

# Alda-1 Protects Against Acrolein-Induced Acute Lung Injury and Endothelial Barrier Dysfunction

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## Abstract

Inhalation of acrolein, a highly reactive aldehyde, causes lung edema. The underlying mechanism is poorly understood and there is no effective treatment. In this study, we demonstrated that acrolein not only dose-dependently induced lung edema but also promoted LPS-induced acute lung injury. Importantly, acrolein-induced lung injury was prevented and rescued by Alda-1, an activator of mitochondrial aldehyde dehydrogenase 2. Acrolein also dose-dependently increased monolayer permeability, disrupted adherens junctions and focal adhesion complexes, and caused intercellular gap formation in primary cultured lung microvascular endothelial cells (LMVECs). These effects were attenuated by Alda-1 and the antioxidant *N*-acetylcysteine, but not by the NADPH inhibitor apocynin. Furthermore, acrolein inhibited AMP-activated protein kinase (AMPK) and increased mitochondrial reactive oxygen species levels in LMVECs—effects that were associated with impaired mitochondrial respiration. AMPK total protein levels were also reduced in lung tissue of mice and LMVECs exposed to acrolein. Activation of AMPK with 5-aminoimidazole-4-carboxamide-1- $\beta$ -4-ribofuranoside blunted an acrolein-induced increase in endothelial monolayer permeability, but not mitochondrial oxidative stress or inhibition of mitochondrial respiration. Our results suggest that acrolein-induced mitochondrial dysfunction may not contribute

to endothelial barrier dysfunction. We speculate that detoxification of acrolein by Alda-1 and activation of AMPK may be novel approaches to prevent and treat acrolein-associated acute lung injury, which may occur after smoke inhalation.

**Keywords:** acrolein; acute lung injury; endothelial cells; mitochondrial aldehyde dehydrogenase 2; mitochondrial respiration

## Clinical Relevance

Inhalation of acrolein, a highly reactive aldehyde, causes lung edema. The underlying mechanism is poorly understood, and there is no effective treatment available. We demonstrated that acrolein-induced lung injury was prevented and rescued by Alda-1, an activator of mitochondrial aldehyde dehydrogenase 2. Acrolein-induced lung microvascular endothelial barrier dysfunction was also attenuated by Alda-1 and 5-aminoimidazole-4-carboxamide-1- $\beta$ -4-ribofuranoside, an activator of AMP-activated protein kinase. We speculate that Alda-1 and AMP-activated protein kinase activators may be novel treatments for acrolein-driven lung injury.

(Received in original form October 26, 2016; accepted in final form July 27, 2017)

Supported by the Providence VA Medical Center, the U.S. Department of Veterans Affairs Merit Review Program (S.R.), a Brown University Dean's Award (S.R.), and National Institutes of Health grants P20GM103652 (S.R.; project 1, Q.L.), RO1 HL130230 (Q.L.), U54GM115677 (S.R.), R25 HL088992 (R.B. and M.O.), and HL128661 (G.C.).

Author Contributions: Conception and design: Q.L. and S.R.; data acquisition and analysis: Q.L., M.M., E.C., T.L., J.N., D.B., H.Y., R.B., and M.O.; data interpretation and drafting of the manuscript: Q.L.; revision of the manuscript: Q.L., G.C., and S.R.

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Am J Respir Cell Mol Biol Vol 57, Iss 6, pp 662–673, Dec 2017

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Originally Published in Press as DOI: 10.1165/rcmb.2016-0342OC on August 1, 2017

Internet address: www.atsjournals.org

Acrolein is a highly reactive  $\alpha,\beta$ -unsaturated aldehyde with an estimated half-life of 12 hours. The safe reference concentration of acrolein for inhalation recommended by the U.S. Environmental Protection Agency is  $0.02 \mu\text{g}/\text{m}^3$ . The major sources of indoor acrolein are tobacco smoke and emissions from overheated oils, fireplace heating, and biomass cooking, whereas outdoor acrolein is mainly from gasoline and diesel exhaust, combustion of organic materials, forest fires, and burn pits used to dispose of waste by the U.S. military (1). As a result, tobacco smokers (2), firefighters (3, 4), restaurant workers (5), certain manufacturing workers (6), and military personnel deployed to military bases in Afghanistan and Iraq (7) can be exposed to toxic levels of acrolein. Lung tissue from mice exposed to cigarette smoke has increased levels of reactive aldehydes (8). In addition to exogenous sources, acrolein is endogenously produced via lipid peroxidation and metabolism (9).

Inhalation of low levels of acrolein causes nasal irritation, bronchial hyperreactivity, and excessive mucus production (10). Exposure to high levels of acrolein elevates blood pressure and causes quick cessation of heart beat (11). Acrolein exposure has been implicated in chronic obstructive pulmonary disease (12) and increases susceptibility to microbial infections (13). Smoke inhalation can cause acute respiratory distress syndrome (14). Acrolein is the second most common inhaled toxin, after carbon monoxide, in smoke inhalation during fires. Similarly to smoke inhalation, acrolein inhalation causes noncardiogenic pulmonary edema and respiratory distress in sheep (15, 16), dogs (17), and mice (18). In addition, acrolein was shown to increase bronchial epithelial barrier permeability (19) and alter the tight-junction protein Claudin 5 in a cultured endothelial cell line (18). However, it is not known whether acrolein directly increases lung microvascular endothelial cell (LMVEC) permeability. The mechanisms underlying acrolein-induced lung edema are not well understood.

Acrolein can be detoxified by glutathione-S-transferase alpha 4 (GST $\alpha$ 4), alkenal/one oxidoreductase, aldose reductase, or aldehyde dehydrogenase 2 (ALDH2) (20, 21). Excessive acrolein is subjected to Michael addition (also termed carbonylation), whereby the acrolein reacts with proteins or nucleic acids to form

aldehyde adducts (1). Protein carbonylation may cause protein misfolding, cross-linking, aggregation, and degradation (22). Acrolein has been reported to deplete cellular glutathione (GSH) (23), increase reactive oxygen species (ROS) production (24, 25), suppress NF- $\kappa$ B-dependent gene transcription (26, 27), inhibit histone acetylation (28), and change metabolome and transcriptome profiling in mouse lungs (29).

In this study, we found that acrolein caused lung edema and promoted LPS-induced acute lung injury (ALI). Alda-1 (*N*-C1,3-benzodioxol-5-ylmethyl)-2,6-dichlorobenzamide, an ALDH2 activator, not only prevented but also rescued acrolein-induced lung injury. Alda-1 also attenuated acrolein-induced endothelial barrier dysfunction. AMP-activated protein kinase (AMPK) is a ubiquitously expressed major sensor of cellular bioenergetics. We found that acrolein-induced lung edema and increased LMVEC permeability were associated with inhibition of AMPK and impaired mitochondrial respiration. The AMPK activator 5-aminoimidazole-4-carboxamide-1- $\beta$ -D-ribofuranoside (AICAR) abrogated acrolein-induced endothelial monolayer permeability. We speculate that Alda-1 and AMPK activators may be novel treatments for acrolein-driven lung injury.

Some of these results were presented at the American Thoracic Society International Conference, May 2015, and published in abstract form (30).

