Deletion of Apoptosis Signal-Regulating Kinase-1 Prevents Ventilator-Induced Lung Injury in Mice

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Both hyperoxia and mechanical ventilation can independently cause lung injury. In combination, these insults produce accelerated and severe lung injury. We recently reported that pre-exposure to hyperoxia for 12 hours, followed by ventilation with large tidal volumes, induced significant lung injury and epithelial cell apoptosis compared with either stimulus alone. We also reported that such injury and apoptosis are inhibited by antioxidant treatment. In this study, we hypothesized that apoptosis signal-regulating kinase-1 (ASK-1), a redox-sensitive, mitogen-activated protein kinase kinase, plays a role in lung injury and apoptosis in this model. To determine the role of ASK-1 in lung injury, the release of inflammatory mediators and apoptosis, attributable to 12 hours of hyperoxia, were followed by large tidal volume mechanical ventilation with hyperoxia. Wild-type and ASK-1 knockout mice were subjected to hyperoxia ($FiO_2 = 0.9$) for 12 hours before 4 hours of large tidal mechanical ventilation (tidal volume = 25 μ l/g) with hyperoxia, and were compared with nonventilated control mice. Lung injury, apoptosis, and cytokine release were measured. The deletion of ASK-1 significantly inhibited lung injury and apoptosis, but did not affect the release of inflammatory mediators, compared with the wild-type mice. ASK-1 is an important regulator of lung injury and apoptosis in this model. Further study is needed to determine the mechanism of lung injury and apoptosis by ASK-1 and its downstream mediators in the lung.

Keywords: ventilator-induced lung injury; acute lung injury; hyperoxia; apoptosis; apoptosis signal-regulating kinase-1

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are destructive disorders that affect approximately 200,000 patients per year in the United States alone, with mortality as high as 60%. The resulting hypoxemic respiratory failure mandates the use of mechanical ventilation, putting patients at risk for ventilator-induced lung injury (VILI). At least 9% excess mortality has been attributed to VILI in these patients (1). Most patients with ARDS are mechanically ventilated using high

CLINICAL RELEVANCE

Ventilator-induced lung injury is an important consequence of mechanical ventilation, resulting in excess mortality in patients with acute lung injury and acute respiratory distress syndrome. Although both mechanical ventilation and hyperoxia can cause acute lung injury, in combination, these two insults produce accelerated and severe lung injury associated with increased oxidant stress and apoptosis. Apoptosis signal-regulating kinase-1 (ASK-1) is a redox-sensitive, mitogen-activated kinase kinase that can regulate both apoptosis and inflammation. In a model of ventilator-induced lung injury (VILI) produced by exposure to hyperoxia, followed by large tidal volume mechanical ventilation, lung injury is associated with the activation of ASK-1. The deletion of ASK-1 significantly mitigates lung injury and apoptosis, but does not affect the release of inflammatory mediators. ASK-1 and its downstream mediators are potential therapeutic targets for the prevention and treatment of VILI.

concentrations of oxygen (mean FiO_2 on Day 1 = 70%) (2). The most severely ill patients require a much higher FiO_2 (100%) for prolonged periods or for frequent intervals (3). Although both mechanical ventilation and hyperoxia (HO) have been extensively studied, surprisingly little attention has been paid to their potential interaction, despite their near ubiquitous concomitant use. Although high concentrations of oxygen combined with mechanical ventilation are often life-saving, hyperoxia in conjunction with mechanical ventilation can augment lung injury (4–9). We reported that pre-exposure to HO ($FiO_2 = 0.9$ for 12 hours), followed by large tidal volume mechanical ventilation (HV = 25 μ l/g for 4 hours) with HO, caused significant lung injury compared with either stimulus alone or combined HV and HO not preceded by exposure to HO (7). This severe lung injury was associated with caspase-mediated epithelial cell apoptosis. We also showed that this increased lung injury and apoptosis are oxidant-mediated and occur primarily via the caspase-mediated mitochondrial pathway (10).

Although both increased oxidative stress and apoptosis have been associated with ALI and ARDS, no direct causative relationship has been established. One possible link between these two phenomena involves apoptosis signal-regulated kinase-1 (ASK-1). ASK-1 is a redox-sensitive, mitogen-activated protein kinase kinase kinase that is known to induce p38-mediated and c-Jun N-terminal protein kinase (JNK)-mediated inflammation and apoptosis, respectively (11, 12).

