Review Article

Pulmonary Circulation

Cigarette smoke alters lung vascular permeability and endothelial barrier function (2017 Grover Conference Series)

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Abstract

Smoking of tobacco products continues to be widespread, despite recent progress in decreasing use. Both in the United States and worldwide, cigarette smoking is a major cause of morbidity and mortality. Growing evidence indicates that acute respiratory distress syndrome (ARDS) is among the consequences of cigarette smoking. Based on the topic from the 2017 Grover Conference, we review evidence that cigarette smoking increases lung vascular permeability using both acute and longer exposures of mice to cigarette smoke (CS). We also review studies indicating that CS extract disrupts cultured lung endothelial cell barrier function through effects on focal adhesion contacts, adherens junctions, actin cytoskeleton, and microtubules. Among the potentially injurious components of CS, the reactive aldehyde, acrolein, similarly increases lung vascular permeability and disrupts barrier function. We speculate that inhibition of aldehyde-induced lung vascular permeability may prevent CS-induced lung injury.

Keywords

tobacco, acute respiratory distress syndrome, paracellular permeability, acrolein, aldehyde dehydrogenase

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Cigarette smoking is a public health problem

Cigarette smoking is the leading cause of preventable disease, disability, and death worldwide. According to the World Health Organization,[1] more than 1 billion individuals smoke and more than 6 million die as a result of tobacco use each year, including 600,000 deaths from secondhand smoke.

Centers for Disease Control statistics[2] indicate that 15.1% of all US adults (36.5 million people) were current cigarette smokers in 2015, 4.7 million teenagers use at least one tobacco product, and 16 million Americans live with smoking-related disease. Cigarette smoking causes around 480,000 deaths per year in the United States, contributing to about one-fifth of deaths. Secondhand smoke exposure is a factor in 41,000 deaths per year among non-smoking adults and 400 infant deaths per year in the US. Nearly $170 billion of medical care cost is spent to treat smoking-related diseases in American adults each year. Smokers tend to be younger, less well educated, and have lower incomes. Thus, smoking-related disease is an important cause of health disparities in the United States and worldwide.

Diseases associated with cigarette smoking

Cancers, cardiovascular disease, and respiratory diseases, such as chronic obstructive pulmonary disease (COPD) and pneumonia, are major causes of mortality among smokers. It is evident that smoking damages many organs, causing multiple problems, ranging from periodontitis to erectile dysfunction. Renal microvascular endothelial injury related to cigarette smoking has recently been recognized to accompany COPD.[3] This supports the concept first raised by Voelkel and others[4,5] that cigarette smoking is a cause of

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endothelial injury both in the lungs as well as the systemic circulation.

Lung diseases associated with cigarette smoking include COPD, idiopathic pulmonary fibrosis, COPD-associated pulmonary hypertension (PH), asthma, and pneumonia. In addition, a growing body of evidence indicates that acute respiratory distress syndrome (ARDS) is also associated with cigarette smoking. Using plasma and urine markers of smoke exposure, Calfee et al. have demonstrated that both active smokers and those exposed to secondhand smoke have an increased risk of developing ARDS after blunt chest trauma[6] and that active cigarette smoke (CS) exposure was more prevalent among ARDSNET enrollees with ARDS than population averages.[7] Studies on ARDS from a Kaiser Permanente database of hospitalizations[8] demonstrated that current heavy smokers (>20 cigarettes per day) had 5.7 times the risk of developing ARDS and cigarette smoking contributes to an estimated 50% of the risk of ARDS. In addition, studies on a cohort of patients admitted to a surgical ICU in Thailand indicated that active smokers had a higher incidence of ARDS than former smokers and never smokers.[9] A history of cigarette smoking was also associated with the later development of ARDS in esophagectomy patients.[10] Ware et al. found that long-term environmental ozone exposure increased the risk of ARDS after trauma in smokers.[11] Ware et al. also demonstrated that explanted lungs from smokers were heavier and were associated with worse clinical outcomes after transplantation.[12] These epidemiological studies indicate that cigarette smoking increases susceptibility and severity of ARDS in patients with clinical risk factors, such as trauma, surgery, infections, or other environmental risk factors.

ARDS is characterized by increased lung endothelial and epithelial permeability, resulting in increased permeability pulmonary edema and consequent hypoxemia, decreased lung compliance, and infiltrates on chest X-ray. Several laboratories have contributed to studies addressing the underlying mechanisms of smoking as a risk factor for ARDS. This review, mainly a summary of outputs and conclusions about this topic from the 2017 Grover Conference, summarizes the effect of CS on pulmonary microvascular permeability and endothelial barrier function.