## Materials and Methods

### Reagents

Acrolein was purchased from Polysciences, Inc. (Warrington, PA). LPS (catalog # L5418, *Escherichia coli* [055:B5]), *N*-acetylcysteine (NAC), apocynin, and vinculin antibody were obtained from Sigma-Aldrich (St. Louis, MO). Alda-1, AICAR, and a keratinocyte chemoattractant ELISA kit were obtained from R&D Systems (Minneapolis, MN). IL-6 and TNF- $\alpha$  ELISA kits and  $\beta$ -catenin antibody were obtained from BD Biosciences (San Diego, CA). Antibodies against AMPK and phosphorylated AMPK at T172 were obtained from Cell Signaling (Danvers, MA). An IL-10 ELISA kit was obtained from Biolegend (San Diego, CA). Rat LMVECs were obtained from VEC

Technologies, Inc. (Rensselaer, NY) and used between passages 2 and 7.

### Mice

Male 8- to 10-week-old C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All animal studies were approved by the Institutional Animal Care and Use Committee of the Providence VA Medical Center. Animals were housed in standard conditions (12 h light/dark cycle, 68–72°F, and humidity of 30–70%) in ventilated racks with automatic watering systems and fed with standard chow *ad libitum*.

### Intratracheal Administration of Acrolein and LPS

Acrolein and LPS ( $\sim 50 \mu\text{l}$ ) were delivered intratracheally to mice anesthetized with 3% isoflurane as previously described (31).

Assessments of bronchoalveolar lavage (BAL) protein content, lung wet-to-dry weight ratio and inflammatory cell count were performed as previously described (32).

An ALDH2 enzymatic activity assay was performed on freshly harvested liver and lung tissue from mice treated, with acrolein in the absence or presence of Alda-1 using an ALDH2 activity assay kit (Abcam, Cambridge, MA). Optical density units of the reaction product were measured at a wavelength of 450 nm. Data are represented as means  $\pm$  SE at the 1-hour time point.

An endothelial monolayer permeability assay and immunofluorescence microscopy were performed as previously described (33).

### Measurement of Mitochondrial ROS

Cells were stained with Hoechst 33342 for 20 minutes and then with MitoSOX (both from ThermoFisher Scientific, Waltham, MA) for 20 minutes. The fluorescence signals of the MitoSOX and Hoechst 33342 were captured by a fluorescence spectrometer at excitations/emissions of 510/580 and 350/461, respectively. The data are presented as the ratio of MitoSOX to Hoechst 33342.

The mitochondrial oxygen consumption rate (OCR) was measured using an XF-96 Extracellular Flux Analyzer from Agilent Technologies (Santa Clara, CA) according to the manufacturer's Mito Stress Test Kit user guide. ATP production was assessed by injection of oligomycin ( $1 \mu\text{M}$ ). Maximal respiration capacity was

measured by injection of carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (0.5  $\mu$ M). Injection of rotenone/antimycin A (0.5  $\mu$ M) was used to determine nonmitochondrial respiration. All values were normalized to viable cells using the CyQUANTdirect cell assay (ThermoFisher Scientific). Each experimental condition included three to four wells that were averaged for one experimental data point.

### Data Analysis

For animal studies, three to 12 mice per group for each experiment were used. All experiments using cultured cells were performed at least three independent times. Data are presented as the mean  $\pm$  SE. The difference between two means was assessed using Student's *t* test. The differences among three or more means were assessed using ANOVA and Fisher's *post hoc* test. Differences among means are considered statistically significant when  $P < 0.05$ .

## Results

### Acrolein Dose-Dependently Increased Lung Edema and Inflammation

We previously showed that acute cigarette smoke exposure caused lung edema in mice (34). To determine whether acrolein has a similar effect, C57BL/6 mice were intratracheally instilled with varying doses of acrolein (0, 1, 2.5, and 5 mg/kg), and lung edema and inflammation were determined 18 hours after acrolein challenge. We found that acrolein dose-dependently increased the BAL protein content (Figure 1A) and BAL cell count (Figure 1B), with a minimal effective dose at 2.5 mg/kg. Intratracheal instillation of acrolein at 2.5 mg/kg also significantly increased the lung wet-to-dry weight ratio (Figure 1C). In addition, acrolein exposure dramatically elevated BAL cytokines, including KC, IL-6, and TNF- $\alpha$  (see Figures 4E–4G). These data indicate that acute acrolein exposure causes lung edema and inflammation. Additionally, mouse body weights were significantly reduced 18 hours after exposure to 2.5 mg/kg of acrolein (Figure 1D), an indication of systemic distress.

### Acrolein Promoted LPS-Induced ALI

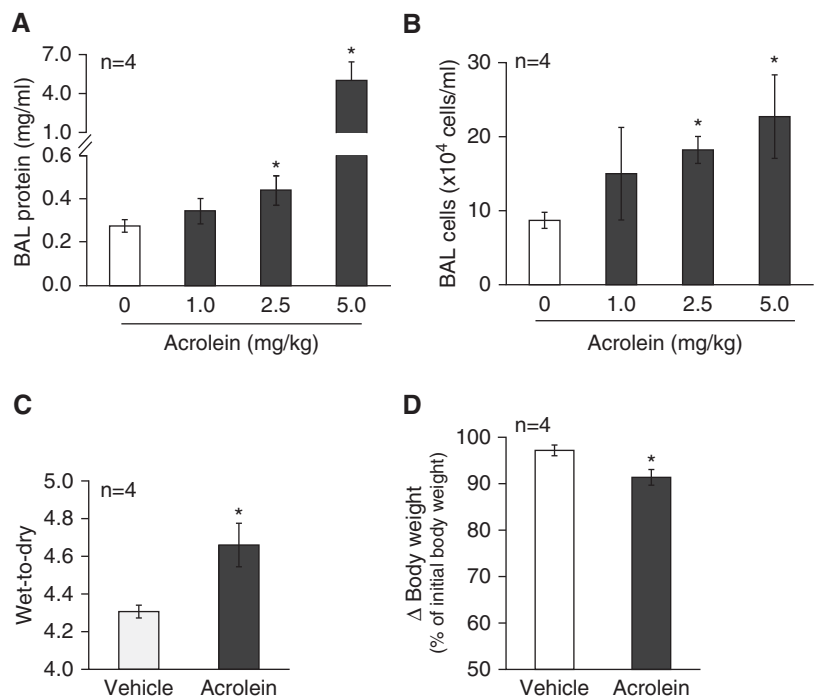
Cigarette smoking increases susceptibility to acute respiratory distress syndrome, as well

as the severity of the disease, in humans (35–38) and animal models (31). Acrolein is an important component of cigarette smoke. Because other investigators and we have found that cigarette smoking increases the inflammatory response to LPS inhalation in both mice (31) and humans (38), we questioned whether preexposure to acrolein would have an effect similar to that of cigarette smoking on LPS-induced lung injury. In the current study, mice were challenged with 2.5 mg/kg of acrolein by intratracheal instillation. After 6 hours of exposure to acrolein, the mice were intratracheally administered 2.5 mg/kg of LPS. Lung injury was assessed 18 hours after instillation of LPS. We found that acrolein alone increased the BAL protein content (Figure 2A) and caused a significant loss of body weight (Figure 2C), similar to the extent observed with LPS. Acrolein alone also increased the BAL inflammatory cell count, but to a lesser extent than LPS (Figure 2B). Interestingly, mice preexposed to acrolein had a greater increase in both the