Although ubiquitously expressed, ASK-1 has received little investigative attention in the lung. Only recently was its potential role in VILI explored (13). In this study, we hypothesized that

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ASK-1 would be activated in our VILI model, and that the deletion of ASK-1 (ASK-1-ko) would protect against lung injury, apoptosis, and the release of inflammatory mediators.

MATERIALS AND METHODS

See the online supplement for further details of our methods.

Animals

ASK-1^(-/-) knockout mice (25–30 g) were generated on a C57BL/6J background as previously described (12), and were provided by Hide-nori Ichijo (University of Tokyo, Tokyo, Japan). Age-matched and sex-matched C57BL/6J mice were used as controls. Animal care was in accordance with the *Guide for the Care and Use of Laboratory Animals* from the National Institutes of Health. Our institutional Animal Care and Use Committee approved this study.

Experimental Conditions

Anesthetized mice were prepared, ventilated, and monitored as previously described (7), and were divided into four groups: (1) wild-type control mice (Control-WT; spontaneously breathing; $FiO_2 = 0.21$); (2) ASK-1 knockout control mice (Control-ko); (3) wild-type mice under lung injury conditions (LIC-WT; exposed to hyperoxia, i.e., $FiO_2 = 0.9 \times 12$ hours, before 4 hours of large tidal mechanical ventilation, i.e., $V_T = 25 \mu\text{L/g}$) with hyperoxia; and (4) ASK-1 knockout mice under lung injury conditions (LIC-ko).

Assessment of Lung Injury

Respiratory system compliance. Quasistatic compliance (C_{st}) was estimated at time = 0 hours and at 4 hours by fitting pressure–volume loop data to the Salazar-Knowles equation (14). Measurements were performed using the Flexivent system (Scireq USA, Inc., Tempe, AZ). Non-ventilated groups were prepared as already described, and measurements were performed after 5 minutes of mechanical ventilation ($V_T = 10 \mu\text{L/g}$; respiratory rate [RR] = 150/minute; positive end-expiratory pressure [PEEP] = 0 cm H_2O ; $FiO_2 = 0.21$).

Bronchoalveolar lavage fluid. Bronchoalveolar lavage fluid (BALF) was obtained and processed, and total protein concentrations and cell counts were measured as previously described (7).

Lung histology. Lungs sections were prepared as previously described (7). Stained sections ($n = 3$ per condition) were scored by a pathologist blinded to experimental conditions. In each section, 10 randomly selected fields were scored for (1) interstitial edema, (2) alveolar edema, (3) hemorrhage, and (4) neutrophil infiltration at $\times 40$ magnification, as previously described (9).

Assessment of ASK-1 Activation

Immunoprecipitation. Immunoprecipitation methods were described elsewhere (15). Phosphothreonine was immunoprecipitated from lung homogenate with rabbit anti-phosphothreonine antibody, and immunoblotted with rabbit anti-total ASK-1 primary antibody and then with horseradish peroxidase-coupled anti-rabbit IgG secondary antibody.

Assessment of Apoptosis

Immunoblot (Western blot) analysis. Lung homogenates were processed as described previously (7). Primary antibodies included rabbit anti-total ASK-1 (GeneTex, Irvine, CA), anti-phosphothreonine (Invitrogen, Carlsbad, CA), anti-caspase-3, cleaved caspase-3, poly [ADP-ribose] polymerase 1 (PARP-1), cleaved PARP-1, and glyceraldehyde 3-phosphate dehydrogenase (Cell Signaling Technology, Beverly, MA).

Terminal deoxynucleotidyl transferase dUTP nick-end labeling staining. An *in situ* cell death detection kit (Roche Applied Science, Indianapolis, IN) was used according to the manufacturer's instructions. Confocal images ($\times 20$ magnification) were used to quantify terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining. Five randomly selected fields from each section were counted, and the mean numbers of TUNEL-positive cells are reported (3 mice/group).

Immunofluorescence and confocal imaging. Lung tissue immunofluorescence was performed as previously described (16), using primary antibodies of anti-mouse PECAM-1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-goat cytokeratin18 (Santa Cruz Biotechnology), and anti-rabbit prosurfactant C (pro-SP-C; Abcam).

