The extrinsic caspase pathway modulates endotoxin-induced diaphragm contractile dysfunction

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Submitted 29 March 2006; accepted in final form 3 December 2006

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-Leu-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 diaphragmatic 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 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 diaphragmatic nuclear DNA damage as assessed by the terminal deoxynucleotidyl transferase (TdT) biotin-DUFP 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-Tnfrsf1αtm1Mak/J, background strain C57BL/6). We compared (n = 4/group) 1) C57BL/6, saline IP 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 (cytovix, 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 C6-12 myotubes evoked caspase activation. For these studies, either saline (35 μl), TNF-α (20 ng/ml in a volume of 35 μl), or cytovix (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 TUNEL staining. TUNEL staining was performed using the Fluorescein-FragEL DNA Fragmentation Detection Kit from Oncogene (Bosto, MA). For this assessment, costal diaphragm was first embedded in paraffin and sectioned (10-μm-thick sections). Sections were mounted on slides, and slides were immersed in xylene (two 5-min incubations at room temperature). Slides were then placed in serial ethanol solutions (100 to 70%), as per the manufacturer’s instructions (Oncogene). The specimen was then permeabilized with Proteinase K and rinsed with PBS. Slides were then placed in TdT equilibration buffer for 30 min followed by immersion in TdT labeling reaction mixture with TdT enzyme. Buffers also contained 4,6-diamidino-2-phenylindole (DAPI; Oncogene), to allow visualization of nuclei. As a control, duplicate specimens were processed in reaction mixture minus the TdT enzyme but in DAPI. As a positive control, HL-60 cells that had been incubated with actinomycin to induce apoptosis were also processed. Slides were then covered with paraffin and placed in a humidified chamber for 1.5 h (37°C). The paraffin was then removed, and slides were washed in PBS. Slides were visualized using fluorescence microscopy using a fluorescein filter (excitation maximum of 494 nm). Sections were then visualized using a filter to detect DAPI nuclear staining. A Retiga camera interfaced with the microscope was used to obtain photomicrographs of slides, which were then analyzed using IP laboratory software.

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. Duplicate determinations 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 spectrophotofluorometer (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 (NEN Life Science Products, Boston, MA). Densitometry of filmed gels was performed using a Microtek scanner (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 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 1 mM, NaHCO₃ 14.9 mM, NaHPO₄ 1 mM, insulin 50 UL, 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.

**Fig. 2.** Diaphragm caspase-8 protein levels. Western blots are shown for procaspase-8 and active caspase-8 for diaphragm samples from control and endotoxin-treated animals. Western blots for α-tubulin were performed as a loading control. Endotoxin administration induced an increase in the active caspase-8 band, as shown for representative experiments (A) and as group mean data (B). *Statistically significant increase.

**Fig. 3.** Diaphragm caspase-9 protein levels. Western blots are shown for procaspase-9 and active caspase-9 for diaphragm samples from control and endotoxin-treated animals. Western blots for α-tubulin were performed as a loading control. Representative blots (A) and group mean data (B) are presented. Endotoxin administration did not alter procaspase-9 band levels. Moreover, no appreciable active caspase-9 band was detected following endotoxin administration.
fluorogenic caspase-3 substrate by muscle homogenates from control and endotoxin-treated animals. Caspase-3 activity levels were $49.3/1100611.9$ nmol AMC$/18528188/10021$ min/$18528188/10021$ mg protein/$18528188/10021$ in diaphragms from control animals and $137/1100623$ nmol AMC$/18528188/10021$ min$/18528188/10021$ mg protein/$18528188/10021$ 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 $24.0/110061.0$, $12.6/110061.1$, and $21.4/110061.4$ 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 procaspase-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

Fig. 4. Diaphragm Bax and Bcl-2 levels. Western blots are shown for Bax, Bcl-2, and α-tubulin for diaphragm samples from control and endotoxin (LPS)-treated animals. α-Tubulin was used as a loading control. Endotoxin did not significantly alter Bcl-2 or Bax levels. Representative Western blots (A) and group mean data (B) are presented.