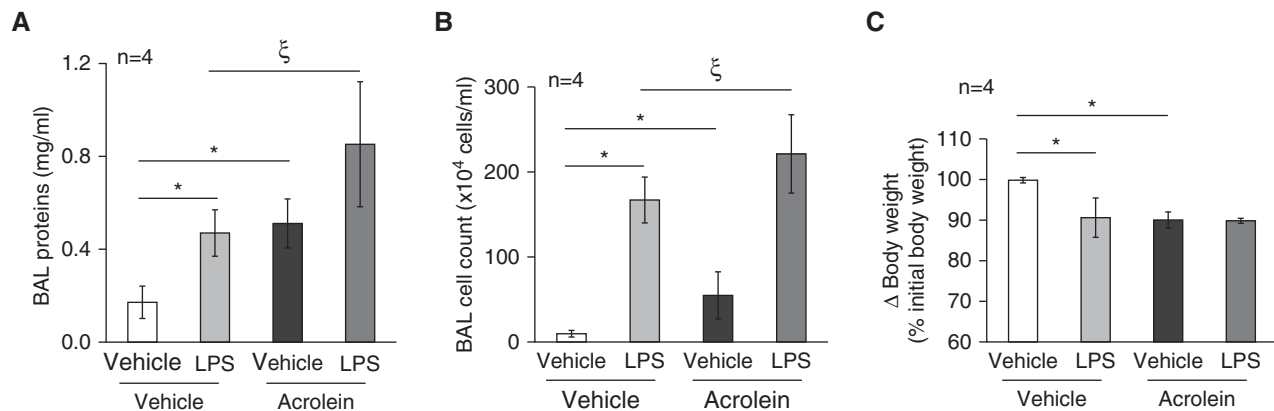
BAL protein content (Figure 2A) and BAL cell count (Figure 2B) after challenge with LPS as compared with LPS alone. These results indicate that acrolein had an additive effect on LPS-induced lung injury, which suggests that populations preexposed to acrolein may experience enhanced lung injury after bacterial infections.

### Alda-1 Prevented Acrolein-Induced Lung Edema and Inflammation

Reactive aldehydes are detoxified by ALDH2 (20, 21). Alda-1, a small-molecule activator of ALDH2, is capable of preventing the formation of cytotoxic reactive aldehydes (38). We found that pretreatment of mice with Alda-1 significantly attenuated the acrolein-induced increase in BAL protein content (Figure 3A). Alda-1 also exhibited a trend toward blunting of the acrolein-induced increase in BAL cell count (Figure 3B), lung wet-to-dry weight ratio (Figure 3C), and loss of body weight (Figure 3D).



**Figure 1.** Effects of acrolein on lung edema and inflammation. (A and B) Mice were intratracheally administered varying doses of acrolein (0–5 mg/kg) dissolved in sterilized saline. Bronchoalveolar lavage (BAL) fluid was collected 18 hours after instillation of acrolein. The total protein content (A) and leukocyte numbers (B) in BAL fluid were assessed. (C and D) Mice were intratracheally administered 2.5 mg/kg of acrolein or an equal volume of saline as vehicle control. The lung wet-to-dry weight ratio (C) and changes ( $\Delta$ ) in mouse body weights (D) were determined 18 hours after instillation of acrolein. Four mice per group were used for each panel. Data are represented as means  $\pm$  SE. \* $P < 0.05$  versus mice treated with vehicle control.



**Figure 2.** Effects of acrolein on LPS-induced lung injury. Mice were intratracheally administered 2.5 mg/kg of acrolein or an equal volume of saline (vehicle control). Six hours after instillation of acrolein, mice were intratracheally challenged with 2.5 mg/kg of LPS or an equal volume of saline. The total BAL protein content (A), BAL cell count (B), and changes in mouse body weights (C) were determined 18 hours after instillation of LPS. Four mice per group were used for each panel. Data are represented as means  $\pm$  SE. \* $P < 0.05$  versus mice treated with vehicle,  $\xi P < 0.05$  versus mice treated with LPS alone.

### Alda-1 Rescued Acrolein-Induced Lung Edema and Inflammation

To test the therapeutic effect of Alda-1 on acrolein-induced lung edema and inflammation, we administered Alda-1 to mice 2 hours after initiating lung injury by acrolein. ALDH2 activity and lung injury were assessed 18 hours after acrolein challenge. We noted that Alda-1 significantly elevated ALDH2 activity in liver tissue (Figure 4A). ALDH2 has been reported to be modestly expressed in lungs (40). However, lung ALDH2 activity was not detectable by our assay (data not shown). Although intratracheal administration of acrolein did not reduce liver ALDH2 activity, it attenuated Alda-1-induced activation of ALDH2 in livers (Figure 4A). Interestingly, Alda-1 significantly rescued the acrolein-induced increase in BAL protein content (Figure 4B), lung wet-to-dry weight ratio (see Figure 3C), and loss of body weight (Figure 4I), and showed a trend toward rescuing the acrolein-induced increase in the BAL cell count (Figure 4C). In addition, Alda-1 dramatically rescued acrolein-induced increases in proinflammatory cytokines, including KC (Figure 4E), IL-6 (Figure 4F), and TNF- $\alpha$  (Figure 4G). On the other hand, Alda-1 did not display a significant effect on acrolein-induced neutrophil infiltration (Figure 4D). Neither acrolein nor Alda-1 affected expression of the antiinflammatory cytokine IL-10 in lung homogenates (Figure 4H).

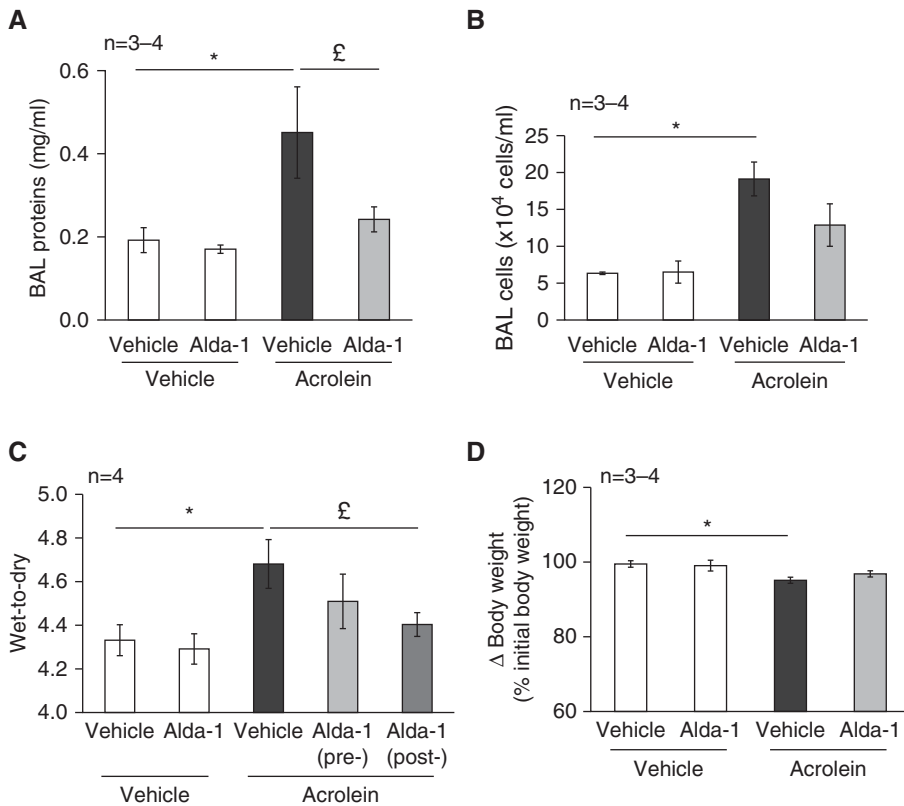
### Acrolein Increased LMVEC Monolayer Permeability

To understand the mechanism of acrolein-induced lung edema, we assessed the direct effect of acrolein on LMVEC monolayer permeability, which has not been reported previously. We found that acrolein dose-dependently decreased electrical resistance across monolayers of rat LMVECs (Figure 5A), indicating increased monolayer permeability. Similar effects were seen in human LMVECs (data not shown). Acrolein also disrupted intercellular adherens junctions (AJs), as indicated by reduced immunofluorescence staining of the AJ component  $\beta$ -catenin, and focal adherens complexes, as shown by loss of staining of the focal adherens complex component vinculin, and caused intercellular gap formation (Figure 5B). These data indicate that acrolein directly increases LMVEC permeability. We also found that the disruptive effect of acrolein on endothelial barrier integrity was recovered after removal of acrolein (Figure 5C), suggesting a reversible effect of acrolein on endothelial barrier function.

