Endothelial Dysfunction and Claudin 5 Regulation during Acrolein-Induced Lung Injury

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An integral membrane protein, Claudin 5 (CLDN5), is a critical component of endothelial tight junctions that control pericellular permeability. Breaching of endothelial barriers is a key event in the development of pulmonary edema during acute lung injury (ALI). A major irritant in smoke, acrolein can induce ALI possibly by altering CLDN5 expression. This study sought to determine the cell signaling mechanism controlling endothelial CLDN5 expression during ALI. To assess susceptibility, 12 mouse strains were exposed to acrolein (10 ppm, 24 h), and survival monitored. Histology, lavage protein, and CLDN5 transcripts were measured in the lung of the most sensitive and resistant strains. CLDN5 transcripts and phosphorylation status of forkhead box O1 (FOXO1) and catenin (cadherin-associated protein) beta 1 (CTNNB1) proteins were determined in control and acrolein-treated human endothelial cells. Mean survival time (MST) varied more than 2-fold among strains with the susceptible (BALB/cBy) and resistant (129X1/Sv) strains (MST, 17.3 ± 1.9 h vs. 41.4 ± 5.1 h, respectively). Histological analysis revealed earlier perivascular enlargement in the BALB/cBy than in 129X1/Sv mouse lung. Lung CLDN5 transcript and protein increased more in the resistant strain than in the susceptible strain. In human endothelial cells, 30 nM acrolein increased CLDN5 transcripts and increased p-FOXO1 protein levels. The phosphatidylinositol 3-kinase inhibitor LY294002 diminished the acrolein-induced increased CLDN5 transcript. Acrolein (300 nM) decreased CLDN5 transcripts, which were accompanied by increased FOXO1 and CTNNB1. The phosphorylation status of these transcription factors was consistent with the observed CLDN5 alteration. Preservation of endothelial CLDN5 may be a novel clinical approach for ALI therapy.

Keywords: ARDS; perivascular edema; vascular permeability; smoke inhalation; carboxyl stress

Adhesive structures between adjacent cells, including tight and adherens junctions, enable the establishment of cell polarity, differentiation, and survival and are critical to the maintenance of tissue integrity (1). Tight junctions consist of a macromolecular complex of numerous adhesive molecules, including occludin, tight junction proteins (zona occludins), junctional adhesion molecules, and claudins (2). Tight junctions are often located apically with respect to adherens junctions (composed mainly of cadherins), as in epithelial cells, but can be intermingled throughout cell-cell contact areas, as in endothelial

CLINICAL RELEVANCE

One of the major histological features of acute lung injury is pulmonary edema, which results from increased epithelial and endothelial permeability and decreased clearance of edema fluid by the alveolar epithelium. In contrast to other claudins (CLDN) in the lung, CLDN5, which is expressed weakly in the epithelium, is expressed strongly in endothelium of normal lung. However, its role in acute lung injury has had modest attention in the past. Acrolein-induced acute lung injury was marked by perivascular edema in mice. This is accompanied by a compensatory increase in CLDN5 transcripts, which was more evident in a resistant than in a sensitive mouse strain. Acrolein (30 nM) stimulated phosphorylation of FOXO1 protein and increased CLDN5 transcripts, whereas 300 nM acrolein stimulated FOXO1 and CTNNB1 protein levels and decreased CLDN5 transcripts. These events are consistent with the rapid increase in vascular permeability and could provide a critical target for future pharmacological intervention during acute lung injury.

cells (3). Tight junction strands serve as a physical barrier to regulate solutes and water movement through the paracellular space between epithelial or endothelial cells. Compromised barrier function of adhesive structures is a common event in several diseases, including ischemic brain disease (4, 5), Crohn's disease (6), and acute lung injury (ALI) (7–9).

In spite of considerable medical achievements, treatment for ALI is limited to supportive care, and recent estimates indicate that mortality remains high (~ 30 – 40% or 74,500 deaths per year in the United States) (10). ALI can be induced indirectly (e.g., by sepsis or trauma) or directly (e.g., by smoke inhalation) (8). One major histological feature of ALI is pulmonary edema, which results from increased epithelial and endothelial permeability and decreased clearance of edema fluid by the alveolar epithelium. These events, when combined with decreased surfactant-associated protein B synthesis, disrupt surfactant surface tension and ultimately produce respiratory failure (11). These barrier functions require adequate control of paracellular tight junctions; however, our current understanding of the structural components and regulation of tight junctions in the lung is insufficient.