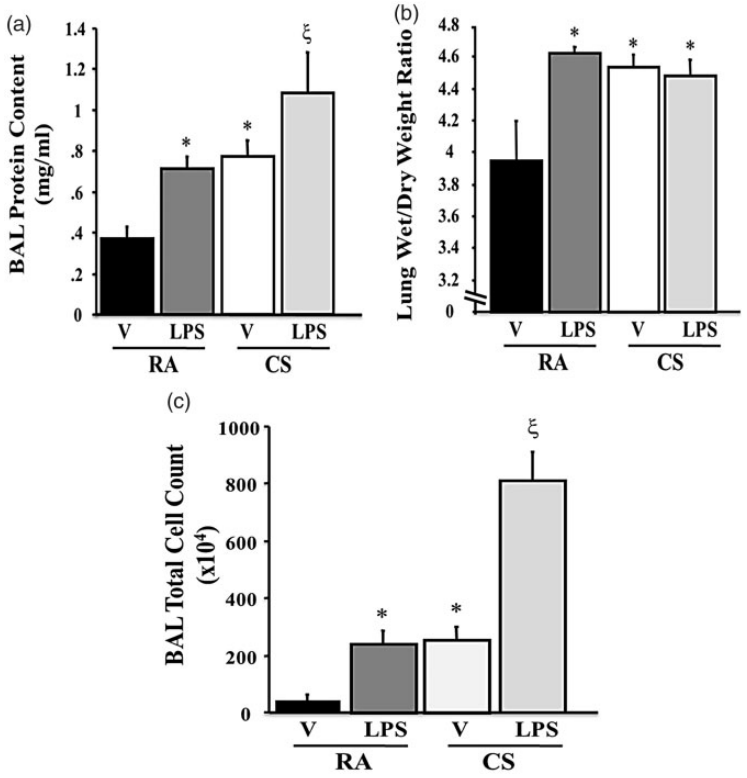

Fig. 1. CS increased lung vascular permeability and exacerbated LPS-induced lung edema. Male six-week-old C57BL/6 mice were exposed to CS or room air (RA) for 6 h and then intratracheally given 2.5 mg/kg LPS or equal volume of 0.9% NaCL (Vehicle, V, ~50 μl). 24 h after LPS or vehicle challenge, the lung was lavaged with 600 μL of saline and the protein content in BAL fluid was assessed (a). Total cell counts in BAL fluid were also assessed (c). Other animals were used in parallel studies for assessment of lung wet-to-dry weight ratio (Wet/dry) (b). The data are presented as means ± SE. Six mice per group (n = 6) in each panel, $^{*}P < 0.05$ vs. mice exposed to room air and treated with vehicle; $^{\xi}P < 0.05$ vs. mice exposed to RA and treated with LPS. Reprinted with permission of the *American Journal of Physiology: Lung Cellular and Molecular Physiology.*[17]

Exposure to cigarette smoke worsens lung edema and inflammation

It has been reported that CS exposure increases alveolar epithelial barrier permeability in guinea pigs[13] and increases pulmonary capillary barrier permeability in rats.[14] Both brief (hours) and subacute (four weeks) CS exposure also increased bronchoalveolar lavage (BAL) protein levels in guinea pigs.[15] Our studies of mouse model showed similar findings.[16,17] We exposed 6–8-week-old C57BL/6 mice to smoke generated in a TE-10 smoking machine by ignition of 3R4F reference cigarettes (University of Kentucky Tobacco Research Institute) that had been rehydrated by exposure to glycerol. Total particulate matter was 120 mg/M^3 with 89% sidestream and 11% mainstream smoke. After 6 h of smoke exposure, mice were anesthetized and administered intra-tracheal lipopolysaccharide (LPS) or an equivalent volume of 0.9% NaCl as vehicle (V) control. After 24 h in room air, mice were anesthetized and BAL protein, lung wet/dry weights, and BAL cell counts were assessed. LPS challenges increased BAL protein (Fig. 1a), lung wet/dry weights (Fig. 1b), and BAL cell count (Fig. 1c). Interestingly, only 6 h of smoke exposure followed by challenge with saline control for 24 h also increased BAL protein (Fig. 1a), lung wet/dry weights (Fig. 1b), and BAL cell count (Fig. 1c). Increases in BAL protein and cell count caused by LPS were exacerbated by CS exposure (Fig. 1a and c). Similar effects of 6 h of CS exposure were observed in mice challenged by intra-tracheal *Pseudomonas aeruginosa* (PA-103 strain).[16]

Lee et al. simultaneously co-exposed AKR/J mice to CS and LPS and demonstrated exacerbated macrophage infiltrate with fewer neutrophils in lungs, enhanced lung cell apoptosis, and reduced levels of lung cytokines.[18] We assessed the effects of subacute CS pre-exposure on LPS-induced acute lung injury.[19] In our experiments, C57BL/6