### NAC and Alda-1 Prevented an Acrolein-Induced Increase in Endothelial Monolayer Permeability

Reactive aldehydes can cause cellular oxidative stress by activating NADPH oxidases (24, 25) and/or depleting the antioxidant GSH (23). As expected, we

found that NAC completely prevented an acrolein-induced increase in endothelial monolayer permeability (Figures 5D and 5E). To test whether NADPH oxidases contribute to acrolein-induced oxidative stress, leading to increased endothelial permeability, we used apocynin, which inhibits the association of p47phox with a membrane-bound heterodimer and thus acts as an NADPH oxidase assembly inhibitor (41). We found that apocynin did not have any protective effect against an acrolein-induced increase in endothelial monolayer permeability (Figures 5F and 5G), suggesting that NADPH oxidases are not important in mediating acrolein-induced lung endothelial barrier dysfunction. On the other hand, consistent with our *in vivo* findings, Alda-1 significantly attenuated the acrolein-induced barrier dysfunction of LMVECs (Figures 5H and 5I). To further investigate the mechanism of acrolein-induced endothelial barrier dysfunction, we assessed mitochondrial oxidative stress, because acrolein has been shown to cause mitochondrial oxidative stress in hepatocytes (42). Consistently, acrolein enhanced mitochondrial ROS levels; an effect that was not prevented by Alda-1 (Figure 5J). Acrolein has been reported to suppress NF- $\kappa$ B-mediated gene transcription (26, 27). Therefore, we assessed NF- $\kappa$ B-driven endothelial activation by probing p65 phosphorylation. We observed a trend toward a decrease in NF- $\kappa$ B activity by acrolein in LMVECs—an



**Figure 3.** Preventive effects of Alda-1 on acrolein-induced lung edema and inflammation. Mice were administered Alda-1 (10 mg/kg) or an equal volume of sterilized saline (control) via intraperitoneal injection. After 1 hour, the mice were intratracheally administered 2.5 mg/kg of acrolein or an equal volume of sterilized saline. BAL protein levels (A), BAL cell count (B), lung wet-to-dry weight ratio (C), and changes in mouse body weights (D) were assessed 18 hours after challenge with acrolein. Some mice were treated first with acrolein for 2 hours, followed by Alda-1 (C). Three to four mice per group were used for each panel. Data are represented as means  $\pm$  SE. \* $P < 0.05$  versus mice treated with saline control,  $^{\#}P < 0.05$  versus mice treated with acrolein alone.

effect that was not prevented by Alda-1 (Figures 5K and 5L).

### Acrolein Decreased AMPK in LMVECs *In Vitro* and in Lung Tissue *In Vivo*

To further elucidate the mechanism by which acrolein increases endothelial cell permeability and causes lung edema, we assessed the effect of acrolein on AMPK activity by measuring AMPK phosphorylation at T172. We found that 30  $\mu$ M of acrolein time-dependently reduced both AMPK phosphorylation at T172 and total AMPK protein in cultured rat LMVECs after as little as 10 minutes of exposure, peaking at 1–3 hours of exposure (Figures 6A–6C). AMPK total protein was also significantly reduced in lung homogenates of mice exposed to acrolein for 18 hours (Figures 6D–6F). These data reveal an association of decreased AMPK activity with increased endothelial

monolayer permeability and lung edema upon acrolein exposure. We further demonstrated that the AMPK activator AICAR significantly attenuated the acrolein-induced increase in endothelial monolayer permeability (Figures 6G and 6H).

### Acrolein Impaired Mitochondrial Oxygen Consumption, and the Effect Was Not Prevented by Alda-1 or AICAR

AMPK is a key signaling molecule that regulates mitochondrial oxidative phosphorylation. Because AMPK was dramatically reduced by acrolein exposure, we assessed the effect of acrolein on mitochondrial oxygen consumption and found that acrolein dose- and time-dependently reduced basal respiration, ATP production, maximal respiration, and spare respiration capacity (Figures 7A–7D).

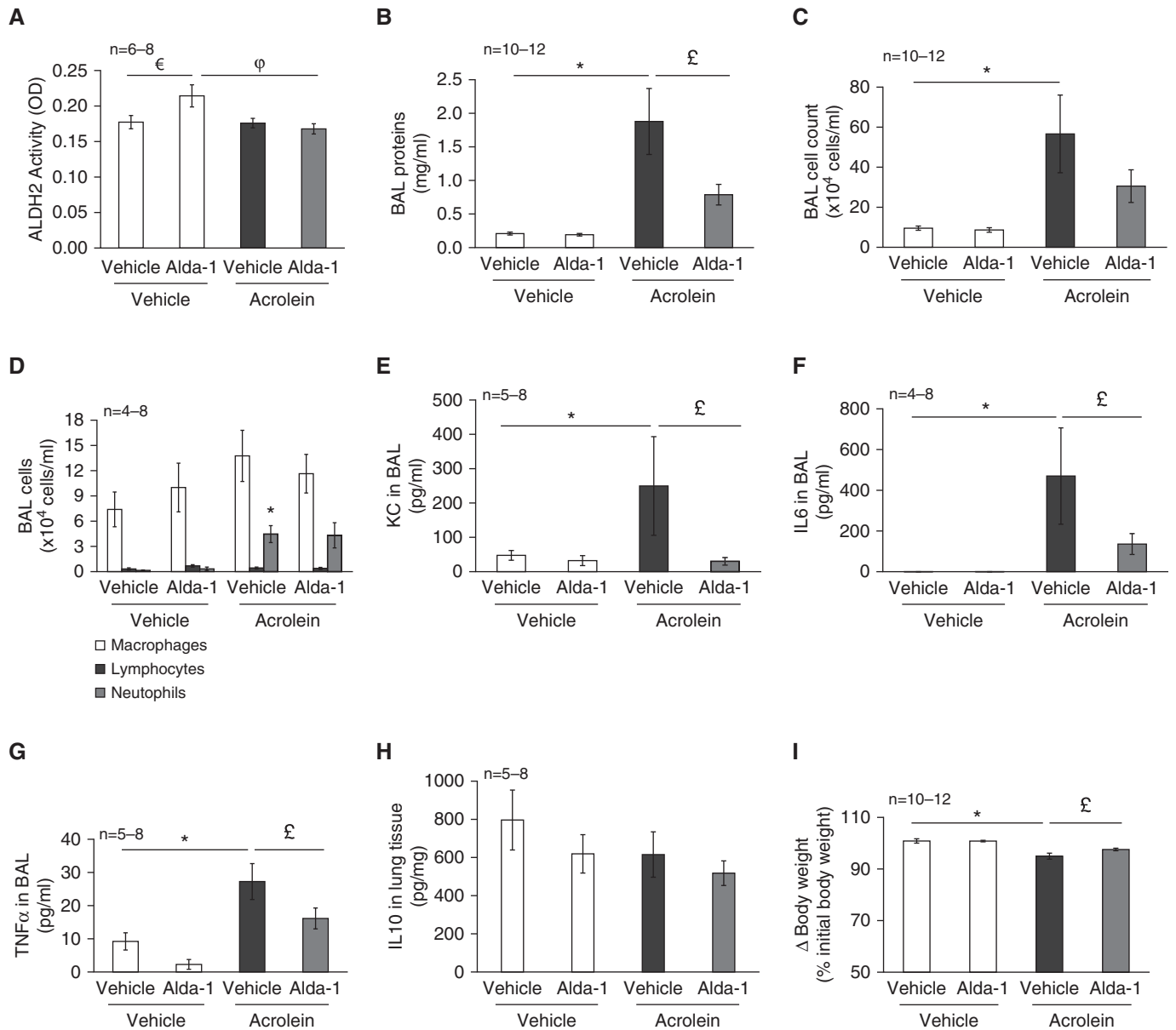
Because both Alda-1 and AICAR attenuated the acrolein-induced increase in endothelial cell permeability, we assessed the effects of Alda-1 and AICAR on the acrolein-induced inhibition of mitochondrial respiration. To our surprise, neither Alda-1 nor AICAR altered acrolein-induced suppression of OCR in cultured LMVECs (Figures 7E and 7F). Our data suggest that Alda-1 and AICAR protect against acrolein-induced endothelial permeability and lung injury via a mechanism that is independent of mitochondrial dysfunction.

