The extrinsic caspase pathway modulates endotoxin-induced diaphragm contractile dysfunction

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Supinski GS, Ji X, Wang W, Callahan LA. The extrinsic caspase pathway modulates endotoxin-induced diaphragm contractile dysfunction. J Appl Physiol 102: 1649–1657, 2007. First published January 11, 2007; doi:10.1152/japplphysiol.00377.2006.—The mechanisms by which infections induce diaphragm dysfunction remain poorly understood. The purpose of this study was to determine which caspase pathways (i.e., the extrinsic, death receptor-linked caspase-8 pathway, and/or the intrinsic, mitochondrial-related caspase-9 pathway) are responsible for endotoxin-induced diaphragm contractile dysfunction. We determined 1) whether endotoxin administration (12 mg/kg IP) to mice induces caspase-8 or -9 activation in the diaphragm; 2) whether administration of a caspase-8 inhibitor (N-acetyl-Ile-Glu-Thr-Asp-CHO, 3 mg/kg iv) or a caspase-9 inhibitor (N-acetyl-Leu-Glu-His-Asp-CHO, 3 mg/kg iv) blocks endotoxin-induced diaphragmatic weakness and caspase-3 activation; 3) whether TNF receptor 1-deficient mice have reduced caspase activation and diaphragm dysfunction following endotoxin; and 4) whether cytokines (TNF-α or cytomix, a mixture of TNF-α, interleukin-1β, interferon-γ, and endotoxin) evoke caspase activation in C2C12 myotubes. Endotoxin markedly reduced diaphragm force generation (P < 0.001) and induced increases in caspase-3 and caspase-8 activity (P < 0.03), but failed to increase caspase-9. Inhibitors of caspase-8, but not of caspase-9, prevented endotoxin-induced reductions in diaphragm force and caspase-3 activation (P < 0.01). Mice deficient in TNF receptor 1 also had reduced caspase-8 activation (P < 0.001) and less contractile dysfunction (P < 0.01) after endotoxin. Furthermore, incubation of C2C12 cells with either TNF-α or cytomix elicited significant caspase-8 activation. The caspase-8 pathway is strongly activated in the diaphragm following endotoxin and is responsible for caspase-3 activation and diaphragm weakness.

Skeletal muscle; sepsis; weakness; tumor necrosis factor-α; tumor necrosis factor receptor 1

STUDIES INDICATE THAT THE respiratory muscles of critically ill patients requiring mechanical ventilation are profoundly weak, generating pressures that are only 20–25% of that measured in healthy controls (13, 28). Severe respiratory muscle weakness, in turn, may limit weaning of patients from mechanical ventilation, increasing the duration that ventilation is required and contributing to the morbidity and mortality of respiratory failure. Animal models have identified several potential phenomena that are likely to contribute to the development of respiratory muscle weakness in critically ill patients: weakness secondary to infection-related systemic inflammation (3, 8, 12, 17, 19), weakness triggered by stress-related hyperglycemia (4), and respiratory muscle weakness induced by the effects of mechanical ventilation per se (2, 18).

The cellular mechanisms by which systemic infection induces respiratory muscle weakness are poorly understood, as discussed by Reid (20). Recently, we found that endotoxin administration potently induces formation of active caspase-3 in the diaphragm (25). These previous data raise the possibility that active caspase-3 may be a major contributor to the genesis of diaphragmatic weakness in response to inflammatory stresses. There are multiple potential upstream initiators of caspase-3 activation (24, 27). In most pathophysiological situations, caspase-3 is activated either by caspase-9 (the intrinsic caspase pathway) or caspase-8 (the extrinsic pathway). Caspase-9 activation is usually triggered by cytochrome c released by mitochondria and is usually associated with changes in the Bax/Bcl-2 ratio (24, 27). On the other hand, caspase-8 activation is usually linked to activation of cell surface receptors [e.g., TNF receptor 1 (TNFR1)] and is influenced by cellular levels of a host of death receptor-associated proteins (e.g., Fas-associated death domain protein) (24, 27).