The claudin (CLDN) family consists of 24 tetraspan transmembrane proteins, each of which has a tissue-restricted expression pattern (12–14). In the normal lung, bronchiolar epithelial cells mainly express CLDN1, -3, -4, -7, and -18, and alveolar type II epithelial cells mainly express CLDN3, -4, -7, and -18 (7, 9, 15). In epithelial cells, transgenic expression of CLDN1 with CLDN3 increased transepithelial resistance and decreased paracellular permeability, whereas CLDN4 confers

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selective ion transport function without effecting paracellular solute permeability (16). Recently, Wray and colleagues reported that CLDN4 inhibition decreased transepithelial resistance without altering paracellular permeability in primary rat and human epithelial cells (9). In addition, *in vivo* CLDN4 epithelial expression was an early event in ALI, leading the authors to conclude that CLDN4 represents a possible mechanism to limit pulmonary edema.

In contrast to other claudins in the lung, CLDN5, while expressed weakly in the epithelium, is expressed strongly in endothelium of normal lung and is intense in endothelium in usual interstitial pneumonia (15). Newborn gene-targeted *Cldn5*^(-/-) mice die within 10 hours of birth possibly due to altered permeability of the blood-brain barrier (17). When CLDN5 was transfected into airway epithelial cells, paracellular permeability increased even in the presence of excessive CLDN1 and CLDN3 (18). Moreover, inducing CLDN5 expression in leaky rat lung endothelial cells can enhance paracellular barrier function against large (but not small) molecules (19). One of the key pathognomic features of ALI is perivascular edema, in part due to endothelial dysfunction (20). In this study, we examine the endothelial dysfunction and CLDN5 expression during lung injury induced by acrolein, a key irritant in smoke.

MATERIALS AND METHODS

Experimental Design

Twelve inbred mouse strains were exposed to filtered air (control) or acrolein (10 ppm, 24 h) generated and monitored as described previously (21–23), and survival time was recorded. Detailed analyses, which included measurement of lung CLDN5 mRNA and protein, lung histology, and bronchoalveolar lavage (BAL), contrasted the response of the most sensitive (BALB/cByJ) and resistant (129X1/SvJ) strains after exposure to filtered air (0 h, control) or acrolein (10 ppm, 6 or 12 h). Additional tests were performed with a cell line derived from the fusion of human umbilical vascular endothelial cells with the lung carcinoma cell line A549 (EA.hy926) or primary human lung microvascular endothelial cells. Confluent cells were washed three times in Dulbecco's modified PBS (DPBS), incubated for 30 minutes, and exposed to acrolein (≤ 4 h) in DPBS. Assays include measurements of Claudin 5 transcripts, catenin (cadherin-associated protein), beta 1 (CTNNB1), phosphorylated CTNNB1 (Ser 552) (p-CTNNB1), forkhead box O1 (FOXO1), phosphorylated FOXO1 (Ser256) (p-FOXO1), thymoma viral proto-oncogene 1 (AKT), and phosphorylated AKT (p-AKT). EA.hy926 cells were also treated with vehicle (DMSO), 30 nM acrolein, or 30 nM acrolein and 10 μ M LY294002 [2-(4-Morpholinyl)-8-phenyl-4H-1-benzopyran-4-one] (LY), a phosphatidylinositol 3-kinase (PI3K) inhibitor. Additional details of the methods are contained in the online supplement.

