Phosphorylation of Caveolin-1 Regulates Oxidant–Induced Pulmonary Vascular Permeability via Paracellular and Transcellular Pathways

Yu Sun, Guochang Hu, Xiumei Zhang, Richard D. Minshall

Rationale: Oxidants are important signaling molecules known to increase endothelial permeability, although the mechanisms underlying permeability regulation are not clear.

Objective: To define the role of caveolin-1 in the mechanism of oxidant-induced pulmonary vascular hyperpermeability and edema formation.

Methods and Results: Using genetic approaches, we show that phosphorylation of caveolin-1 Tyr14 is required for increased pulmonary microvessel permeability induced by hydrogen peroxide (H₂O₂). Caveolin-1–deficient mice (cav-1⁻/⁻) were resistant to H₂O₂-induced pulmonary vascular albumin hyperpermeability and edema formation. Furthermore, the vascular hyperpermeability response to H₂O₂ was completely rescued by expression of caveolin-1 in cav-1⁻/⁻ mouse lung microvessels but was not restored by the phosphorylation-defective caveolin-1 mutant. The increase in caveolin-1 phosphorylation induced by H₂O₂ was dose-dependently coupled to both increased ¹²⁵I-albumin transcytosis and decreased transendothelial electric resistance in pulmonary endothelial cells. Phosphorylation of caveolin-1 following H₂O₂ exposure resulted in the dissociation of vascular endothelial cadherin/β-catenin complexes and resultant endothelial barrier disruption.

Conclusions: Caveolin-1 phosphorylation–dependent signaling plays a crucial role in oxidative stress-induced pulmonary vascular hyperpermeability via transcellular and paracellular pathways. Thus, caveolin-1 phosphorylation may be an important therapeutic target for limiting oxidant-mediated vascular hyperpermeability, protein-rich edema formation, and acute lung injury. (Circ Res. 2009;105:676-685.)

Key Words: vascular endothelial barrier ■ transcytosis ■ adherens junctions ■ caveolin-1 ■ lung edema

An increase in vascular permeability is a key hallmark of inflammation of many disease states including acute lung injury, ischemia-reperfusion injury, atherosclerosis, and diabetes.1 Under physiological conditions, adherens junctions predominate in endothelial cell–cell contacts and control pulmonary endothelial barrier integrity. Stabilization of adherens junctions is dependent on the association of vascular endothelial (VE)-cadherin, β-catenin, p120-catenin, and α-catenin proteins and their linkage to the actin cytoskeleton2 wherein VE-cadherin association with the actin cytoskeleton is thought to be dependent on the β-catenin linkage.3 Oxidants including superoxide and hydrogen peroxide (H₂O₂) generated by activated neutrophils and endothelial cells in response to inflammatory stimuli increase paracellular endothelial permeability by promoting the loss of cell–cell adhesion and activation of actin-myosin based cell retraction.1,4,5

We recently demonstrated that caveolae-mediated transendothelial transport (transcytosis) of macromolecules through the microvascular endothelial barrier is also an important mechanism responsible for inflammation-evoked pulmonary vascular hyperpermeability and protein-rich edema formation.6 We showed that an increase in transcellular (caveolae-mediated) permeability, triggered by the binding of neutrophils to endothelial cell surface intercellular adhesion molecule (ICAM)-1, was mediated by Src activation and phosphorylation of caveolin (Cav)-1,6 which thereby stimulates caveolae formation and trafficking.7–11 ICAM-1 expression and activation is mediated by oxidant signaling in lung endothelial cells.1 In addition, oxidants have also been shown to directly induce Src-dependent phosphorylation of Cav-1 at Tyr14 in endothelial cells.12–14 Thus, oxidants generated by neutrophils and/or endothelial cells may serve as signal transduction mediators that regulate transcellular permeability through a Cav-1–dependent mechanism.

Cav-1 expression is required for caveolae-mediated endocytosis and transcytosis in endothelial cells.4,6,7,11,15–17 Recent evidence also points to the potential role of Cav-1 in cell–cell
H2O2-induced stimulation of transcytosis and destabilization of cell–cell junctions and hence propose that tyrosine phosphorylation of Cav-1 plays an important role in the pathogenesis of oxidant-induced pulmonary vascular hyperpermeability.

**Methods**

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Briefly, Cav-1–null (cav-1−/−) mice, wild-type (WT) B6/129SJ2 mice, and rat lung microvascular endothelial cells (RLMVECs) were used. Animal protocols received institutional review and committee approval. Endocytosis and transendothelial transport of 125I-albumin, fluorescent albumin uptake, transendothelial electrical resistance (TER), siRNA transfection, Western blotting and immunoprecipitation were performed as described previously. Rescue studies were made in mouse lungs from cav-1−/− mice by liposome-mediated plasmid cDNA transfection. 125I-albumin permeability surface area (PS) product was measured in lungs perfused with Krebs solution.

