Deletion of Caveolin-1 Protects against Oxidative Lung Injury via Up-Regulation of Heme Oxygenase-1

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Acute lung injury (ALI) is a major cause of morbidity and mortality in critically ill patients. Hyperoxia causes lung injury in animals and humans, and is an established model of ALI. Caveolin-1, a major constituent of caveolae, regulates numerous biological processes, including cell death and proliferation. Here we demonstrate that caveolin-1-null mice (*cav-1*^{-/-}) were resistant to hyperoxia-induced death and lung injury. *Cav-1*^{-/-} mice sustained reduced lung injury after hyperoxia as determined by protein levels in bronchoalveolar lavage fluid and histologic analysis. Furthermore, *cav-1*^{-/-} fibroblasts and endothelial cells and *cav-1* knockdown epithelial cells resisted hyperoxia-induced cell death *in vitro*. Basal and inducible expression of the stress protein heme oxygenase-1 (HO-1) were markedly elevated in lung tissue or fibroblasts from *cav-1*^{-/-} mice. Hyperoxia induced the physical interaction between *cav-1* and HO-1 in fibroblasts assessed by co-immunoprecipitation studies, which resulted in attenuation of HO activity. Inhibition of HO activity with tin protoporphyrin-IX abolished the survival benefits of *cav-1*^{-/-} cells and *cav-1*^{-/-} mice exposed to hyperoxia. The *cav-1*^{-/-} mice displayed elevated phospho-p38 mitogen-activated protein kinase (MAPK) and p38 β expression in lung tissue/cells under basal conditions and during hyperoxia. Treatment with SB202190, an inhibitor of p38 MAPK, decreased hyperoxia-inducible HO-1 expression in wild-type and *cav-1*^{-/-} fibroblasts. Taken together, our data demonstrated that *cav-1* deletion protects against hyperoxia-induced lung injury, involving in part the modulation of the HO-1-*cav-1* interaction, and the enhanced induction of HO-1 through a p38 MAPK-mediated pathway. These studies identify caveolin-1 as a novel component involved in hyperoxia-induced lung injury.

Keywords: acute lung injury; acute respiratory distress syndrome; caveolin-1; heme oxygenase-1

The clinical treatment of respiratory failure often requires supplemental oxygen (O₂) therapy, which paradoxically can cause acute lung injury (ALI). In its most severe manifestation, ALI can progress to acute respiratory distress syndrome (ARDS). ALI and ARDS are devastating syndromes that account for high morbidity and mortality among critically ill patients (1, 2). In rodent models, exposure to high O₂ tension (hyperoxia) causes well-characterized lung injury that resembles ARDS. Hyperoxia triggers an extensive inflammatory response in the lung that degrades the alveolar-capillary barrier, leading to impaired gas exchange and pulmonary edema (3, 4). The pathological changes in hyperoxia-injured lungs coincide with the injury or death of pulmonary capillary endothelial cells

CLINICAL RELEVANCE

This novel study demonstrates that deletion of caveolin-1 confers protection against hyperoxia-induced lung injury and promotes survival in mice via heme oxygenase-1. This may provide additional therapeutic targets in the management or prevention of acute lung injury/acute respiratory distress syndrome.

and alveolar epithelial cells (4–7). Compromised epithelial cell function may permit fluid and macromolecules to leak into the airspace, resulting in clinical respiratory failure and death (5, 8, 9). Lung tissue damage may result from the direct action of increased intracellular reactive oxygen species, or as a consequence of inflammation (5, 6). Increased pulmonary oxidant stress appears to play an important pathogenic role in several lung diseases and conditions, including ALI/ARDS (10, 11). The mechanisms underlying lung cell injury and death in response to hyperoxia remain incompletely understood, though recently both necrosis and apoptosis pathways have been demonstrated to play important roles (12, 13).

Caveolae are 50- to 100-nm omega-shaped invaginations of the cell surface plasma membrane that are enriched in glycosphingolipids and cholesterol (14). Caveolae occur in a variety of cell types including epithelial, endothelial cells, fibroblasts, smooth muscle cells, and adipocytes (14). The caveolae mediate non-clathrin-dependent endocytosis, and regulate the internalization of particles such as viruses and bacteria (15, 16). Caveolin-1 (*cav-1*), a 21- to 24-kD protein, is a major resident scaffolding protein constituent of caveolae that participates in vesicular trafficking and signal transduction events (14, 17, 18). *Cav-1* has been reported to reduce cell growth and increase apoptosis by inhibiting the activation of growth factor receptors and their downstream signaling pathways (14, 17, 19). In addition, *cav-1* negatively regulates smooth muscle cell proliferation (20, 21), and arrests mouse embryonic fibroblasts in the G0/G1 phase (22). *Cav-1* expression is increased in senescent cells and aged animals (23, 24). Furthermore, *cav-1*-null mice (*cav-1*^{-/-}) develop hypercellularity in the lungs, mammary gland, and heart (25, 26). These reports, taken together, indicate that *cav-1* plays important roles in the regulation of cell proliferation and death. Although emerging evidence indicates that *cav-1* also plays a role in the regulation of apoptosis *in vitro* (21, 27), its role *in vivo* remains unclear. No studies to date have focused on the potential function of *cav-1* in human diseases, such as ALI/ARDS.