Mouse Strains Vary in Sensitivity to Acrolein-Induced ALI

The mean survival time (MST) varied among the 12 mouse strains exposed to acrolein (10 ppm, 24 h) (Figure 1A). The most susceptible (BALB/cByJ) and resistant (129X1/SvJ) mouse strains differed by more than 2-fold (MST 17.3 ± 1.9 h vs. 41.4 ± 5.1 h, respectively). Survival curves for the sensitive (BALB/cByJ) and resistant (129X1/SvJ) mouse strains were significantly different ($P < 0.001$) (Figure 1B). Thus, the BALB/cByJ and 129X1/SvJ were selected for subsequent analyses. Histological assessment of lung tissue from the sensitive BALB/cByJ strain demonstrated perivascular enlargement present within 12 hours of acrolein exposure (Figure 2C), as compared with strain-matched control (Figure 2A). In acrolein-treated BALB/cByJ mouse lung, leukocytes were present in the perivascular space (Figure 2C) and focal areas in the alveolar interstitium (Figure 2E), with thickening of alveolar wall (Figure 2E). The perivascular enlargement included an increased distance between the tunica media and the tunica adventitia in lung of BALB/cByJ mice (Figure 2G). Neither perivascular enlargement nor leukocytes was evident in the resistant 129X1/SvJ strain after 17 hours of acrolein exposure (Figures 2D and

2F) compared with strain-matched control mice exposed to filtered air (Figure 2B). Similarly, BAL protein increased more in sensitive (BALB/cByJ) mice than in resistant (129X1/SvJ) mice after 12 hours of acrolein exposure ($P < 0.001$) (see Figure E1 in the online supplement).

Lung CLDN5 Transcript Levels Increased during Acrolein-Induced ALI

Because the endothelial tight junction protein CLDN5 had previously been associated with endothelial permeability (18, 19), CLDN5 transcript and protein levels were measured after 6 and 12 hours of acrolein exposure (Figure 3). At 12 hours, lung CLDN5 transcript (Figure 3A) and protein (Figure 3C) increased more in resistant (129X1/SvJ) mice compared with strain-matched control mice ($P < 0.001$), whereas the lung CLDN5 protein increased less (Figure 3C), and transcript levels in the sensitive (BALB/cByJ) mice were not significantly different from control mice.

Figure 1. (A) Mean survival time (MST) of 12 mouse strains after acrolein exposure. MST varied among mouse strains, with the most susceptible (BALB/cByJ) and resistant (129X1/SvJ) mouse strains varying more than 2-fold (MST, 17.3 ± 1.9 h vs. 41.4 ± 5.1 h, respectively). Mice ($n = 9$ mice per strain) were exposed to acrolein (10 ppm, 24 h) under specific pathogen-free conditions. (B) Survival curves for the sensitive (BALB/cByJ) and resistant (129X1/SvJ) mouse strains. Between-strain survival time was significantly different using the Kaplan-Meier method ($P < 0.001$).

Acrolein Alters CLDN5 Transcript Levels in EA.hy926 Hybrid Cells and Human Lung Microvascular Endothelial Cells

To begin to determine a mechanism by which acrolein altered CLDN5 transcripts, we exposed EA.hy926 cells (a hybrid cell line) or human lung microvascular endothelial cells to acrolein and measured CLDN5 transcript levels (Figure 4). Within 1 hour of exposure to 30 nM acrolein, CLDN5 transcripts increased and were significantly ($P < 0.001$) different from control (DPBS) in EA.hy926 cells (Figure 4A). This effect was dose dependent (Figure 4B), with CLDN5 mRNA increasing at doses from 1 to 30 nM acrolein (4 h) and decreasing at doses of 100 or 300 nM as compared with cells exposed to DPBS (control) alone. Similarly, in human lung microvascular endothelial cells, CLDN5 mRNA increased after 30 nM acrolein and CLDN5 mRNA decreased after 100 or 300 nM acrolein treatment (Figure 4C).

Acrolein Increased p-FOXO1 Protein in EA.hy926 Cells

Acrolein could activate a variety of signaling pathways that regulate the expression of junctional proteins. CLDN5 expression is normally enhanced by CTNNB1 or repressed by nonphosphorylated FOXO1

