Alveolar cell apoptosis is dependent on p38 MAP kinase-mediated activation of xanthine oxidoreductase in ventilator-induced lung injury

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Le A, Damico R, Damarla M, Boueiz A, Pae HH, Skirball J, Hasan E, Peng X, Chesley A, Crow MT, Reddy SP, Tuder RM, Hassoun PM. Alveolar cell apoptosis is dependent on p38 MAP kinase-mediated activation of xanthine oxidoreductase in ventilator-induced lung injury. J Appl Physiol 105: 1282–1290, 2008. First published July 31, 2008; doi:10.1152/japplphysiol.90689.2008.—Signaling via p38 MAP kinase has been implicated in the mechanotransduction associated with mechanical stress and ventilator-induced lung injury (VILI). However, the critical downstream mediators of alveolar injury remain incompletely defined. We provide evidence that high-tidal volume mechanical ventilation (HVT MV) rapidly activates caspases within the lung, resulting in increased alveolar cell apoptosis. Antagonism of MV-induced p38 MAP kinase activity with SB-203580 suppresses both MV-induced caspase activity and alveolar apoptosis, placing p38 MAP kinase upstream of MV-induced caspase activation and programmed cell death. The reactive oxygen species (ROS)-producing enzyme xanthine oxidoreductase (XOR) is activated in a p38 MAP kinase-dependent manner following HVT MV. Allopurinol, a XOR inhibitor, also suppresses HVT MV-induced apoptosis, implicating HVT MV-induced ROS in the induction of alveolar cell apoptosis. Finally, systemic administration of the pan-caspase inhibitor, z-VAD-fmk, but not its inactive peptidyl analog, z-FA-fmk, blocks ventilator-induced apoptosis of alveolar cells and alveolar-capillary leak, indicating that caspase-dependent cell death is necessary for VILI-associated barrier dysfunction in vivo.

ACUTE LUNG INJURY (ALI) is a clinically devastating pulmonary complication of infection and systemic inflammation characterized by noncardiogenic pulmonary edema leading to extreme hypoxemia. On the basis of recent estimates, nearly 200,000 patients will develop ALI each year in the United States, requiring greater than 3 million hospital days. The high mortality rate (30–60%) of this syndrome accounts for roughly 75,000 deaths, making ALI a substantial public health issue (26). Treatment for ALI remains largely supportive and almost invariably includes mechanical ventilation (MV). However, it has long been recognized that mechanical ventilation itself can directly contribute to lung injury, a phenomenon referred to as ventilator-induced lung injury (VILI) (1). Large clinical trials demonstrate that a lung protective ventilatory strategy aimed at minimizing mechanical stress and alveolar overdistension attenuates the systemic inflammatory response (25) and substantially improves survival (2). The high cost of this disease in terms of both human lives and medical resources necessitates the identification of new therapeutic targets for the treatment of ALI and VILI.

Apoptosis has been implicated in the pathogenesis of numerous disease states, including VILI (10, 16, 30, 33), and is dependent on the activity of cysteine proteases, which act to both amplify death stimuli via initiator caspases-2 and -9 (intrinsic pathway) and caspases-8 and -10 (extrinsic pathway) and ultimately dismantle the cell via the executor caspases-3 and -7 (5). Apoptotic cell death is an active, energy-mediated process dependent on intracellular stores of ATP. Inadequate ATP stores lead cells instead to passive necrosis. Poly(adenosine-ribose) polymerase-1 (PARP-1) is a downstream target of the executor caspases-3 and -7 that consumes ATP in the repair of DNA in response to cellular insult. Cleavage of PARP-1 by caspases prevents ATP depletion and is a hallmark of apoptotic cell death. Activation of p38 MAP kinase has been linked to the initiation of proapoptotic cascades leading to cell death (10, 27, 35). In addition, reactive oxygen species (ROS), possibly generated by xanthine oxidoreductase (XOR), have been implicated in cell signaling leading to cell death (19). However, data demonstrating a causal relationship between apoptotic cell death and VILI are still lacking.