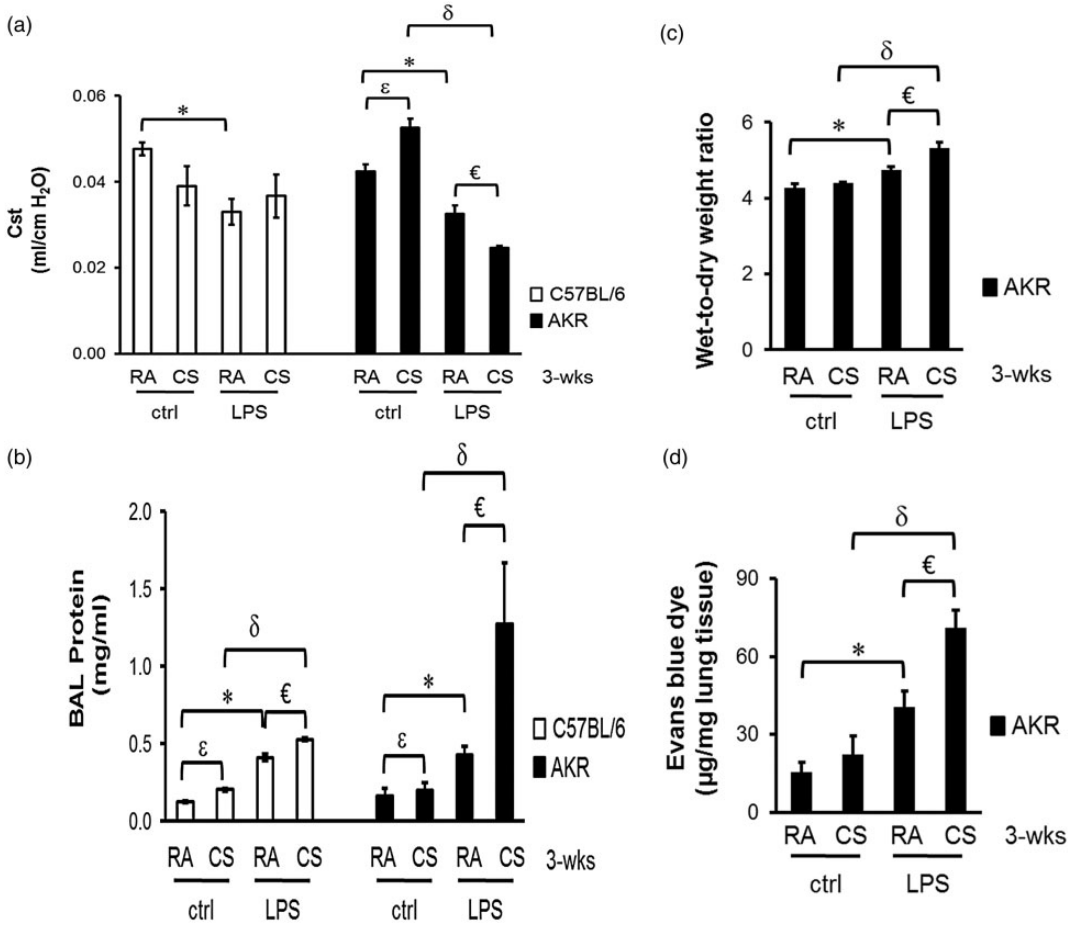

Fig. 2. Effects of prolonged CS exposure on LPS-induced lung edema in two strains of mice. Male six-week-old C57BL/6 and AKR mice were exposed to room air (RA) or CS for three weeks. One hour after the last CS exposure, mice were intratracheally administered with 2.5 mg/kg of LPS or equal volume of saline as a control (ctrl). After 18 h, lung static compliance (Cst) was assessed using FlexiVent system (a). BAL fluid was collected for assessment of BAL protein content (b). Lung wet-to-dry weight ratio (c) and lung extravasation of albumin-conjugated Evans blue dye (EBD) (d) were assessed in additional sets of AKR mice that were subjected to the same treatments. (a) 9–10 C57BL/6 mice per group and 9–11 AKR mice per group were used; (b) 3–4 C57BL/6 mice per group and 4–6 AKR mice per group were used; (c) 3 AKR mice per group were used; (d) 4–5 AKR mice per group were used. $^{\varepsilon}P < 0.05$ CS/ctrl vs. RA/ctrl; $^{*}P < 0.05$ RA/LPS vs. RA/ctrl; $^{\delta}P < 0.05$ CS/LPS vs. CS/ctrl; $^{€}P < 0.05$ CS/LPS vs. RA/LPS. Reprinted with permission of the *American Journal of Physiology: Lung Cellular and Molecular Physiology.*[19]

and AKR mice were exposed to CS for 6 h per day, four days per week for three weeks, followed by intra-tracheal LPS or vehicle control, and assessed for changes in lung compliance, BAL protein, and wet/dry lung weights 18 h after LPS administration. Figure 2 illustrates that after prolonged CS exposure, expected LPS-induced decreases in compliance and LPS-induced increased lung vascular permeability (BAL protein, wet/dry weights, and Evans blue dye [EBD]) were exacerbated after CS exposure in the AKR mouse strain, known to be more susceptible to CS-induced injury. Assessment of LPS-and CS-induced lung inflammation indicated that three weeks of CS