Among the innate protective mechanisms of the lung, the low-molecular-weight stress protein heme oxygenase-1 (HO-1) represents a major inducible cellular and tissue defense against oxidative stress (28–31). HO-1, the rate-limiting step in heme degradation, oxidizes the α -methene bridge carbon of the heme molecule to generate equimolar biliverdin-IX α , iron, and carbon monoxide (CO) (32). NADPH biliverdin reductase completes the heme metabolic pathway by converting biliverdin-IX α to

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bilirubin-IX α (32). HO-1 confers cytoprotection involving anti-inflammatory, anti-apoptotic, and anti-proliferative effects in multiple models of cellular and tissue injury, including endotoxemia, oxidative lung injury, vascular injury, and ischemia/reperfusion injury (29). Mice deficient in HO-1, and endothelial cells derived from such mice, are prone to oxidant-mediated injury, and display aberrations in intracellular and tissue iron metabolism (33, 34). Recent studies have suggested that HO-1 protects against cell death induced by various injurious stimuli through the generation of its reaction products (29, 35–38). Our recent studies have indicated that HO-1 localizes in part to plasmalemmal caveolae in endothelial cells, in response to various injurious stimuli, and that this localization may have functional significance with respect to cellular adaptation to stress (39).

In this study, we investigated the role of *cav-1* in hyperoxia-induced acute lung injury and death. We demonstrate that *cav-1*^{-/-} mice significantly resisted hyperoxia-induced lung injury and cell death, and furthermore, that pulmonary cells derived from these animals were resistant to hyperoxia-induced cytotoxicity. We describe a novel mechanism by which *cav-1* deficiency confers cytoprotection, involving the up-regulated expression of HO-1.

MATERIALS AND METHODS

Chemicals

SB202190, 4-[4-(4-Fluorophenyl)-5-(4-pyridinyl)-1H-imidazol-2-yl]phenol, was from Calbiochem (San Diego, CA), and tin protoporphyrin-IX (SnPP) was from Frontier Scientific (Logan, UT). All other reagent chemicals were from Sigma (St. Louis, MO).

Cell Culture and Treatments

Primary mouse lung fibroblasts and pulmonary endothelial cells were cultured as described (12, 13) and used for experiments as subconfluent monolayers at passages 7 to 12. Cells were cultured from the lungs of wild-type C57BL/6 mice and *cav-1*^{-/-} mice as previously described (12, 13). Beas-2B lung epithelial cells were purchased from American Type Culture Collection (ATCC, Manassas, VA) and cultured in the serum-free medium BEGM (Cambrex, East Rutherford, NJ). All cells were grown in humidified incubators containing an atmosphere of 5% CO₂ and 95% air at 37°C. Chemical inhibitors including SB202190 (10 μ M) and tin protoporphyrin-IX (SnPP, 20 μ M), were prepared as concentrated DMSO stock solutions and applied to the culture media at the indicated final concentrations. Cell cultures were exposed to hyperoxia in modular exposure chambers as described (12), using 95% oxygen with 5% CO₂.

Animal Exposures

Wild-type C57BL/6 mice and inbred *cav-1*^{-/-} mice, 8 to 12 weeks old, were maintained in laminar flow cages in a pathogen-free facility at the University of Pittsburgh. All procedures were performed in accordance with the Council on Animal Care at the University of Pittsburgh and the National Research Council's Guide for the Humane Care and Use of Laboratory animals. The *cav-1*^{-/-} mice were from Dr. M. Drab (Max Planck Institute for Infection Biology, Berlin, Germany) and inbred in our laboratories. For *in vivo* experiments, SnPP was dissolved in aqueous solution as previously described (35). SnPP was administered to mice by injection (20 μ mol/kg/d, intraperitoneally). PBS with the same volume was used as control. The animals were exposed to room air or hyperoxia (95% O₂, 5% N₂). For biochemical and histologic analysis, animals were killed at 96 hours of exposure. Histologic analysis was done in a blind manner. For survival experiments, animal mortality was evaluated twice a day for up to 7 days of continuous exposure.

Bronchoalveolar lavage (BAL) was performed as previously described (40). Briefly, mice were killed and tracheas were cannulated. BAL was performed by injection and withdrawal of three aliquots of 0.6 ml of saline. After gentle but thorough mixing of the BAL fluid, the sample was centrifuged for cell counts. Approximately 1.5 ml of BAL fluid per mouse was obtained. The BAL fluid was centrifuged (800 $\times$ g

for 10 min at 4°C), and the cell-free supernatant analyzed for total protein. The total cells in the BAL fluid were quantified by hemocytometric counting.

