p38 Mitogen-Activated Protein Kinase Modulates Endotoxin-Induced Diaphragm Caspase Activation

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We postulated that the p38 pathway is activated in the diaphragm during sepsis and contributes to sepsis-induced diaphragm caspase activation and contractile dysfunction. This study determined whether: (1) endotoxin administration elicits p38 activation in the diaphragm; (2) cytokines activate p38 in isolated muscle cells; (3) activation of p38 is accompanied by caspase 8 activation; (4) inhibition of p38 prevents caspase 8 activation and; (5) inhibition of p38 prevents diaphragm dysfunction in endotoxin-treated animals. We first evaluated the time course of diaphragm p38 activation after endotoxin in mice. We then determined if p38 inhibitor administration could prevent caspase 8 activation in endotoxin-treated mice. We also assessed p38 and caspase 8 activation in C2C12 muscle cells treated with control media or a cytokine mixture, with or without concomitant chemical inhibition of p38 (using SB203580, 25 μ M) or loss of p38 function due to cell transfection with a dominant negative p38 genetic construct. Endotoxin administration activated diaphragm p38 ($P < 0.001$), and cytokines activated p38 in C2C12 cells ($P < 0.05$). In both the diaphragm and cells, p38 activation was accompanied by increases in active caspase 8 ($P < 0.01$). Inhibition of p38 with either SB203580 or with a dominant negative p38 construct prevented caspase activation ($P < 0.001$). p38 inhibitors also prevented endotoxin-induced diaphragm weakness ($P < 0.001$). p38 modulates cytokine-induced skeletal muscle caspase activation.

Keywords: diaphragm; endotoxin; caspase; proteolysis; sepsis

Two recent studies performed with the magnetic twitch stimulation technique found that critically ill patients develop severe diaphragm weakness, averaging diaphragm twitch forces that are only 20–25% of those observed in normal subjects (1, 2). This severe weakness is thought to account, in part, for the difficulty weaning many patients in intensive care units (ICUs) from mechanical ventilation (3). The mechanisms responsible for this severe respiratory muscle weakness in patients in the ICU are poorly understood, but many of these patients have infections, and infections have been repeatedly shown to induce severe respiratory muscle weakness (4–6). In recent studies, we have shown that endotoxin-induced sepsis potentially activates diaphragmatic caspase 8, and that caspase 8 activation is linked, in turn, to the development of diaphragmatic weakness (7, 8). It is also known that infections strongly activate the mitogen-activated protein kinase (MAPK) pathways in many tissues, and that the p38 MAPK pathway can elicit caspase activation and cellular dysfunction in a number of organs (9–11). However, the role of the p38 kinase pathway in modulating infection-induced diaphragm muscle caspase activation has never been studied.

CLINICAL RELEVANCE

Infections produce severe diaphragm weakness, and this weakness predisposes infected patients to respiratory failure. This article provides the first evidence that activation of the p38 kinase pathway plays an important role in mediating the development of infection-related diaphragm weakness.

The purpose of the present group of experiments, therefore, was to test the hypothesis that endotoxin-induced sepsis leads to p38 pathway activation and p38-mediated activation of diaphragmatic caspase 8. In whole-animal studies, sepsis was induced by endotoxin injection into mice, diaphragm caspase 8 activation was assessed by Western blot techniques, and the effects of inhibiting p38 with SB203580 on diaphragm muscle caspase activation and function was determined. In additional experiments, we evaluated the direct effects of addition of cytokines to a mouse muscle cell line (C2C12 cells), and determined the effects of chemical and genetic inhibition of p38 activation (i.e., chemical inhibition of p38 with SB203580 and transfection with a dominant negative p38 construct) on caspase activation in these isolated cells.

MATERIALS AND METHODS

Experimental Models and Protocols

All animal experiments were performed with male mice weighing between 25 and 35 g. Mice had unrestrained access to food and water throughout the period of experimentation. Experiments were approved by the University of Kentucky Institutional Animal Care and Use Committee, and animals were monitored throughout experiments by the University Animal Center staff.