Assessment of Cytokine Release

BALF was analyzed for proinflammatory mediators (IL-6, keratinocyte-derived chemokine [KC], macrophage inflammatory protein [MIP]-2, and monocyte chemoattractant protein [MCP]-1), using the Luminex system and a custom multiplex kit (Millipore, Billerica, MA) according to the manufacturer's instructions.

Statistical Analysis

One-way ANOVA was used for multiple group comparisons, followed by the Dunn method for pairwise comparisons between groups. For non-parametric data, we used Kruskal-Wallis one-way ANOVA on ranks, and then the Holm-Sidak method for pairwise comparisons. Significance was set at $P < 0.05$.

RESULTS

Lung Injury Is Prevented in ASK-1 Knockout Mice

We compared the severity of lung injury in wild-type and ASK-1 knockout mice by evaluating lung histology (hematoxylin and eosin stains, Figures 1A–1D), composite lung injury scores (Figure 2A), lung injury subtype scores (Figure 2B), heart rate and oxygen saturation (Table 1), C_{st} (Figure 1E), total BALF cell counts (Figure 2C), and protein concentrations (Figure 2D). Lung sections of Control-WT (Figure 1A) and Control-ko mice (Figure 1C) showed normal lung parenchyma, whereas LIC-WT mice (Figure 1B) showed severe and diffuse alveolar and interstitial edema, neutrophil infiltration, and especially hemorrhage. In contrast, LIC-ko mice (Figure 1D) exhibited decreased alveolar and interstitial edema, neutrophil infiltration, and especially hemorrhages (Figure 2B). Although some injury was observed in the LIC-ko group, composite lung injury scores (Figure 2A) were significantly lower than in the LIC-WT group, and did not differ statistically from either control group. The most significant differences were seen in alveolar edema and hemorrhage scores (Figure 2B).

LIC-WT mice had significantly increased heart rates and reduced SAO_2 during the 4 hours of mechanical ventilation, whereas in LIC-ko mice, both these parameters remained stable. Lung mechanics were similar in all experimental groups at 0 hours, but C_{st} significantly decreased in LIC-WT mice after 4 hours of mechanical ventilation, compared with both control groups and LIC-ko mice (Figure 1E).

LIC-WT mice showed a significant increase in total BALF cells (Figure 2C) and protein concentrations (Figure 2D) compared with control mice. In LIC-ko mice, concentrations of BALF protein were greater than in control mice, but significantly lower than in LIC-WT mice, whereas BALF cell counts did not differ significantly from those in control mice.

Lung Injury Conditions Activated ASK-1

To confirm the expression of ASK-1 protein, we documented that total ASK-1 was only present in wild-type lungs but not in ASK-1 knockouts (Figure 3A). The densitometric analysis of Western blots (Figure 3B) indicated no significant change in the total expression of ASK-1 protein in LIC-WT compared with Control-WT mice, and no detectable ASK-1 protein in ASK-1 knockouts.

To determine whether lung injury was attributable to the activation of ASK-1, we needed to confirm the phosphorylation of ASK-1 to its active form (phosphorylation at threonine 845). No

Figure 1. The deletion of apoptosis signal-regulating kinase-1 (ASK-1) protects against lung injury in mice subjected to lung injury conditions (LIC; $n = 3$ mice per group). (A) Hematoxylin and eosin-stained, formalin-fixed, paraffin-embedded lung tissues from wild-type (Control-WT) and ASK-1-ko mice (Control-ko) spontaneously breathing room air; LIC; wild-type (LIC-WT); and ASK-1-ko (LIC-ko) mice pre-exposed to hyperoxia ($F_{O_2} = 0.9$) for 12 hours followed by high tidal volume mechanical ventilation with hyperoxia for 4 hours (scale = 100 μ M). LIC-WT shows evidence of extensive lung injury with interstitial and alveolar edema (thin arrows), and acute inflammatory cell infiltration (thick arrows), and marked hemorrhage (arrowheads), whereas LIC-ko mice are protected

against lung injury. (B) Quasistatic compliance (Q_{st}) of the four experimental conditions at time = 0 hours and at the end of the experiment (time = 4 hours). The Q_{st} was significantly decreased in LIC-WT, compatible with the evident increased lung injury, whereas ASK-1-ko prevented this decrease in Q_{st} . * $P < 0.05$, compared with Control-WT. # $P < 0.05$, compared with LIC-WT.