Transfections

Beas-2B cells were transfected with *cav-1* siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Transiently transfected cells were incubated for an additional 24 hours and exposed to hyperoxia. After 72 hours, cell viability was determined with Cell viability assay (Promega, Madison, WI) and the rest of cells were harvested for the determination of caveolin-1 expression. Small interfering RNA (siRNA) was designed against the coding sequence of caveolin-1 cDNA by using software by Dharmacon Research (Lafayette, CO). Sequences corresponding to the siRNAs were as follows: human caveolin-1, (CCAG AAGGGACACACAGTT), GenBank accession no.NM_001753 (41).

Cav-1 Adenovirus Infection

Infections with *cav-1* and lacZ adenovirus were performed as previously described (41). Briefly, 2×10^5 fibroblasts were cultured in 6-well plates and exposed to 2×10^7 plaque-forming units of each virus in 1 ml of serum-free medium for 4 hours. The cells were washed and incubated in serum-containing media for 36 hours. The cells were then exposed to hyperoxia for 24 hours, as shown in figure legends.

Enzyme Activity Assays

The HO-1 activity was measured by the spectrophotometric determination of bilirubin production, as described previously (35, 39). For HO-1 activity, final reaction concentrations were: 25 μ M heme, 2 mM glucose 6-phosphate, 2 U glucose 6-phosphate dehydrogenase (Type XV from Baker's Yeast; Sigma), 1 mM β -NADPH, 1 mg/ml cell extract, and 2 mg/ml partially purified rat liver biliverdin reductase preparation.

Reaction mixtures were incubated for 60 minutes in a 37°C water bath in the dark. The reactions were terminated by addition of 2 volumes of chloroform (Aldrich, Milwaukee, WI). Bilirubin concentration in the chloroform extracts was determined on a Beckman DU640 scanning spectrophotometer (Beckman Instruments, Fullerton, CA) by measuring O.D. (464–530 nm). HO activity was reported as pmol BR/mg protein/h assuming an extinction coefficient of 40 mM⁻¹ cm⁻¹ for bilirubin in chloroform.

Western Blot Analysis, Immunocytochemistry, and Immunoprecipitation

The following antibodies were used for immunoblotting: monoclonal anti-caveolin-1, (BD Transduction Laboratories, Lexington, KY); monoclonal anti-HO-1 (Stressgene, Ann Arbor, MI); polyclonal anti-caveolin-1, anti-p38 mitogen-activated protein kinase (MAPK) (total and isoforms), and polyclonal anti-HO-1 (Santa Cruz, Santa Cruz, CA); and anti-phosphor p38 MAPK (Cell Signaling, Beverly, MA). Western blot analysis or immunocytochemistry were performed as described previously (12, 13).

Cell Viability Assay

Cell viability assays were performed using the CellTiter-Glo Luminescent Cell Viability Assay according to the manufacturer's protocol (Promega, Madison, WI). Briefly, cells were plated into 96-well plates. After transfection and exposure to hyperoxia, cells were washed twice with cold PBS. One hundred microliters of PBS was added into each well, followed by 100 μ l CellTiter-Glo Substrate. Cells were incubated at room temperature for at least 10 minutes; luminescent signal was then measured using an Lmax luminometer (Molecular Devices, Sunnyvale, CA). Trypan Blue Exclusion Test of Cell Viability was also performed according to the standard protocol (Warren Strober, National Institute of Allergy and Infectious Diseases, Bethesda, MD).

Statistical Analysis

All values were expressed as the mean $\pm$ SD from at least three independent experiments. Differences in measured variables between experimental and control group were assessed by using the Student's

t test (comparing numerical data, two groups) or chi-square test (comparing categorical data) for mouse survival study. Statistically significant difference was accepted at $P < 0.05$.

RESULTS

Cav-1^{-/-} Cells Are Resistant to Hyperoxia-Induced Cell Death

To assess the role of caveolin-1 in cellular sensitivity to high oxygen stress, we isolated pulmonary fibroblasts from *cav-1*^{-/-} mice or corresponding C57BL/6 wild-type mice and exposed them *in vitro* to a hyperoxic environment (95% O₂, 5% CO₂). After 72 hours of hyperoxia exposure, we found that *cav-1*^{-/-} fibroblasts were resistant to hyperoxia-induced cell death, with 60% survival observed in *cav-1*^{-/-} fibroblasts compared with less than 20% survival in the wild-type fibroblasts (Figure 1A). To determine whether this observation could be generalized to other cell types, we also evaluated cell survival in response to hyperoxia in mouse lung endothelial cells (MLEC) isolated from *cav-1*^{-/-} mice or corresponding C57BL/6 wild-type mice. Similar results were observed in MLEC cells (Figure 1B). Approximately 50% survival after hyperoxia in *cav-1*^{-/-} MLEC was found, compared with 20% survival in wild-type MLEC. We also transfected bronchial epithelial (Beas-2B) cells with *cav-1* siRNA to generate *cav-1* knockdown epithelial cells as described in MATERIALS AND METHODS. As expected, the *cav-1* knockdown epithelial cells were more resistant to hyperoxia compared with cells transfected with control siRNA (Figure 1C). Collectively, these results indicate that *cav-1*^{-/-} enhances cellular survival against hyperoxia in various pulmonary cell types. Given that we had used fibroblasts and Beas2B cells as main cell models in this study, we performed Trypan Blue Exclusion Test to evaluate cell viability in these two cells to further verify our observation. Representative results from three independent experiments were illustrated in Figures 1D and 1E. Student's *t* tests were used to analyze the statistic significance. We re-confirmed the resistance to hyperoxia induced cell death with trypan blue exclusion assays.