Three sets of studies were performed. In the first study, we determined the time course of diaphragm p38 activation in animals after endotoxin administration. For this study, animals were killed at 2, 4, 8, and 24 hours after 12 mg/kg endotoxin (*Escherichia coli* 055:B5 LPS [Sigma, St. Louis, MO], injected intraperitoneally in 0.3 ml saline; $n = 3$ /group). Saline-injected animals (0.3 ml given intraperitoneally; $n = 3$) served as controls for these studies. To provide hydration, all groups were also injected subcutaneously with 60 ml/kg saline at the time of saline or endotoxin administration. At the time of death, animals were anesthetized with pentobarbital (50 mg/kg); diaphragms were then removed and used for assessment of force generation and p38 activation, as described subsequently here.

In the second set of studies, we determined if administration of p38 inhibitor could prevent endotoxin-induced caspase 8 activation and diaphragm dysfunction in animals. Four groups ($n = 4$ –5/group) were studied: (1) control animals injected with saline (intraperitoneally, 0.3 ml); (2) animals injected with endotoxin (12 mg/kg *E. Coli* LPS 055: B5 intraperitoneally in 0.3 ml saline); (3) animals injected intraperitoneally with both 12 mg/kg endotoxin and 25 μ M/kg SB 203580, a p38 inhibitor; and (4) animals intraperitoneally injected with 25 μ M/kg SB 203,580 alone. All groups were also injected subcutaneously with 60 ml/kg saline. At 24 hours after these injections, animals were anesthetized with pentobarbital and killed; diaphragms were removed and used for assessment of force generation and caspase 8 protein levels. In our

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previous work examining caspase activation in the diaphragm (7, 8), we found that endotoxin administration was followed by increases in active caspase 8 protein, active caspase 3 protein, increased caspase 8 function assessed with an activity assay, increased caspase 3 function assessed with an activity assay, and increased formation of caspase cleavage-specific spectrin degradation products. Endotoxin did not, however, increase caspase 9 protein, did not increase caspase 9 activity, did not significantly increase Bcl-2-associated X protein, and did not reduce B-cell lymphoma 2 protein levels. We also found that administration of a caspase 8 inhibitor completely blocked all indices of caspase 3 activation, implicating formation of caspase 8 protein as the critical diaphragm caspase component activated after endotoxin administration (7, 8). For these reasons, we chose to monitor active caspase 8 protein levels in the present study.

In a third set of studies, we determined if caspase was activated in C2C12 cells in response to incubation with a mixture of cytokines, and if inhibitors of p38 prevented caspase 8 activation. The reason we chose to expose cells to a mixture of cytokines instead of endotoxin alone (as was used in intact animal studies) is that endotoxin administration to intact animals results in elaboration of a mixture of cytokines (including TNF- α , IL-1 β , and IFN- γ) due to the effects of endotoxin on white cells. As a result, the diaphragm is exposed to a mixture of cytokines *in vivo* after endotoxin administration, and we therefore thought it appropriate to also expose cultured myotubes *in vitro* to a mixture of cytokines. Studies were conducted on the following groups ($n = 4$ plates of cells/group): (1) C2C12 myotubes to which sterile saline was added (35 μ l); (2) myotubes treated with a cytokine mixture (termed cytomix; final concentrations in media of 10 μ g/ml LPS, 20 ng/ml TNF- α , 50 U/ml IL-1 β , and 100 U/ml IFN- γ in a volume of 35 μ l); (3) myotubes treated with cytomix and 25 μ M/kg SB 203580, a chemical p38 incubated in Dulbecco's modified Eagles medium (DMEM) with 2% horse serum during these exposures. Details about cell growth and differentiation are provided in the CELL PREPARATIONS AND TRANSFECTIONS section. After 24 hours, cells were harvested and assessment made of cell caspase concentrations. In cell studies, we also determined if genetic inhibition of p38 activation would alter the activation of caspase in C2C12 cells. Studies were conducted on the following groups ($n = 4$ –5 plates of cells/group): (1) control sham transfected cells treated with saline (35 μ l); (2) sham construct transfected cells treated with cytomix (35 μ l); (3) cells transfected with a p38 dominant negative construct, then treated with saline (35 μ l); and (4) p38 dominant negative transfected cells treated with cytomix (35 μ l). At 24 hours after application of saline or cytomix, cells were harvested and assessment made of cell caspase 8 concentrations. Details regarding transfection are provided subsequently here.