commercially available anti-phospho-ASK-1 (Thr845) antibodies provided adequate specificity. Therefore, we alternately immunoprecipitated whole-lung homogenates (control-WT and LIC-WT, as well as 4 hours of HV alone, 16 hours of HO alone, and *N*-acetylcysteine [NAC], 150 μ g/g body weight, intraperitoneal, before LIC) with an antibody directed against phosphorylated threonine, followed by immunoblotting with anti-total ASK-1 antibody (Figure 3C). A densitometric analysis (Figure 3D) indicated that LIC-WT increased ASK-1 threonine phosphorylation compared with all other groups. Minimal ASK-1 phosphorylation was seen with 16 hours of HO, whereas HV alone for 4 hours produced more than 16 hours of HO

alone, but significantly less ASK-1 phosphorylation than LIC. Moreover, mice treated with the antioxidant NAC before LIC showed reduced ASK-1 phosphorylation. These results confirm that lung injury in this model was associated with the activation of ASK-1, and this activation was oxidant-dependent.

Deletion of ASK-1 Inhibited Apoptosis

To determine whether the deletion of ASK-1 reduces apoptosis during exposure to LIC, we performed Western blot analyses of cleaved caspase-3 and PARP-1 (Figure 4A). LIC-WT mice exhibited significantly increased cleavage of both caspase-3

Figure 2. The deletion of ASK-1 reduces lung injury and barrier dysfunction in mice subjected to LIC ($n = 3$ mice per group). (A) Composite lung injury scores (LIS in D) represent the sum of the mean injury subtype scores (0–4) for each condition on a scale of 0–16. (B) Lung injury subtype scores (LIS subtypes) of alveolar edema, interstitial edema, neutrophil infiltration, and hemorrhage are presented on a scale of 0–4 (0 = none, and 4 = diffuse and severe). Wild-type mice subjected to LIC (LIC-WT) showed a significant increase in alveolar edema, interstitial edema, neutrophil infiltration, and hemorrhage, compared with the Control-WT, Control-ko, and LIC-ko groups. (C) Total bronchoalveolar lavage fluid (BALF) cells and (D) BALF protein concentrations were significantly higher in LIC. LIC-ko decreased both BALF total cell counts and protein concentrations. * $P < 0.05$, compared with Control-WT. # $P < 0.05$, compared with LIC-WT.

TABLE 1. MEAN HEART RATES AND OXYGEN SATURATIONS OF LIC-WT AND LIC-KO AT 0 HOURS AND 4 HOURS OF VENTILATION

	HR (beats/min), 0 Hours	HR (beats/min), 4 Hours	SA _O ₂ (% ± SD), 0 Hours	SA _O ₂ (% ± SD), 4 Hours
LIC-WT	242 ± 19	487 ± 6	98 ± 0.7	83 ± 2.9
LIC-ko	250 ± 45	262 ± 14	99 ± 0.3	96 ± 4.0

Definition of abbreviations: ko, knockout; HR, heart rate; LIC, lung injury conditions; WT, wild-type.

(Figure 4B) and PARP-1 (Figure 4C). In LIC-ko mice, the cleavage of caspase-3 and PARP-1 was completely inhibited.

In addition, we performed TUNEL staining on fixed lung tissue from each group, to identify any evidence of apoptosis (Figure 5A). Minimal TUNEL-positive cells were seen in control groups, but significantly fewer in Control-ko mice compared with Control-WT mice (Figures 5A and 5B). LIC-WT mice showed significant and diffuse TUNEL-positive cells. LIC-ko mice exhibited significantly fewer TUNEL-positive cells, and did not differ significantly from Control-WT mice. LIC-ko mice had significantly more TUNEL-positive cells than did Control-ko mice.