**Results**

H2O2 Induced Cav-1 Phosphorylation via Src and c-Abl Kinases

H2O2 in a concentration-dependent manner (0.05 to 0.8 mmol/L) increased Cav-1 Tyr14 phosphorylation (p[Y14]-Cav-1; Figure 1A). Quantitative analysis revealed that the level of phospho–Cav-1 was increased by 2- to 9-fold more, Cav-1 phosphorylation increased within 5 minutes, peaked at 30 minutes, and then returned to basal levels 60 minutes after H2O2 treatment (Figure 1B; Online Figure I, B). Coincident with the increase in p(Y14)-Cav-1 levels, we also observed activation of c-Src and c-Abl and result -

adhesion and thus paracellular permeability regulation. Cav-1 colocalizes with adherens junction proteins E-cadherin, β-catenin and γ-catenin in MDCK (Madin–Darby canine kidney) epithelial cells. Downregulation of Cav-1 leads to a loss and redistribution of tight junction proteins (occludin and ZO-1) in brain microvascular endothelial cells resulting in an increase in paracellular permeability. Furthermore, small interfering (si)RNA-mediated depletion of Cav-1 in the mouse lung induced an increase in the number of interendothelial gaps in pulmonary capillaries and veins. In contrast, deletion of Cav-1 attenuated protein kinase C–induced interendothelial gap formation in myocardial microvascular endothelial cells. Whether and how Cav-1 regulates endothelial barrier function during oxidative stress remains an important question.

In the present study, using genetic approaches, we investigated the role of Cav-1 in pulmonary microvascular permeability regulation through both transcellular and paracellular pathways. We found that Cav-1 phosphorylation is required for
fibroblasts. Therefore, we examined the potential role of c-Abl kinase in the regulation of Cav-1 phosphorylation in pulmonary endothelial cells. As shown in Figure 1D and Online Figure 1 (D), H2O2 induced the phosphorylation (activation) of c-Abl kinase in a concentration-dependent manner. Downregulation of c-Abl with specific siRNA reduced Cav-1 phosphorylation induced by high but not low concentration of H2O2 (Figure 1E; Online Figure I, E). To determine whether Src and c-Abl are activated by H2O2 independently of one another, we measured H2O2-induced c-Abl activation in the presence and absence of PP2. As shown in Figure 1F, PP2 significantly blocked H2O2-induced (0.6 mmol/L) activation of c-Abl kinase (Online Figure I, F), suggesting that c-Abl activation by oxidants occurs at least in part via activation of Src kinase.

Tyrosine Phosphorylation of Cav-1 Signals H2O2-Induced Transcellular Albumin Hyperpermeability

To evaluate whether H2O2 stimulates transcellular albumin permeability, we first addressed the possibility that H2O2 facilitates caveolae-mediated endocytosis of albumin in endothelial cells, the initial step in albumin transport via transcytosis. As shown in Figure 2A and 2B, H2O2 caused a concentration-dependent increase in Alexa 488-albumin endocytosis in endothelial cells (Figure 2A, B). Confocal images (Figure 2A) indicated that albumin was internalized by endocytic vesicles and that exposure of endothelial cells to H2O2 induced a significant increase in albumin uptake. In parallel, tracer 125I-albumin uptake by endothelial cells following exposure to low dose H2O2 (0.05 to 0.2 mmol/L) was significantly increased (Figure 2B). We next determined the effect of H2O2 on transcytosis of 125I-albumin using a well-established technique. The concentration range of H2O2 (0.05 to 0.2 mmol/L) was chosen because at these levels, H2O2 did not disrupt the endothelial barrier (Figure 3A). Consistent with the activation of endocytosis, H2O2 induced a concentration-dependent increase in transendothelial 125I-albumin transport (Figure 2C). These data suggest that H2O2 stimulates transcellular albumin transport via a vesicular pathway.
To further assess the mechanism of vesicular albumin transport, cells were pretreated with the caveolae disrupting agent methyl-β-cyclodextrin and Cav-1 siRNA. As shown in Online Figure II (A and B), methyl-β-cyclodextrin prevented H$_2$O$_2$-induced increase in endocytosis and transcytosis of $^{125}$I-albumin. Furthermore, H$_2$O$_2$ increased albumin endocytosis and transendothelial $^{125}$I-albumin flux in endothelial cells transduced with scrambled siRNA, whereas Cav-1 knockdown by ≥90% abolished these effects.

To address the role of Cav-1 phosphorylation in H$_2$O$_2$-induced increase in transendothelial albumin permeability, we measured H$_2$O$_2$-induced increase in transendothelial albumin permeability. We measured H$_2$O$_2$-induced increase in transendothelial albumin transport of $^{125}$I-albumin in RLMVEC lines overexpressing phosphorylation-defective Y14F Cav-1 mutant (Y14F-Cav-1) and as a control, WT-Cav-1. H$_2$O$_2$-induced (0.2 mmol/L) increase in endocytosis and transcytosis of $^{125}$I-albumin was significantly attenuated by Y14F-Cav-1 expression compared to WT-Cav-1-expressing cells (Figure 2D and 2E). Moreover, inhibition of Cav-1 phosphorylation by Src inhibitor PP2 reduced H$_2$O$_2$-stimulated endocytosis and transendothelial albumin permeability (Figure 2F and 2G), whereas downregulation of c-Abl kinase did not affect the endocytosis or transcytosis of albumin (Figure 2F and 2G).

Tyrosine Phosphorylation of Cav-1 Signals H$_2$O$_2$-Induced Paracellular Hyperpermeability

H$_2$O$_2$ at higher concentrations (0.4 to 0.8 mmol/L) induced interendothelial cell gap formation, detected as a reduction in TER, whereas H$_2$O$_2$ at lower concentrations (0.05 to 0.2 mmol/L) did not affect TER (Figure 3A). The effect of 0.4 and 0.6 mmol/L H$_2$O$_2$ was reversible in that TER returned to basal levels (4.7±0.7 Ω·cm$^2$) within 5 hours, whereas endothelial cells treated with 0.8 mmol/L H$_2$O$_2$ did not fully recover (Figure 3A). Accordingly, 0.6 mmol/L H$_2$O$_2$ was chosen for subsequent experiments to explore the mechanism of H$_2$O$_2$-induced loss of monolayer integrity (increase in paracellular permeability).