Figure 2. Mouse strains vary in pathophysiological response to acrolein-induced lung pathology. Histological assessment of lung tissue from (A) control (filtered air) BALB/cByJ mice, (B) control (filtered air) 129X1/SvJ mice, (C, E) acrolein (10 ppm, 12 h) exposed BALB/cByJ mice, or (D, F) acrolein (10 ppm, 17 h) exposed 129X1/SvJ mice. Compare (arrow) area around blood vessel from (C) acrolein-treated with (A) control. In the sensitive BALB/cByJ strain, perivascular enlargement was present at 12 hours of exposure. Leukocytes were present in the (C) perivascular space and (E) alveolus in acrolein-treated BALB/cByJ mouse lung. (D) Perivascular enlargement was not as evident in the lungs of the resistant 129X1/SvJ strain as compared with the BALB/cByJ strain after acrolein exposure. (F) Leukocytes present in alveolus were less in the 129X1/SvJ strain as compared with the BALB/cByJ strain. Bar indicates magnification of original image obtained from 5- μm sections prepared with hematoxylin and eosin stain (50 μm in A–D and 10 μm in E and F). (G) The perivascular interstitial space increased more in lung of BALB/cByJ than of 129X1/SvJ mouse strains after acrolein exposure. After acrolein exposure, the length of the distance between the tunica media and the tunica adventitia (median with 25 and 75% confidence intervals in parentheses) increased from control = 2.6 (1.8–3.3) μm to exposed = 16.6 (11.3–24.7) μm in the lung of BALB/cByJ mice and from control = 1.9 (1.4–2.9) μm to exposed = 4.1 (2.8–5.8) μm in the lung of 129X1/SvJ mice. Values plotted indicate the median (line in box) with 25 and 75% confidence intervals (borders of the box) and 95% confidence intervals (error bars). *Statistically ($P < 0.001$) different from strain-matched control mice (filtered air) as determined by Kruskal-Wallis ANOVA on ranks followed by pairwise comparison with the Tukey method.

and CTNNB1 binding at sites in the proximal promoter (28). To determine whether acrolein could alter FOXO1 or CTNNB1 status, time- and dose-dependent effects on FOXO1 and CTNNB1 and their corresponding phosphorylated forms were measured in cells treated with acrolein. After 4 hours of exposure to 30 nM acrolein, p-FOXO1 protein increased, whereas FOXO1 protein levels were not significantly different from DPBS (control)-treated EA.hy926 cells (Figure 5A). Thus, the FOXO1 to p-FOXO1 protein ratio decreased after 30 nM acrolein as compared with control ($P < 0.001$) (Figure 5C). Western immunoblots for CTNNB1 or p-CTNNB1 protein were unchanged by treatment with 30 nM acrolein (Figures 5A and 5B).

Acrolein Increased FOXO1 or CTNNB1, whereas p-FOXO1 or p-CTNNB1 Was Unchanged after Acrolein in EA.hy926 Cells

After 1 to 4 hours of exposure to 300 nM acrolein, FOXO1 protein increased, whereas p-FOXO1 protein levels were not significantly different from DPBS (control)-treated EA.hy926 cells (Figure 6). Cell viability as measured by a MTS-dehydrogenase enzyme assay of EA.hy926 cells treated with 3,000 nM acrolein or less was not significantly different from vehicle control (Figure E2). Similarly, CTNNB1 but not p-CTNNB1 increased with treatment (Figure 6B), and the ratio of CTNNB1 to p-CTNNB1 increased as compared with control ($P < 0.001$) (Figure 6B). The ratio of FOXO1 to p-FOXO1 increased as compared with control ($P < 0.001$) (Figure 6C).

The Phosphatidylinositol 3-Kinase Inhibitor Diminishes Acrolein-Induced Increased CLDN5 Transcript in EA.hy926 Cells

Induction of CLDN5 and p-FOXO1 was observed in EA.hy926 cells treated with 30 nM acrolein (see Figures 4B and 5C). PI3K phosphorylates AKT, which in turn phosphorylates FOXO1 (and other transcription factors), and this signaling pathway can be inhibited by LY. To examine PI3K inhibition after acrolein treatment of EA.hy926 cells, cells were treated with 10 μM LY (30 min) and exposed to 30 nM acrolein (4 h). LY treatment diminished acrolein-induced CLDN5 mRNA (Figure 7). We also examined whether LY could inhibit p-AKT formation after acrolein treatment of EA.hy926. Acrolein increased p-AKT, and this effect was inhibited by LY addition (Figure E3).