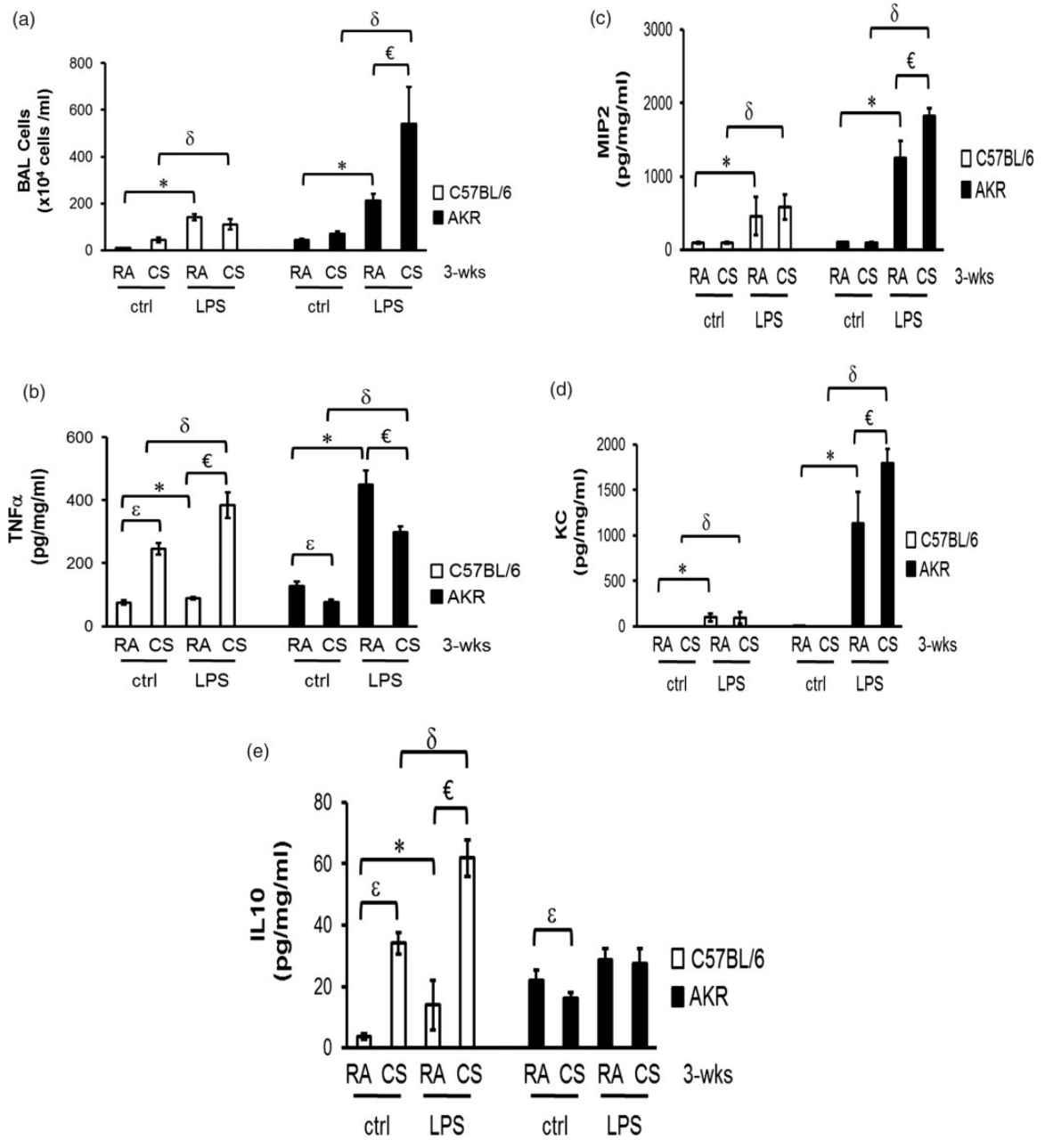

Fig. 3. Effects of prolonged CS exposure on LPS-induced lung inflammation in two strains of mice. Male 6-week-old C57BL/6 and AKR mice were exposed to room air (RA) or CS for three weeks. One hour after the last CS exposure, mice were intratracheally administered with 2.5 mg/kg of LPS or equal volume of saline as a control (ctrl). After 18 h, the number of the total inflammatory cells in BAL fluid was assessed (a). Lung tissue was collected and lung homogenates were prepared for assessments of levels of TNFα (b), MIP2 (c), KC (d), and IL10 (e) by ELISA. (a) 3–4 C57BL/6 mice per group and 4–6 AKR mice per group were used; (b–e) 4 C57BL/6 mice per group and 4 AKR mice per group were used. $^{\varepsilon}P < 0.05$ CS/ctrl vs. RA/ctrl; $^{*}P < 0.05$ RA/LPS vs. RA/ctrl; $^{\delta}P < 0.05$ CS/LPS vs. CS/ctrl; $^{€}P < 0.05$ CS/LPS vs. RA/LPS. Reprinted with permission of the *American Journal of Physiology: Lung Cellular and Molecular Physiology.*[19]

exposure enhanced LPS-induced increases in BAL cell counts and cytokines (MIP2 and KC) in lung homogenates (Fig. 3), an effect that was exaggerated in the AKR mouse strain. Of note, CS decreased lung tissue levels of IL-10, an anti-inflammatory cytokine, in the more susceptible AKR mouse strain (Fig. 3). Cell counts in lung tissue in AKR mice showed that LPS-induced increases in polymorphonuclear neutrophils, alveolar macrophages, and M2 macrophages were enhanced by CS exposure.[19] The different results in neutrophil infiltration and lung cytokine levels observed between our study and the Lee study may be due to the difference in experimental models. Our study used a smoke priming double-hit model, whereas they used smoke-LPS co-exposure model. Similar to our findings, Gotts et al. also reported that CS pre-exposure of C57BL/6 mice for 12 days exacerbated LPS-induced increase in pulmonary edema, BAL neutrophilia, lung IL-6, and plasma CXCL9.[20] Taken together, these results indicate that CS primes lungs for enhanced lung edema and inflammatory lung injury, despite acclimatization with longer-term smoke exposure. Furthermore, the magnitude of this effect varied among mouse strains.

Cigarette smoke directly impairs endothelial barrier function

Inhaled CS has complex effects on epithelium and airway inflammatory cells. Smoking-induced increased lung vascular permeability suggested that CS might also directly alter lung vascular endothelial cell barrier function. In order to investigate this possibility, we cultured pulmonary artery endothelial cells on gold electrodes and assessed the effects of an aqueous extract of CS on transendothelial electrical resistance, a measure of paracellular permeability.[17] We found that CS extract (CSE) increased endothelial monolayer permeability in a dose-dependent manner, an effect that was blunted by the anti-oxidant, N-acetyl cysteine (Fig. 4). Furthermore, CSE exacerbated barrier disruption caused by endothelial cell incubation with LPS.[17] In addition, we found that lung microvascular endothelial cells isolated from mice exposed to CS, displayed enhanced barrier dysfunction and incomplete recovery upon exposure to either LPS or thrombin.[16]

We investigated potential mechanisms of CSE-induced endothelial barrier dysfunction and found that structures that regulate paracellular permeability were disrupted by exposure to CSE.[17] Figure 5 illustrates immunofluorescence microscopy of cultured bovine pulmonary arterial endothelial cells. Exposure to CSE disrupted focal adhesion contacts (vinculin), actin stress fibers (phalloidin), and adherens junctions (beta-catenin). These effects were blunted by co-incubation with N-acetyl cysteine, suggesting that the changes were caused by an oxidant injury caused by CSE. In further studies of mechanisms of CS-induced changes in permeability, we found that CSE decreased expression of phosphorylated focal adhesion kinase and activation of RhoA GTPase, signaling molecules important in regulation of paracellular endothelial permeability.