## Discussion

Acrolein inhalation causes pulmonary edema in animals (16–18). In addition, acrolein has been reported to suppress LPS-induced T-helper cell type 1 cytokine responses (43). In this study, we demonstrated that acrolein not only caused lung edema but also worsened LPS-induced lung injury. Acrolein has been shown to increase bronchial epithelial barrier permeability (19) and to alter a tight-junction protein, Claudin 5, in an endothelial cell line (18). In this study, we showed that acrolein dose-dependently increased the permeability of primary cultured LMVECs, disrupted AJs and focal adhesion complexes *in vitro*, and caused lung edema *in vivo*. Our data suggest that the injurious effect of acrolein on LMVECs may contribute to smoke inhalation-induced lung edema. To our knowledge, this finding has not been reported previously.

Mice were intratracheally administered 2.5 mg/kg body weight of acrolein (molecular mass = 56.06 g/mole) in  $\sim 50$   $\mu$ l. This is  $\sim 1$   $\mu$ mole instilled in a concentration of 20 mM. Sidestream smoke is estimated to expose lungs to 25.2 mg of acrolein per pack or 450  $\mu$ moles per pack, whereas mainstream smoke exposes lungs to 1.5 mg of acrolein per pack or 188  $\mu$ moles per pack (10). Thus, the *in vivo* exposures to acrolein used in this study are comparable to pathophysiologic levels. Cell cultures were exposed to 10–50  $\mu$ M of acrolein, which is a much smaller amount than we used in the *in vivo* study, but similar to those used in previous *in vitro* studies (19).

Kitaguchi and colleagues previously reported that acrolein caused lung septal cell apoptosis and airspace enlargement after

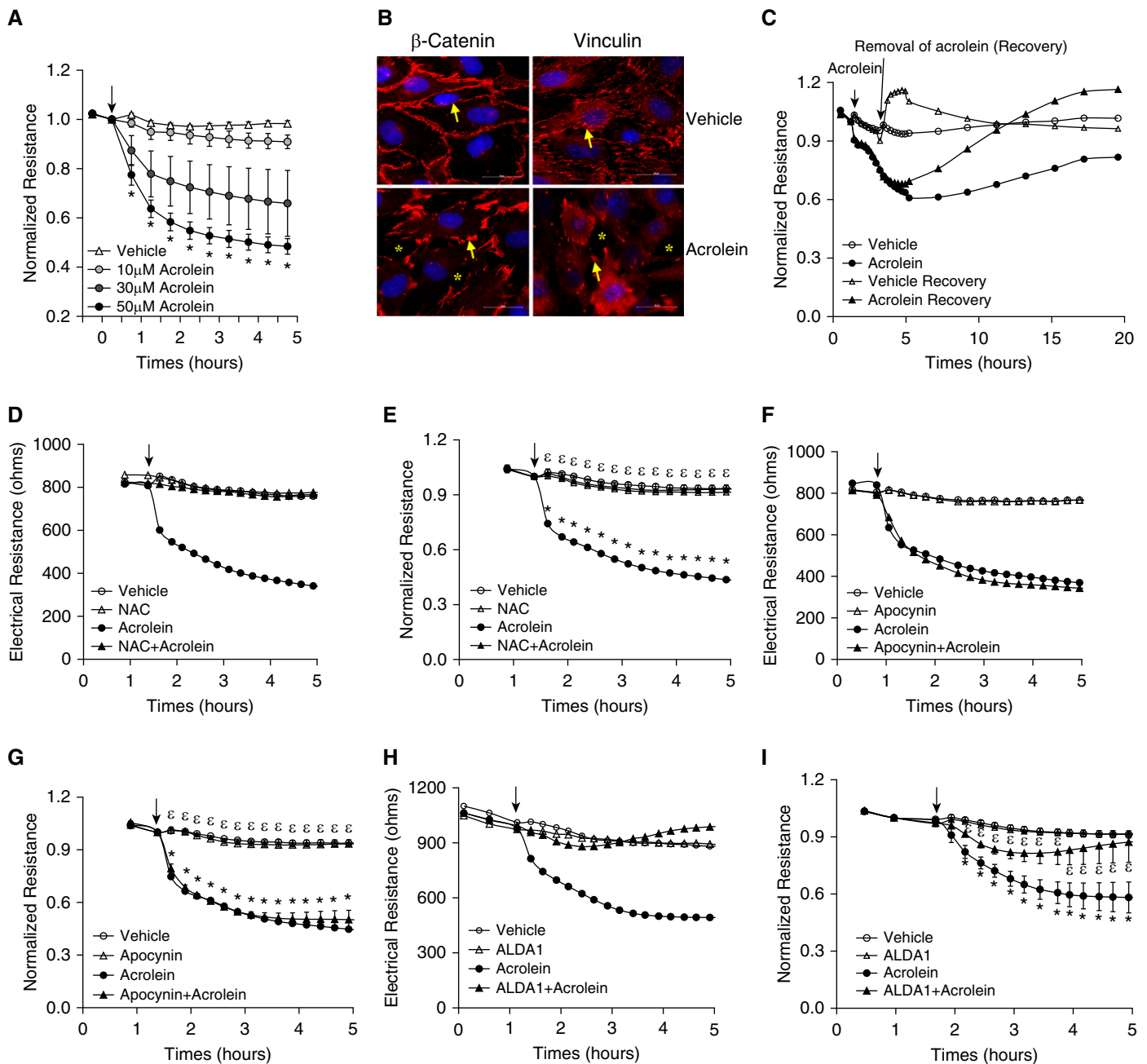


**Figure 4.** Treatment effects of Alda-1 on acrolein-induced lung edema and inflammation. Mice were intratracheally administered 2.5 mg/kg of acrolein or saline. Two hours after instillation of acrolein, the mice were given Alda-1 (10 mg/kg) or an equal volume of sterilized saline via intraperitoneal injection. Liver aldehyde dehydrogenase 2 (ALDH2) activity (A), BAL protein levels (B), BAL cell count (C), BAL cell differentiation (D), BAL cytokines (E–H), and changes in mouse body weights (I) were determined 18 hours after acrolein administration. Four to 12 mice per group were used for each panel. Data are represented as means  $\pm$  SE. €*P* < 0.05 versus mice treated with vehicle/vehicle, \**P* < 0.05 versus mice treated with saline control, £*P* < 0.05 versus mice treated with acrolein alone, φ*P* < 0.05 versus mice treated with Alda-1 alone. KC, keratinocyte chemoattractant; OD, optical density.