We have previously demonstrated that mechanical stress induced by cyclic stretch of pulmonary microvascular endothelial cells in culture or high-tidal volume (HVT) MV leads to upregulation of XOR (3), an enzyme known to cause oxidative stress. The induction of XOR activity is coincident and dependent on the activation of p38 MAP kinase. Furthermore, pharmacological inhibition of the p38 MAP kinase-XOR pathway prevents VILI (3). Thus p38 MAP kinase and subsequent XOR activation appear to be key components in the pathogenesis of VILI. How the activities of these enzymes ultimately manifest in alveolar-capillary dysfunction is incompletely understood. As such, we tested the hypothesis that p38 MAP kinase and its downstream effector XOR are key determinants of alveolar cell apoptosis and that alveolar cell apoptosis mediates alveolar-capillary dysfunction seen in VILI.

MATERIALS AND METHODS

The Johns Hopkins University Institutional Animal Care and Use Committee approved all animal protocols.
Animal model of ALI induced by MV. C57BL/6J male mice were anesthetized, underwent tracheal intubation, and were then subjected to MV (Harvard Apparatus, Boston, MA) at room air with low-(7 ml/kg, LVR) and high-tidal volume (20 ml/kg, HVR) for 0 (sham), 2, or 4 h. In certain experiments, animals were pretreated before MV with drugs (as detailed below) or the equivalent volume of vehicle (untreated controls). The respiratory rate was set at 160 breaths/min for all tidal volumes, and the dead space was adjusted by increasing the length of ventilator tubing outside the animal to maintain similar alveolar ventilation between mice ventilated at LVR and HVR. The resulting arterial pH was between 7.35 and 7.45 for all animals. Airway pressures continuously measured during MV revealed that end-expiratory pressures remained ~0–2 cmH2O throughout the 4-h period for both LVR and HVR. A 500-μl bolus of lactated Ringer solution was given intravenously at the start of each experiment. Mean arterial blood pressure was continuously monitored via catheterization of a femoral artery in preliminary experiments using a blood pressure monitor (Cardiomax-III) and a data acquisition system (Columbus Instruments, Columbus, OH) and remained typically 80 Torr. The adequacy of MV settings on gas exchange was confirmed in preliminary experiments in which arterial blood gases, obtained via catheterization of a femoral artery and analyzed by automated blood gas analyzer (Instrumentation Laboratories, Lexington, MA), revealed stable levels of arterial oxygen (PaO2 of 90–108 Torr) and carbon dioxide (PaCO2 of 32–42 Torr). At the end of MV, the animals were administered an intraperitoneal lethal dose of the anesthetic agent before the lungs were harvested.

Where indicated, mice were treated with an intratracheal injection of 0.25 mg of z-VAD-fmk [carbenoxozy-valyl-alamyl-aspartyl(O-methyl)-fluoromethylketone], a pan-caspase inhibitor, and z-FK-fmk (benzoyloxycarbonyl-phenyl-alamyl-fluoromethylketone), a caspase inhibitor negative control (R&D Systems, Minneapolis, MN) or vehicle (1% DMSO) 15 min before MV. This was followed with two subsequent injections (0.1 and 0.15 mg) at 1 and 2 h of MV for inhibition of p38 MAP kinase, mice were pretreated 1 h before MV with an intraperitoneal injection of 2 mg/kg SB 203580 (Promega, Madison, WI) dissolved at 0.4 mg/ml in 1% DMSO in sterile saline vs. carrier alone (vehicle). For inhibition of XOR, allopurinol was given by gavage at a dose of 200 mg/kg 12 h before MV as previously described (3). All doses, routes of administration, and timing of delivery of pharmacological agents were based on the known half-life of agents and preliminary experiments demonstrating efficacy.

Assessment of pulmonary capillary permeability. Evans blue dye (EGB) (20 mg/kg) was injected into the external jugular vein 60 min before termination of the experiment to assess vascular leak as previously described (3). To determine wet-to-dry lung weight ratio, the whole right lung was immediately weighed after excision from the animal (wet weight) and then dried at 60°C for 24 h and weighed again (dry weight).