p38, ATF-2, and Caspase Protein Identification by Western Blots

Western blotting was employed to measure diaphragm and myotube levels of phospho-p38, total p38, ATF-2, and caspase 8. We also measured levels of α -tubulin as a loading control. For these determinations, muscle samples were first homogenized in buffer (10 mM β -glycerophosphate, 50 mM sodium fluoride, 1 mM sodium, 20 mM Hepes, 2 mM EDTA, 250 mM sodium chloride, 2 μ g/ml leupeptin, 2 μ g/ml aprotinin, 1 mM PMSF, 0.5 μ g/ml benzamidine, and 1 mM DTT) in a 1 g:10 ml ratio, centrifuged at $3,000 \times g$ for 10 minutes, the supernatant saved, and its protein content determined. Supernatant samples of equal protein content were then diluted 1:1 with loading buffer (126 mM Tris-HCl, 20% glycerol, 4% SDS, 1.0% 2-mercaptoethanol, 0.005% bromophenol blue [pH 6.8]), placed in boiling water for 5–7 minutes, and then loaded onto Tris glycine polyacrylamide gels. Protein mixtures were separated by electrophoresis (Novex Minicell II; Invitrogen, Carlsbad, CA), transferred to polyvinylidene fluoride membranes, and incubated overnight at 4°C with primary antibodies to targeted proteins (phospho-p38, total p38, phospho-ATF-2, total ATF-2 [Cell Signaling, Danvers, MA], and caspase 8 [Santa Cruz Biotechnology, Santa Cruz, CA]). Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies, and antibody binding detected on film by enhanced chemiluminescence (NEN Life Science Products, Boston, MA). Densitometry of filmed gels was performed with 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) 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 with a Microtek scanner; these values were used to normalize densitometry values. All blots had both control and experimental lanes. After controlling for protein loading, we then additionally normalized the data from each blot to the value for the density of the control samples (i.e., the density of the control sample was taken as 1.0, and the other experimental lanes were expressed as a ratio to the value for the control sample) to allow averaging of data from different blots.

p38 Kinase Activity Assay

We measured p38 kinase activity directly by immunoprecipitation of p38 from diaphragm sample homogenates and subsequent incubation of the immunoprecipitate with a synthetic p38 substrate with the p38 kinase assay kit from Cell Signaling (Danvers, MA). For this assay, muscle samples were homogenized in lysis buffer (supplied in the Cell Signaling p38 kit, in a proportion of 1:10 [i.e., 1 g tissue/10 ml buffer]) with a polytron. Homogenates were then centrifuged in the cold at $3,000 \times g$ for 10 minutes, the supernatant taken, and its protein concentration determined. Aliquots of homogenates containing 100 μ g (processed from muscles from control and endotoxin-treated animals) were then placed in tubes containing immobilized phospho-p38 MAPK monoclonal antibody to immunoprecipitate the p38 kinase. After washing, ATF-2 was added to serve as an exogenous p38 substrate. This mixture was allowed to incubate, and, subsequently, the reaction products were run on a gel and detected by probing for ATF-2 phosphorylation (i.e., with phospho-ATF-2(Thr71) antibody). Densities of the ATF-2 phosphorylation product were quantitated with UN-SCAN-it software.