To further confirm our observations, we tested the effects of *cav-1* on cell survival under hyperoxia with gain of function experiment. Using the *ad-cav-1* to up-regulate *cav-1* expression in wild-type fibroblasts, we found that over expressing *cav-1* significantly suppressed cell survival in these cells treated with hyperoxia (24 h) (Figure 1D), suggesting the pro-apoptotic function of *cav-1* in this model.

Cav-1^{-/-} Mice Resisted the Lethal Effects of Hyperoxia

We evaluated whether *cav-1*^{-/-} mice were resistant to hyperoxia-induced lung injury and death *in vivo*. *Cav-1* expression in the lung tissue of wild-type and *cav-1*^{-/-} strains was evaluated by Western blot analysis and immunofluorescence microscopy, as illustrated in Figure 2A. The *cav-1*^{-/-} mice and wild-type C57BL/6 mice were exposed to hyperoxia (95% O₂, 5% N₂) in modular animal exposure chambers as described previously (42). As shown in Figure 2B, 50% of the wild-type mice had died after 108 hours (4.5 d), and 100% of wild-type mice had died by 120 hours (5 d) of exposure to continuous hyperoxia. In contrast, all *cav-1*^{-/-} mice remained alive after 156 hours. There were 60% *cav-1*^{-/-} mice remain alive for up to 7 days of continuous hyperoxia (168 h). We had to terminate the experiment after 7 days to comply with IACUC guidelines of our institution.

Cav-1^{-/-} Mice Were Resistant to Hyperoxia-Induced Lung Injury

Next we examined histologic characteristics of the lungs from wild-type and *cav-1*^{-/-} mice. Using hematoxylin and eosin

staining, we observed thickened alveolar septa and hypercellularity in the lungs of *cav-1*^{-/-} mice relative to wild-type mice as previously reported (26). However, there were no significant differences in morphology between untreated and hyperoxia-treated *cav-1*^{-/-} mice (data not shown). To determine whether *cav-1*^{-/-} mice sustain less lung injury in response to hyperoxia, we exposed *cav-1*^{-/-} mice and corresponding C57BL/6 wild-type mice to continuous hyperoxia or room air until killing at 96 hours, at which time the whole lungs were lavaged. The total cell counts in the BAL fluid did not differ among the treatment groups (data not shown). Total protein content in the BAL was compared among the four groups, as illustrated in Figure 3A. In wild-type mice, significantly higher levels of protein in the BAL were found in the hyperoxia-treated group, relative to the room air-treated group (Figure 3A). In *cav-1*^{-/-} mice, there were no significant differences in total BAL protein content between hyperoxia and air-treated mice (Figure 3A). Four mice were used in each group. Next, the lungs were excised from each animal, fixed, and analyzed by transition electronic microscopy (TEM). Significant morphologic changes at the cellular level were observed in the lungs of hyperoxia-treated wild-type mice relative to air-treated controls, including nuclear shrinkage (pyknosis), a characteristic of apoptotic cell death (Figure 3B). However, no morphologic differences were observed in hyperoxia-treated *cav-1*^{-/-} mice, relative to air-treated *cav-1*^{-/-} mice (Figure 3B).

Hyperoxia Induced Significantly Higher HO-1 Expression in *Cav-1*^{-/-} Mice

We sought to determine the mechanisms by which the *cav-1*^{-/-} genotype conferred protection against hyperoxia-induced lung injury. We found that the stress protein HO-1 was induced by hyperoxia as previously reported (42). Interestingly, basal and hyperoxia-inducible HO-1 levels were dramatically higher in *cav-1*^{-/-} mice than in corresponding wild-type mice (Figure 4A). A time course of HO-1 expression after hyperoxia in wild-type mice and *cav-1*^{-/-} mice was also illustrated in Figure 4B. We further confirmed this observation using *cav-1*^{-/-} fibroblasts. Again, significantly higher levels of HO-1 induced by hyperoxia were found in *cav-1*^{-/-} fibroblasts relative to wild-type fibroblasts (Figure 4C), indicating that hyperoxia induced the time-dependent elevation of HO-1 protein *in vitro*. We previously described a physical interaction between HO-1 and *cav-1* in endothelial cells that functionally suppressed HO activity (39). To investigate the relationship between *cav-1* and HO-1 in hyperoxia, we evaluated the physical interaction between *cav-1* and HO-1 in fibroblasts by co-immunoprecipitation. Interestingly, hyperoxia induced the formation of a complex between *cav-1* and HO-1 as illustrated in Figure 4D. We also found that hyperoxia had no effects on *cav-1* expression *in vitro* and *in vivo* (data not shown). Furthermore, we performed the HO-1 activity assay to directly verify our observations. A representative figure from one out of three assays was illustrated in Figure 4E. Significantly higher level of HO-1 activity was observed in *cav-1*^{-/-} cells treated with hyperoxia (Figure 4E).