In other studies, we found that CSE caused disruption of endothelial cell microtubules, decreased acetylation of α-tubulin, and decreased tubulin polymer formation (Fig. 6).[16] Furthermore, the microtubule stabilizer, taxol, prevented monolayer permeability changes caused by CSE. These effects on microtubule integrity were prevented by inhibitors of histone deacetylase 6 (HDAC6), phosphorylation of which was enhanced by oxidant-induced changes in GSK-3β activity.

Schweitzer et al. also reported that CSE increased rat lung microvascular endothelial cell monolayer permeability and disrupted structures involved in maintenance of paracellular permeability; this effect was mimicked by

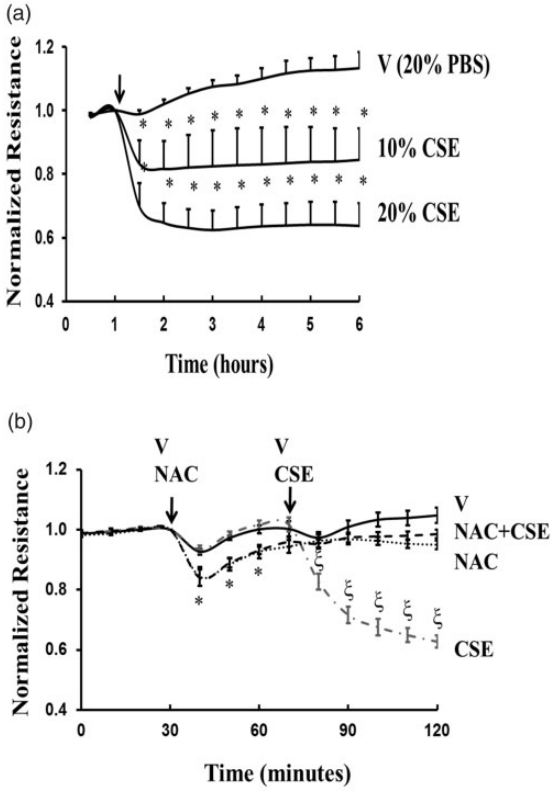

Fig. 4. CSE increased endothelial monolayer permeability via oxidative stress. Bovine pulmonary artery endothelial cells (PAEC) were treated with vehicle (V, 20% sham PBS) or varying concentrations of CSE (10, 20%) for indicated times (a), or preincubated with vehicle (V, HEPES) or 25 mM N-acetylcysteine (NAC) for 40 min and then treated with vehicle (V, 20% sham PBS) or 20% CSE in the absence or presence of 25 mM NAC for indicated times (b). Endothelial monolayer permeability was assessed by Electric Cell-substrate Impedance Sensing (ECIS). The data are presented as means ± SE of the normalized electrical resistance relative to the time when agents were added (at 1 h in (a); at 30 min in (b)). Arrows indicate the times for addition of treatments. (a) n = 10, $^{*}P < 0.05$ vs. vehicle-treated cells; (b) n = 6, $^{*}P < 0.05$ vs. vehicle-treated cells; $^{\xi}P < 0.05$ vs. vehicle, NAC, and NAC+CSE-treated cells. Reprinted with permission of the *American Journal of Physiology: Lung Cellular and Molecular Physiology.*[17]