Measurement of Force Generation in Isolated Diaphragm Muscle Strips

For assessment of force generation, diaphragm strips were dissected from the left costal diaphragm and mounted vertically in water-jacketed organ baths (Radnoti Glass, Monrovia, CA) containing curarized Krebs-Henseleit solution at 22°C, bubbled with 95% O₂/5% CO₂ (12). The rib end of strips was secured to the bottom of baths by silk ties, and the tendinous end to a Grass FT10 force transducer (West Warwick, RI). Platinum electrodes were placed about strips and connected to an amplifier (Biomedical Technology of America, Cleveland, OH) attached to a Grass S48 stimulator. After a 15-minute equilibrium period, muscle length was adjusted to Lo (the length of maximum force generation), stimulation current adjusted to supramaximal levels, and a force-frequency curve was constructed by sequentially stimulating strips at 1, 10, 20, 50, 75, 100, 125, and 150 Hz (train duration, 800 ms) with a 30-second rest period between adjacent stimulus trains. At the conclusion of force measurements, transducers were calibrated with standard weights, and force/cross-sectional area calculated by the method of Close (13).

Cell Preparations and Transfections

For these experiments, C2C12 myoblasts (ATCC, Manassas, VA) were grown to 70% confluence in plastic Petri dishes in DMEM with 10% FBS. To induce differentiation, media were switched to DMEM with 2% horse serum for 5 days.

For transfection experiments, C2C12 myoblasts were first transfected with a genetic construct containing Cre flanked by tamoxifen-responsive elements downstream of a muscle-specific creatine kinase (MCK) promoter. This construct was made by ligating an MCK promoter (pMCKG, 4.9 kb [a gift of Dr. Suzanne Porszasz-Reisz] to tamoxifen-inducible cyclization recombination (Cre) (pAN-MCM [from Dr. Michael Reth]; this contains a G418 selection cassette). The resultant MCK-MCM was transfected into C2C12 cells with Ambion Xpress transfection reagent (Ambion, Foster City, CA), cells containing MCK-MCM were selected by growth in media containing G418, and verification of transfection was performed by PCR with primers for MCK-MCM. Cells were then transfected with a second construct containing a cytomegalovirus (CMV) promoter linked to a floxed stop codon connected, in turn, to a p38 dominant negative gene. This construct was produced by ligating a floxed stop element (LSL-TOPO, from Dr. Tyler Jacks) to a p38 dominant negative element (p38 DN; from Dr. Phillip Scherer; this construct contains a purinomycin selection element). The resultant CMV-LSL-p38 DN was transfected into cells with Ambion Xpress

reagent, cells were selected by growth in purinomycin containing media, and verification of transfection was performed by PCR with primers to LSL. For sham controls, we used cells transfected with MCK-MCM, but not CMV-LSL-p38 DN. For experimental use, transfected cells were differentiated for 4 days, then exposed to tamoxifen (50 nM) for 3 days to induce Cre activation, and then subsequently exposed to either saline or cytomix (see Figure 1).

Statistical Analysis

ANOVA was used to compare variables (e.g., force) across groups of animals treated with different agents, with *post hoc* testing (Tukey) to determine differences between groups. A *P* value of less than 0.05 was taken as indicating statistical significance for all experiments. Data are presented as means $\pm$ 1 SEM.

RESULTS

Effect of Endotoxin Administration on Diaphragm p38 Activity

Endotoxin administration increased diaphragm levels of phospho-p38 protein, the active form of p38, as shown in Figure 2. Diaphragm phospho-p38 protein levels increased gradually after endotoxin administration, peaking at 8 hours, and then subsequently declined. Total p38 protein levels were constant over the 24 hours after endotoxin administration.

The kinase activity of p38 was directly measured by addition of a p38-specific substrate to p38 immunoprecipitated from diaphragm samples, with representative and group mean results shown in Figure 3. Muscle samples from endotoxin-treated animals demonstrated a marked increase in p38-specific kinase activity, with threefold greater phosphorylation of synthetic substrate when compared with control samples ($P < 0.001$).