Lack of HO-1 Activity Abolished the Survival Benefits of *Cav-1*^{-/-} Mice Treated with Hyperoxia

To test whether HO-1 plays an important role in the apparent hyperoxia resistance of *cav-1*^{-/-} mice, we treated wild-type or *cav-1*^{-/-} fibroblasts with SnPP, a competitive inhibitor of HO activity, and then exposed the cells to hyperoxia *in vitro*. After 72 hours, cell viability was measured. We found that *cav-1*^{-/-} cells pretreated with SnPP lost their resistance to hyperoxia-induced cell death. As shown in Figure 5A, after 72 hours of hyperoxia,

60% of *cav-1*^{-/-} cells remained alive, whereas only 20% of SnPP-treated *cav-1*^{-/-} cells were viable, essentially comparable with wild-type cells. Transfection with HO-1 siRNA also reduced the survival advantage of *cav-1*^{-/-} cells (data not shown). To further verify this hypothesis, we treated *cav-1*^{-/-} or wild-type mice with SnPP by daily injection. The *cav-1*^{-/-} mice treated with SnPP died much earlier during continuous hyperoxia exposure, relative to *cav-1*^{-/-} mice treated with vehicle (PBS). SnPP treatment, however, did not affect the survival of wild-type mice. As shown in Figure 5B, *cav-1*^{-/-} mice treated with SnPP had no survival benefit compared with wild-type mice. These results indicated that the survival advantage of the *cav-1*^{-/-} genotype in hyperoxia was attributed, at least in part, to differential HO-1 expression and activity, both *in vitro* and *in vivo*.

Figure 1. Deletion of caveolin-1 (cav-1) protected against hyperoxia *in vitro*. Various cell types were exposed to hyperoxia for 72 hours, and cell viability was measured using CellTiter-Glo Luminescent Cell Viability Assay. (A) Fibroblasts were isolated from wild-type and *cav-1*^{-/-} mice. (B) Endothelial cells were isolated from wild-type and *cav-1*^{-/-} mice. (C) Beas-2B cells were transfected with control siRNA or *cav-1* siRNA. Shaded bars: wild-type cells or Beas2B cells transfected with control siRNA; open bars: *cav-1*^{-/-} cells; striped bar: cells transfected with *cav-1* siRNA. Insets in A and B: Western blot confirming genotype of fibroblasts and endothelial cells. Inset in C: Western blot analysis indicating the efficacy of *cav-1* “knock down” in Beas-2B cells. Similar results were found in at least three independent experiments and one representative figure was shown here. Student’s *t*-tests were used to determine statistical significance, and the statistical significance was defined as *P* < 0.05. Furthermore, cell viability was measured using trypan blue exclusion assays. (D) The same fibroblasts from experiment in A were used. (E) Beas-2B cells were transfected with control siRNA or *cav-1* siRNA as described above in C. Shaded bars: wild-type cells or Beas2B cells transfected with control siRNA; open bar: *cav-1*^{-/-} cells; striped bar: cells transfected with *cav-1* siRNA. All figures represented at least three independent experiments. (F) Gain-of-function experiment. Wild-type fibroblasts were infected with *ad-cav-1* and *ad-lacZ* as described in MATERIALS AND METHODS. After 24 hours of hyperoxia, cell viability was measured. Shaded bar: cells infected with *ad-lacZ*; open bar: cells infected with *ad-cav-1*. Inset: Western blot analysis indicating the overexpressing *cav-1* in wild-type fibroblasts infected with *ad-cav-1*. Figures represented at least three independent experiments. In each experiment, each point had triplicate samples. *Statistically significant (*P* < 0.05, Student’s *t* test).

Elevated HO-1 Expression in *Cav-1*^{-/-} Mice Involves the p38 MAPK Pathway

We investigated the role of p38 MAPK in hyperoxia-induced HO-1 expression. As shown in Figure 6A, the basal level of phospho-p38 MAPK was elevated in *cav-1*^{-/-} mice but not detectable in wild-type mice. Although hyperoxia-treatment induced the phosphorylation of p38 MAPK in both strains, the level of phospho-p38 MAPK expression was dramatically higher in *cav-1*^{-/-} mice relative to wild-type mice. Next we evaluated whether p38 MAPK was involved in hyperoxia-induced HO-1 expression. One representative figure from three similar results was illustrated in Figure 6B: pretreatment with SB202190, a chemical inhibitor of p38 MAPK, reduced the apparent elevation in hyperoxia-inducible HO-1 expression in *cav-1*^{-/-} cells, relative to vehicle controls (DMSO). We further evaluated the isoforms of p38 MAPK in wild-type and *cav-1*^{-/-} cells and homogenized lung tissue. Increased p38β expression was observed in *cav-1*^{-/-} cells and tissue (Figure 6C and data not shown). One out of three independent assays was illustrated in Figure 6C. These results indicate that the differential elevations in basal and inducible HO-1 expression observed in *cav-1*^{-/-} cells can be attributed in part to enhanced activation of the p38 MAPK pathway.