The purpose of the present experiment was to determine which of these upstream caspase pathways is responsible for caspase-3 activation in the diaphragm following endotoxin injection. Studies were conducted to determine: 1) whether endotoxin induces increases in diaphragm caspase-8 or -9 activity and/or alterations in diaphragm Bax and Bcl-2 protein levels; 2) whether administration of either specific inhibitors of caspase-8 or caspase-9 alters endotoxin-induced diaphragmatic weakness and/or prevents activation of caspase-3; 3) whether mice deficient in TNFR1 have reduced caspase activation and contractile dysfunction following endotoxin administration; and 4) whether exposure of C2C12 cells, a skeletal muscle cell line, to cytokines evokes caspase-8 and/or -9 activation.

Data were examined to test the alternative hypotheses that either caspase-8 or caspase-9 activation is linked to caspase-3 activation and diaphragm weakness following endotoxin administration. Our data would support a role for caspase-8 1) if endotoxin induces an increase in diaphragm active caspase-8 protein and/or activity; 2) if administration of caspase-8 inhibitors prevents diaphragm dysfunction and caspase-3 activation; 3) if TNFR1-deficient mice have reduced diaphragm caspase activation and contractile dysfunction following endotoxin administration; and 4) if exposure of isolated skeletal muscle myotubes to cytokines activates caspase-8. On the other hand, a role for caspase-9 would be supported 1) if endotoxin induces an increase in diaphragm active caspase-9 protein/activity; 2) if administration of caspase-9 inhibitors prevents diaphragm dysfunction and caspase-3 activation; 3) if endotoxin administration is associated with increases in diaphragm Bax levels and reductions in Bcl-2; and 4) if exposure of isolated skeletal muscle myotubes to cytokines activates caspase-9.

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METHODS

Experimental protocols. Experiments were performed using mice 20–35 g in weight (n = 50 total). Approval for this work was granted by the Medical College of Georgia Institutional Animal Care and Use Committee. Animals were given food and water ad libitum and housed in university facilities. Saline (60 mg·kg⁻¹·day⁻¹) was administered subcutaneously to maintain fluid volume status. Animals were sedated with pentobarbital [50 mg·kg⁻¹ intraperitoneally (IP)] before euthanasia.

Four experiments were performed. In the first experiment, we determined which caspase pathway (caspase-9 pathway or the caspase-8 pathway) was activated in endotoxin-treated animals and if caspase activation was accompanied by diaphragm nuclear DNA damage as assessed by the terminal deoxynucleotidyl transferase (TdT) biotin-DUTP nick-end labeling (TUNEL) technique. For these studies, control mice (male, ICR strain) were injected IP with saline (0.3 ml), and endotoxin-treated mice were injected IP with 12 mg/kg of E. coli lipopolysaccharide (LPS) in 0.3 ml (Sigma Chemical, St. Louis, MO). Animals were euthanized 24 h after injections. For five control and five endotoxin-treated animals, diaphragms were immediately frozen, stored at −80°C, and assayed for caspase-3, -8, and -9 activities and caspase-8 and -9 protein levels. In four control and four endotoxin-treated animals, diaphragms were embedded in paraffin and TUNEL stained.

In the second experiment, we determined whether caspase-8 or -9 inhibitors could prevent endotoxin-induced diaphragm dysfunction and/or caspase-3 activation. We compared 1) control, saline-injected mice (0.3 ml IP), 2) endotoxin-injected mice (12 mg/kg IP in 0.3 ml), 3) mice given endotoxin (12 mg/kg IP in 0.3 ml) and caspase-8 inhibitor Ac-IETD-CHO (N-acetyl-Ile-Glu-Thr-Asp-CHO, 3 mg/kg iv via tail vein), and 4) mice given endotoxin and caspase-9 inhibitor Ac-LEHD-CHO (N-acetyl-Leu-His-Asp-CHO, 3 mg/kg iv via tail vein); n = 4/group. Animals were euthanized 24 h after injections. Diaphragm force and caspase-3 activity levels were determined.