To determine whether Cav-1 phosphorylation signals H$_2$O$_2$-induced increase in paracellular permeability, we mea-
sured H$_2$O$_2$-induced changes in TER in WT and Y14F-Cav-1–expressing RLMVEC lines. The effect of low and high levels of Cav-1 phosphorylation on TER, induced by 0.2 and 0.6 mmol/L H$_2$O$_2$ respectively, was evaluated in endothelial monolayers. As shown in Figure 3A, 0.2 mmol/L H$_2$O$_2$ did not alter TER in native endothelial cells (nontransfected) but remarkably decreased TER in cells overexpressing WT-Cav-1 (Figure 3B). Importantly, 0.2 mmol/L H$_2$O$_2$ had no effect on TER in cells expressing the same level of mutant Y14F-Cav-1. The decrease in TER induced by 0.6 mmol/L H$_2$O$_2$ in endothelial cells expressing WT-Cav-1 was also significantly greater than the response observed in native cells and endothelial cells expressing Y14F-Cav-1 (Figure 3A and 3B). Inhibition of Cav-1 phosphorylation by Src inhibitor PP2 or c-Abl siRNA similarly reduced the magnitude of the H$_2$O$_2$-induced (0.6 mmol/L) decrease in TER and resembled the response observed in cells expressing the phosphorylation-defective Cav-1 mutant. One difference noted was that the response was more transient in cells treated with c-Abl siRNA compared to PP2-treated cells (Figure 3C).

**Cav-1 Phosphorylation Mediates H$_2$O$_2$-Induced Dissociation of VE-Cadherin and β-Catenin**

To gain further insight into the regulatory mechanism of oxidant-induced endothelial barrier disruption and increase in paracellular permeability, we investigated the effect of H$_2$O$_2$-induced Cav-1 phosphorylation on the stability of adherens junctions by assessing VE-cadherin/β-catenin complexes using confocal microscopy and immunoprecipitation analysis. Confocal images showed significant colocalization of Cav-1 and β-catenin at cell–cell borders (Figure 4A) and immunoprecipitation studies also demonstrated an association between Cav-1 and β-catenin at baseline (Figure 4B). However, the association between Cav-1 and β-catenin at the cell borders observed by immunostaining, as well as the communoprecipitated proteins measured in the immunoblots, was reduced by ~25% and 75% following exposure of endothelial cells to 0.2 and 0.6 mmol/L H$_2$O$_2$, respectively (Figure 4B).

To assess the role of Cav-1 phosphorylation in the mechanism of H$_2$O$_2$-induced dissociation of Cav-1 and β-catenin, RLMVEC lines expressing Myc-tagged WT-Cav-1 or Y14F-
Cav-1 were stimulated, lysed, and immunoprecipitated with anti-Myc or anti-β-catenin antibodies. As shown in Figure 4C, β-catenin was associated with WT-Cav-1 and to a greater extent with Y14F-Cav-1 in untreated cells, suggesting that β-catenin binds Cav-1 in the nonphosphorylated state. Stimulation of cells with 0.2 mmol/L H2O2 led to a decrease in association between β-catenin and WT-Cav-1, whereas H2O2 had no effect on the association between β-catenin and Y14F-Cav-1 (Figure 4C). These data strongly argue that phosphorylation of Cav-1 leads to its dissociation from β-catenin.

We next determined whether the dissociation of Cav-1 from β-catenin induced by Cav-1 phosphorylation also led to the disruption of VE-cadherin/β-catenin complexes. As shown in Figure 5A, association between VE-cadherin and β-catenin was reduced by 15% and 80% on exposure to 0.2 and 0.6 mmol/L H2O2 compared to untreated endothelial cells. Furthermore, H2O2-induced dissociation of VE-cadherin and β-catenin observed in WT-Cav-1–expressing cells was abolished in Y14F Cav-1–expressing cells (Figure 5B).

To further assess whether β-catenin translocates from the membrane following H2O2 stimulation at the higher concentration (0.6 mmol/L), cells were treated, lysed, fractionated, and immunoblotted. Figure 6A shows β-catenin predominantly localized in the membrane basally and then in the cytosolic compartment after treatment of endothelial cells with 0.6 mmol/L H2O2. Moreover, we observed H2O2-induced β-catenin translocation in WT-Cav-1–expressing cells but not in Y14F-Cav-1–expressing cells (Figure 6B). Similarly, confocal imaging showed widespread gap formation, increased β-catenin translocation into the cytosol, and a loss of β-catenin staining in membranes at sites of cell-cell contact in WT-Cav-1–expressing cells, whereas β-catenin localization was unaffected and gaps in the monolayer did not form in Y14F-Cav-1 mutant–expressing cells following exposure to 0.2 mmol/L H2O2 (Online Figure III). Thus, Cav-1 phosphorylation leads to its dissociation from β-catenin, disruption of β-catenin/VE-cadherin complexes, and β-catenin translocation from the membrane to cytosolic compartments.