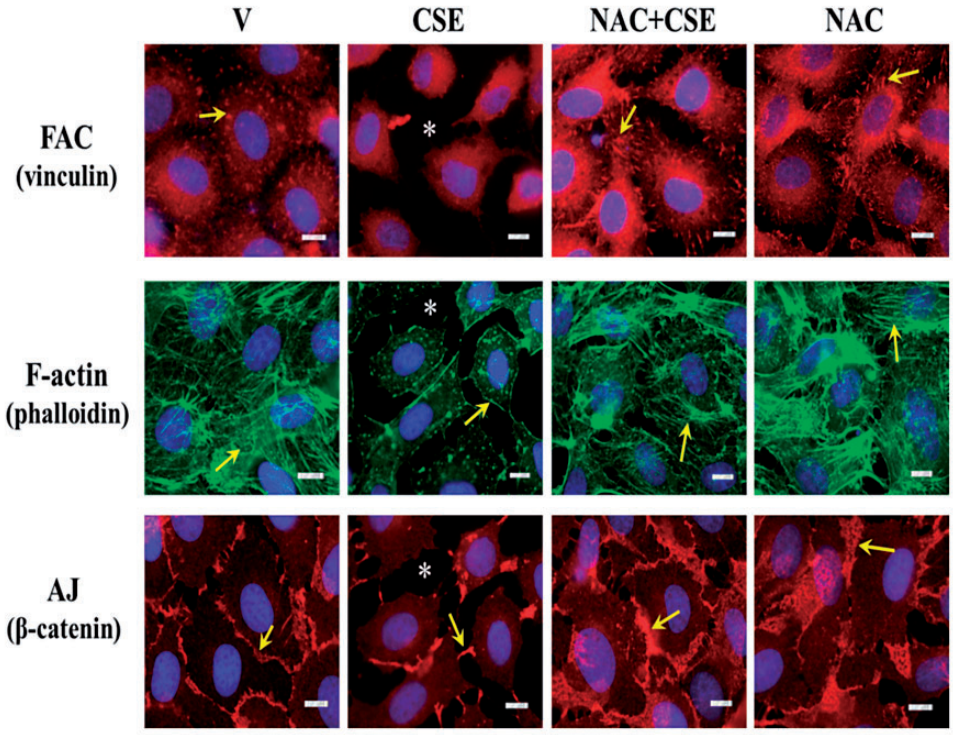

Fig. 5. CSE disrupted focal adhesion complexes (FAC), F-actin fibers, and adherens junctions (AJ) via oxidative stress. Bovine PAECs were preincubated with vehicle (V) or 25 mM N-acetylcysteine (NAC) for 1 h and then exposed to vehicle (10% sham PBS) or 10% CSE in the absence or presence of 25 mM NAC for 4 h. FAC and AJ were assessed by immunofluorescence staining of vinculin and β-catenin, respectively, and visualized by fluorescence microscopy. F-actin fibers were assessed by phalloidin staining of F-actin. Arrows indicate vinculin, F-actin, and β-catenin staining. Asterisks indicate intercellular gaps. Scale bar = 25 μm. Representative images from four independent experiments for each panel are shown. Reprinted with permission of the *American Journal of Physiology: Lung Cellular and Molecular Physiology.*[17]

exogenous ceramide.[21] Ceramide has been shown to directly increase endothelial cell permeability.[22] Additionally, intratracheal administration of ceramide significantly increased lung vascular permeability in rats.[23] These results suggest that CS-induced increases in ceramides may significantly contribute to CS-enhanced lung microvascular permeability.

Acrolein also enhances lung microvascular permeability

CS is a complex mixture of about 4500 gaseous, lipophilic, hydrophilic, and particulate materials. Acrolein, a highly reactive, α,β-unsaturated aldehyde, is one of the many potentially injurious components of CS. The U.S. Environmental Protection Agency has established a safe Reference Concentration (RfC) of 0.02 μg/m^3 for inhalation of acrolein and a safe RfC of 0.02 mg/kg per day for ingestion. Acrolein concentrations in ambient air can reach to 8.2–24.6 μg/m^3. The major sources of acrolein in the indoor environment are smoking of tobacco and tobacco additives, e.g. glycerol and carbohydrates, overheating oils, cooking with biomass fuels, and fireplace heating.[24] Acrolein in the outdoor environment is mainly from automobile gasoline and diesel exhausts, forest fires, and other combustion of organic materials. Tobacco smokers have significantly elevated levels of acrolein metabolites in their serum, exhaled breath condensates,[25] and urine.[26] Lungs from mice exposed to CS also had increased levels of acetaldehyde and malondialdehyde.[27] Firefighters and certain manufacturing and restaurant workers are often exposed to high levels of acrolein. Acrolein also exists in high concentration in "burn pits" in Afghanistan and Iraq (OEF/OIF) military bases. Therefore, acrolein exposure is a significant health hazard. In addition to external inhalation and ingestion, acrolein can be endogenously produced via lipid peroxidation, metabolism of certain amino acids (e.g. polyamine, spermidine) and anti-cancer drugs (e.g. cyclophosphamide), and neutrophil myeloperoxidase action at sites of inflammation and injury.[28]

Acrolein can be detoxified by glutathione-S-transferase alpha 4 (GSTA4), which catalyzes the conjugation of acrolein to glutathione. Acrolein-glutathione conjugates are removed from cells by the glutathione conjugate transporter, RLIP76. Acrolein can also be converted into less toxic molecules via oxidation by aldehyde dehydrogenases (ALDHs). In addition, acrolein can be reduced and thus detoxified by NADPH-dependent acrolein-reducing