In a third experiment, we examined the effect of endotoxin administration to TNFR1-deficient animals, since TNFR1 activation is a major trigger for cleavage of procaspase-8 to active caspase-8. TNFR1 knockout mice were obtained from Jackson Laboratories (Bar Harbor, ME; strain B6.129-Tnfrsf1atm1Mak/J, background strain C57BL/6). We compared (n = 4/group) 1) C57BL/6, saline injected mice, 2) C57BL/6 mice injected with endotoxin (12 mg/kg IP), 3) TNFR1-deficient, saline IP injected mice, and 4) TNFR1-deficient mice injected with endotoxin (12 mg/kg IP). Animals were euthanized 24 h after injections. Diaphragm force and caspase-3 activity levels were assessed.

In a fourth experiment, we determined whether administration of TNF-α or a mixture of cytokines (cytome, a mixture of TNF-α 20 ng/ml, interleukin-1β 50 U/ml, IFN-γ 100 U/ml, and endotoxin 10 μg/ml) to isolated C6/C12 myotubes evoked caspase activation. For these studies, either saline (35 μl), TNF-α (20 ng/ml in a volume of 35 μl), or cytome (volume 35 μl) was added to plates of differentiated myotubes, cells were harvested 24 h after exposure, and caspase levels were determined on cell homogenates.

Assessment of caspase activity levels. A modified BIOMOL assay (BIOMOL International, Plymouth Meeting, PA) was used to determine caspase activity for muscle homogenates (14). For this assay, muscle homogenate (100 μg of protein) was added to assay buffer and a caspase-specific fluorogenic substrate. For caspase-3 activity determination, we used a caspase-3-specific fluorogenic substrate, 30 μM N-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (AMC). To determine caspase-8 activity, we used 30 μM N-acetyl-Ile-Glu-Thr-Asp-7-AMC, and to determine caspase-9 activity, we used 30 μM N-acetyl-Leu-His-Asp-7-AMC. Diaphragms were made with muscle homogenate, assay buffer, substrates, and specific caspase inhibitors for each sample. For inhibitors, we used 20 nM Asp-Glu-Val-Asp-CHO to inhibit caspase-3, 20 nM Ile-Glu-Thr-Asp-CHO to inhibit caspase-8, and 20 nM Leu-Glu-His-Asp-CHO to inhibit caspase-9. For each set of measurements, immediately after substrate was added, a baseline fluorescent measurement of AMC was performed using a Molecular Devices spectrophuorophotometer (excitation frequency of 360 nm and an emission frequency of 460 nm). This measurement was then repeated after 0.5 h of incubation at 30°C. AMC and caspase standards were used to quantitate activity levels.

Caspase, Bcl-2, and Bax protein levels. Western blotting was employed to measure diaphragm and myotube levels of caspase-8, caspase-9, Bcl-2, and Bax. We also measured levels of α-tubulin as a loading control. For these determinations, muscle 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) and loaded onto Tris glycine polyacrylamide gels, and protein mixtures were separated by electrophoresis (Novex MiniCell II, Carlsbad, CA). Proteins were then transferred to polyvinylidene fluoride membranes and incubated overnight at 4°C with primary antibodies to targeted proteins (anti-caspase-8, anti-Bcl-2, and anti-Bax from Santa Cruz Biotechnology, Santa Cruz, CA; anti-caspase-9 from Cell Signaling, Danvers, MA). Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies, and antibody binding was detected on film using enhanced chemiluminescence (NEL Life Science Products, Boston, MA). Densitometry of filmed gels was performed using a Microtek scanner (Carson, CA) and UN-SCAN-IT software (SIlent 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 among lanes. We chose α-tubulin for this normalization, because previous experiments indicate this protein is not altered in skeletal muscle by sepsis. Densities of the α-tubulin blots were determined using a Microtek scanner; these values were used to normalize densitometry values for caspase-8, caspase-9, Bcl-2, and Bax Westerns.