**H2O2-Induced Vascular Albumin Hyperpermeability and Lung Edema Formation Requires Cav-1 Phosphorylation in Mouse Lungs**

To address whether phosphorylation of Cav-1 mediates H2O2-induced increase in pulmonary vascular hyperpermeability and edema formation, we infused H2O2 into isolated, perfused mouse lungs. As shown in Figure 7A, H2O2 (0.5 mmol/L) induced a robust increase in Cav-1 phosphorylation in WT mouse lungs. We chose 0.5 mmol/L H2O2 as this dose reliably induced an increase in 125I-albumin permeability (PS product) (Figure 7B) and lung edema formation (wet/dry ratio) (Figure 7C) in the isolated lung. In contrast, in cav-1−/− mouse lungs, which exhibit reduced basal albumin PS (Figure 7B), H2O2 did not induce an increase in albumin and fluid permeability. To assess the role of Cav-1 phosphorylation in these permeability enhancing-effects of H2O2 in vivo, we used liposome-mediated Cav-1 cDNA delivery to rescue vascular endothelial Cav-1 expression in cav-1−/− mouse lungs with either WT-Cav-1 or the phosphorylation-defective Y14F-Cav-1 mutant (Figure 7A). Consistent with the in vitro data, H2O2 induced an increase in albumin PS product and lung wet/dry weight ratio in WT-Cav-1–expressing mouse lungs, whereas these effects were not reconstituted on expression of Y14F-Cav-1 in cav-1−/− lungs (Figure 7B and 7C).

**Discussion**

This study provides strong evidence indicating that Cav-1 phosphorylation in endothelial cells plays a fundamental role in the mechanism of oxidant-induced pulmonary vascular
hyperpermeability. Oxidant-induced increase in caveolae-mediated albumin transport and decrease in endothelial barrier integrity were both dependent on tyrosine phosphorylation of Cav-1, suggesting Cav-1 phosphorylation is a common signal regulating transcellular and paracellular permeability pathways in lung microvessels. The molecular mechanisms regulating transcellular and paracellular permeability induced by Cav-1 phosphorylation following oxidant stress appear to be different. The increase in transcellular permeability in response to low concentration of oxidant is entirely mediated by Src-dependent Cav-1 phosphorylation, whereas increased paracellular permeability induced by high concentration of oxidant depends on both Src- and c-Abl-mediated Cav-1 phosphorylation and subsequent regulation of β-catenin localization.

We established that exposure of pulmonary microvascular endothelial cells to pathophysiological concentrations of H$_2$O$_2$ (0.05 to 0.8 mmol/L) stimulated Cav-1 phosphorylation in a concentration-dependent manner. Our results are distinct from previous reports that showed that higher concentrations of H$_2$O$_2$ (>1 mmol/L) were required to induce an increase in Cav-1 phosphorylation in cultured bovine pulmonary artery, aortic, and human umbilical vein endothelial cells.

Cav-1 Tyr14 is a principal target for Src kinase phosphorylation in response to oxidative stress, and thus we predicted that treatment of endothelial cells with Src inhibitor...
PP2 to inactivate Src kinase would block H₂O₂-induced Cav-1 Tyr14 phosphorylation. However, our results demonstrated that the level of phospho-Cav-1 following PP2 treatment was only decreased by approximately 65%, suggesting the existence of a Src-independent pathway, particularly when higher concentrations of H₂O₂ were used to stimulate Cav-1 phosphorylation. Depletion of c-Abl with specific siRNA demonstrated that c-Abl also contributed to H₂O₂-induced Cav-1 phosphorylation in pulmonary microvascular endothelial cells. In contrast to previous findings that c-Abl phosphorylates Cav-1 independently of Src,22,29 our results showed that c-Abl activation and subsequent Cav-1 phosphorylation was in part dependent on Src activity. It is likely that Src family kinases directly phosphorylate tyrosine residues in the kinase domain of c-Abl, leading to enhanced activity.30

Studies from our laboratory6,7,9–11 and others16,17 have suggested that Cav-1 phosphorylation plays an essential role in the mechanisms regulating caveolae formation and caveolae-mediated endocytosis and transcytosis of albumin in microvascular endothelial cells. Using 125I-albumin and Alexa 488–albumin tracers, we observed that H₂O₂ (0.05 to 0.2 mmol/L) increased both the endocytosis and transcytosis of albumin. The cholesterol-depleting agent methyl-β-cyclodextrin and siRNA-induced depletion of Cav-1 prevented H₂O₂-induced endocytosis and transcytosis of albumin, indicative of a caveolae-dependent mechanism. Furthermore, the H₂O₂-induced increase in endocytosis and transcytosis of 125I-albumin in WT-Cav-1–expressing cells was abolished in phospho-defective Cav-1 mutant–overexpressing endothelial cell line. Noteworthy is that Src inhibitor PP2 but not c-Abl siRNA inhibited endocytosis and transcytosis of albumin. These findings provide further support for the concept that Src-dependent phosphorylation of Cav-1 mediates oxidant-induced transepithelial transport via caveolae in pulmonary microvascular endothelial cells.

The present data show, for the first time, that Cav-1 phosphorylation, which is an important mechanism regulating caveolae-mediated transepithelial permeability,7–11 is also a crucial regulator of oxidant-induced paracellular hyperpermeability. Expression of a Cav-1 mutant lacking the tyrosine phosphorylation site resulted in the inhibition of H₂O₂-induced decrease in TER, a measure of loss of endothelial junctional integrity. Furthermore, overexpression of WT-Cav-1 in endothelial cells reduced the threshold concentration of H₂O₂ needed to disrupt endothelial cell–cell junctions, presumably because of an increase in the total amount of phosphorylated Cav-1.6,22 In agreement with these findings, inhibition of Cav-1 phosphorylation by either Src inhibitor PP2 or c-Abl siRNA partially attenuated H₂O₂-induced decrease in TER. These results highlight the importance of Cav-1 Tyr14 phosphorylation in the regulation of H₂O₂-induced endothelial barrier disruption. A recent study showed that oxidized phospholipid-induced Cav-1 phosphorylation is associated with sphingosine 1-phosphate receptor signaling in caveolae that leads to endothelial barrier enhancement.31 However, it remains unclear whether Cav-1 phosphorylation per se plays an important signaling role in strengthening the endothelial barrier in this scenario.