Diaphragm force fell over time after endotoxin administration in this initial series of experiments. Specifically, maximum diaphragm force (in response to 150-Hz stimulation) averaged 24.9 (± 1.9), 24.3 (± 1.4), 23.3 (± 1.9), 21.6 (± 1.1), 19.0 (± 2.2), and 13.3 (± 0.4) N/cm², respectively, for muscles from control animals at 2, 4, 8, and 24 hours after endotoxin ($P < 0.01$ for comparison of the 24-h group to the control group).

Response of Diaphragm Force Generation and Caspase 8 Levels to Endotoxin and p38 Inhibitor Administration

Endotoxin administration induced a marked reduction in diaphragm muscle-specific force generation (Figure 4, $P < 0.001$

Figure 1. Constructs used For C2C12 Cell transfections. Cells were transfected first with a construct containing the Cre gene flanked by tamoxifen-responsive elements (TR) downstream of a muscle-specific creatine kinase (MCK) promoter (*left*). Cells were then transfected with a construct containing a cytomegalovirus (CMV) promoter linked to a floxed stop codon (Flox-X-Flox) connected, in turn, to a p38 dominant negative gene (DN p38). Addition of tamoxifen to cells containing both constructs results in production of Cre by the *left* gene construct: Cre then elicits removal of the floxed stop codon from the *right* construct, permitting expression of the dominant negative p38 gene.

for comparison of control to endotoxin-treated animals). Systemic administration of SB203580, a p38-specific inhibitor, ablated the effects of endotoxin administration, with force generated by diaphragms from endotoxin/SB203580-treated animals similar to control levels ($P < 0.001$ for comparison of both endotoxin to endotoxin/SB203580 and endotoxin to control).

Endotoxin administration also evoked a small, nonsignificant reduction in diaphragm procaspase 8 levels, and a large, 46-fold increase in active caspase 8 protein levels (Figure 5, $P < 0.001$). Inhibition of p38 with SB203580 prevented active caspase 8 formation in endotoxin-treated animals, with active caspase 8 levels similar to control levels and significantly lower than levels for the endotoxin alone-treated group ($P < 0.01$).

To evaluate the ability of SB203580, in the doses employed, to inhibit p38 activity *in vivo* we measured diaphragm levels of phospho-ATF-2 in the four experimental groups (control, endotoxin, endotoxin/SB203580, SB203580), because ATF-2 is a naturally occurring substrate for activated p38. As a loading

Figure 2. (A) Time course of p38 activation for representative diaphragm samples from endotoxin-treated animals. Western blotting was used to determine protein levels of phospho-p38 and total p38 as a function of time after endotoxin administration. Phospho-p38 protein levels increased above control levels between 4 and 8 hours after endotoxin administration, and then fell. Total p38 was similar for the five time points. (B) Mean phospho-p38 protein levels over time. Diaphragm phospho-p38 levels increased significantly at 8 hours after endotoxin administration as compared with control levels ($n = 3$ /point; $P < 0.001$). Total p38 levels did not change over time. *Statistically significant difference compared with control levels.

Figure 3. p38 Kinase activity assays for diaphragms from endotoxin-treated animals. *A* presents p38 Kinase assay data for diaphragm samples from controls and at 8 hours after endotoxin administration ($n = 4/\text{group}$), whereas *B* presents mean densitometric analysis of assay product levels. Endotoxin administration was associated with a large increase in p38 kinase activity ($P < 0.001$). *Statistically significant difference compared with control levels.

control, we also assessed total ATF-2 levels in the four experimental groups. We found that endotoxin administration increased ATF-2 phosphorylation, and SB203580 blocked this increase. Specifically, phospho-ATF-2 levels averaged 1.00, 1.84 (± 0.17), 0.93 (± 0.11), and 1.16 (± 0.14) normalized densitometry units, respectively, in control, endotoxin, endotoxin/SB203580, and SB203580 groups ($P < 0.01$ for comparison of phospho-ATF-2 levels in the endotoxin-treated to the other experimental groups). Total ATF-2 levels were similar in the four groups of animals.