Assessment of caspase-3/7 activity. Frozen lungs were homogenized on ice in 1,500 μl of lysis buffer (50 mM HEPES, pH 7.4, 0.1% CHAPS, 0.1 mM EDTA, 1 mM DTT, and 100 mM NaCl), sonicated at cold temperature (4°C) for 10 s, and centrifuged at 12,000 rpm for 10 min. Next, 100 μl of the supernatants from lung homogenates were used for the assay, which was performed according to the manufacturer’s instructions. The caspase-3/7 activity was determined using the Apo-ONE homogeneous caspase-3/7 assay (Promega).

Processing of lung tissue for immunofluorescent terminal deoxy-nucleotidyl transferase-mediated dUDP nick-end labeling staining. After being flushed free of blood, the lungs were inflated at a pressure not exceeding 25 cmH2O with 0.6% low-melting agarose, harvested, and fixed overnight in 10% buffered formalin at room temperature before being embedded in paraffin. After deparaffinization and hydration, antigen retrieval was performed by steaming tissue sections in Borg Decloaker (Biocare Medical, Concord, CA) for 10 min. Antigen retrieval of tissue sections did not affect the terminal deoxynucleotidyl transferase-mediated dUDP nick-end labeling (TUNEL) staining. Endogenous biotin was blocked using the Biotin-Blocking System (Dako, Carpinteria, CA). The TUNEL reaction was carried out by incubating the sections with 96 μl of equilibration buffer, 2 μl of biotinylated nucleotide mix, and 2 μl of rTdT enzyme (Promega) for 1 h at 37°C following incubation with Alexa Fluor 647-streptavidin conjugate (Invitrogen, Carlsbad, CA) for 1 h at room temperature. Type I and II epithelial cells and endothelial cells were detected by hamster monoclonal anti-Ti alpha (diluted 1: 10), rabbit anti-prosurfactant protein C (Chemicon, San Diego, CA; diluted 1: 500), and polyclonal goat anti-thrombomodulin (R&D Systems; diluted 1: 200), respectively. All primary antibodies were diluted in antibody diluent purchased from DakoCytomation. The secondary antibody was biotin-goat polyclonal anti-hamster IgG (Abcam, Cambridge, MA) for type I cells, carried out using Texas red streptavidin (Vector Laboratories, Burlingame, CA); Alexa Fluor 594 donkey anti-rabbit IgG (Invitrogen) for type II epithelial cells; and Alexa Fluor 488 donkey anti-goat (Invitrogen, Carlsbad, CA) for endothelial cells. All secondary antibodies were diluted 1:200 in PBS. 4’,6-Diamidino-2-phenylindole (Invitrogen), a fluorescent dye that strongly binds to DNA, was used to stain the nuclei. Identification of the sections was done at 100 magnification (Tokyo, Japan) and analyzed using National Institutes of Health Image J 1.44 software. Statistical analysis. Values are means ± SD. Data were analyzed using one-way ANOVA. Significance was defined as P < 0.05.

RESULTS

HVR MV increases total lung caspase-3/7 activity. We have previously demonstrated that MV with high tidal volumes causes significant alveolar-capillary leakage in a mouse model of VILI (3). To examine the potential role of apoptosis in this lung injury model, we allowed adult wild-type C57BL/6J mice to breathe spontaneously (sham) or exposed them to MV at 7 (LVR) and 20 ml/kg (HVR) for 4 h as detailed in MATERIALS AND METHODS. At the end of exposure, lungs homogenates were analyzed for caspase-3/7 activity. As shown in Fig. 1A, HVR MV caused a significant increase in lung caspase-3/7 activity. Lungs of mice exposed to 2 and 4 h of HVR MV had a 1.5- and 1.7-fold increase, respectively, in caspase-3/7 activity relative to lungs of sham-treated animals. The caspase-3/7 activity was tidal volume dependent, since lungs of mice ventilated with LVR demonstrated no significant change at 2 or 4 h of exposure compared with sham-treated mice. To demonstrate that the increase in protease activity observed with HVR MV was...
specific for caspase, we treated animals with the substrate analog z-VAD-fmk. This peptide analog irreversibly binds to and inhibits the activity of all caspases. Treatment with z-VAD-fmk prevented the increase in lung caspase-3/7 activity observed following mechanical stress. In contrast, z-FA-fmk, a cathespin inhibitor and negative control for z-VAD-fmk, had no demonstrable effect (Fig. 1B).