In cav-1−/− and Cav-1 siRNA–treated mice, an increase in the number of open interendothelial junctions in pulmonary capillaries and veins was observed, indicating that there is a compensatory mechanism that increases basal paracellular permeability in the absence of the vesicular (transcellular) permeability pathway.33 In our study, however, both transepithelial and paracellular permeability pathways were stimulated on Cav-1 phosphorylation, suggesting these 2 pathways are affected in a coordinate manner in response to oxidative stress. Consistent with previous findings,6,34 the present study demonstrated that the increase in transepithelial permeability occurred before an increase in paracellular permeability. These data suggest that the mechanism mediating increased transepithelial permeability, namely Src phosphorylation of Cav-1, is also a trigger signal for increasing paracellular permeability. Thus, it is likely that the cooperative mechanism between the 2 pathways via Cav-1 phosphorylation may play an important role in the development and progression of vascular hyperpermeability and inflammatory lung injury.

Recent studies indicate the important role of Cav-1 in stabilization of adherens junctions.19,35 Knockdown of Cav-1 was shown to be associated with a significant decrease in both VE-cadherin and β-catenin localized at interendothelial junctions.19 In our studies with confluent endothelial cell monolayers, we observed that Cav-1 colocalized with adherens junction–associated protein β-catenin, consistent with a previous study conducted in MDCK cells.19 Confocal imaging and immunoprecipitation studies indicated that expression of endothelial cells to higher concentrations of H₂O₂ caused a marked dissociation of β-catenin from Cav-1, as well as VE-cadherin. H₂O₂-induced Src activation may also promote direct phosphorylation of VE-cadherin and/or β-catenin, inducing their dissociation from cytoskeletal anchors.1,4,36 However, using a Cav-1–overexpressing endothelial cell line, we showed that dissociation of β-catenin from Cav-1 and VE-cadherin following exposure to H₂O₂ was dependent on Cav-1 Tyr14 phosphorylation as these changes did not occur in endothelial cells expressing the phosphorylation-defective Cav-1 mutant. Furthermore, Cav-1 phosphorylation promoted the translocation of β-catenin from the membrane to cytosol. These data support a model in which, when phospho–Cav-1 is present in cell–cell junctions at significant levels, it negatively regulates adherens junction stability by decreasing the association between VE-cadherin and β-catenin. At this stage, we cannot rule out the possibility that Cav-1–mediated endocytosis of VE-cadherin and/or β-catenin may lead to the disassembly of adherens junction complexes and thereby contribute to the increase in paracellular permeability.37 Although Cav-1 phosphorylation is known to regulate caveolae-mediated endocytosis, as also shown herein, this explanation of β-catenin internalization is less plausible, given that the coimmunoprecipitation of β-catenin with Cav-1 decreased on Cav-1 phosphorylation.

The significance of Cav-1 phosphorylation as a molecular signal in endothelial permeability regulation is not clear. Phosphorylation of Cav-1 may reduce interactions between
Cav-1 and other signaling proteins that are negatively regulated by association with Cav-1. Increased Cav-1 phosphorylation may also be necessary for translocation of proteins normally associated with Cav-1 at the plasma membrane. Our data support these hypotheses, as indicated by the dissociation of Cav-1 and β-catenin and subsequent translocation of β-catenin from the membrane to the cytosol in a manner dependent on increased Cav-1 phosphorylation induced by high concentration of H₂O₂.

We also found that H₂O₂-induced increase in pulmonary vascular permeability was dependent on Cav-1 Tyr14 phosphorylation in vivo, consistent with our findings from pulmonary endothelial monolayers. Mice lacking Cav-1 expression were resistant to H₂O₂-induced increase in pulmonary vascular hyperpermeability and edema formation. The pulmonary vascular response to H₂O₂ was rescued by adding back WT-Cav-1 to the cav-1−/− pulmonary vasculature but not by expression of the phospho-defective Cav-1 mutant. These findings, together with those from pulmonary endothelial cells, strongly suggest that Cav-1 phosphorylation plays a pivotal role in the mechanism of increased pulmonary vascular permeability and edema formation evoked by oxidants.

In summary, our findings implicate Cav-1 phosphorylation as a critical mechanism mediating oxidant-induced pulmonary vascular hyperpermeability. H₂O₂ stimulated caveolae-mediated transcellular transport and the opening of interendothelial junctions in a manner dependent on Cav-1 phosphorylation. Although these studies focused on oxidative stress as a means of increasing endothelial permeability and inducing acute lung injury, it is possible that our findings have broader applicability in understanding the mechanisms and development of inflammatory pulmonary vascular hyperpermeability. Therefore, therapeutic inhibition of Cav-1 phosphorylation may be an effective means of limiting lung vascular injury by preventing increased transcellular albumin permeability and stabilizing the endothelial junctional barrier.

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Disclosures
None.

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Supplement Material

Animals

Caveolin-1 null (cav-1⁻⁻) mice and wild-type B6/129SJ mice were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were housed in microisolator cages under specific pathogen-free conditions, fed with autoclaved food, and used in experiments at 8-12 weeks of age. All studies using mice were approved by the University of Illinois Institutional Animal Care and Use Committee.