Measurement of force generation. Diaphragm force generation was assessed, as our laboratory has previously reported (5). 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-Henseleit solution (22°C, curare 50 mg/L, pH 7.40, NaCl 135 mM, KCl 5 mM, dextrose 11.1 mM, CaCl2 2.5 mM, MgSO4·7H2O 1 mM, NaHCO3 14.9 mM, NaHPO4·2H2O 1 mM, insulin 50 U/mL, 95% O2–5% CO2). One end of each strip was tied to the base of the organ bath, and the other end to a SI force transducer (Heidelberg, Germany). Platinum mesh field electrodes were used to deliver supramaximal currents using a biphasic constant-
current amplifier driven by a Grass S48 stimulator. After a 15-min equilibration period, muscle strip length was adjusted to optimal length, i.e., the length at which strip force generation in response to single stimuli 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 was recorded with a Gould 2600 strip chart recorder (Cleveland, OH). 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.

C2C12 cell studies. For these experiments, C2C12 myoblasts obtained from ATCC (Manassas, VA) were grown to 70% confluency in plastic petri dishes in DMEM with 10% fetal bovine serum. Media was then switched to DMEM with 2% horse serum for differentiation into myotubes. After 5 days of differentiation, we added either sterile saline (35 μl), TNF-α (volume 35 μl, with a final concentration of 20 nM of TNF-α in the dish), or a cytokine mixture (termed cytomix, final concentrations in media of 10 μg/ml LPS, 20 ng/ml TNF-α, 50 U/ml IL-1β, and 100 U/ml IFN-γ concentrated in a delivered volume of 35 μl). We examined 11 plates of cells for each of the three experimental conditions. After 24 h of exposure, four plates/condition were washed with PBS, and Trypan blue was added (0.4% solution) to assess viability of cells remaining attached to plates. After 30 min of Trypan blue incubation, the Trypan blue suspension was removed, and the still attached cells were washed with PBS. Cells were then photographed (three fields/plate, 12 fields/condition) using a Retiga camera interfaced with an Olympus microscope. Photomicrographs were inspected using Digimizer software (Mariakerke, Belgium) to calculate the percentage of Trypan blue-positive myotubes. The remaining plates of cells (7 plates/condition) were harvested at 24 h for determination of active caspase-8 and active caspase-9 protein levels. At the time of harvest, cells were rinsed in PBS containing 1 mM vanadate, 200 μl cell lysis buffer/plate (10 mM β-glycerophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 20 mM HEPES, 2 mM EDTA, 250 mM sodium chloride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 mM PMSF, 0.5 μg/ml benzamidine, 1 mM DTT, pH to 7.4) were then added for 30 min, and cells were collected and frozen at −80°C for subsequent analysis of caspase protein levels. Note that, for both Trypan blue determinations and cell harvesting to assess caspase-8/9 levels, plates were first washed; this procedure removes detached cells and reduces potential artifactual nonspecific increases in caspase activation related to these detached cells.

Statistical analysis. Unpaired t-tests (Sigma-Stat Software) were used to compare parameters across experimental groups for the first group of experiments. ANOVA was used for comparison of caspase levels and diaphragm forces across experimental groups for the second, third, and fourth groups of experiments. Tukey’s test was used to determine differences between individual groups following ANOVA. A P value of < 0.05 was taken as indicating statistical significance. Data are presented as means ± SE.

RESULTS

General response to endotoxin (LPS) administration. All animals receiving endotoxin (LPS) injections appeared systemically ill, with discharge around the eyes, mild diarrhea, and reduced movement around the cage. In contrast, control animals behaved normally. Animals receiving caspase inhibitors...
(inhibitors to caspase-8 and caspase-9) also had reduced movement, some diarrhea, and eye discharge.