**HVr MV-induced PARP-1 cleavage.** To measure activation of the apoptotic cascade further downstream of the executioner caspases, we explored the role of HVr MV on inactivation of PARP-1 (as a result of its degradation), an enzyme that promotes energy failure necrosis. PARP-1 cleavage was significantly increased in total lung homogenates from animals exposed to HVr MV for 4 h (Fig. 2, A and B). To demonstrate that PARP-1 cleavage was in fact caspase dependent, we treated a subset of animals with z-VAD-fmk, the pan-caspase inhibitor. As shown in Fig. 2, treatment with z-VAD-fmk significantly prevented HVr MV-induced PARP-1 cleavage.

Since p38 MAP kinase activation has been linked to the initiation of proapoptotic cascades, we investigated the role of this signaling pathway on caspase-dependent PARP-1 cleavage. Pretreatment of animals with the p38 MAP kinase inhibitor SB 203580 suppressed HVr MV-induced PARP-1 cleavage (Fig. 2, A and B). Since we had previously shown that XOR activity is downstream of, and dependent on, p38 MAP kinase activation, we investigated the role of XOR inhibition on PARP-1 cleavage. As shown in Fig. 2, the XOR inhibitor allopurinol prevented PARP-1 cleavage in response to HVr MV (Fig. 2B).

**HVr MV induces alveolar cell apoptosis.** Although caspase-3 activation and PARP-1 cleavage are typical of apoptotic cell death, they are not synonymous with apoptosis and need to be interpreted in conjunction with other data demonstrating apoptotic changes. In addition, these assays in total lung homogenates cannot discriminate the cellular sites of apoptosis. Other groups have provided evidence of apoptosis within epithelial cells lining large airways following ventilation with HVr (17). Since the alveolar-capillary interface and not the large airways is the presumed site of leakage during ALI, we set out to determine whether alveolar cell death played a role in our model of HVr MV-induced apoptosis. Therefore, we set out to evaluate a molecular event distal to the activation of the executioner caspases and PARP-1 cleavage specifically within the alveolar compartment. A terminal event in the apoptotic cascade is activation of endonucleases. These enzymes cleave chromosomal DNA resulting in nicked or fragmented DNA. Nicked DNA was identified in situ using TUNEL staining, as detailed in MATERIALS AND METHODS. All three alveolar cell types (endothelial cells and type I and II epithelial cells) were counterstained to define and quantify alveolar cells undergoing apoptosis under the experimental conditions indicated. As demonstrated in Figs. 3 and 4, baseline DNA fragmentation in control mice was evident in 5.7, 5.8, and 8.6% of endothelial cells and type I and II epithelial cells, respectively. The number of cells demonstrating nicked DNA was not significantly different in mice exposed to HVr compared with sham-treated animals. In contrast, mice exposed to 4 h of HVr MV demonstrated a significant increase in TUNEL positivity within all three alveolar cell types. Importantly, treatment of mice with z-VAD-fmk prevented DNA nicking in the three alveolar cell types examined (Figs. 3 and 4), demonstrating a dependence on active caspases.

