzyme system alone is unlikely to be responsible for the profound and rapid reductions in respiratory muscle force-generating capacity that have been reported in several models of infection. First, according to the model of proteasomally mediated muscle dysfunction, this multicatalytic system degrades proteins, inducing muscle atrophy, and thereby secondarily inducing reductions in muscle force generation (23, 24). In contrast, after endotoxin administration, diaphragm force falls rapidly (within 12–24 h), and force loss precedes loss of diaphragm protein content or muscle mass (25). Second, studies examining the effect of the proteasome on intact myofibrillar complexes have shown that the proteasome is unable to directly degrade protein elements when these complexes are intact (26). As a result, some alternative process must first disupt and destabilize the skeletal muscle contractile protein matrix in sepsis, releasing myofibrillar component proteins that can then be broken down by the proteasomal complex (11).

The findings of the current study suggest that calpain may play a role in causing this initial loss of specific skeletal muscle force after the induction of infection, as administration of calpain inhibitor III prevented diaphragm force loss after endotoxin administration. Our data demonstrate that calpain inhibitor III, moreover, not only prevented endotoxin-induced reductions in diaphragm force, but also completely blocked endotoxin-induced calpain I activation, calpain II activation, and talin cleavage.

Although no chemical inhibitors are completely specific, the particular agent used in this study, calpain inhibitor III, has been shown to potently inhibit both calpain I and calpain II with a high level of activity (27–29), and is without inhibitory effects on the proteasome or caspase proteolytic systems. Specifically, three studies have shown that calpain inhibitor III produces no inhibition of the proteasome complex (27–29). We also specifically examined the effect of incubation of calpain inhibitor III