Caspase pathway activation in response to endotoxin (LPS). TUNEL staining was used to determine whether endotoxin (LPS) administration elicited an increase in apoptotic nuclei in the diaphragm. We found rare nuclei staining positive by the TUNEL technique in diaphragms from control animals, as shown for representative samples in Fig. 1. We also found a small number of TUNEL-positive nuclei in diaphragms from endotoxin-treated animals (far right panels, Fig. 1), and these TUNEL-positive nuclei were primarily located on the outer peritoneal surface of the diaphragm. On average, TUNEL staining was positive for 0.21 ± 0.08% of nuclei for sections from control animals and was positive in 0.62 ± 0.14% of nuclei from endotoxin-treated animals ($P < 0.045$).

Although endotoxin administration did not elicit large amounts of TUNEL staining in the diaphragm, this stress nevertheless induced a marked increase in diaphragm active caspase-8 levels, as shown in Fig. 2. On average, endotoxin administration evoked a sevenfold increase in active caspase-8 protein levels (711% of control, $P < 0.001$). In contrast, we observed no change in diaphragm procaspase-9 levels following endotoxin administration and no evidence of active caspase-9 formation in response to endotoxin (Fig. 3). Since activation of caspase-9 and the other components of the intrinsic caspase pathway are usually accompanied by reductions in Bcl-2 and increases in Bax protein levels, we assessed diaphragms from control and endotoxin-treated animals for these proteins. Endotoxin administration did not significantly alter either Bcl-2 or Bax protein levels, as shown in Fig. 4.

Endotoxin significantly increased diaphragm caspase-3 activity levels, measured as in vitro cleavage of a specific
fluorogenic caspase-3 substrate by muscle homogenates from control and endotoxin-treated animals. Caspase-3 activity levels were 49.3 ± 11.9 nmol AMC·min⁻¹·mg protein⁻¹ in diaphragms from control animals and 137 ± 23 nmol AMC·min⁻¹·mg protein⁻¹ in diaphragms from endotoxin-treated animals (P < 0.01). We also assessed the ability of muscle homogenates to cleave specific caspase-8 (IETD-AMC) and caspase-9 (LEHD-AMC) substrates (Fig. 5). Endotoxin resulted in a marked increase in diaphragm caspase-8 activity (P < 0.03), but caspase-9 activity did not increase significantly for endotoxin group samples (Fig. 5).

Response to administration of caspase inhibitors. Endotoxin administration markedly reduced diaphragm force generation over a range of muscle stimulation frequencies (1–150 Hz), as shown in Fig. 6. For example, endotoxin administration elicited a 40.3% reduction in the diaphragm force generated in response to 20-Hz stimulation and a 47.5% reduction in the force generated in response to 150-Hz stimulation (P < 0.001 and P < 0.001, respectively, for comparison to controls). Concomitant administration of a caspase-8 inhibitor (Ac-IETD-CHO) to endotoxin-treated animals almost completely prevented endotoxin-induced reductions in diaphragm force generation (Fig. 6). For example, force generation in response to 150-Hz stimulation averaged 24.0 ± 1.0, 12.6 ± 1.1, and 21.4 ± 1.4 N/cm², respectively, for diaphragm muscles taken from control animals, endotoxin-treated animals, and animals given both endotoxin and Ac-IETD-CHO (P < 0.001 for this comparison, with the endotoxin-treated group different from the other two groups). On the other hand, administration of a caspase-9 inhibitor (Ac-LEHD-CHO) did not alter the effect of endotoxin, with the forces generated by muscles from animals given both endotoxin and Ac-LEHD-CHO similar to forces generated by samples from animals given endotoxin alone (Fig. 6).