Apoptosis Primarily Involved the Alveolar Epithelium

To determine the cell types in which apoptosis occurred, we performed immunofluorescence of lung sections exposed to antibodies specific for epithelial cells (cytokeratin 18), alveolar Type II cells (pro-SP-C), and endothelial cells (PECAM-1), and determined colocalization with TUNEL staining. Figure 6 shows that the majority of TUNEL-positive cells are alveolar Type II (positive for both cytokeratin 18 and pro-SP-C). A few TUNEL-positive cells were positive only for cytokeratin 18,

indicating non-Type II epithelial cells. These were located in alveolar structures, indicating that they were Type I epithelial cells. Very few TUNEL-positive cells were observed that colocalized with PECAM-1, indicating a small number of endothelial cells undergoing apoptosis.

Deletion of ASK-1 Did Not Affect VILI-Associated Release of Cytokines

To determine whether the deletion of ASK-1 affects the release of inflammatory mediators, we measured concentrations of IL-6, KC, MCP-1, and MIP-1 in BALF. Despite significant differences in indicators of lung injury, significant elevations of all four inflammatory mediators were seen in both LIC-WT and LIC-ko mice, compared with control mice (Figures 7A–7D).

DISCUSSION

This study produced three unique findings: (1) the deletion of ASK-1 inhibited lung injury, compared with wild-type mice; (2) injury conditions in this model activated ASK-1; and (3) the deletion of ASK-1 inhibited apoptosis compared with wild-type mice, but did not affect release of inflammatory mediators.

The Experimental Model

We chose this large tidal volume model of mechanical ventilation-induced lung injury for several reasons. First, it is stable, reproducible, and compatible with many previously described models in this field. Second, it allows for the isolation of a single additional variable (hyperoxia) and its impact on the development of lung injury, without clouding the results with other potential variables (e.g., saline lavage, oleic acid, LPS, bacterial infection, or PEEP level), and third, in contrast to patients with

Figure 3. Protein expression and augmented threonine (Thr) phosphorylation of ASK-1 in whole-lung homogenates of wild-type mice subjected to LIC ($n = 3$ mice per group). (A) Protein expression of total ASK-1 in Control-WT (lane 1), Control-ko (lane 2), LIC-WT (lane 3), and LIC-ko (lane 4). (B) Densitometric analysis of data in A shows no significant change in ASK-1 protein expression between Control-WT and LIC-WT. ASK-1-ko mice demonstrated no ASK-1 protein. (C) Immunoprecipitation (IP) of phospho-threonine was followed by immunoblotting (IB) of total ASK-1 in whole-lung homogenates from mice subjected to Control-WT (lane 1), LIC-WT (lane 2), 4 hours of high tidal volume mechanical ventilation with normoxia (HVNO-4 hr; lane 3), 16 hours of hyperoxia (SVHO-16 hr; lane 4), pretreatment with *N*-acetylcysteine (NAC) followed by LIC (NAC + LIC-WT; lane 5), and control IgG (lane 6). (D) Densitometric analysis of data in C shows increased Thr phosphorylation of ASK-1 in LIC-WT compared with HVNO-4 hr and SVHO-16 hr. NAC pretreatment significantly reduced the Thr phosphorylation of ASK-1 (activation). * $P < 0.05$, compared with Control-WT mice. # $P < 0.05$, compared with LIC mice. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ND, none detected.

lowed by LIC (NAC + LIC-WT; lane 5), and control IgG (lane 6). (D) Densitometric analysis of data in C shows increased Thr phosphorylation of ASK-1 in LIC-WT compared with HVNO-4 hr and SVHO-16 hr. NAC pretreatment significantly reduced the Thr phosphorylation of ASK-1 (activation). * $P < 0.05$, compared with Control-WT mice. # $P < 0.05$, compared with LIC mice. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ND, none detected.

Figure 4. Activation of executioner caspase, caspase-3, and its downstream substrate poly [ADP-ribose] polymerase 1 (PARP)-1 in wild-type and ASK-1-ko mice subjected to LIC ($n = 3$ mice per group). (A) Activation (cleavage) of caspase-3 and PARP-1 (numbers on the left are molecular weights). (B and C) Densitometric analysis of (B) cleaved caspase-3 and (C) cleaved PARP-1. No cleavage of caspase-3 or PARP-1 was observed in LIC-ko, whereas LIC-WT induced significant cleavage of both caspase-3 and PARP-1 compared with control samples. * $P < 0.05$, compared with Control-WT. Casp, caspase; CI, control; ND, none detected; T, total.