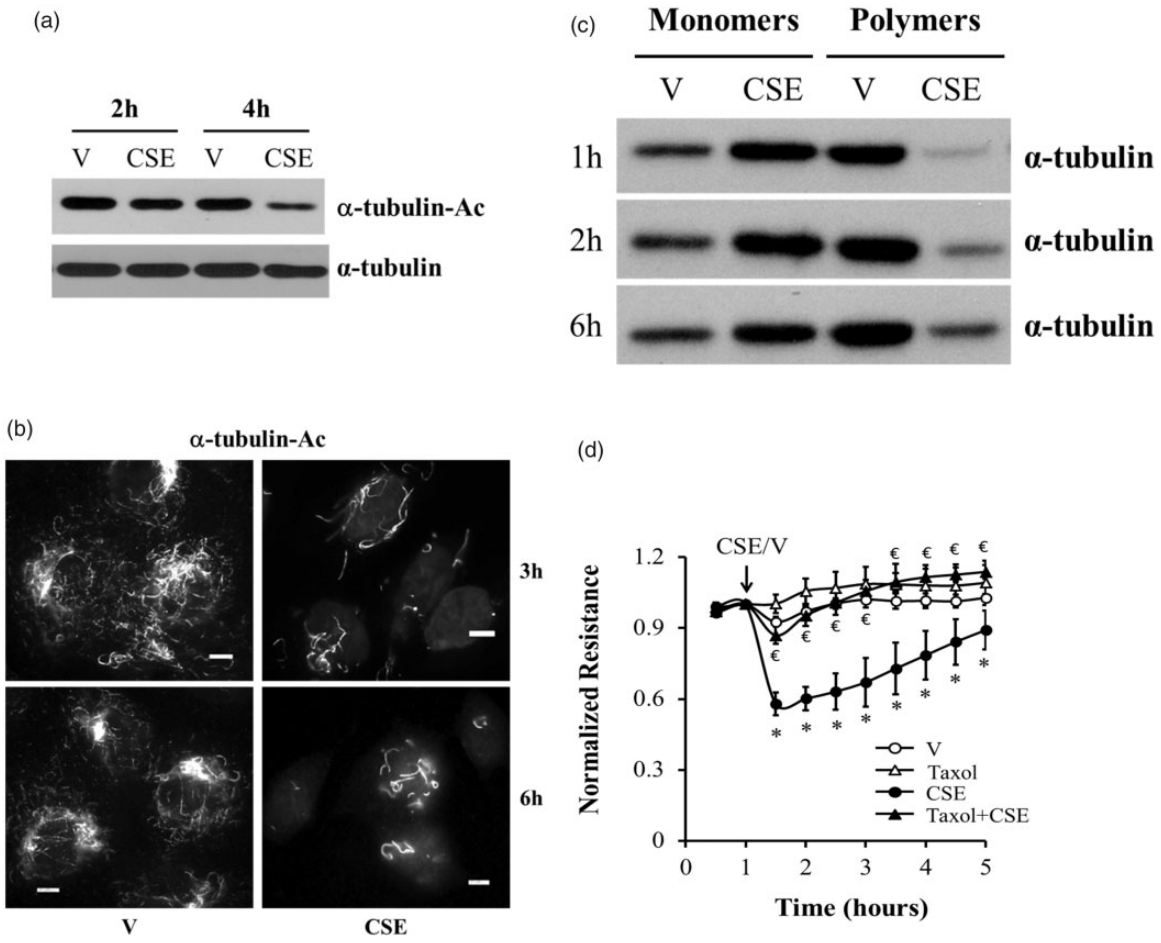

Fig. 6. CSE caused lung endothelial barrier dysfunction, α-tubulin deacetylation, and microtubule disassembly. (a, b) Bovine PAECs were treated with vehicle (V, 10% PBS) or 10% CSE for indicated time. Acetylated α-tubulin (α-tubulin-Ac) was assessed by western blot (a) and immunofluorescence microscopy (b), using anti-acetylated α-tubulin antibody. α-tubulin was also assessed. (c) Bovine PAECs were treated with vehicle (V, 10% PBS) or 10% CSE for indicated time. Microtubule depolymerization was assessed by examining the levels of monomers and polymers of α-tubulin using microtubule extraction assay. (d) Bovine PAECs were pretreated with 5 μM Taxol for 30 min and then treated with vehicle (V, 10% PBS) or 10% CSE in the absence or presence of 5 μM Taxol for indicated time and monolayer permeability was assessed. Arrows indicate the time of addition of treatments. (a–c) Three independent experiments. (d) The data are presented as the mean ± SE of the normalized electrical resistance at each time point relative to initial resistance. n = 4, ANOVA and Tukey–Kramer post-hoc test was used to determine statistically significant difference across means among groups. $^{*}P < 0.05$ vs. V; $^{€}P < 0.05$ vs. CSE. Reprinted with permission of the *American Journal of Respiratory Cell and Molecular Biology*.[16]

enzymes, alkenal/one oxidoreductase (AOR) and aldose reductase.[29,30] Like other reactive aldehydes, acrolein that is not metabolized or detoxified is subjected to *Michael addition* reaction by which acrolein reacts with the side chains of lysine, histidine, or cysteine residues of proteins or nucleic acid to form covalent bonds (aldehyde adducts),[24] a process termed carbonylation.[31] Carbonylation of proteins may cause protein mis-folding, cross-linking, or aggregation, followed by proteasomal degradation. The aldehyde-modified proteins are removed by autophagy.[32] Increased aldehyde-adducted proteins have been found in lungs of patients with COPD,[33] serum of patients with COPD,[34] and serum of animals exposed to CS.[35]

Acrolein is the second most common toxin from fires, after carbon monoxide. Similar to smoke inhalation, acrolein inhalation has been shown to cause non-cardiogenic pulmonary edema and respiratory distress in sheep[36,37] and dogs[38] and perivascular cuffing in susceptible mouse strains.[39] We found that acrolein increases lung microvascular endothelial cell permeability in vitro and causes lung edema as well as exacerbating LPS-induced lung injury in mice,[40] similar to the effects of CS. We found that pretreatment of mice with Alda-1 (NC1,3-benzodioxol-5-ylmethyl)-2,6-dichlorobenzamide), a selective ALDH2 activator, significantly attenuated acrolein-induced increase in BAL protein content (Fig. 7a) and lung wet-to-dry weight ratio (Fig. 7b). More importantly, Alda-1 significantly rescued acrolein-induced increase in BAL protein content (Fig. 7c), lung wet-to-dry weight ratio (Fig. 7b), and pro-inflammatory cytokines, KC, IL6, and TNFα.[40] Alda-1 also