Figure 3. Lung Claudin 5 (CLDN5) transcript and protein increased more in the resistant mouse strain (129X1/SvJ) than in the sensitive mouse strain (BALB/cByJ) after acrolein exposure. (A) CLDN5 transcript increased more at 12 hours in 129X1/SvJ than in BALB/cByJ mouse lung. Mice were exposed to control (filtered air, 0 h) or 10 ppm acrolein for 6 or 12 hours, and lung mRNA was analyzed by qRT-PCR. Values are mean $\pm$ SE ($n = 8$ mice per group). At 12 hours, lung CLDN5 transcript levels increased in resistant (129X1/SvJ) mice compared with strain-matched control mice, whereas the lung CLDN5 transcript levels in the sensitive (BALB/cByJ) mice were not significantly different from control mice. *Significantly different ($P < 0.0001$) from strain-matched control mice as determined by ANOVA with an all pairwise multiple comparison procedure (Holm-Sidak method). (B) CLDN5 protein increased more at 12 hours in 129X1/SvJ than in BALB/cByJ mouse lung as determined by Western blot. Each lane represents protein from a single mouse. (C) CLDN5 protein increased more at 12 hours in 129X1/SvJ than in BALB/cByJ mouse lung. Each test was repeated four times and quantified using ImageQuant 5.2 software (Typhoon 9410; GE Healthcare, Piscataway, NJ). Values are mean $\pm$ SE ($n = 4$) normalized to β -actin. *Significantly different ($P < 0.05$) from strain-matched control mice (0 h) as determined by ANOVA with an all pairwise multiple comparison procedure (Holm-Sidak method). **Significantly different ($P < 0.001$) between 12 hour-exposed 129X1/SvJ and 12 hour-exposed BALB/cByJ mice as determined by ANOVA with an all pairwise multiple comparison procedure (Holm-Sidak method).

DISCUSSION

A major adverse event after smoke inhalation is delayed pulmonary edema and respiratory failure due to ALI (24). In fire victims surviving carbon monoxide poisoning, progressive pulmonary failure and cardiovascular dysfunction are important determinants of morbidity and mortality (25). ALI is marked by perivascular edema (20). This study focused on acrolein, which previously has been demonstrated to be the chemical responsible for pulmonary edema in smoke inhalation (26, 27). Endothelial junctional proteins play critical roles in tissue integrity and can regulate vascular permeability, leukocyte diapedesis, and angiogenesis (28). Endothelial cells express cell type-specific transmembrane junction proteins, including CLDN5 in tight junctions (15, 19) and cadherin 5, type 2 (vascular endothelium) (CDH5, a.k.a. VE-cadherin) at adhesion junctions (28). In concert with CDH5 (1, 29, 30) and other junctional proteins, CLDN5 controls vascular permeability *in vitro* and *in vivo* (12–14). In the normal lung, CLDN5 is expressed strongly in endothelium and is considered a major contributor to the formation of tight junctions in these cells (15). Inducing CLDN5 expression in leaky rat lung endothelial cells can help to restore paracellular barrier function (19). The objective of this study was to determine the cell signal mechanism controlling endothelial CLDN5 expression during ALI.

We determined that the sensitivity of 12 inbred mouse strains to acrolein-induced ALI varied. The polar strains (sensitive: BALB/cByJ; resistant: 129X1/SvJ) differed more than 2-fold in survival time. After acrolein exposure, histological changes included perivascular edema and increased BAL protein, features consistent with ALI. These signs occurred earlier and were demonstrably greater in the sensitive strain compared with the resistant strain. This finding strongly supports the likelihood that an underlying genetic difference is linked to these phenotypes, much like we and others have found for other models of ALI (11, 31–41). Future studies to expand the number of strains examined for single nucleotide polymorphisms mapping or to use crosses from these strains for quantitative trait loci analysis are clearly warranted.

Having obtained histological evidence that acrolein altered vascular permeability, we next measured CLDN5 transcripts in mouse lung and confluent endothelial cells. *In vitro* assays with human lung microvascular endothelial cells or EA.hy926 cells indicated that the CLDN5 transcript regulation was concentration dependent. At 30 nM acrolein, CLDN5 transcript levels increased. A similar increase was noted in mouse lung, which was greater in the resistant strain than in the sensitive strain. This initial CLDN5 induction can be considered a compensatory mechanism to mend junctional complexes and restore barrier function. At 300 nM acrolein *in vitro*, CLDN5 transcripts decreased, which would be detrimental to the maintenance of tight junction function.