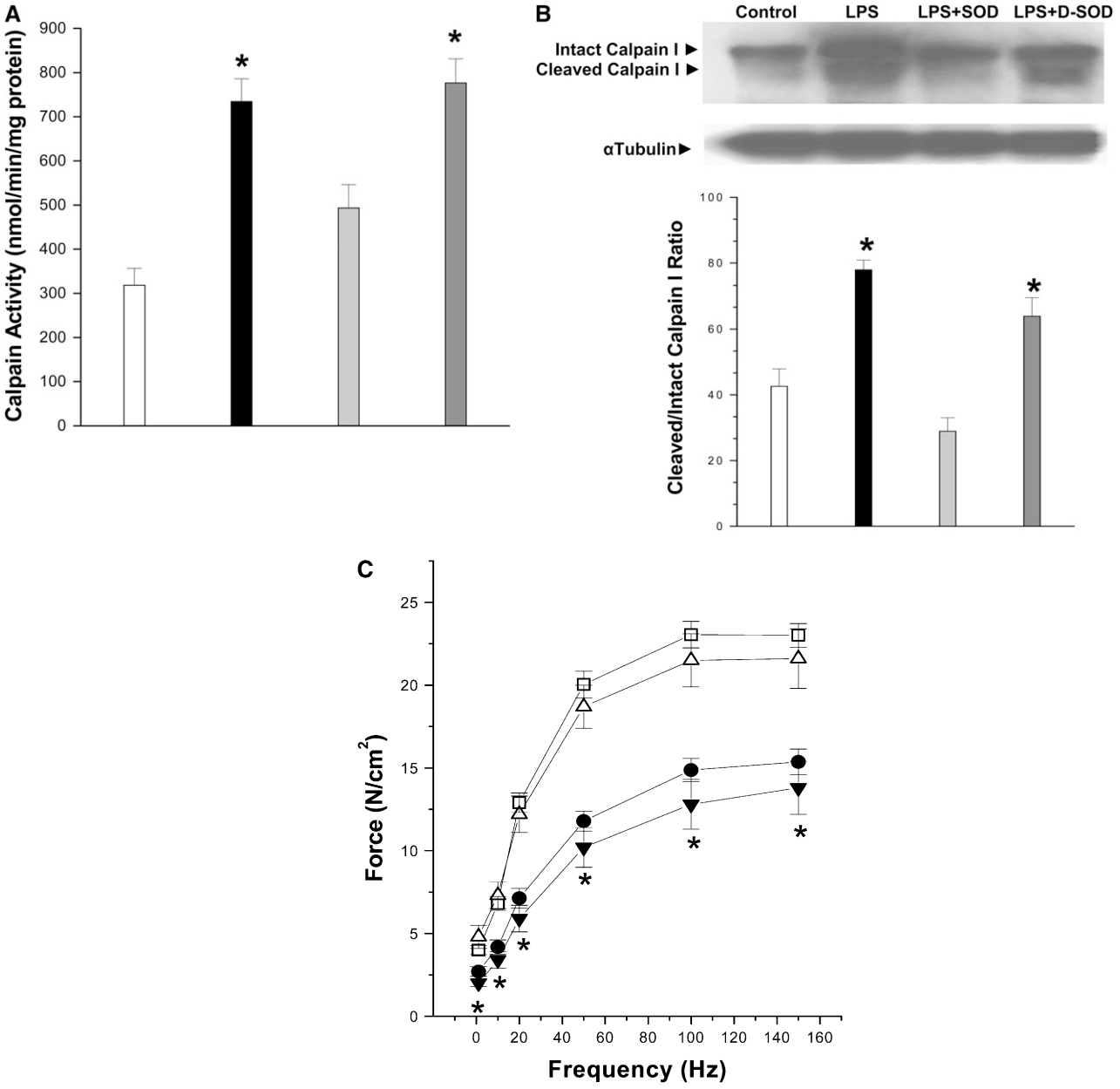

Figure 7. Effect of superoxide dismutase (SOD) on calpain and force in endotoxin-treated animals. (*A*) Calpain activity, measured by assessing cleavage of a fluorogenic substrate by diaphragm homogenates, for groups of control animals (*open bar*), LPS-treated animals (*closed bar*), animals treated with both LPS and polyethylene glycol (PEG)-SOD (*light gray bar*), and animals treated with LPS and denatured PEG-SOD (*dark gray bar*). Calpain activity was higher in the LPS group than in the control animals. Administration of PEG-SOD prevented the endotoxin-induced increase in calpain activity ($P < 0.02$ for comparison of endotoxin plus PEG-SOD to endotoxin alone groups). Administration of denatured endotoxin had no effect. *Significant statistical difference compared with control. (*B*) Western blots stained for calpain I protein, with *top panels* presenting blots for representative samples, and the *bottom panel* providing group mean densitometry data. Blots were reprobed with α-tubulin to verify equal loading of lanes. Levels of cleaved calpain I (78 kD protein) were higher for samples from endotoxin-treated animals (*closed bar*) than for samples from control animals (*open bar*) or animals given both endotoxin and PEG-SOD (*light gray bar*). Cleaved calpain I levels were similar for samples given endotoxin or endotoxin plus denatured PEG-SOD (*dark gray bar*). *Significant statistical difference. (*C*) Administration of PEG-SOD prevented endotoxin-induced reductions in diaphragm force. Specifically, forces generated at stimulation frequencies from 1 to 150 Hz were higher for diaphragms from control (*open squares*) and endotoxin plus PEG-SOD (*open triangles*)-treated animals than for forces in diaphragms from endotoxin (*closed circles*) and endotoxin plus denatured PEG-SOD groups (*closed triangles*). *Significant statistical difference in comparison with the control group (*open squares*).

with caspase 3 *in vitro*, and found that this agent has no effect on caspase activity *per se*, as measured by assessment of cleavage of a caspase-specific fluorogenic substrate. In addition, we also think it unlikely that the observed effects of calpain inhibitor III were due to a nonspecific systemic effect to reduce inflammation, because diaphragmatic TNF-α levels remained elevated and similar for animals given endotoxin alone and animals given both endotoxin and calpain inhibitor III in the present study.

If calpain does degrade the diaphragm myofibrillar matrix after endotoxin administration, then calpain I, calpain II, or

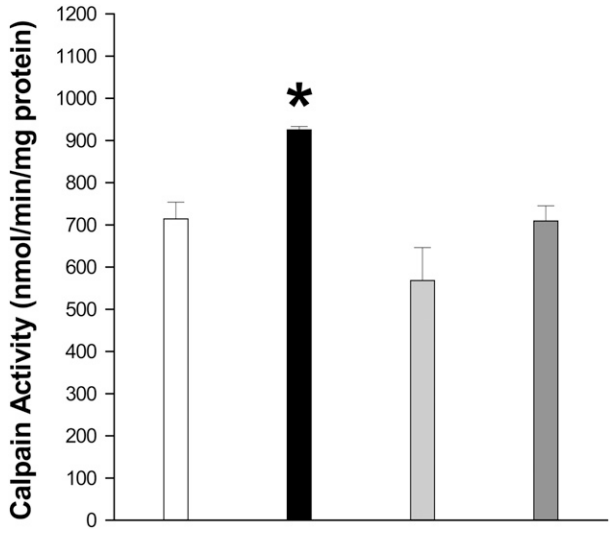

Figure 8. Effect of superoxide scavengers on calpain activation in C2C12 cells. Calpain activity measured as the cleavage of a fluorogenic substrate by cell homogenates. C2C12 cells incubated with cytomix (*closed bars*) had higher calpain activities than cells incubated with saline (*open bar*). Addition of either PEG-SOD (*light gray bar*) or tiron (*dark gray bar*) prevented this cytomix-induced increase in calpain activity ($P < 0.02$ for comparison of cytomix alone to the other two groups; *significant statistical difference).

both enzymes must be capable of moving to sites within the contractile protein matrix. In addition, one would expect this process to result in a measureable release of myofibrillar components after endotoxin administration. In support of these possibilities, both calpain I and calpain II are reasonably small proteins, and the cleavage forms of these proteins, which increase in the diaphragm after endotoxin administration, are even smaller and more active than the intact forms of these enzymes. Work by Goll and colleagues (30) has suggested that calpains are capable of diffusing to areas adjacent to or within myofibrillar complexes, and, thus, at least theoretically, being positioned at sites capable of cleaving key proteins within the contractile protein complex. Our finding, that the levels of ERM (*see* Figure 5) increase after endotoxin administration, is also in keeping with this concept. It is thought that ERMs represent myofibrillar components that are either not tightly integrated within the contractile protein lattice, or have recently become dissociated from the lattice (9). We also found that administration of calpain inhibitor peptide III reduced ERM levels, in keeping with the possibility that calpain activation is responsible, in part, for ERM increases.