**p38 MAP kinase activity is necessary for HVr ventilator-induced apoptosis.** We previously demonstrated that p38 MAP kinase signaling is a critical component of mechanotransduction in VILI (3). This work demonstrated that mechanical stress imparted by cyclic stretch in vitro or HVr ventilation in vivo induces the rapid phosphorylation of p38 MAP kinase in both alveolar epithelial and endothelial cells. Therefore, we investigated the contribution of VILI-induced p38 MAP kinase activation on HVr MV-induced alveolar cell apoptosis. Mice pretreated with SB-203580, a p38 MAP kinase inhibitor, demonstrated significantly less caspase-3/7 activity following HVr MV than animals receiving vehicle (DMSO), placing p38 MAP kinase activity upstream of caspase-3/7 activation (Fig. 5) and PARP-1 cleavage (Fig. 2). Furthermore, TUNEL staining revealed substantial suppression of HVr MV-induced alveolar epithelial and endothelial cell apoptosis in animals treated with SB-203580 (Figs. 3 and 5). Thus the activation of p38 MAP kinase is necessary for HVr MV-induced alveolar cell apoptosis.
kinase is upstream of caspase activation and subsequent alveolar cell apoptosis.

**XOR inhibition attenuates HVT MV-induced alveolar cell apoptosis.** We previously demonstrated that mechanical stress and HVT MV-induced phosphorylation and activation of p38 MAP kinase precede (and are required for) cyclic stretch and VILI-associated XOR activation (3). Moreover, XOR inhibition prevents alveolar-capillary leakage. To investigate further potential downstream effects of p38 MAP kinase in HVT MV-induced cell death, we evaluated the effects of XOR inhibition on HVT MV-induced apoptotic pathway. Pretreatment of mice with the XOR inhibitor allopurinol resulted in a small, yet statistically significant, decrease in HVT MV-associated caspase-3/7 activity (Fig. 6). Interestingly, allopurinol efficiently suppressed HVT MV-induced PARP-1 cleavage (Fig. 2) and DNA nicking in all three cell alveolar cell types as

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**Fig. 2.** HVT MV-induced poly(adenosine-ribose) polymerase-1 (PARP-1) cleavage is antagonized by caspase, p38 MAP kinase, and xanthine oxidoreductase (XOR) inhibition. **A:** full-length (116 kDa) and cleaved fragment (89 kDa) PARP-1 were detected using immunoblot analysis of whole lung homogenates from animals exposed to sham therapy or HVT MV alone or with SB 203580, allopurinol, or z-VAD-fmk treatment. **B:** there was a significant increase in cleaved PARP-1 relative to actin after 4-h HVT MV. This was completely prevented by inhibition of p38 MAP kinase (by SB-203580) and XOR (by allopurinol). Cleavage of PARP-1 was caspase dependent and inhibited by z-VAD-fmk treatment. *P < 0.05 vs. all other conditions (n = 3–6 mice/group). Z-VAD, z-VAD-fmk peptide.

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**Fig. 3.** HVT MV induced DNA nicking within alveolar cells. **A:** C57BL/6J mice were randomized to spontaneous breathing (sham; a and b) or to LVT (7 ml/kg; c and d) or HVT MV (20 ml/kg; e and f) for 4 h. Nicked DNA was detected using terminal deoxynucleotidyl transferase-mediated dUDP nick-end labeling (TUNEL; green) within the lung parenchyma. Endothelial cells were recognized by thrombomodulin staining (white; long arrows); type I and II epithelial cells were recognized by staining with T1 alpha and surfactant protein C (SPC; red; short arrows). Apoptotic cells were quantified after nuclear staining (4’,6-diamidino-2-phenylindole; blue) as described in MATERIALS AND METHODS. **B:** a subset of animals exposed to HVT MV were treated with z-VAD-fmk (g and h), SB 203580 (i and j), or allopurinol (k and l), all of which significantly prevented DNA nicking (TUNEL positivity; green).

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**Fig. 6.**
assessed by TUNEL staining (Figs. 3 and 6). Together with our previous results (3), these experiments indicate that XOR contributes to capillary leakage in VILI, in part through increasing alveolar cell apoptosis.