ARDS/ALI, we are ventilating animals with normal lungs before undergoing mechanical ventilation (no significant injury was seen with 12 hours of hyperoxia pre-exposure). In addition, the experimental protocol was limited to 4 hours of mechanical ventilation, which may be insufficient time to document prevention versus delay in the development of VILI. Furthermore, given that patients admitted to the hospital with respiratory failure are usually treated with supplemental oxygen for hours

to days before the initiation of mechanical ventilation, we believe that this model is more clinically relevant than many reported VILI models that do not account for hyperoxia before or during mechanical ventilation.

The large tidal volumes used in this study are clearly not clinically applicable, especially in light of the proven mortality benefit with low tidal volume ventilation in ARDS (1). In the lung with ARDS, the spatial heterogeneity of injury results in very

Figure 5. Apoptotic cell death in wild-type and ASK-1-ko mice subjected to LIC ($n = 3$ mice per group). (A) Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay shows positive apoptotic cells (green) in lung sections of mice subjected to Control-WT, control-ko, LIC-WT, and LIC-ko. DRAQ5 was used to stain nuclei (blue). All confocal images were obtained at $\times 20$ magnification. (B) Mean TUNEL-positive cells per field (five fields/section) in lung sections of mice subjected to Control-WT, control-ko, LIC-WT, and LIC-ko. A significant increase in numbers of TUNEL-positive cells was seen in LIC-WT and LIC-ko compared with other Control groups, but LIC-ko showed significantly fewer TUNEL-positive cells compared with LIC-WT. * $P < 0.05$, compared with Control-WT mice. † $P < 0.05$, compared with Control-ko mice. ‡ $P < 0.05$, compared with LIC-WT mice.

Figure 6. Cellular distribution of apoptosis in mice subjected to LIC. Immunofluorescence staining for TUNEL (green), prosurfactant C (pro-SP-C; yellow), platelet/endothelial cell adhesion molecule-1 (PECAM-1) (orange), and cytokeratin-18 (red) in lung sections of mice subjected to LIC ($n = 3$). Arrows denote cells staining positive for each particular antibody. The predominant TUNEL-positive stained cells appeared to colocalize with pro-SP-C–positive cells, and to a lesser extent with cytokeratin-18–positive cells. Very few TUNEL-positive cells appeared to colocalize with PECAM-1–stained cells.

diseased lung regions, primarily in dependent regions, along with relatively normal lung regions (17). During mechanical ventilation, the relatively unaffected lung, with its higher regional compliance, will be more distended for a given tidal volume than its diseased counterparts. Higher tidal volumes are therefore required to mimic these conditions in uninjured lungs. Moreover, a patient with ALI is unlikely to be ventilated without the benefit of positive end-expiratory pressure. In addition, most patients with ARDS or ALI have another risk factor (e.g., sepsis, trauma, or aspiration) for lung injury besides mechanical ventilation.

ASK-1 and Lung Injury

When overexpressed in certain cell lines, ASK-1 can induce apoptosis (18, 19), and the activation of ASK-1 is associated with apoptosis in response to treatment with TNF- α , Fas, and H₂O₂ (18, 20). Thioredoxin (Trx) is a ubiquitous thiol-reducing system in the cytosol of all cells (21). Saitoh and colleagues found that

Trx binds directly to the noncatalytic N-terminal end of ASK-1 (22). Trx binding to ASK-1 blocks its kinase activity as well as ASK-1–dependent apoptosis. In the presence of reactive oxygen species (ROS), Trx can be oxidized and dissociate from ASK-1. This frees the catalytic domain of ASK-1, which can then be activated via phosphorylation. The exact mechanism by which ASK-1 is phosphorylated to its active form remains unclear, but recent studies suggest that the formation of homo-oligomers with TNF-associated factor (TRAF)2 and TRAF6 are a necessary step (23). The phosphorylation of threonine (Thr) 845 in the activation loop at the carboxy-terminal end then occurs via auto-phosphorylation or via an as yet undetermined pathway (24, 25). Phosphorylation is also involved in the down-regulation of ASK-1 activity. The phosphorylation at serine (Ser) 967 is essential for binding to its inhibitor protein, 14-3-3, and the suppression of cell death (26) and phosphorylation of Ser83 by Akt also attenuates the activity of ASK-1 (27).