chronic administration in rats (44). We noted that acrolein did not cause LMVEC apoptosis after 6 hours of exposure (data not shown). The difference between our results and those of Kitaguchi and colleagues may be due to the different durations (chronic versus acute) of acrolein exposure. Intranasal exposure of mice to acrolein (1–5  $\mu$ mol/kg, similar to the concentrations used in this study) activates

alveolar macrophages and increases proinflammatory cytokines, including TNF- $\alpha$ , IL-6, IL-12, and Chi3L3 (45). In the current study, we found that acrolein instillation increased lung neutrophil infiltration, with a mild effect on alveolar macrophages. Acrolein also enhanced lung proinflammatory cytokines, including TNF- $\alpha$ , IL-6, and KC. Although acrolein and LPS had a similar effect on BAL protein

levels (0.51 mg/ml versus 0.47 mg/ml; Figure 2A), acrolein had a three times smaller effect on BAL cells than LPS ( $0.55 \times 10^6$  leukocytes/ml versus  $1.67 \times 10^6$  leukocytes/ml; Figure 2B). In other words, acrolein was more effective in promoting endothelial cell permeability and lung edema, and had a relatively milder effect on inflammation. Acrolein has been shown to inhibit NF- $\kappa$ B-mediated gene transcription



**Figure 5.** Effects of antioxidants and Alda-1 on an acrolein-induced increase in endothelial monolayer permeability. (A) Rat lung microvascular endothelial cells (LMVECs) were treated with vehicle or varying doses of acrolein for the indicated times. Monolayer permeability was assessed by measuring electrical resistance across the monolayers by electric cell-substrate impedance sensing (ECIS). Data are represented as the normalized electrical resistance of means  $\pm$  SE of three independent experiments. (B) Rat LMVECs were treated with vehicle or 30  $\mu$ M of acrolein for 2 hours, and  $\beta$ -catenin and vinculin were assessed by immunofluorescence microscopy. Arrows indicate  $\beta$ -catenin or vinculin, and asterisks indicate intercellular gaps. Representative images from three independent experiments are shown. Scale bars: 20  $\mu$ m. (C) Rat LMVECs were treated with vehicle or 30  $\mu$ M of acrolein for 2 hours. The acrolein was then removed or not removed as a control. The arrow indicates the time of acrolein removal. Monolayer permeability was assessed over time by ECIS. Representative tracings from three independent experiments are shown. (D–I) Rat LMVECs were preincubated with vehicle, 1 mM *N*-acetylcysteine (NAC), 10  $\mu$ M apocynin, or 50  $\mu$ M Alda-1 for 30 minutes and then exposed to vehicle or 30  $\mu$ M acrolein in the absence or presence of NAC, apocynin, or Alda-1 for the indicated times. Monolayer permeability was assessed by ECIS. (D, F, and H) Representative tracings from three to five independent experiments. Arrows indicate the time of addition of treatments. \**P* < 0.05 versus vehicle-treated cells, <sup>#</sup>*P* < 0.05 versus cells treated with acrolein. (J–L) Rat LMVECs were preincubated with vehicle, 80  $\mu$ M Alda-1, or 500  $\mu$ M 5-aminoimidazole-4-carboxamide-1- $\beta$ -D-ribofuranoside (AICAR) for 30 minutes and then exposed to vehicle or 50  $\mu$ M acrolein in the absence or presence of Alda-1 or AICAR for 2 hours. Mitochondrial reactive oxygen species was assessed by mitoSOX staining (J). (K and L) NF- $\kappa$ B activity was assessed by probing p65 phosphorylation. (K) Representative immunoblots. (L) Densitometry of immunoblot signals, means  $\pm$  SE of three independent experiments. \**P* < 0.05 versus vehicle-treated cells. Actin was used as a protein loading control.

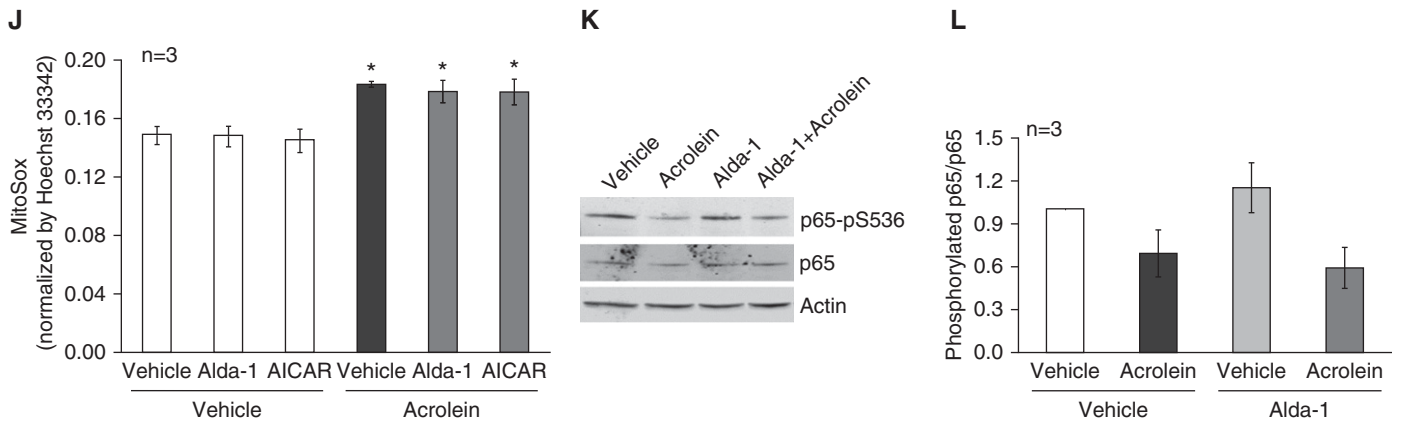


Figure 5. (Continued).

(26, 27). We observed a trend toward a decrease in NF- $\kappa$ B activity in LMVECs exposed to acrolein. Taken together, these results suggest that acrolein-induced alveolar barrier dysfunction and lung edema may be more important than proinflammatory effects in contributing to ALI.

Kasahara and colleagues demonstrated that the effect of acrolein on LPS-induced lung injury and inflammation was dependent on the timing of acrolein exposure (43). They reported that inhalation of the vapor phase of acrolein 18 hours after LPS instillation suppressed LPS-induced lung edema and proinflammatory responses (43). However, acrolein inhalation immediately after LPS instillation enhanced the LPS-induced increase in BAL protein levels and lung proinflammatory cytokines (43). In the current study, we showed that preexposure to intratracheally instilled acrolein 6 hours before LPS administration exaggerated LPS-induced lung edema and BAL inflammatory cell numbers. The difference between our results and those of Kasahara and colleagues may be due to the different doses of acrolein, the methods of exposure, and the timing of exposures relative to LPS used in the studies.