The fact, however, that administration of calpain inhibitor III did not completely restore ERM levels to baseline, and did not entirely prevent endotoxin-induced reductions in diaphragm force generation, despite totally preventing calpain I and calpain II activation, suggests that another factor or process may also be contributing to myofibrillar matrix disruption after endotoxin. A likely candidate for this additional process is caspase activation (31). Like calpain, caspase is a low molecular weight protein capable of entering the contractile protein lattice. In addition, recent studies indicate that caspase is activated in the diaphragm in animal models of infection, cleaves diaphragm cytoskeleton proteins in these models, and contributes to a loss in diaphragm force generation (31). Of interest, calpain and caspase activation are known to occur in parallel in many other pathophysiological conditions. For example, ischemia-induced brain injury is associated with coordinate activation of both caspase and calpain in neural tissues, with these two enzymes acting together to cleave cellular proteins and produce tissue damage (32). Caspase and calpain interactions in producing alterations in muscle force can, therefore, theoretically exist at three levels: (*1*) both caspase and calpain may be activated in parallel by some upstream process (e.g., activation of a family of signaling kinases or activation by oxidant-mediated processes); (*2*) caspase may directly activate calpain (e.g., by degrading calpastatin), or calpain may directly activate caspase (by cleaving procaspase to active caspase); or (*3*) contractile protein matrices exposed to activated calpain may be more susceptible to caspase-mediated proteolysis, or, conversely, caspase exposure may lead to enhanced susceptibility to calpain-mediated degradation. Additional work will be needed to determine if any or all of these potential sites of interaction between caspase and calpain occur in the diaphragm in sepsis.

The present study focused on measurement of active tension development in diaphragm fibers assessed at optimal lengths for force generation, and we did not specifically analyze the passive tension characteristics of diaphragm muscle strips. If, however, calpain does disrupt z-disks, then loss of transmission of active force thru these structures may play a role in loss of active force, and we would also expect to see alterations in muscle structure and passive tension characteristics. Additional work will be needed to explore these possibilities.

Free Radicals and Calpain Activation

Previous work has suggested that calpain activation in many tissues is influenced by levels of oxidative stress (33). Moreover, it is known that endotoxin administration results in a significant increase in indices of oxidative stress in the diaphragm (12). Taking these two facts together, it seemed reasonable to ask whether or not calpain activation in the diaphragm in sepsis was linked, in some way, to levels of oxidative stress. To examine this issue, we examined the effects of administration of SOD, an enzyme that catalyzes the removal of superoxide anions, on calpain activation after endotoxin administration. We found that SOD largely prevented calpain activation in intact animals, blocking both endotoxin-induced increases in calpain activity, assessed using a fluorogenic assay, and preventing autocatalytic cleavage of calpain, as indicated by assessment of calpain I protein levels. To further examine this issue, we examined the effects of SOD and tiron, another superoxide scavenger, on cytokine-induced calpain activation in the C2C12 muscle cell line. Both superoxide scavengers prevented increases in calpain activity in this cell line, supporting the concept that the process of cytokine-mediated calpain activation in muscle cells is modulated by free radicals. Determination of the exact mechanisms by which free radicals may exert this effect is beyond the scope of the current study, but it is worth noting that the diaphragm muscle NADPH oxidase complex has been shown to markedly increase its superoxide generation in animal models of sepsis (34). It is also known that the NADPH oxidase complex in muscle may play a role in regulating cell calcium levels (35). Future studies will therefore be needed to determine if free radicals modulate calpain activation in skeletal muscle by influencing cytosolic calcium levels.

Implications

Severe diaphragm weakness is known to be present in many patients that are critically ill. For example, two recent studies have shown that patients requiring sustained mechanical ventilation in ICUs have 75–80% reductions in the strength of the diaphragm (1, 2). Although several factors contribute to this profound weakness, the majority of critically ill patients have infections that are either primarily responsible for their need for ICU care or contribute to their illness. Currently, there are no

therapies available to prevent this devastating loss of diaphragm strength in the sickest patients in the ICU. The present study identifies an important potential treatment for these patients—namely, pharmacological inhibition of calpain. Diaphragm weakness is a major factor responsible for morbidity and mortality in this patient population, predisposing these patients to a prolonged or indefinite need for mechanical ventilation. As a result, calpain inhibition may represent an important tool to reduce morbidity, decrease mortality, and improve costs in infected, critically ill patients.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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