Mechanical stimuli can also modulate the activation of ASK-1. In the endothelium, laminar flow inhibits TNF-induced ASK-1 activation in endothelial cells by preventing its dissociation from its inhibitory protein (21, 28, 29). High tidal volume ventilation was also reported to activate ASK-1 (13). Both reports link the activation of ASK-1 to mechanical stress, yet the response to this stress varies with cell type.

The role of ASK-1 in VILI was only recently explored. Li and colleagues found that mice ventilated with large tidal volumes (30 cm³/kg) and room air increased the production of MIP-2 and airway epithelial cell apoptosis that was dependent on the activation of ASK-1 and its downstream activation of the JNK pathway (13). Although we observed minimal ASK-1 activation with HO alone, HV alone for 4 hours did produce demonstrable activation of ASK-1 (Figures 3B and 3D). We did not, however, observe significant lung injury or apoptosis with either of these conditions alone (7), suggesting that a critical level of ASK-1 activation is necessary to produce physiologically relevant lung injury in this model. The activation of ASK-1 observed in LIC-WT mice appears to be an additive effect of combined HO pre-exposure and 4 hours of HV (Figure 3D). The discrepancies between the findings in these studies are likely explained by experimental differences (30 cm³/kg V_T for up to 5 hours with room air, versus 25 cm³/kg V_T for 4 hours after 12 hours of exposure to hyperoxia). Furthermore, in the study by Li and colleagues (13), the antibody used for phospho-ASK-1 was directed against Ser967 rather than Thr845 (D.A. Quinn, personal communication). Oxidative stress induces the dephosphorylation of Ser967 and phosphorylation of Thr845 in the activation loop of ASK-1, and both are correlated with the activity of ASK-1 and ASK-1–dependent apoptosis (25, 30).

We previously showed that the conditions of lung injury in the present study produce excess oxidative stress, and the resulting lung injury and apoptosis are significantly attenuated with NAC treatment (31). This increase in oxidative stress is the most likely initiator of ASK-1 activation in this model. In the present study, we observed that ASK-1 phosphorylation is significantly inhibited when mice are treated with NAC before LIC (Figures 3B and 3D). This finding supports the hypothesis that the activation of ASK-1, through the excess production of ROS, plays an important role in the development of lung injury in this model.

ASK-1 and Apoptosis

The deletion of ASK-1 significantly reduced apoptosis compared with LIC-WT (Figures 4 and 5). Activated ASK-1 phosphorylates p38 and JNK (11, 12). JNK phosphorylates not only c-Jun (32) but also other transcription (33–36) and nontranscription factors, such as the B cell lymphoma-2 (Bcl-2) family

Figure 7. Concentration of inflammatory mediators in BALF of wild-type and ASK-1-ko mice subjected to LIC ($n = 3$ mice per group). (A–D) Protein expression (pg/ml) of (A) IL-6, (B) keratinocyte-derived chemokine (KC), (C) monocyte chemoattractant protein (MCP)-1, and (D) macrophage inflammatory protein (MIP)-2. No significant difference is evident in concentrations of IL-6, KC, MCP-1, and MIP-2 in the BALF of LIC-WT mice compared with LIC-ko mice, but both are significantly increased compared with control mice. $*P < 0.05$, compared with Control-WT mice. ND, none detected.

of proteins (Bcl-2, Bcl-xL, Bcl-2-interacting mediator of cell death [Bim], and Bcl-2-associated death promoter [BAD]) (37–39). Through its actions on Bcl-2 proteins, JNK can either inhibit (39) or promote (38, 40) apoptosis. Recent studies suggest that JNK may induce apoptosis via modulating the proapoptotic Bcl-2 protein BIM (41). Bcl-2 itself, an inhibitor of apoptosis, is phosphorylated by the ASK-1/JNK pathway to its inactive form, resulting in increased susceptibility to apoptosis in Jurkat cells (38, 40). We observed significant caspase cleavage (Figures 4A and 4B), PARP-1 cleavage (Figures 4A and 4C), and TUNEL-positive cells (Figures 5A and 5B) in the LIC-WT condition that were prevented in LIC-ko mice.