As shown in Fig. 7, administration of a caspase-8 inhibitor completely prevented endotoxin-induced caspase-3 activation, while administration of a caspase-9 inhibitor failed to block caspase-3 activation (P < 0.007 for comparison for caspase-3 activity between these groups, with caspase-3 activity for the caspase-8 inhibitor/endotoxin group similar to control levels and significantly lower than caspase-3 activity in the endotoxin group).

Caspase-8 responses in TNFR1 deficient animals. Pro-caspase-8 in cells is normally activated after binding to one of several death receptors, including the cell surface TNFR1. To determine whether diaphragm pro-caspase-8 activation is dependent on TNFR1 activation, we examined the response of TNFR1 knockout mice to administration of endotoxin. As shown for representative samples in Fig. 8A, mice deficient in the TNFR1 demonstrated minimal formation of active caspase-8 protein in response to endotoxin administration. Group mean data were similar, with essentially no evidence of active caspase-8 protein formation in any TNFR1 mice treated with endotoxin (n = 4/group, Fig. 8B). TNFR1-deficient mice
also demonstrated only a minor reduction in diaphragm force generation in response to endotoxin administration, as shown in Fig. 9. For example, the force generated in response to 150-Hz stimulation averaged 24.2 ± 1.2, 12.5 ± 1.5, 23.4 ± 1.2, and 20.4 ± 1.9 N/cm², respectively, in wild-type (i.e., C57BL/6) saline-treated control animals, endotoxin-treated wild-type animals, TNFR1-deficient saline-treated animals, and TNFR1-deficient endotoxin-treated animals (P < 0.004 for comparison of force for endotoxin-treated wild-type animals to the other three groups).

Muscle cell line studies. If caspase-8 activation is a consequence of cytokine-induced activation of muscle cell death receptors, then incubation of isolated muscle cells with cytokines should also result in caspase-8 activation. We, therefore, examined the effect of addition of cytokines to a muscle cell line, C2C12 myotubes, on caspase-8 levels. Since previous work has suggested a central role for TNF-α in the induction of skeletal muscle dysfunction, we examined this particular cytokine (7). In vivo, however, the circulating and regional levels of multiple cytokines increase in response to systemic inflammatory stimuli, so we also examined the response of cells to a mixture of cytokines (i.e., termed “cytomix”, which consists of TNF-α, IL-1β, IFN-γ, and endotoxin). As shown in Fig. 10, we found that both TNF-α and cytomix induced marked increases in levels of active caspase-8 following incubation with C2C12 cells for 24 h. Cell homogenates were also probed to assess caspase-9 levels. Neither TNF-α nor cytomix induced formation of caspase-9 in C2C12 myotubes.

We also found that the percentage of Trypan blue-positive cells remained small after 24 h of incubation with TNF-α and cytomix, arguing that this duration of these specific cytokine concentrations did not induce significant nonspecific cellular damage. Specifically, Trypan blue-positive cells averaged 3.1 ± 0.5, 4.6 ± 1.4, and 5.5 ± 1.3%, respectively, for control C2C12 cells, TNF-α-exposed cells, and cytomix-treated cells (n = 12 fields/condition, nonsignificant).