Endothelial cell culture

Rat lung microvascular endothelial cells (RLMVECs) were obtained from Vec Technologies (Rensselaer, NY). For monolayer cultures, the cells were plated at a density of 1 × 10⁵ cells/cm² on fibronectin-coated dishes in MCDB-131 complete medium supplemented with 10% FBS, incubated (37°C) under a humidified atmosphere of 5% CO₂-95% air, and used at passages 3-5. Confluent monolayers formed on culture dishes, microporous Transwell filter inserts, or glass coverslips within 24-48 h. RLMVEC lines stably expressing myc-tagged wild-type caveolin-1 (WT-Cav-1) or non-phosphorylatable caveolin-1 mutant (Y14F-Cav-1) were generated as described previously.¹² Endogenous caveolin-1 levels were not altered in these cell lines, but Y14F-Cav-1 acts as a dominant-negative and reduces caveolae formation by ~50%⁴⁻⁵. RLMVEC monolayers were serum-deprived for 2 h prior to experiments.

Antibodies and fluorescent probes

The antibodies (Abs) and fluorescent probes used were obtained from the following sources. Caveolin-1 and β-actin monoclonal Abs and pY418-Src polyclonal Ab were from Transduction Laboratories and Cell Signaling, respectively; c-Src polyclonal Ab, and horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG, VE-cadherin, β-catenin, and c-Abl polyclonal Abs
were from Santa Cruz Biotechnology; DAPI, goat anti-mouse and anti-rabbit IgG labeled with Alexa 488-conjugated albumin and Alexa 546 phalloidin were from Molecular Probes.

**Treatment with drugs**

Confluent RLMVEC monolayers were incubated with the caveolae disrupting agent methyl-β-cyclodextrin (2.0 mmol/L)\(^1\), or Src inhibitor PP2 (15 μmol/L)\(^1\) in HBSS for 15 min at 37 °C, followed by two washes with HBSS.

**siRNA transfection**

Caveolin-1 siRNA duplex oligonucleotides and c-Abl siGENOME SMART pool were purchased from Dharmacia (Lafayette, CO). The sequences of caveolin-1 siRNA and negative control duplex were 5’-UCUGUGAUCCACUCUUUGAUU-3’ and 5’-UAAGGCUAUGAAGAGAUAC-3’, respectively. Depletion of caveolin-1 and c-Abl in RLMVECs was optimal using 50 and 80 nmol/L siRNA, respectively, at 50-70% confluent monolayers in complete MCDB-131 medium + 10% FBS as described by the manufacturer. All experiments were performed 48 h after siRNA transfection.

**Endocytosis of \(^{125}\text{I-}\text{albumin}\)**

Endocytosis of \(^{125}\text{I-}\text{albumin}\) by RLMVECs was assayed as described previously.\(^1\) Briefly, confluent RLMVEC monolayers were seeded in six-well plates (1.0-1.2 × 10\(^6\) cells/35mm). Unlabeled albumin in HBSS was added at a final concentration of 0.1 mg/mL in four wells and 100 mg/mL in the other two wells. \(^{125}\text{I-}\text{albumin}\) (1 × 10\(^6\) cpm) was then added into each well and endocytosis of \(^{125}\text{I-}\text{albumin}\) assessed after incubating at 37°C for 30 min. Cells were washed three times with ice-cold acid wash buffer (0.5 mol/L NaCl and 0.2 mol/L acetic acid, pH 2.5) and another three times in cold HBSS to remove \(^{125}\text{I-}\text{albumin}\) attached to the cell surface. The cells were lysed with 1 mL Tris-HCl buffer (0.05 mol/L Tris-HCl, 1% Triton X-100 and 0.5%
SDS, pH 7.4) and the radioactivity determined using a gamma counter (Packard Instruments, Downers Grove, IL). Specific uptake of $^{125}$I-albumin was estimated by subtracting the non-specific cell-associated activity (determined in the presence of 100 mg/mL unlabeled albumin) from the total (determined in the presence of 0.1 mg/mL unlabeled albumin). Specific uptake was normalized to cell protein (determined by Bicinchoninic Acid method) and expressed in units of cpm/mg cell protein.

**Determination of fluorescent albumin endocytosis and immunostaining by confocal microscopy**

For determination of fluorescent albumin uptake by RLMVECs, we utilized confluent RLMVEC monolayers grown on glass coverslips. Monolayers were treated with fluorescently-tagged albumin (Alexa 488-labeled bovine serum albumin, at 50 µg/mL, plus 0.5 mg/mL unlabeled albumin in HBSS). Unincorporated probe was removed by rinsing with HBSS. Internalized fluorescent albumin was viewed by confocal microscopy in optical sections midway through the cell.

To determine the co-localization of caveolin-1 and β-catenin, cells were exposed to H$_2$O$_2$ for 30 min, rinsed quickly with ice-cold HBSS, and fixed with 3% paraformaldehyde. Cells were permeabilized for 30 min with 0.1% Triton X-100 in HBSS and then incubated with anti-caveolin-1 or β-catenin antibody followed by incubation with Alexa-labeled secondary antibody for another 2 h. Cell nuclei were labeled with 4',6-diamidino-2-phenyl indole dihydrochloride (DAPI). Briefly, the monolayer cultures were rinsed and fixed with 3% paraformaldehyde in HBSS; DAPI (1 µg/ml) was added immediately after cell fixation for 20 min, the cells were rinsed three-times, and finally mounted on glass slides using ProLong antifade mounting medium (Molecular Probes). Confocal images were acquired with a laser-scanning confocal
microscope (Zeiss LSM 510 META) using Hg lamp and UV-filter set to detect DAPI [band pass (BP) 385-470 nm emission], 488 nm excitation laser line to Alexa 488 (BP505-550 nm emission), respectively. Optical sections had a thickness of <1 µm (pinhole set to achieve 1 Airy unit).