Response of C2C12 Myotubes to Cytokine Incubation

Incubation of C2C12 myotubes with a commonly employed cytokine mixture (i.e., LPS, TNF- α , IL-1 β , and IFN- γ , termed cytomix) induced significant caspase 8 activation, as shown in Figure 6 ($P < 0.001$ for comparison of control to cytomix-treated cells). Concomitant administration of both cytomix and a chemical p38 inhibitor, SB203580, however, resulted in virtually no caspase 8 activation.

To further examine the effect of silencing p38 activity on cytokine-mediated caspase 8 activation in C2C12 cells, we doubly transfected C2C12 cells with a tamoxifen-inducible Cre construct and a construct containing dominant negative p38 distal to a floxed stop codon. Upon exposure to tamoxifen, Cre is activated in this cell line, resulting in expression of the dominant negative p38 (Figure 1). We then compared the ability of cytomix exposure to activate p38 and caspase 8 in control construct transfected cells with the effects of cytomix on cells doubly transfected with the Cre and dominant negative p38 constructs. As shown in Figure 7, cells transfected with the Cre and dominant negative p38 constructs did not have formation of

Figure 4. Effect of SB203580 on the diaphragm force-frequency curve. Force generation was significantly lower for diaphragms from LPS-treated animals (filled squares) than for control animals (open circles) ($n = 4\text{--}5/\text{group}$; $P < 0.001$ for all frequencies tested). Diaphragms from animals given both SB203580 ($n = 5$, a p38 inhibitor, open triangles) and LPS generated forces higher than diaphragms from animals given LPS alone ($P < 0.001$). Force generation for muscles taken from animals given SB203580 alone ($n = 4$) were similar to control levels. *Statistically significant difference between LPS and all other groups.

phospho-p38, the active form of p38, after cytomix exposure, whereas cells transfected with a control construct demonstrated pronounced p38 activation, that is, increases in phospho-p38, after cytomix exposure. Control cells also demonstrated significant caspase 8 activation after cytomix exposure (see Figure 8), whereas cells transfected with the dominant negative p38 construct had essentially no caspase 8 activation after cytomix ($P < 0.001$ for comparison of active caspase 8 levels in control cells treated with cytomix to dominant negative p38 construct transfected cells treated with cytomix).

DISCUSSION

Recent work by our group implicates caspase 8 activation as a key factor involved in sepsis-induced diaphragm dysfunction, as we found that endotoxin administration rapidly increases diaphragm caspase 8 activation and that caspase 8 inhibitors prevent endotoxin-induced diaphragm dysfunction (7, 8). The current study extends this previous work, examining one of the upstream signaling pathways, p38, that is thought to modulate death receptor activation and death receptor mediated caspase 8 formation. The main observations of the present study are that endotoxin-induced sepsis elicits an activation of p38 kinase in the diaphragm and that administration of a specific p38 kinase inhibitor to intact animals prevents endotoxin-induced diaphragm caspase 8 activation and endotoxin-induced reductions in diaphragm force generation. Our data also indicates that p38 is required for cytokine-induced activation of caspase 8 in isolated skeletal muscle cells.

A Procaspase 8 and Active Caspase 8 Protein

Figure 5. (A) Effect of p38 inhibition on caspase 8 protein levels. Procaspase 8 and active caspase 8 protein are shown in the *top panel* for representative diaphragm samples; the *bottom panel blot* represents α -tubulin protein levels. LPS administration elicited a large increase in active cleaved caspase 8 protein compared with the control, and administration of a p38 chemical inhibitor, SB203580, prevented this increase. (B) Mean data examining the effect of SB203580 on diaphragm caspase activation. Procaspase 8 levels were similar for the four experimental groups ($n = 4$ –5/group, *left panel*). In contrast, diaphragm active caspase 8 protein levels increased dramatically for LPS-treated animals ($P < 0.001$), and administration of SB203580 to LPS-treated animals prevented this increase ($P < 0.01$ for comparison between LPS and LPS plus SB203580 groups). *Statistically significant difference between LPS and all other groups.