DISCUSSION

Infection induced respiratory skeletal muscle weakness. A number of previous publications have shown that infections lead to significant reductions in the force/pressure-generating capacity of the respiratory muscles (3, 8, 17). This work includes studies showing that humans (both normal human volunteers and patients with respiratory disorders) have 30–40% reductions in inspiratory muscle pressure-generating capacity in response to viral infections (17). In addition, animal models of infection have consistently demonstrated 50–60% reductions in diaphragm muscle force-generating capacity within 4–96 h of application of the inflammatory stimulus (3, 9, 19). While it is often assumed that infection-induced reductions in muscle function are a consequence of muscle wasting due to wholesale protein loss (21), several studies
indicate that muscle force falls before appreciable muscle protein loss (3, 19). In keeping with this observation, Solomon and Goldberg (23) have shown that intact myofibrillar complexes are not susceptible to breakdown by the proteasome complex, the major proteolytic system in muscle. As a result, it has been suggested that muscle protein loss in response to catabolic stimuli is a two-step process, with an initial process that disrupts the contractile protein lattice and a subsequent step (the proteosomal system) that degrades the freed myofibrillar components (11). The mechanisms responsible for initial disruption of myofibrillar complexes in catabolic conditions are poorly understood, albeit recent studies suggest caspase may be responsible (11). Of interest, a number of recent investigations suggest that an analogous process occurs in another striated muscle, the heart (6, 16, 22, 26). Importantly, this cardiac work indicates that reductions in cardiac contractility due to caspase activation can occur in the absence of cardiomyocyte apoptosis (22).

Recently, we found that endotoxin administration to rodents rapidly induces marked increases in diaphragm levels of active caspase-3 protein and caspase-3 activity (25). We also found that activation of caspase-3 was accompanied by significant reductions in diaphragm force generation following endotoxin, and that coadministration of caspase-3 inhibitors along with endotoxin prevented endotoxin-induced reductions in diaphragm force. Taken together, these previous data strongly argue that caspase-3 activation is a major contributor to the development of endotoxin-induced diaphragmatic dysfunction.

Pathways of diaphragmatic caspase activation following endotoxin administration. In most tissues, caspase-3 is thought to be a downstream “effector” caspase responsible for directly cleaving proteins and DNA to induce apoptosis and alterations in cellular structure. Formation of active caspase-3 from pro-caspase-3 can be initiated by several upstream pathways, including several “activator” caspases, such as caspase-9 and caspase-8 (24, 27). Caspase-9 activation, in turn, is primarily linked to molecules (e.g., cytochrome c) released by mitochondria. This pathway is termed the mitochondrially dependent or intrinsic caspase activation pathway, and its activity is influenced by levels of Bax, Bcl-2, and related BH3 protein family members.
members. Activation of caspase-9 is usually connected with increased mitochondrial release of cytochrome c and is often associated with increases in Bax and reductions in Bcl-2. On the other hand, caspase-8 activation is generally the result of ligand attachment to one of several death receptors, including the TNFR1, Fas, and direct report-4/5 receptors (1, 27). Once ligands attach to death receptors, procaspase-8 is autocatalytically cleaved to form active caspase-8.

The present series of experiments are the first to examine the pathways of diaphragm caspase activation in an animal model of systemic infection and/or inflammation. Our findings are entirely consistent with a primary role for activation of the extrinsic, caspase-8-linked pathway as the principal mechanism responsible for downstream activation of caspase-3 following endotoxin administration. Specifically, we found that endotoxin administration to intact animals induced a large increase in diaphragm levels of active caspase-8 protein and caspase-8 activity. In addition, administration of a selective caspase-8 inhibitor blocked endotoxin-induced increases in caspase-3 activity and also largely prevented endotoxin-induced reductions in diaphragm force generation. In contrast, diaphragm caspase-9 protein levels and/or activity did not increase 24 h after administration of endotoxin to animals, and also endotoxin failed to elicit significant changes in either diaphragm Bax or Bcl-2 protein levels. Administration of a selective caspase-9 inhibitor also failed to prevent endotoxin-induced increases in caspases-3 activity and failed to prevent endotoxin-induced reductions in diaphragm force generation. As has been reported previously in cardiac tissues, we observed marked increases in diaphragm caspase activation in the absence of a major degree of nuclear apoptosis, as indicated by the only small increase in nuclear TUNEL staining observed following endotoxin administration. While it is possible that we may have observed more marked evidence of apoptosis had we performed additional studies at later time points, the present data, nevertheless, indicate that substantial caspase-dependent reductions in diaphragm force generation can occur with only small increases in nuclear evidence of apoptosis, as assessed using TUNEL.