DISCUSSION

Cav-1, the major structural component of caveolae, exerts a variety of biological functions, including the regulation of cholesterol homeostasis, vesicular transport, proliferation and apoptosis in a diversity of cell types, as well as the regulation of cardiovascular function *in vivo* (14). *cav-1*^{-/-} mice develop cardiac and pulmonary hypertrophy and fibrosis (25, 26). Furthermore, cardiac fibroblasts derived from *cav-1*^{-/-} mice display deregulated signaling pathways, with hyperactivation of

Figure 2. Deletion of cav-1 protected against hyperoxia *in vivo*. (A) Immunofluorescence and Western blot analysis were performed to evaluate the expression of cav-1 in lung tissue from wild-type and cav-1^{-/-} mice. Red stain indicates cav-1. (B) Survival of mice after continuous hyperoxia treatment. X-axis: days after hyperoxia; y-axis: percentage of survived mice. Solid line: wild-type mice; dotted line: cav-1^{-/-} mice ($n = 22$; 11 wild-type mice and 11 cav-1^{-/-} mice exposed to hyperoxia). The ages of cav-1^{-/-} mice and wild-type mice were between 8 and 12 weeks. In each experiment, we matched the age of wild-type mice and cav-1^{-/-} mice. The difference of ages between the wild-type mice and cav-1^{-/-} mice was 2 days. All wild-type mice died before 120 hours (5 d), no exclusions. *Statistically significant (* $P < 0.05$; Chi-square test).

ERK1/2 MAPK, and nitric oxide synthase activity (25). Such observations have indicated a critical role for caveolae and cav-1 in cellular signal transduction. Indeed, numerous transmembrane growth factor receptors (e.g., platelet-derived growth factor, epidermal growth factor, and nerve growth factor receptors) and other diverse signaling molecules (e.g., GTPases) localize to caveolae (19). Cav-1 can regulate membrane receptor signaling either by direct binding to the receptor, or binding to the downstream molecules, through interactions mediated by its scaffolding domain (19).

Recently, cav-1 has also been implicated as a modulator of innate immunity and inflammation (43–45). The cav-1^{-/-} mice displayed increased susceptibility to bacterial infection, whereas macrophages derived from these mice exhibited enhanced inflammatory responses to bacterial endotoxin (43). Previously, we have demonstrated an anti-inflammatory function of cav-1 expression *in vitro* with respect to the inhibition of pro-inflammatory cytokines production during endotoxin-induced inflammation in macrophages (46). Although emerging evidence indicates that cav-1 may also regulate apoptosis *in vitro* (21, 27), no studies have examined the role of cav-1 in cell death *in vivo*. We report here for the first time a potential role of cav-1 in cell death

Figure 3. Deletion of cav-1 reduced lung injury after hyperoxia. (A) Mice were exposed to hyperoxia for 96 hours. Bronchoalveolar lavage fluid was obtained from wild-type and hyperoxia-treated mice, and protein concentration was determined. Shaded bars: mice treated with room air; open bars: mice treated with hyperoxia. * $P < 0.05$. Four mice in each group were used. Student's *t* tests were used. Statistical significance was defined as $P < 0.05$. (B) Lung cellular morphology was examined by transition electronic microscopy (TEM). Upper panels: mice treated with room air. Lower panels: mice exposed to hyperoxia. Left panels: wild-type mice. Right panels: cav-1^{-/-} mice. Black arrows: nuclei. Representative figures are shown.

in vivo, using a model of hyperoxia-induced acute lung injury, given that cell death is a prominent feature in this syndrome.

In our previous studies of cav-1 in lung injury models, we have shown that cav-1 exerts an anti-fibrotic effect, associated with anti-proliferative effects in fibroblasts (41). The primary defects in cav-1^{-/-} mice occur in the lungs and vasculature, mainly present as thickening of alveolar septa and hypercellularity (25, 26). Presumably, these morphological characteristics may impair gas exchange in these animals. On the basis of these observations we initially hypothesized that cav-1^{-/-} mice would be more susceptible to hyperoxia-induced lung injury and death. Surprisingly, and in contrast to our initial hypothesis, we found