Apoptosis is necessary for alveolar-capillary leakage induced by HVT MV. In conjunction, caspase activation, PARP-1 cleavage, and DNA nicking demonstrate MV-induced alveolar apoptosis. However, the relationship between this form of cellular injury and dysfunction has not been defined. To address this question, we globally antagonized caspase activity with the pan-caspase inhibitor z-VAD-fmk and assessed animals for evidence of HVT MV-induced capillary leak. Wet-to-dry lung weight ratios and EBD extravasation were analyzed in animals receiving sham treatment and HVT MV as described in MATERIALS AND METHODS. Mice exposed to HVT MV had significantly increased wet-to-dry lung weight ratios and EBD extravasation 1.27- and 1.68-fold greater than sham treatment, respectively (Fig. 7). This increase in wet-to-dry lung weight ratio and EBD extravasation was completely prevented by treatment with z-VAD-fmk but not with the inactive peptidyl analog z-FA-fmk. These results demonstrate that HVT MV-induced caspase activation is not merely coincident with injury but plays an obligatory role in alveolar-capillary leakage in this model of VILI.

DISCUSSION

Using a murine model of VILI, we have demonstrated that HVT ventilation alone is sufficient to induce both alveolar cell apoptosis and alveolar-capillary leak. Furthermore, ventilator-induced activation of p38 MAP kinase and one of its downstream effectors, XOR, is required for both alveolar cell death and vascular leak, placing the p38 MAP kinase-XOR pathway upstream of HVT ventilation-induced apoptosis. More importantly, we have demonstrated that pharmacological antagonism of the enzymatic mediators of apoptosis, caspases, blocks ventilator-induced capillary leak. This is the first time demonstrates that the activity of caspases is an obligatory upstream event required for vascular dysfunction in vivo. In summary, this work, coupled with results from prior studies, demonstrates that alveolar apoptosis is a compulsory upstream mediator of VILI and is mediated in part via a p38 MAP kinase-XOR dependent pathway.

In the present study, we focused on the role of apoptosis in ventilator-associated alveolar-capillary injury and dysfunction in a murine model. Our animal model does not rely on a second injurious stimulus, such as endotoxin or hyperoxia, and allows direct evaluation of specific biochemical pathways in mechanical stress-induced injury.

We initially demonstrated that mechanical stress with HVT ventilation is sufficient to increase lung caspase activity in vivo (Fig. 1). Classic apoptotic cell death is dependent on the activity of caspases, which act to both amplify death stimuli and ultimately dismantle the cell. The increased enzymatic activity observed was lost in animals pretreated with z-VAD-fmk, but not with z-FA-fmk, the inactive peptidyl analog. Our results differ from recent published reports (33). Vaschetto et al. (33) failed to demonstrate a difference in caspase-3 activity between rats ventilated at high vs. low tidal volumes. However, caspase-3 activity in spontaneously breathing controls was not reported, making it difficult to assess
the specific effect of tidal volume rather than mechanical ventilation on caspase-3 activation. Our data demonstrates that MV at LVT does not increase caspase activity compared with spontaneous breathing controls. Thus MV alone is not sufficient to induce caspase activity; rather, MV with injurious tidal volumes is necessary.

Next, we began exploring events downstream of caspases-3 and -7. We focused on PARP-1, a nuclear enzyme involved in...
DNA repair, DNA stability, and transcriptional regulation, which catalyzes covalent attachment of long branched chains of poly(ADP-ribose) and is a target for cleavage by caspases. Excessive activation of PARP-1 depletes cellular reserve of NAD⁺ (substrate and precursor of ATP) leading to cell death via energy failure (31). In vitro, PARP-1 can be cleaved by all caspases. However, it is targeted in vivo by the executioner caspases-3 and -7 (28). Our data indicate that HVT ventilation induces cleavage of full-length PARP-1 (116 kDa) to its cleaved COOH-terminal fragment (89 kDa). Furthermore, animals pretreated with z-VAD-fmk and exposed to HVT had a significant reduction in the cleaved COOH-terminal fragment, confirming HVT induction of PARP-1 cleavage is caspase dependent (Fig. 2).