Fig. 5. Caspase-8 and caspase-9 activity assays. Diaphragm caspase-8 activity (left) and caspase-9 activity (right) measured by incubating samples from control and endotoxin-treated animals with fluorogenic substrates are shown. As an additional control, we also performed duplicate assays with addition of either inhibitors of caspase-8 (for left panel assays) or inhibitors of caspase-9 (for right panel assays). Endotoxin markedly increased caspase-8 activity ($P < 0.03$), but not caspase-9 activity. AMC, amino-4-methyl coumarin. *Statistically significant increase.
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...
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 proteosome 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 suggest 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 procaspase-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 mitochondrial dependent or intrinsic caspase activation pathway, and its activity is influenced by levels of Bax, Bcl-2, and related BH3 protein family members.

**Fig. 8.** Caspase-8 responses in TNF receptor 1 (TNFR1) knockout (TNFR1X) mice. Western blotting is shown for procaspase-8 and active caspase-8 levels for diaphragm samples (from left to right) from wild-type control animals given saline, wild-type animals given endotoxin (LPS), TNFR1X, and TNFR1X mice given endotoxin. Representative blots (A) and group mean data (B) are shown. Endotoxin induced significant active caspase-8 formation in the wild-type mice, but did not elicit caspase-8 activation in the TNFR1X mice. *Statistically different from the control group.

**Fig. 9.** Diaphragm force generation in TNFR1X mice. Diaphragm force-frequency relationships are shown for wild-type saline-treated control animals, wild-type endotoxin (LPS)-treated animals, TNFR1X mice, and TNFR1X mice given endotoxin. Endotoxin induced a significant reduction in force generation at all frequencies of stimulation in wild-type mice. TNFR1X mice were resistant to the effect of endotoxin, with force generation in TNFR1X given endotoxin for frequencies 20–150 Hz significantly higher than forces generated by diaphragms from wild-type animals given endotoxin. *A statistically significant reduction from control levels; #A significant difference from the wild-type-endotoxin-treated group.
from control levels. Large increases in active caspase-8 levels. *A statistically significant increase procaspase-8 and active caspase-8 levels in C2C12 myotubes exposed to control selective caspase-8 inhibitor blocked endotoxin-induced in-

tein and caspase-8 activity. In addition, administration of a large increase in diaphragm levels of active caspase-8 pro-

that endotoxin administration to intact animals induced a following endotoxin administration. Specifically, we found 

anism responsible for downstream activation of caspase-3 extrinsic, caspase-8-linked pathway as the principal mech-

of systemic infection and/or inflammation. Our findings are pathways of diaphragm caspase activation in an animal model 

cytoplasmic cleavage to form active caspase-8. Ligands attach to death receptors, procaspase-8 is autocatalyt-

the TNFR1, Fas, and direct report-4/5 receptors (1, 27). Once 

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 in-

creases 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 admin-

istration 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., cytomix, 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 cytomix 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 phosphatidylinositol 3-kinase activity (10, 11). As a result, caspase-3 activation may represent a common down-

stream 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

Fig. 10. Caspase activation in C2C12 cells. Western blots are shown for procaspase-8 and active caspase-8 levels in C2C12 myotubes exposed to control media (containing saline), media containing TNF-α, and media containing cytomix (TNF-α, IL-1β, IFN-γ, and LPS). Samples were reprobed with α-tubulin as a loading control. Representative blots (A) and group mean data (B) are shown. Exposure of C2C12 cells to either TNF-α or cytomix induced large increases in active caspase-8 levels. *A statistically significant increase from control levels.
condition, depending on the precise mixture of factors (cytokine exposure, lack of insulin, etc.) to which muscle is exposed.

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 mechanical ventilation, are present in a high percentage of patients with adult respiratory distress syndrome, and often develop 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 population.

**GRANTS**

This study was supported by National Heart, Lung, and Blood Institute Grants 69821, 63698, and 80429.

**REFERENCES**