Figure 4. Expression and regulation of heme oxygenase-1 (HO-1) in wild-type and *cav-1*^{-/-} mice. (A) Wild-type and *cav-1*^{-/-} mice were subjected to hyperoxia *in vivo*. After 96 hours, lung tissue was excised, homogenized, and analyzed for HO-1 expression by Western blot analysis. Figures represented three independent experiments. **P* < 0.05 (statistical analysis was performed using *t* tests). (B) Time course of hyperoxia-induced HO-1 expression in wild-type and *cav-1*^{-/-} mice. Wild-type and *cav-1*^{-/-} mice were subjected to hyperoxia *in vivo* for different days as indicated. Lung tissue was excised, homogenized, and analyzed for HO-1 expression by Western blot analysis. There were no wild-type mice that survived on Day 5 from which to collect lung tissue for Western blot analysis. Figures represented three independent experiments. **P* < 0.05 (statistics performed using *t* tests on time [day]-matched samples). (C) Wild-type and *cav-1*^{-/-} fibroblasts were exposed to hyperoxia (95% O₂, 5% CO₂) *in vitro* for 0 to 72 hours. HO-1 expression was determined by Western blot analysis. Figures represented three independent experiments. **P* < 0.05 (statistics performed using *t* test on time-matched samples). (D) Wild-type fibroblasts were treated with hyperoxia (72 h). The interaction of HO-1 and cav-1 was detected by immunoprecipitation with HO-1 polyclonal antibodies followed by blotting with cav-1 monoclonal antibodies. Figure represented three independent

experiments. (E) HO-1 activity in wild-type (open bars) and *cav-1*^{-/-} (shaded bars) fibroblasts with and without hyperoxia. Wild-type and *cav-1*^{-/-} fibroblasts were exposed to hyperoxia (95% O₂, 5% CO₂) *in vitro*. After 72 hours, HO-1 activity was measured as described in MATERIALS AND METHODS. Open bars: wild-type cells; shaded bars: *cav-1*^{-/-} cells. Figure represents three independent experiments. **P* < 0.05 (statistics performed using *t* tests).

that *cav-1*^{-/-} mice were resistant to hyperoxia-induced lung injury and death (Figure 2), and furthermore, that various lung cells, including fibroblasts and endothelial cells derived from these animals, were resistant to hyperoxia-induced cell death *in vitro* (Figure 1). Alveolar protein content, a marker of lung injury, was significantly higher in wild-type mice after hyperoxia as previously reported (47), whereas it did not increase in *cav-1*^{-/-} mice after hyperoxia (Figure 3A). These results indicated that lung injury occurred much earlier in wild-type mice, evident at 96 hours after hyperoxia, compared with *cav-1*^{-/-} mice, which were more tolerant to the hyperoxia. However, the results shown in Figure 3A were based on the assumption that

BAL protein concentration was a surrogate for respiratory tract lining fluid (RTLFL) protein, given that intact RTLFL is difficult to sample. In this study, we further assumed that the RTLFL → BAL dilution factors were similar within each animal. Further experiments will be necessary to confirm this assumption. In addition, given that all our experimental mice were at age 8 to 12 weeks, we cannot extrapolate this conclusion to mice beyond age 12 weeks or under 8 weeks. There are studies indicating that *cav-1* is involved in senescence (24). Therefore, to answer whether the above observation can be applied to older or younger mice, further studies using older and younger mice should be performed.

Figure 5. HO activity is required for hyperoxia resistance in *cav-1*-deficient cells and mice. (A) Wild-type and *cav-1*^{-/-} cells were subjected to hyperoxia (95% O₂, 5% CO₂) after pretreatment with SnPP (20 μM; open bars) or vehicle (DMSO; shaded bars). After 72 hours, cell viability was evaluated as previously described. The percentage of surviving cells after hyperoxia was determined. Shaded bars: cells pre-treated with DMSO; open bars: cells pre-treated with SnPP. Results reflect the mean and standard deviation of three determinations (**P* <

0.05). Figure represents three independent experiments. Statistical analysis was performed using *t* tests. (B) SnPP was administered to wild-type or *cav-1*^{-/-} mice by injection (20 μmol/kg/d, intraperitoneally). PBS with the same volume was used as control. The animals were exposed to room air or hyperoxia (95% O₂, 5% N₂) and time-dependent survival was determined (*n* = 7 in each group). **P* < 0.05 (statistics performed using chi-square tests [categorical data]).

We examined the underlying mechanisms by which *cav-1*^{-/-} mice displayed prolonged survival and reduced lung injury after hyperoxia. Our data indicate that the apparent resistance to hyperoxia in *cav-1*^{-/-} pulmonary cells and tissues is due to increased activation of the stress protein HO-1. We observed an increased HO-1 expression at basal and induced level (Figure 4). Given that *cav-1*^{-/-} mice had baseline thickening of alveolar septa/hypercellularity (25–27), we suspected that the increased basal level of HO-1 in *cav-1*^{-/-} cells and mice might indicate a chronic stress status as stated previously (25, 26, 41, 44). The HO-1/CO pathway has emerged as a major inhibitor of inflammation and apoptosis *in vitro* and *in vivo* (29). Overexpression of HO-1 or exogenous application of CO protected against hyperoxia-induced lung injury and lung cell death in rodent models (36, 47–49). We cannot exclude the possibility that the apparent hyperoxia resistance of *cav-1*^{-/-} mice involves the activation or up-regulation of additional stress proteins (e.g., heat shock proteins) or antioxidant enzymes.