Reactive aldehydes can cause cellular oxidative stress by activating NADPH oxidase (24, 25) and/or depleting the antioxidant GSH (46) via the formation of aldehyde adducts (19, 47). Acrolein has been shown to deplete GSH and cause mitochondrial oxidative stress in hepatocytes (42). In contrast, acrolein has been shown to increase the antioxidant capacity of cells (48). In this study, we

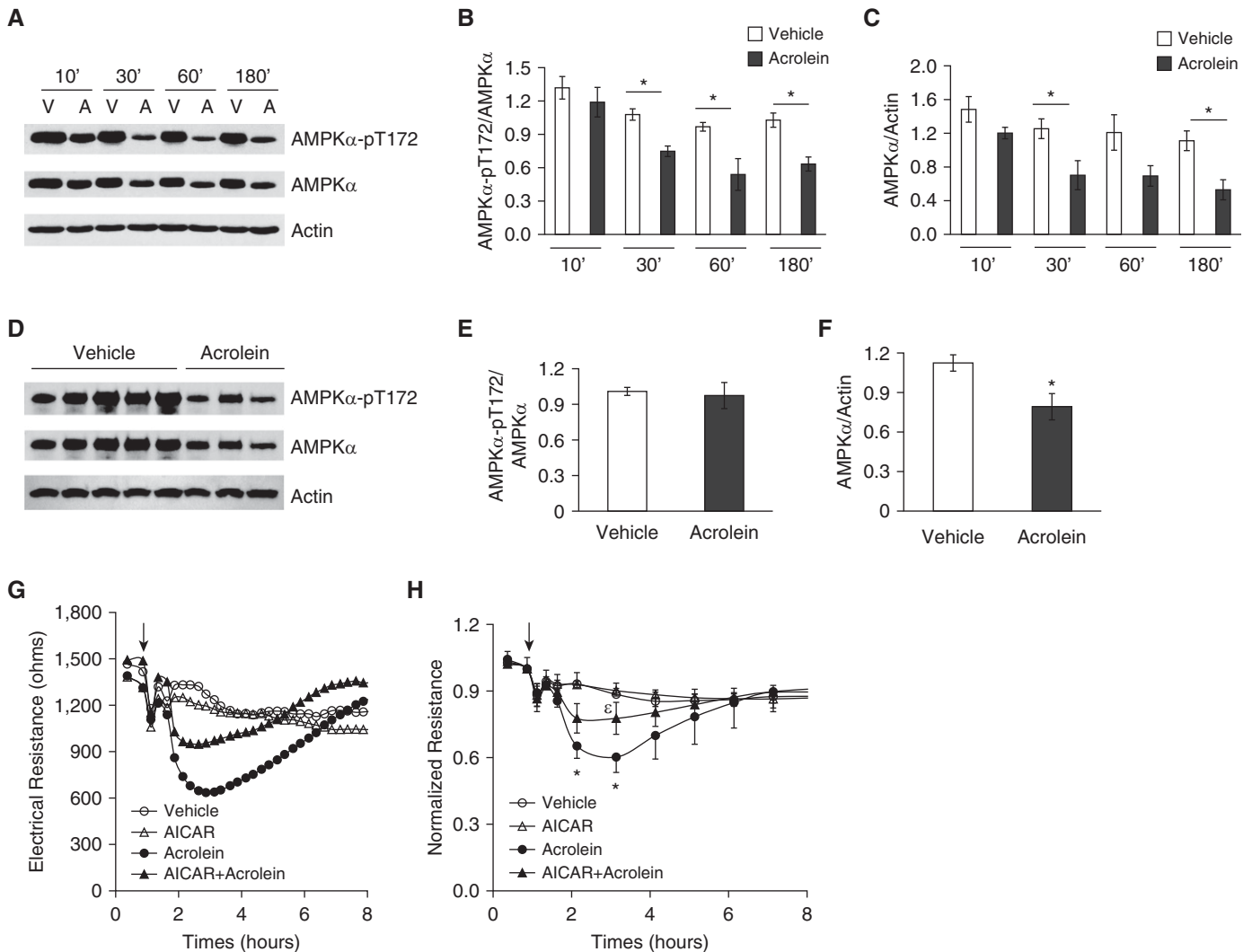
observed an increase of mitochondrial ROS in LMVECs treated with acrolein. We also found that acrolein-induced endothelial barrier dysfunction was completely prevented by the antioxidant NAC, a precursor of GSH, but not by apocynin, an NADPH oxidase inhibitor. Our results suggest that acrolein disrupts LMVEC barrier integrity, likely by depleting GSH rather than by activating NADPH oxidase.

Acrolein can be oxidized and thus detoxified by ALDH2, which is modestly expressed in lungs (40). Alda-1 is a small-molecule activator of ALDH2 (39). We confirmed that Alda-1 significantly elevated ALDH2 activity in liver. However, lung ALDH2 activity remained undetectable in our assay. Acrolein is also a potent inhibitor of both cytosolic and mitochondrial ALDH isoforms (49). We noted that liver ALDH2 activity did not decrease in mice intratracheally challenged with acrolein. This may be due to the instillation route used to administer the acrolein, and limited accumulation of acrolein in the liver, which would prevent a reduction in the baseline levels of ALDH2 in liver. However, acrolein prevented Alda-1-induced activation of ALDH2 in liver. We do not know whether acrolein inhibits basal or Alda-1-induced ALDH2 activity in lungs. We speculate that acrolein inhibits ALDH2 activity in lungs, leading to oxidative stress and lung injury. We show that Alda-1 not only prevented but also rescued acrolein-induced lung edema and lung inflammation *in vivo*. Alda-1 also attenuated acrolein-induced LMVEC barrier dysfunction *in vitro*. Although we still do not know whether Alda-1 protects against lung injury by preventing acrolein-induced inhibition of

ALDH2, our data suggest that Alda-1 may be a novel strategy to treat smoke inhalation and other acrolein-associated lung injuries.

Activation of AMPK has been shown to attenuate lung inflammation and the severity of lung injuries induced by LPS (50) and sepsis (51), as well as LPS-induced lung vascular endothelial permeability (52). AMPK is activated via phosphorylation of T172. The reactive aldehyde 4-hydroxy-2-nonenal (4-HNE) has been shown to inactivate AMPK via carbonylation and thus dephosphorylation of AMPK at T172 (53). Similar to what was observed for 4-HNE, acrolein also inhibited AMPK activity, as indicated by reduced AMPK phosphorylation at T172. It is possible that acrolein causes AMPK carbonylation and thus dephosphorylation at T172. Additionally, lung AMPK expression levels were also reduced in mice exposed to acrolein—an effect that was associated with lung edema. Importantly, activation of AMPK with AICAR attenuated acrolein-induced LMVEC monolayer permeability. Our results suggest that inhibition of AMPK signaling may contribute to acrolein-induced lung injury. The mechanism by which inhibition of AMPK leads to endothelial barrier dysfunction and lung injury is not understood. High-mobility group box 1 protein (HMGB1), a component of damage-associated molecular patterns, is released during tissue injury and is one of the crucial proinflammatory cytokines that mediate the response to infection and inflammation (54). AMPK activators have been shown to prevent lethal endotoxin-induced lung inflammation and improve survival in mice





**Figure 6.** Role of AMP-activated protein kinase (AMPK) in acrolein-induced endothelial monolayer permeability. (A–C) Rat LMVECs were treated with vehicle (V) or 30  $\mu$ M acrolein (A) for the indicated times. (D–F) Mice were intratracheally administered with 2.5 mg/kg of acrolein or saline for 18 hours. AMPK activation (AMPK $\alpha$ -pT172) and total AMPK protein levels were assessed in cell lysates and lung tissue. Actin was used as a protein loading control. Representative immunoblots are shown in A and D. Densitometry was performed on four independent experiments (B and C) or three to five mice (E and F). The ratio of AMPK $\alpha$ -pT172 to AMPK (B and C) and the ratio of AMPK to actin (E and F) are presented as means  $\pm$  SE. \* $P < 0.05$  versus respective vehicle control. (G and H) Rat LMVECs were preincubated with vehicle or 500  $\mu$ M AICAR for 30 minutes and then exposed to vehicle or 30  $\mu$ M acrolein in the absence or presence of AICAR for the indicated times. Monolayer permeability was assessed by ECIS. (G) Representative tracing from three independent experiments. (H) Normalized electrical resistance of means  $\pm$  SE of three independent experiments. Arrows indicate the time of addition of treatments. \* $P < 0.05$  versus vehicle-treated cells.  $^{\#}P < 0.05$  versus cells treated with acrolein.