**Transendothelial ¹²⁵I-albumin permeability**

RLMVECs were grown on fibronectin-coated microporous polyester Transwell membranes (12 wells, 1 cm² growth area, 0.4 µm pore size; Corning Costar, Cambridge, MA). The membrane inserts (inner well) were filled with a total of 0.5 mL HBSS containing ¹²⁵I-albumin (1 × 10⁶ cpm) in the presence of unlabeled albumin (0.1 mg/mL or 100mg/mL). The lower well was filled with 1.5 mL of HBSS containing unlabeled albumin of the same osmolarity as the inner well, thus fluid levels and osmotic pressure in the "upper" and "lower" wells were equalized to eliminate hydrostatic and osmotic pressure differences across the monolayer. Aliquots of 200 µL were sampled from the lower chamber and gamma radioactivity of the samples was measured. Transendothelial ¹²⁵I-albumin permeability was calculated from the flux of radiolabeled albumin across the cell monolayer, as described previously⁴, where caveolae-mediated transcytosis is determined as the displaceable fraction of the total flux. Albumin permeability is expressed in units of µL·min⁻¹·cm⁻².

**Assessment of barrier function by transendothelial electrical resistance**

Endothelial barrier function was assessed using an electric cell substrate impedance sensor to measure real-time changes in electrical resistance across endothelial monolayers as described previously.¹⁵ RLMVECs were grown to confluence in wells containing gold-plated microelectrodes. The small electrode and the larger counter electrode were connected to a phase-sensitive lock-in amplifier, and the voltage difference between the large electrodes was
monitored, stored, and processed with a personal computer. Data are presented as the change in the resistive (in-phase) portion of the impedance normalized to its initial value at time zero.

**Subcellular fractionation**

Confluent cells were exposed to H$_2$O$_2$ for 30 min and washed with HBSS, and lysed in a detergent-free buffer (50 mmol/L Tris-HCl, pH 7.4, 0.1 mmol/L EDTA, and protease inhibitor mixture). Samples were sonicated on ice four times using 30-s bursts with 30-s intervals and centrifuged at 100,000 x g for 1 h in a TLA55 rotor (Beckman Instruments, Palo Alto, Ca) at 4 °C. The supernatant (cytosolic fraction) was compared with the pellet (membrane fraction). Equal amounts of protein were subjected to SDS-PAGE and Western blotting.

**Western blot analysis and immunoprecipitation**

Cells were lysed in lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 0.25% sodium deoxycholate, 1.0% NP-40, 0.1% SDS, 1 mmol/L Na$_3$VO$_4$, 1 mmol/L NaF, 1mmol/L PMSF, and protease inhibitor mixture). Protein concentrations were determined by the Bicinchoninic Acid assay. For immunoprecipitation, cells were lysed in ODG buffer containing 50 mmol/L Tris-HCl (PH 8.0), 150 mmol/L NaCl, 1 mmol/L EDTA, 20 mmol/L NaF, 2% Octyl-D-glucoside, 5% glycerol, 1 mmol/L PMSF, 1 mmol/L Na$_3$VO$_4$ and protease inhibitor mixture. Samples were precleared for 1 h at 4 °C using 1μg of control IgG (normal mouse IgG) together with protein A/G PLUS-Agarose, and then incubated overnight at 4 °C with primary antibody, followed by addition of 25 μL protein A/GPLUS-agarose and further incubation at 4°C for 2 h. Equal amounts of lysate protein were separated by SDS-PAGE and transferred to nitrocellulose membranes, blocked, and then incubated with relevant blotting Ab. The protein bands were determined using the ECL reagent (Pierce). Relative band densities of the various proteins were measured from scanned films using ImageJ Software (NIH).
Liposome preparation and in vivo gene delivery

Rescue studies were made in mouse lungs from cav-1−/− mice by liposome-mediated plasmid DNA transfection.1,7 Myc-tagged WT-Cav-1 and Y14F mutant Cav-1 cDNA in pcDNA6 plasmid vector were used for caveolin-1 repletion studies. The liposome was prepared as previously described and intravenously injected in mice.1,7 Briefly, the mixture consisting of dimethyldioctadecylammonium bromide and cholesterol (1:1 molar ratio) was dried using the Rotavaporator (Brinkmann, Westbury, NY) and dissolved in 5% glucose. The complex consisting of the transgene (50 µg per mouse) and liposomes was combined at the ratio of 1 µg of DNA to 8 nmol/L of liposomes. Successful expression of caveolin-1 was confirmed by Western blot of lung homogenates.

Lung preparation

Mouse lungs were isolated as described.1,8 Briefly, after the animals were anesthetized and ventilated, a thoracotomy was performed and the pulmonary artery was cannulated in situ for perfusion of the lungs with a modified Krebs-Henseleit solution and mounted on a perfusion apparatus. The composition of the solution was as follows: 118 mmol/L NaCl, 4.7 mmol/L KCl, 1.0 mmol/L CaCl₂, 1.0 mmol/L MgCl₂, 5.0 mmol/L HEPES, 11 mmol/L glucose, and 0.025 mmol/L EDTA (pH 7.35-7.45), and BSA (5 g/100 mL). The lung preparation was perfused via the pulmonary artery at a constant flow (2 mL/min) and venous pressure (3 cmH₂O), and pulmonary artery pressures of 8 ± 2 cm H₂O. Pulmonary arterial pressure and lung weight were continuously monitored during experiments. Labtech software (Andover, MA) was used to control data acquisition and storage.