To better understand the mechanism of CLDN5 regulation, we examined FOXO1 and CTNNB1 at 30 or 300 nM acrolein concentrations. FOXO1 integrates various cell signals critical to endothelial cell function at the transcriptional level (42). CLDN5 is expressed in the absence of nuclear accumulation of FOXO1 transcription factor. This is controlled by PI3K-mediated phosphorylation of AKT, which in turn mediates downstream phosphorylation of FOXO1 (p-FOXO1). p-FOXO1 can be retained in the cytoplasm or degraded after ubiquitination mediated, in part, by ring finger and WD repeat domain 2 (43). Consistent with this mechanism, treatment with a PI3K inhibitor (LY) diminished acrolein-induced AKT phosphorylation and increased CLDN5 transcript levels. This signaling can be enhanced by growth factors, including VEGFA, which has previously been

Figure 4. Acrolein alters CLDN5 transcript *in vitro*. (A) Time course of acrolein-induced CLDN5 transcript increases in EA.hy926 cells. Cells were exposed to 30 nM acrolein for the indicated times, and mRNA was analyzed by qRT-PCR. Tests were repeated on three occasions. Values are mean $\pm$ SE ($n = 12$ dishes). *Significantly different ($P < 0.001$) from Dulbecco's PBS (control) as determined by ANOVA with an all pairwise multiple comparison procedure (Holm-Sidak method). (B) Dose response of acrolein-induced CLDN5 transcript in hybrid EA.hy926 cells. Cells were exposed to acrolein for 4 hours, and mRNA expression levels were analyzed by qRT-PCR. Tests were repeated on three occasions. Values are mean $\pm$ SE ($n = 12$ dishes). *Significantly different ($P < 0.001$) from control as determined by ANOVA with an all pairwise multiple comparison procedure (Holm-Sidak method). (C) Dose response of acrolein-induced CLDN5 transcript in human lung microvascular endothelial cells. Cells were exposed to acrolein for 4 hours, and mRNA was analyzed by qRT-PCR. Tests were repeated on three occasions. Values are mean $\pm$ SE ($n = 12$ dishes). *Significantly different ($P < 0.001$) from control as determined by ANOVA with an all pairwise multiple comparison procedure (Holm-Sidak method).

ratio of p-FOXO1 to FOXO1 increased in EA.hy926 cells, which is consistent with such an outcome. With mounting stress, FOXO1 and CTNNB1 proteins accumulate in the cell, translocate to the nucleus, and partner to regulate target genes that promote stress resistance, cell cycle arrest, or apoptosis (48–51). At 300 nM acrolein *in vitro*, CLDN5 transcripts decreased and FOXO1 and CTNNB1 increased in EA.hy926 cells, which is consistent with more severe outcome. The ultimate loss of CLDN5 in tight junctions by this mechanism can lead to disassembly of adhesive structures, endothelial barrier dysfunction, and ultimately increased vascular permeability.

The acrolein levels used in this study, 10 ppm *in vivo* and 1 to 300 nM *in vitro*, are relevant to human exposure. As an α,β -unsaturated 2-alkenal, acrolein is highly reactive in biological systems and can be extremely irritating (e.g., 0.06 ppm can cause eye irritation within 5 min) (52–54). Acrolein can rapidly bind with macromolecules and disrupt critical cellular functions (55–60). Acrolein is generated by combustion and is the major irritant in grassland and forest fires, high temperature cooking with oils (especially in woks), and diesel exhaust (26, 27, 61, 62). Over 30 million nonsmokers in the United States are exposed to acrolein concentrations in indoor air ranging from 0.8 to 1.5 ppm, and levels between 0.1 to 10 ppm have been detected in bars and restaurants (63–67). Acrolein levels are elevated in second-hand smoke compared with mainstream smoke because side-stream smoke is generated at lower combustion temperatures (61, 64, 68–70). The nanomolar acrolein concentrations used in this study also are relevant to endogenously generated levels in injured tissues (55, 56), which can result from amine oxidase-mediated catabolism of spermine or spermidine (71–75), myeloperoxidase catabolism of threonine (76–78), or, albeit less likely, oxidative degradation of membrane fatty acids (61, 79–81). Acrolein-protein adducts accumulate in ischemic tissue (81, 82) and in atherosclerotic lesions (78, 83).