Infection-Induced Respiratory Muscle Weakness

Several different models of infection (*Pseudomonas*-induced pneumonia, cecal ligation perforation induced sepsis, *E. coli* injection, endotoxin administration) have been shown to induce severe reductions in the force-generating capacity of the diaphragm and other respiratory muscles (e.g., intercostals) in a variety of animal species (4–8, 12, 14, 15). Studies of human

A Caspase 8 Protein Levels in C2C12 Myotubes

Figure 6. (A) Effect of p38 inhibition on caspase 8 protein levels in C2C12 cells. Procaspase 8 and cleaved, active caspase 8 protein (*top panel*) for representative C2C12 myotube samples; the *bottom panel blot* represents α -tubulin protein levels. Cytomix administration elicited a large increase in active cleaved caspase 8 protein and administration of a p38 chemical inhibitor, SB203580, prevented this increase. (B) Mean data examining the effect of SB203580 on C2C12 caspase activation procaspase 8 levels were similar for the four experimental groups ($n = 4$ –5/group, *left panel*). In contrast, myotube active caspase 8 protein levels increased dramatically with cytomix administration ($P < 0.001$), and administration of SB203580 prevented this increase ($P < 0.01$ for comparison between cytomix and cytomix plus SB203580 groups). *Statistically significant difference compared with the other groups.

patients are consistent with these reports examining animal models of infection, with several studies finding significant reductions in respiratory muscle strength in patients with infections (16, 17).

**p38 Activation in Control (CT) and Inducible
p38 Dominant Negative (p38 DN) C2C12 Myotubes**

Figure 7. Effect of dominant negative p38 transfection on phospho-p38 protein levels in C2C12 cells. Phospho-p38 and total p38 (*top two panels*) for representative C2C12 myotube samples; the *bottom panel* represents α -tubulin protein levels. Cytomix administration elicited a large increase in active phospho-p38 protein compared with the control, whereas cytomix administration to cells transfected with dominant negative p38 did not produce an increase in active phospho-p38 protein.

The mechanisms by which infection produces these reductions in respiratory muscle strength are incompletely understood. Studies of animal models of infection have indicated that several pathophysiological processes play a role in infection-induced respiratory muscle weakness, including cytokine (e.g., TNF- α)-mediated activation of muscle receptors, and excessive generation of reactive oxygen species within skeletal muscle (18–20). In addition, recent work by our group implicates caspase activation as a key factor involved in sepsis-induced diaphragm dysfunction, as we found that endotoxin administration rapidly increases diaphragm caspase activation, and that caspase inhibitors prevent endotoxin-induced diaphragm dysfunction (7, 8). In this past work, we found that the extrinsic pathway of caspase 3 activation, involving death receptor-linked activation of caspase 8, was the main pathway of caspase 3 activation, because endotoxin potently and rapidly increased diaphragm caspase 8 activity, and administration of selective caspase 8 inhibitors prevented endotoxin-induced muscle weakness (8).

Although caspase 8 activation requires attachment of specific ligands to several classes of death receptors (e.g., attachment of FAS ligand to the FAS receptor), the generation of active caspase 8 in response to ligand–receptor attachment is not an all-or-none phenomenon, but appears to be modulated by the activity of a number of accessory proteins that associate with the death receptor, as well as death receptor–death receptor interactions. Accessory protein function and death receptor interactions are thought to be influenced by a number of factors, including the level of p38 activation. Although the precise manner in which p38 activation alters death receptor function is not known, inhibition of p38 activation can prevent caspase 3 activation despite availability of death receptor ligands at the cell surface (11).