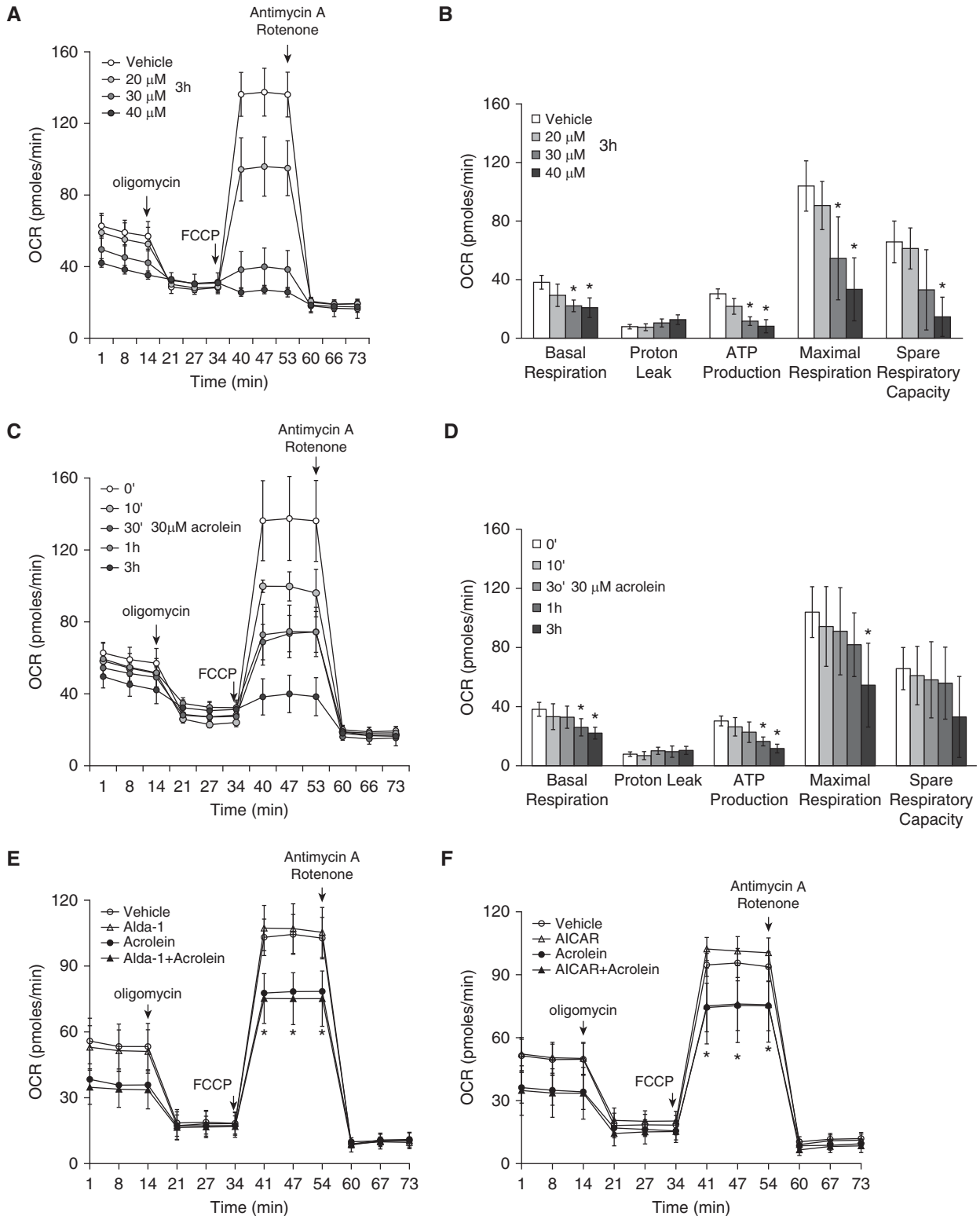
by inhibiting LPS-induced HMGB1 release (55, 56). Whether HMGB1 release contributes to acrolein-induced alveolar-capillary barrier permeability and lung injury remains to be determined.

We showed that acrolein reduced total AMPK protein levels *in vitro* and *in vivo*. The underlying mechanism remains unknown. Protein carbonylation may cause protein misfolding, cross-linking, aggregation, and degradation (22). We

noted that the acrolein-induced decrease in total AMPK in LMVECs was not altered by MG132 (data not shown), suggesting that the decrease in AMPK protein is independent of proteasome-mediated protein degradation. It is possible that carbonylated AMPK is degraded via the unfolded protein response, followed by protein degradation or autophagy/lysosome-associated protein degradation. It is also possible that acrolein reduced

AMPK gene transcription. The exact mechanism of the acrolein-induced reduction in AMPK protein levels remains unknown and warrants further investigation.

Acrolein has been shown to impair mitochondrial respiration in alveolar type 2 epithelial cells (57). Similarly, we found that acrolein dose- and time-dependently impaired LMVEC mitochondrial respiration. Although Alda-1 and AICAR



**Figure 7.** Effects of Alda-1 and AICAR on acrolein-induced impairment of mitochondrial respiration. (A–D) Rat LMVECs were treated with vehicle or the indicated doses of acrolein for the indicated times or (E and F) preincubated with vehicle, 50 μM Alda-1, or 500 μM AICAR for 30 minutes and then exposed to vehicle or 30 μM acrolein in the absence or presence of Alda-1 or AICAR for 1 hour. The mitochondrial oxygen consumption rate (OCR) was assessed with an XF Seahorse analyzer. (A and C) Representative experiments in which three to four wells were averaged for each data point. (B and D–F) Data represent the means ± SE of three to four independent experiments for each panel. \* $P < 0.05$  versus the respective vehicle. FCCP, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone.

attenuated acrolein-induced LMVEC permeability, neither Alda-1 nor AICAR prevented acrolein-induced inhibition of mitochondrial respiration. It has been reported that increased monolayer permeability precedes ATP loss in acrolein-exposed Calu-3 lung adenocarcinoma cells (58). Taken together, our results suggest that acrolein increased LMVEC permeability and caused lung edema via a mechanism that is independent of mitochondrial dysfunction. Because

acrolein not only reduced AMPK activity but also decreased AMPK total protein levels, the inability of the AMPK activator AICAR to prevent acrolein-induced mitochondrial dysfunction may be due to overall diminished amounts of AMPK in acrolein-treated cells. Our results also suggest that AMPK activity is essential for endothelial barrier function, whereas the amount of AMPK protein is important for mitochondrial function.

We recognize that *in vivo* acrolein has complex effects on multiple cell types. Alda-1 may target other cells in addition to LMVECs. Nevertheless, our results suggest that detoxification of acrolein by Alda-1 and activation of AMPK by AICAR may be novel approaches to prevent and treat acrolein-associated ALI. ■

**Author disclosures** are available with the text of this article at [www.atsjournals.org](http://www.atsjournals.org).

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