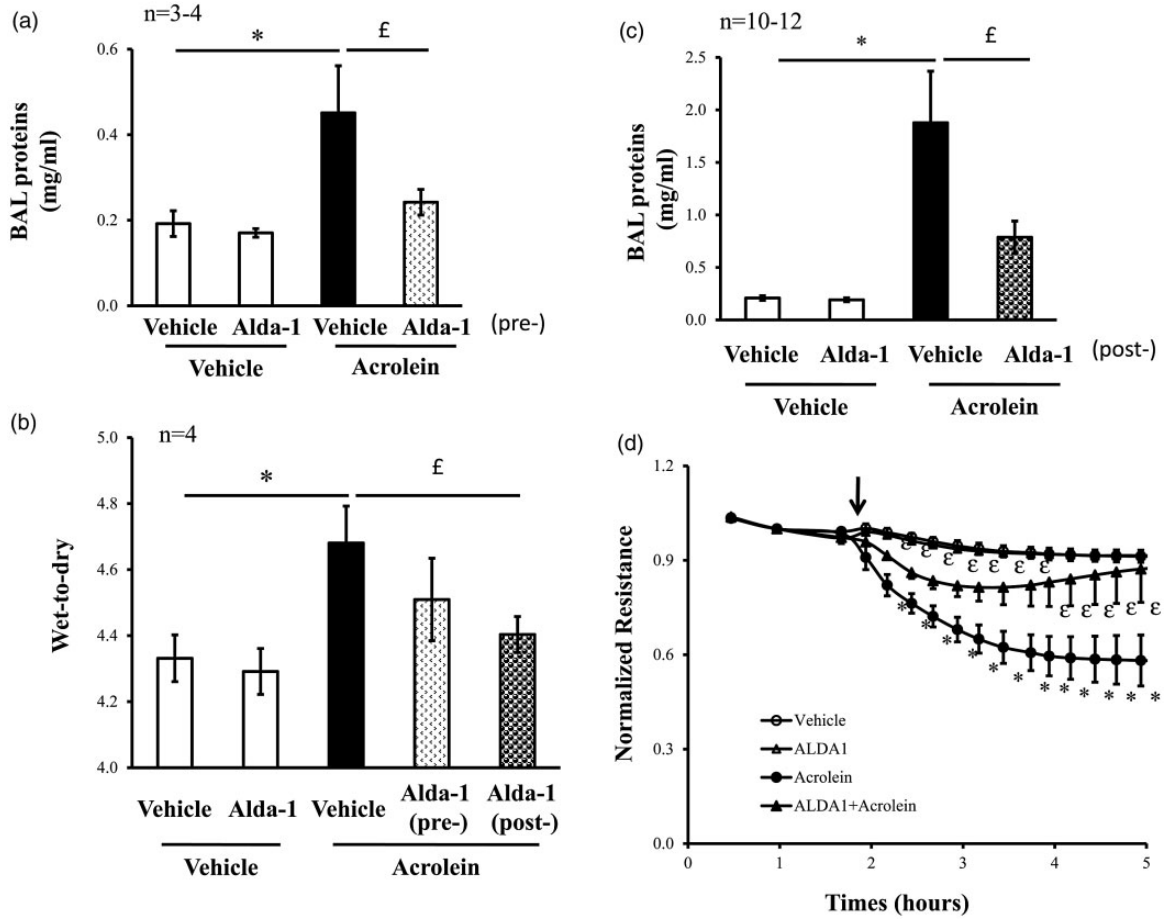

Fig. 7. Effects of Alda-1 on acrolein-induced lung injury and endothelial permeability. Mice were treated with Alda-1 (10 mg/kg) or an equal volume of sterilized saline (control) via i.p injection 1 h before (a, c) or 2 h after (b, c) intratracheally administered with 2.5 mg/kg of acrolein or equal volume of sterilized saline. BAL protein levels (a, b) and lung wet-to-dry weight ratio (c) were assessed 18 h after challenge of acrolein. 3–12 mice per group were used for each panel. Data are represented as means ± SE. *$P < 0.05$ vs. mice treated with saline control; $P < 0.05$ vs. mice treated with acrolein alone. (d) Rat lung microvascular endothelial cells (LMVEC) were pre-incubated with vehicle or 50 μM Alda-1 for 30 min and then exposed to vehicle or 30 μM acrolein in the absence or presence of Alda-1 for indicated times. Monolayer permeability was assessed by measuring electrical resistance across monolayers by ECIS. Data are normalized electrical resistance with means ± SE of three independent experiments. Arrows indicate the time for addition of treatments. *$P < 0.05$ vs. vehicle-treated cells. $^{\varepsilon}P < 0.05$ vs. cells treated with acrolein.

attenuated acrolein-induced barrier dysfunction in endothelial cell monolayers (Fig. 7d). These results suggest that acrolein may be important in CS-induced enhancement of lung vascular permeability and that Alda-1 may be an innovative approach to prevention of CS-induced lung microvascular permeability.

Summary

Growing epidemiological data indicate that cigarette smoking predisposes to development of ARDS. Work from our laboratory and others using mouse models and cultured pulmonary endothelial cells indicates that CS increases vascular permeability and directly causes endothelial monolayer permeability through altered regulation of paracellular permeability. Exposure to acrolein, an aldehyde present in CS, similarly increases lung vascular permeability and primes for a second hit-induced ARDS. It is possible that components of CS, such as acrolein and reactive oxidants, impair alveolar-capillary barrier function, resulting in lung inflammation, thereby increasing susceptibility to ARDS following a second insult. Future studies should develop strategies to protect endothelial barrier function damaged by cigarette smoking. Furthermore, strengthening of the pulmonary endothelial barrier may protect the systemic circulation from injurious agents in CS.

Conflict of interest

The author(s) declare that there is no conflict of interest.

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