However, the role of p38 in modulating skeletal muscle caspase 8 activation has not previously been evaluated. The present study provides the first evidence of p38 activation in the

**A Caspase 8 in Control (CT) and Inducible
Dominant Negative p38 (p38 DN) C2C12 Myotubes**

B

Figure 8. (A) Effect of dominant negative p38 transfection on caspase 8 protein levels in C2C12 cells. Procaspase 8 and cleaved, active caspase 8 protein (*top panel*) for representative C2C12 myotube samples; the *bottom panel* blot represents α -tubulin protein levels. Cytomix administration elicited a large increase in active, cleaved caspase 8 protein, whereas cytomix administration to cells transfected with dominant negative p38 did not manifest an increase in active caspase 8 protein. CT, control. (B) Mean data examining the effect of dominant negative p38 on C2C12 caspase activation. Procaspase 8 levels were similar for the four experimental groups ($n = 4$ –5/group, *left panel*). In contrast, myotube active caspase 8 protein levels increased dramatically with cytomix administration to control cells ($P < 0.001$), whereas administration of cytomix to dominant negative p38 cells did not cause an increase in active caspase 8 protein ($P < 0.01$ for comparison between cytomix in control cells and cytomix in p38 dominant negative cells). *Statistically significant difference compared with the other groups.

diaphragm in an animal model of infection, and the first evidence that p38 activation is required for skeletal muscle caspase 8 activation. We found that endotoxin elicited a marked increase in diaphragm p38 activation, as evidenced by an

increase in both levels of phospho-p38 protein, the active form of this kinase, and an increase in p38 kinase activity measured by detection of phosphorylation of a p38-specific substrate by diaphragm homogenates. Moreover, our data indicate that p38 activation precedes the development of major diaphragm weakness in this experimental model.

Our finding, that inhibition of p38 prevented caspase 8 activation in endotoxin-treated animals, supports the concept that p38 activation is essential for caspase activation and caspase-mediated reductions in muscle function. In parallel experiments, we also demonstrated that cytokine administration to isolated C2C12 cells evoked caspase 8 activation, and that inhibition of p38 by use of either a chemical inhibitor (administration of SB203580) or transfection of cells with a dominant negative p38 construct, blocked cytokine-induced caspase 8 activation.

Taking the observations made in the current study with the reports of other investigators, it is reasonable to speculate that the following pathophysiological sequence may be responsible for endotoxin-mediated diaphragm dysfunction. First, an infectious stimulus leads to the production of cytokines, including TNF- α and IL. These cytokines/ligands may then activate muscle receptors, leading to generation of free radicals within muscle cells (20). Free radicals may then initiate activation of members of the MAPK signaling pathways, including p38 (21). Active p38, in turn, may induce FAS ligand and FAS receptor formation, leading to cleavage of FAS receptor-bound procaspase 8 to active caspase 8, which then activates downstream effector caspases (caspase 3) (7, 8). Finally, caspase 3 may cleave critical elements of the contractile proteins, leading to a destabilization of the contractile protein matrix, release of myofilaments from the matrix, and a reduction in contractile protein force-generating capacity (7, 8, 22).

Potential Implications

Recent studies have revealed that profound respiratory muscle weakness is present in the vast majority of critically ill patients (1, 2). Many of these patients are infected, with infection either precipitating ICU admission (e.g., pneumonia, peritonitis, cellulitis, etc.) or complicating it (nosocomial line infections, hospital acquired pneumonias, etc.). Infection-induced muscle weakness, therefore, is likely a significant contributor to the development of respiratory muscle weakness in critically ill patients. The present data present a new explanation for this process. Caspase 8 activation may be a key factor, with caspase 8 activation dependent on upstream activation of diaphragmatic p38 kinase by cytokines. A complete understanding of the process by which p38 is activated may lead to novel treatments to prevent or reverse weakness of the diaphragm and other skeletal muscles in 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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