Although apoptosis, as assessed by TUNEL staining, was significantly inhibited in LIC-ko mice, these mice did show increased numbers of TUNEL-positive cells compared with control-ko mice. Neither of these groups produced measurable caspase-3 cleavage, which is suggestive of non-caspase-mediated apoptosis. Further investigation is needed to understand the relevant signaling pathways leading to apoptosis downstream from ASK-1 in the lung.

A recently described MAP3K, ASK-2, is closely related to ASK-1, but can only activate p38 and JNK when it forms a heteromeric complex with ASK-1 (42). It was shown to play a critical role in the initiation phase of tumorigenesis in skin (43). Because it requires a complex formation with ASK-1 to be activated, we chose not to evaluate it in this study on the effects of deleting ASK-1.

Apoptosis and Cell type

All Western blot analyses were performed in whole-lung homogenates, and therefore they provide no details regarding which cell types are most important in the development of lung injury in this model. We therefore performed combined immunofluorescence and TUNEL assays of lung sections to determine the cell types undergoing apoptosis. TUNEL-positive cells were primarily alveolar epithelial cells, with Type II cells (pro-SP-C-positive) far exceeding Type I cells. A very small number of endothelial cells were also TUNEL-positive. These findings are consistent with our previous report that the apoptotic cell

death seen in this model is primarily localized to the lung epithelium (7). Likewise, the exposure of a murine alveolar epithelial cell line (MLE-12) to similar conditions of hyperoxia pre-exposure and subsequent stretch produced mitochondrially mediated apoptosis, comparable to our *in vivo* findings (7, 31).

ASK-1 and Cytokine Release

A strong link has been established between p38 and inflammation. Multiple inflammatory diseases are potentially regulated, at least in part, by the p38 pathway. Rheumatoid arthritis, Alzheimer disease, and inflammatory bowel disease are all regulated in part by the p38 pathway (44–46). The activation of the p38 pathway plays an essential role in the production of proinflammatory cytokines (IL-1, TNF, and IL-6) (47), the expression of inducible nitric oxide synthase (48, 49), and the induction of vascular cell adhesion protein-1 and other adhesion molecules, along with other inflammatory mediators (50). In the present study, the deletion of ASK-1 exerted no effect on the cytokine release associated with lung injury (Figures 7A–7D). This result suggests an ASK-1-independent proinflammatory pathway.

The similar proinflammatory mediator concentrations were associated with a similar degree of neutrophil infiltration in both wild type and ASK-1-ko mice (Figure 2B). However, we observed a marked reduction in alveolar hemorrhage and alveolar edema (Figure 2B) as well as concentrations of BALF protein (Figure 2D) with ASK-1 deletion. This is likely attributable to differences in the degree of barrier dysfunction. The differences in barrier dysfunction may be explained by increased epithelial apoptosis. We previously reported increased epithelial apoptosis in this model (7). Cell loss via apoptosis was implicated as a cause of barrier dysfunction in the gut epithelium (51–53), as well as in the lung in response to injury associated with the Fas–Fas ligand-mediated apoptosis of the alveolar epithelium (54–56). Injurious mechanical ventilation in a model of acid aspiration was also associated with epithelial apoptosis in the kidney and small intestine, suggesting a connection between VILI and non-pulmonary organ dysfunction (57). Alternately, the deletion of ASK-1 may alter the mechanical properties of the alveolar

epithelium, making it more resistant to damage by mechanical stretch. In addition, we have yet to investigate the effect of deleting ASK-1 on the pulmonary capillary endothelium, which could have profound effects on barrier function and lung injury. Further study is necessary to investigate these possible mechanisms.

Conclusions

In this model of VILI attributable to hyperoxia followed by high tidal volume mechanical ventilation, lung injury was associated with the activation of ASK-1 in wild-type mice. The deletion of ASK-1 inhibited lung injury, barrier dysfunction, and apoptosis, but surprisingly had no effect on cytokine release. The apoptosis observed in wild-type mice occurred primarily in Type II alveolar epithelial cells. These findings reveal that ASK-1 and its downstream signaling pathways are potential therapeutic targets for patients with ALI who require mechanical ventilation and supplemental oxygen therapy. Further studies are needed to elucidate fully the signaling pathways responsible for this protective effect.

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