In summary, acrolein can induce ALI with perivascular edema in mice. This is accompanied by a compensatory increase in CLDN5 transcript and protein, which was more evident in a resistant than a sensitive mouse strain. *In vitro*, 30 nM acrolein stimulated phosphorylation of FOXO1 protein and increased CLDN5 transcripts, whereas 300 nM acrolein stimulated FOXO1 and CTNNB1 protein levels and decreased CLDN5 transcripts. These events are consistent with the rapid increase in vascular permeability and could provide a critical target for future pharmacological intervention during ALI.

demonstrated to increase markedly and may initiate edema during ALI (29, 44–46).

In addition, CTNNB1 can be released from adhesion complexes during stress (47), relocates to the nucleus, and activates numerous critical survival events, including the transcription of *CLDN5* (1). At 30 nM acrolein, CLDN5 transcripts and the

Figure 5. Phospho-forkhead box O1 (p-FOXO1) increased after 30 nM of acrolein treatment. (A) Western immunoblot with catenin (cadherin-associated protein), beta 1 (CTNNB1), p-CTNNB1, FOXO1, p-FOXO1, or β-actin antibody in EA.hy926 cells treated with Dulbecco's PBS (control) or 30 nM acrolein. p-FOXO1 increased after 4 hours of 30 nM acrolein treatment. (B) Mean transcription factor CTNNB1 protein levels after 30 nM acrolein treatment. Open bar: CTNNB1. Closed bar: p-CTNNB1. Hatched bar: CTNNB1/p-CTNNB1 ratio. (C) Mean transcription factor FOXO1 protein levels after 30 nM acrolein treatment. Open bar: FOXO1. Closed bar: p-FOXO1; Hatched bar: FOXO1/p-FOXO ratio. Each test was repeated four times and quantified using ImageQuant 5.2 software (Typhoon 9410; GE Healthcare). Values are mean ± SE ($n = 4$) normalized to β-actin. *Significantly different ($P < 0.001$) from control as determined by ANOVA with an all pairwise multiple comparison procedure (Holm-Sidak method).

Figure 6. Catenin (cadherin-associated protein), beta 1 (CTNNB1), and Forkhead box O1 (FOXO1) increased, whereas phospho-FOXO1 (p-FOXO1) and phospho-CTNNB1 (p-CTNNB1) were unchanged after treatment with 300 nM acrolein. (A) Western immunoblot with CTNNB1, p-CTNNB1, FOXO1, p-FOXO1, or β-actin antibody in EA.hy926 cells treated with Dulbecco's PBS (control) or 30 nM acrolein. CTNNB1 and FOXO1 increased at 1, 2, or 4 hours of 300-nM acrolein treatment. (B) Mean transcription factor CTNNB1 proteins after 300 nM acrolein treatment. Open bar: CTNNB1. Closed bar: p-CTNNB1. Hatched bar: CTNNB1/p-CTNNB1 ratio. (C) Mean transcription factor FOXO1 proteins after 300 nM acrolein treatment. Open bar: FOXO1. Closed bar: p-FOXO1. Hatched bar: FOXO1/p-FOXO ratio. Each test was repeated four times and quantified using ImageQuant 5.2 software (Typhoon 9410; GE Healthcare). Values are mean ± SE ($n = 4$) normalized to β-actin. *Significantly different ($P < 0.01$) from control as determined by ANOVA with an all pairwise multiple comparison procedure (Holm-Sidak method).

Figure 7. Phosphatidylinositol 3-kinase inhibitor, LY294002 (LY), diminishes 30 nM acrolein-induced increased claudin 5 (CLDN5) transcript in EA.hy926 cells untreated (control) or cells treated with DMSO (vehicle), 30 nM acrolein, or 30 nM acrolein and LY. Cells were treated with vehicle (DMSO) or 10 μ M LY 30 minutes before 30 nM acrolein treatment, and mRNA was analyzed by qRT-PCR. Tests were repeated four times on two occasions. Values are mean $\pm$ SE ($n = 8$ dishes). *Significantly different ($P < 0.01$) from vehicle (Dulbecco's buffered saline or DMSO) control as determined by ANOVA with an all pairwise multiple comparison procedure (Holm-Sidak method). **Significantly different ($P < 0.01$) from acrolein (without LY) as determined by ANOVA with an all pairwise multiple comparison procedure (Sidak-Scheffe method).

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