Using in situ immunofluorescent staining, we identified the specific cellular targets of apoptosis within the alveoli. HVT ventilation induced significant DNA nicking (TUNEL positivity) within pulmonary epithelial (type I and II) and endothelial cells (Fig. 3). Importantly, the number of TUNEL-positive alveolar cells was dramatically decreased in animals pretreated with z-VAD-fmk, demonstrating that the observed staining was caspase dependent. Although DNA nicking may be observed in DNA damage alone, independently of caspase-induced apoptosis, the concomitant demonstration of PARP-1 cleavage and the dependence on caspase activity indicates that, in fact, HVT induces caspase-dependent alveolar cell apoptosis and not other modes of cell death, i.e., oncosis, caspase-independent programmed cell death, or necrosis (32).

Recent publications have suggested pulmonary apoptosis in response to MV (10, 16). However, the evidence for apoptosis was largely based only on the demonstration of TUNEL positivity. Dolinay et al. (10) demonstrated upregulation of DNA nicking in response to moderate tidal volume (10 ml/kg) ventilation as well as lung-protective positive end-expiratory pressure (2 cmH₂O) only after 8 h of MV. However, with their protocol, mice experienced marked relative hypotension at the eighth hour of MV compared with baseline. This raises the possibility of profound metabolic derangements leading to anaerobic metabolism, depletion of ATP stores, and passive necrosis yielding the increased DNA damage and TUNEL positivity (10). The study by Li et al. (16) confirms TUNEL staining to be a result of apoptosis and not necrosis by using electron microscopy but limits analysis to cells within large airways, an anatomical site unlikely to directly contribute to leak, without reporting any effects on alveolar cells. Our data indicate that HVT ventilation induces apoptosis of all three cell types (endothelial cells and type I and II epithelial cells) within the alveolar-capillary unit, i.e., the site of vascular permeability in response to HVT MV (21) (Figs. 3 and 4). More importantly, our study directly defines the contribution of the apoptotic cascade to alveolar-capillary leak.

We also explored potential upstream mediators of MV-induced apoptosis. The p38 MAP kinase has been implicated in both the mechanotransduction associated with mechanical stress and MV (3) and the induction of apoptosis (8, 12, 15). Therefore, we evaluated the effects of antagonizing this kinase in HVT-induced apoptosis. Our results indicate that p38 MAP kinase is both upstream and necessary for HVT-induced activation of the executioner caspases-3 and -7, PARP-1 cleavage, and eventual apoptosis (Figs. 1, 2, and 5). Using mice harboring null mutations of the MAPK kinase 3 (MKK3) or JNK1, Dolinay et al. (10) demonstrated decreased DNA nicking in response to MV. The contribution of p38 MAP kinase to HVT-induced apoptosis was not directly addressed in their study, since p38 MAP kinase is activated by both MKK6 and MKK3 (9). Moreover, MKK3 deficiency alone was not protective of lung injury compared with wild-type mice upon exposure to injurious MV, suggesting that DNA nicking does not correlate with lung injury in that study (10). Although JNK1⁻/⁻ mice appeared to be protected in their protocol, the specific contributions of the other isoforms remain unclear. We have previously failed to demonstrate upregulation of JNK activity in our model (3). Finally, our study demonstrates that p38 MAP kinase inhibition using the specific inhibitor SB-203580 is sufficient to prevent caspase activation, PARP-1 cleavage, and apoptosis, suggesting a primary role of p38 MAP kinase in this model.