Permeability-surface area (PS) product

Albumin PS was determined following 30 min of administration of either saline or H₂O₂ (0.5
mmol/L) using the "single-sample" technique. Briefly, the isolated lung was perfused with $^{125}$I-albumin (~80,000 counts/mL) for 3 min followed by a 6-min washout with Krebs solution containing unlabeled albumin (5 g/100 mL) to remove cell surface and circulating $^{125}$I albumin. At the end of the experiment, the lungs were immediately removed, cleaned of connective tissue, weighed, and counted for gamma radioactivity. $PS$ product (in mL·min$^{-1}$·g$^{-1}$ dry lung) was calculated with the formula $A/(Cp \cdot t)$, where $A$ and $Cp$ are concentrations of tracer albumin in the tissue (in counts/g) and in the perfusate (in counts/mL), respectively, and $t$ is the perfusion time for tracer albumin (3 min).

**Wet/dry lung weight ratio**

At the end of the experiment, lungs were weighed, dried, and reweighed. Wet/dry lung weight ratio was used as an index of accumulated lung water. For determination of lung dry weight, lung tissue was dried in an oven to a constant weight (60 °C for 72 h).

**Drugs and reagents**

The chemicals and reagents used were obtained from Sigma Chemical Company (St. Louis, MO) unless otherwise stated. HBSS containing NaHCO$_3$ (4.2 mmol/L) and HEPES (10 mmol/L) was adjusted to pH 7.4. Bovine serum albumin (fraction V, 99% pure, endotoxin free, cold alcohol precipitated) and methyl-β-cyclodextrin were dissolved in HBSS. Complete MCDB-131 medium and fetal bovine serum were obtained from Vec Technologies (Rensselaer, NY), and from Hyclone (Logan, UT), respectively. $^{125}$I-albumin was purchased from PerkinElmer, Inc. (Wellesley, MA). Trichloroacetic acid precipitation analysis was used to confirm the purity; free $^{125}$I was removed from $^{125}$I-labeled albumin with a Sephadex G25 column such that the contaminant free $^{125}$I in the tracer used contributed <0.3% of the total counts.

**Statistical analysis**
One-way analysis of variance and Student’s Newman-Keuls test for post-hoc comparisons were used to determine differences between control and experimental groups. Student’s t-test was performed for paired samples. Data were expressed as mean ± SEM. Differences were considered significant at $P < 0.05$. 
References


Legends to Supplemental Figures

**Online Figure I.** H$_2$O$_2$ induced $Src$ and c-Abl activation and caveolin-1 (Cav-1) phosphorylation. Densitometric analysis of phosphorylated caveolin-1 (p(Y14) Cav-1), $Src$ (p(Y418) $Src$) and c-Abl (c-Abl-pY) in RLMVEs after different treatments (n = 3 samples). The density of proteins in each control (untreated) group was used as a standard (1 arbitrary unit) to compare relative densities in the other groups. $^*P < 0.05$, compared to control groups (untreated); $^†P < 0.05$, compared to H$_2$O$_2$ (0.6 mM) groups. (A) Relative densities of the bands of p(Y14) Cav-1 and p(Y418) $Src$ expression, as shown in Figure 1A. (B) Relative densities of the bands of p(Y14) Cav-1 expression, as shown in Figure 1B. (C) Relative densities of the bands of p(Y14) Cav-1 expression, as shown in Figure 1C. (D) Relative densities of the bands of c-Abl-pY expression, as shown in Figure 1D. (E) Relative densities of the bands of p(Y14) Cav-1 expression, as shown in Figure 1E. (F) Relative densities of the bands of c-Abl-pY expression, as shown in Figure 1F.

**Online Figure II.** H$_2$O$_2$ increases caveolae-mediated transendothelial $^{125}$I-albumin permeability in Rat lung microvascular endothelial cell (RLMVEC) monolayers. (A) Effects of pretreatment of RLMVECs with methyl-β-cyclodextrin (MβCD) and downregulation of caveolin-1 (Cav-1) with siRNA on transendothelial $^{125}$I-albumin permeability. (B) Effects of pretreatment of RLMVECs with MβCD and downregulation of Cav-1 with siRNA on endothelial endocytosis of $^{125}$I-albumin. (C) Western blots showing Cav-1 knockdown. Endothelial cells were transfected with Cav-1 siRNA or scrambled (Sc) siRNA. Results are typical of 3 experiments. n = 6-8/group (A and B). $^*P < 0.05$, compared to control (untreated) groups; $^†P < 0.05$ compared with respective H$_2$O$_2$ (0.2 mM) groups.
Online Figure III. Immunofluorescent staining of β-catenin and F-actin in RLMVECs over-expressing WT vs. Y14F-Cav-1. Cells were grown to confluence and treated with H₂O₂ (0.2 mM) for 30 min. After fixation and permeabilization, cells were incubated with anti-β-catenin primary antibody followed by FITC-conjugated secondary antibody (green). The F-actin and nucleus were stained with Alexa 546 phalloidin (red) and DAPI (blue), respectively. In the third panel (WT + 0.2 mM H₂O₂), note gap formation (arrow), increased β-catenin translocation into the cytosol (arrow head), and absence of β-catenin in areas where gaps in the monolayer have formed. The fourth panel (Y14F-Cav-1 + 0.2 mM H₂O₂) shows no gap formation as well as β-catenin translocation in response to H₂O₂. Scale bars =10 µm. WT = wild type-caveolin-1 expressing cells; Y14F = Y14F-caveolin-1 mutant expressing cells
Online Figure II

A. 125I-Albumin Endocytosis (% of control)

B. Transendothelial 125I-Albumin Permeability (% of control)

C. Western Blot Analysis

- Cav-1
- β-actin
Online Figure III

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