The mechanisms underlying lung cell injury and death in response to hyperoxia vary in a cell type-specific manner and can involve apoptosis, necrosis, or mixed cell-death phenotypes (12, 13). Whether the protection afforded by the *cav-1*^{-/-} genotype is preferentially anti-apoptotic or anti-necrotic requires further investigation, though the anti-apoptotic potential of HO-1 has been well documented (29).

Our previous studies have identified several functional interactions of *cav-1* with the HO-1/CO pathway. In endothelial cells, *cav-1* negatively regulated caveolae-specific HO activity (39). Furthermore, *cav-1* was required for the anti-proliferative effects of CO in vascular smooth muscle cells (20). Since application of SnPP abolished the survival advantage of *cav-1*^{-/-}, this result was consistent with our previous observations that *cav-1* acts as a negative regulator of HO-1 activity in endothelial cells (39). We previously demonstrated the occurrence of a physical interaction between HO-1 and *cav-1* in endothelial cells (39). Our current data further confirmed that the *cav-1*/HO-1 interaction occurred in pulmonary fibroblast cells and was

stimulated by hyperoxic stress. Thus, the apparent cytoprotection observed in *cav-1*^{-/-} mice may be due in part to loss of negative regulation of HO activity due to the absence of *cav-1*. Interestingly, in current study we also show that *cav-1* negatively regulates basal and hyperoxia-inducible HO-1 expression. HO-1 expression was found dramatically up-regulated in *cav-1*^{-/-} cells and tissues (Figure 4).

We therefore hypothesized that the effect of *cav-1* on HO-1 expression was related to *cav-1*-specific effects on intracellular signaling pathways involved in HO-1 gene activation. MAPK signaling pathways have previously been implicated in the regulation of the HO-1 gene in response to diverse environmental stimuli (29). The MAPK superfamily includes three primary signaling cascades: the ERK1/2, JNK, and p38 MAPK pathways. The specific MAPKs involved in HO-1 gene regulation apparently vary in a cell type- and inducer-specific fashion (29). For example, IL-10- and TGF-β-mediated induction of HO-1 expression involved activation of p38 MAPK (50, 51). The regulation of HO-1 by hypoxia preferentially involved p38 MAPK signaling in cardiomyocytes (52). The up-regulation of HO-1 by photosensitizer-mediated oxidative stress in cancer cells and its associated anti-apoptotic activity was mediated in part by p38 MAPK (53). We investigated the potential role of p38 MAPK in the context of *cav-1*- and hyperoxia-dependent regulation of HO-1. Our results demonstrate that the up-regulation of p38 MAPK signaling pathways mediates the increased basal and inducible induction of HO-1 in *cav-1*^{-/-} cells relative to wild-type cells. We cannot exclude the possibility that other MAPK pathways are up-regulated in the lungs of *cav-1*^{-/-} mice (e.g., JNK, ERK1/2), which may also contribute to HO-1 regulation, and this warrants further investigation. In addition, our future directions will include studying the effects of *cav-1*^{-/-} on apoptosis pathways involved in hyperoxia-induced cell death.

Taken together, these novel results demonstrate that *cav-1* deficiency confers protection against hyperoxia-induced lung injury and promotes survival after hyperoxia in mice via HO-1-mediated pathways. However, given the differences in HO-1

Figure 6. Hyperoxia-inducible HO-1 expression requires p38 mitogen-activated protein kinase (MAPK) in wild-type and *cav-1*^{-/-} mice. (A) Expression of phosphor-p38 (p-p38) in wild-type or *cav-1*^{-/-} mice. Wild-type and *cav-1*^{-/-} mice were exposed to room air (RA) or hyperoxia (95% O₂, 5% N₂) for 96 hours. Total or phospho-p38 MAPK levels were determined by Western blot analysis of lung tissue homogenates. Figure represents three independent experiments (statistics performed using *t* tests; **P* < 0.05). (B) Wild-type or *cav-1*^{-/-} fibroblasts were pretreated with DMSO or SB202190 (10 μM) and then exposed to hyperoxia (95% O₂, 5% CO₂) or room air (RA) for 72 hours. HO-1 expression was determined by Western blot analysis. Figure represents three independent experiments (statistics performed using *t* tests; **P* < 0.05). (C) Expression of p38 isoforms in wild-type or *cav-1*^{-/-} fibroblasts. Wild-type and *cav-1*^{-/-} fibroblasts were exposed to room air (RA) or hyperoxia (95% O₂, 5% N₂) for 24 hours. Western blot analysis was performed to determine the expression of p-38 isoforms. Figures represent three independent experiments (statistics performed using *t* tests; **P* < 0.05).

regulation between mice and humans, whether this conclusion can be applied to humans remains unclear.

These studies may provide additional therapeutic targets in the management or prevention of ALI/ARDS.

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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