We have previously demonstrated XOR phosphorylation and activation by p38 MAP kinase (14) in response to mechanical stress in vitro and in vivo and have implicated this signaling pathway in the capillary leakage of our VILI model.
(3). In contrast to treatment with SB 203580, caspase-3/7 activity was only partially prevented by allopurinol, an inhibitor of the enzyme XOR (Fig. 6). This suggests that other effectors of p38 MAP kinase likely contribute to MV-induced caspase activation. However, allopurinol treatment was sufficient to suppress MV-induced DNA nicking of both alveolar epithelial and endothelial cells in situ and the caspase-dependent cleavage of PARP-1, suggesting that allopurinol exerts its effects either downstream or independently of caspase-3/7 (Figs. 2 and 6). Together, these results indicate that 1) the p38 MAP kinase-XOR signaling pathway is important in the activation of the executioner caspases; 2) p38 MAP kinase signaling is an early nodal event in this activation and the resulting injury; and 3) XOR contributes to the apoptotic cascade as a late downstream effector. Its role in capillary leakage and injury may be multifactorial [i.e., production of ROS, as we suggested previously (3, 22), or caspase-independent actions as suggested by the present studies].

Having demonstrated increased caspase activity in response to MV and identified the specific cellular targets of apoptosis, we next sought to directly link alveolar cell apoptosis to capillary leakage. The pan-caspase inhibitor z-VAD-fmk, but not its inactive analog z-FA-fmk, was sufficient to completely prevent alveolar leak (Fig. 7). Thus our results directly link caspase activity with capillary leakage induced by HV7 MV. The contribution of other modes of cell death such as caspase-independent programmed cell death and/or necrosis in HV7-induced alveolar-vascular leak has not yet been evaluated, but the capacity of caspase inhibition to efficiently block DNA nicking and capillary leakage suggests a negligible role of other modes of cell death in this model of ALI.

The exact mechanisms by which caspase activation and apoptosis contribute to alveolar-capillary dysfunction remain unknown. A number of phenotypic changes have been attributed to endothelial cells undergoing apoptosis in vitro, which could contribute to the loss of vascular homeostasis and increased vascular leak in VILI. Endothelial cells undergoing apoptosis appear to produce altered cytokines, such as transformation growth factor-β, and thus could contribute directly to vascular leak and the proinflammatory cascade (29). Furthermore, caspases have the capacity to cleave cytoskeletal proteins within endothelial cells, altering their interactions with the extracellular matrix or cell-cell contacts (4, 24). Apoptotic endothelial cells have abnormal platelet binding capacity and expression of leukocyte adhesion molecules, potentially contributing to a procoagulant microenvironment and leukocyte recruitment characteristic of ALI (6, 7). Since endothelial cells undergoing apoptosis eventually detach from the basement membrane and are released into the circulation, some authors have postulated that apoptotic endothelial cells may become a nidus for microthrombi formation (11, 18). Furthermore, we speculate that cells undergoing apoptosis fail to maintain homeostatic transvascular transport function, either because of the creation of fenestrations allowing leakage of small solutes or via cell retraction and formation of intercellular gaps that allow passage of macromolecules (34). Clearly, the cellular and molecular mechanisms by which alveolar cell apoptosis contributes to alveolar-capillary leak are important but are beyond the scope of the present study.

Among the potential limitations of our study is the use of nonspecific chemical inhibitors and the limited number of apoptotic pathways studied. However, the use of physiologically inactive substances (z-FA-fmk) in addition to active compounds (z-VAD-fmk) minimizes the effects of nonspecific actions. Furthermore, distinct effects downstream of caspase-3/7 (e.g., PARP-1 cleavage) confirm the specificity of chemical inhibition in vivo. Another potentially confounding factor is the multiplicity of functions of caspases (5), adding to the complexity of deciphering specific functions (e.g., induction of apoptosis). However, our data convincingly demonstrate prevention of apoptosis with inhibition of caspases and link this event to subsequent prevention of lung injury. Recognizing the complexity of the apoptotic cascade and multiplicity of mechanisms involved, we believe our studies represent an important step toward defining the role of apoptosis in VILI, which has not been previously explored.

In conclusion, this study demonstrates that VILI involves caspase activation and endothelial and epithelial cell apoptosis, which is required for alveolar-capillary leakage. We have identified obligatory pathways upstream of pulmonary alveolar cell apoptosis and capillary leakage in response to mechanical stress. Caspases and the regulators of their activity may represent novel therapeutic targets, as demonstrated with the use of pharmacological inhibitors in our in vivo model of VILI.

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