Since caspase-8 formation is usually linked to death receptor activation (1, 15), we also determined the effect of endotoxin administration in transgenic animals deficient in TNFR1, one of the death receptors responsible for caspase-8 activation. We found that administration of endotoxin to these animals resulted in minimal diaphragm caspase-8 activation and only a small reduction in diaphragm force generation. These findings argue that attachment of TNF-α to the TNFR1 may be a critical step in initiation of caspase-8 activation and caspase-8-mediated caspase-3 activation in the diaphragm and other skeletal muscles following endotoxin administration. The alternative possibility is that TNF-α does not directly evoke caspase-8 activation in skeletal muscle, but, rather, lack of TNFR1 so alters the systemic inflammatory response to endotoxin administration that some indirect process contributing to diaphragm caspase activation is blocked. To address this latter concern, we directly exposed isolated skeletal muscle myotubes to either TNF-α or a mixture of cytokines (i.e., cytokin, a commonly used mixture containing a group of cytokines known to increase in response to infections, including TNF-α, endotoxin, IL-1β, and IFN-γ). We found that exposure of myotubes to either TNF-α or cytokin evoked strong activation of caspase-8, mirroring the responses observed in the diaphragms of intact animals given endotoxin. Taken together, these animal and myotube data support the concept that cytokine-induced activation of skeletal muscle death receptors can potently trigger activation of skeletal muscle caspases (8 and 3) and induce caspase-mediated reductions in muscle force generation.

We should note that this mechanism of caspase-3 activation differs from that observed in other pathological conditions associated with muscle dysfunction and/or muscle wasting. Specifically, previous studies have shown that caspase-3 activation in limb skeletal muscle in animal models of uremia and diabetes is largely linked to caspase-9 activation, which, in turn, is linked to inhibition of insulin receptor substrate-1-associated phosphatidilinositol 3-kinase activity (10, 11). As a result, caspase-3 activation may represent a common downstream event in a variety of clinical conditions associated with muscle dysfunction and muscle wasting, with the particular mechanism of caspase-3 activation varying from condition to
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condition, depending on the precise mixture of factors (cyto-
kine exposure, lack of insulin, etc.) to which muscle is ex-
posed.

It is also important to note that previous papers have shown that a variety of cellular pathways, e.g., superoxide and nitric
oxide free radicals (3), are involved in the pathogenesis of
endotoxin-induced diaphragm dysfunction. It seems very likely
that there may be an interaction between these other pathways
and caspase activation in the diaphragm. Additional studies
will be needed to elucidate the interactions between caspase
activation and these other factors.

Potential implications. Critically ill patients have profoundly
weak respiratory muscles (13, 28). Such severe reductions in
diaphragm function contribute to the persistence of respiratory
failure and increase the difficulty in weaning these patients
from mechanical ventilation. Several factors may contribute to
diaphragmatic weakness in this patient population, including
the effects of mechanical ventilation per se, the effects of
uncontrolled hyperglycemia on diaphragm function, and the
effects produced by systemic infections (2, 3, 4, 18, 19). Infections are clearly present in patients requiring mechanical
ventilation for pneumonia, are the most common precipitant
for exacerbations of obstructive lung disease requiring me-
chanical ventilation, are present in a high percentage of pa-
tients with adult respiratory distress syndrome, and often de-
velop nosocomially in patients initially intubated for other
reasons. The present work provides a potential mechanism by
which respiratory muscle weakness may develop in these
patient populations. In keeping with this possibility, one recent
report found evidence of strong caspase activation in the limb
muscles of critically ill patients diagnosed with the critical
illness myopathy syndrome (7). Additional work will be
needed to determine whether the phenomena reported in the
present study also occur in the respiratory and limb muscles of
critically ill patients. If so, inhibition of caspase activation
pathways may provide a therapeutic means of preventing
skeletal muscle weakness and wasting in this patient popula-
tion.

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