

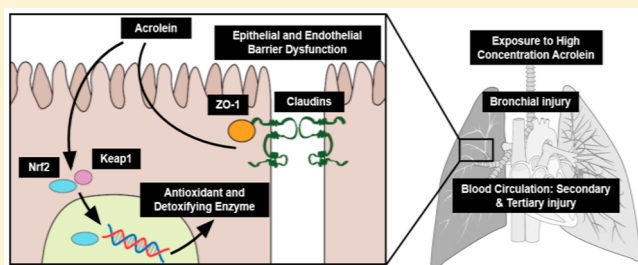
Claudins, VEGF, Nrf2, Keap1, and Nonspecific Airway Hyper-Reactivity Are Increased in Mice Co-Exposed to Allergen and Acrolein

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ABSTRACT: Acrolein, an α/β -unsaturated aldehyde, is volatile at room temperature. It is a respiratory irritant found in environmental tobacco smoke, which can be generated during cooking or endogenously at sites of injury. An acute high concentration of uncontrolled irritant exposure can lead to an asthma-like syndrome known as reactive airways dysfunction syndrome (RADS). However, whether acrolein can induce RADS remains poorly understood. The aim of study is to develop a RADS model of acrolein inhalation in mice and to clarify the mechanism of RADS.

Mice were treated with ovalbumin (OVA) and exposed to acrolein (5 ppm/10 min). Airway hyper-responsiveness (AHR) was measured on days 24 and 56, and samples were collected on days 25 and 57. Tight junction protein, antioxidant-associated protein, and vascular endothelial growth factor (VEGF) levels were estimated by Western blotting and immunohistochemical staining. Reactive oxygen species (ROS) was calculated using enzyme linked immunosorbent assays. Acrolein or OVA groups exhibited an increase in airway inflammatory cells and AHR compared to a sham group. These effects were further increased in mice in the OVA + acrolein exposure group than in the OVA exposure group and persisted in the acrolein exposure group for 8 weeks. CLDNs, carbonyls, VEGF, Nrf2, and Keap1 were observed in the lungs. Our data demonstrate that acrolein induces RADS and that ROS, angiogenesis, and tight junction proteins are involved in RADS in a mouse model.



INTRODUCTION

The well-defined subset of irritant-induced asthma was first used by Brooks (1985)¹ and termed reactive airways dysfunction syndrome (RADS). According to original definition of RADS,^{1–3} the patient symptoms develop quickly, within minutes or hours after high-concentration exposure. Asthma similar symptoms (chest pain/tightness, coughing, or dyspnea) persist for a long time period,⁴ and the nonspecific challenge test is usually positive with positive histopathological results. Recently recommended cardinal diagnostic features of RADS include: (1) identification of the date, time, frequency, and extent of exposure; (2) symptoms occurring within 24 h; (3) no latency period between inhalation and symptoms;⁵ (4) symptoms less likely to improve away from workplace;^{6,7} and (5) objective (pulmonary function) tests (e.g., the methacholine challenge test) demonstrating an obstruction⁸ and the presence and persistence of nonspecific airway hyper-responsiveness (AHR). RADS usually follows inhalation to a corrosive or irritating gas, vapor, fume, or dust; thus, it is also categorized as irritant-induced occupational asthma.⁵

Acrolein is a volatile α/β -unsaturated aldehyde at room temperature. It is a respiratory irritant found in environmental tobacco smoke that can be generated during cooking or endogenously at sites of injury.⁶

An acute high concentration of uncontrolled chemical exposure can cause RADS and lead to long-term sequelae,

chronic airway disease.⁸ However, whether acrolein induces RADS and its mechanism in terms of tight junctional protein, vascular endothelial growth factor (VEGF), reactive oxygen species (ROS), antioxidant enzyme is unknown.

The current study examined whether short-term acrolein exposure induces bronchial hyper-reactivity, whether that hyper-reactivity persists following a single high concentration exposure to acrolein, and the mechanism of RADS in mouse model of asthma.

MATERIALS AND METHODS

Experimental Design. OVA sensitized and challenged mice (female, 6-week, BALB/c, each group, $n = 8$) protocol was used as previously described (Figure 1).⁹ Mice in acrolein or OVA + acrolein group were administered 5 ppm by inhalation at 10 min for 1 day. On day 23 and after 6 weeks (total, recovery and dysfunction group), AHR and bronchoalveolar-lavage fluid (BALF) and lung tissues were obtained, and ROS, cytokine measurement, and tissue staining were done. The mouse experiment was approved by the affiliation University's animal Institutional review board (SCHBC-Animal 2016-02).

AHR and Morphology analyses. Measurement of AHR and morphology of BALF procedures were performed as previously described.¹⁰

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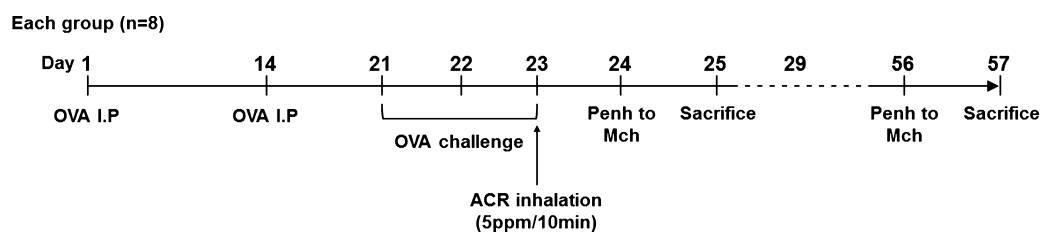


Figure 1. Experiment protocol of acrolein exposure model.

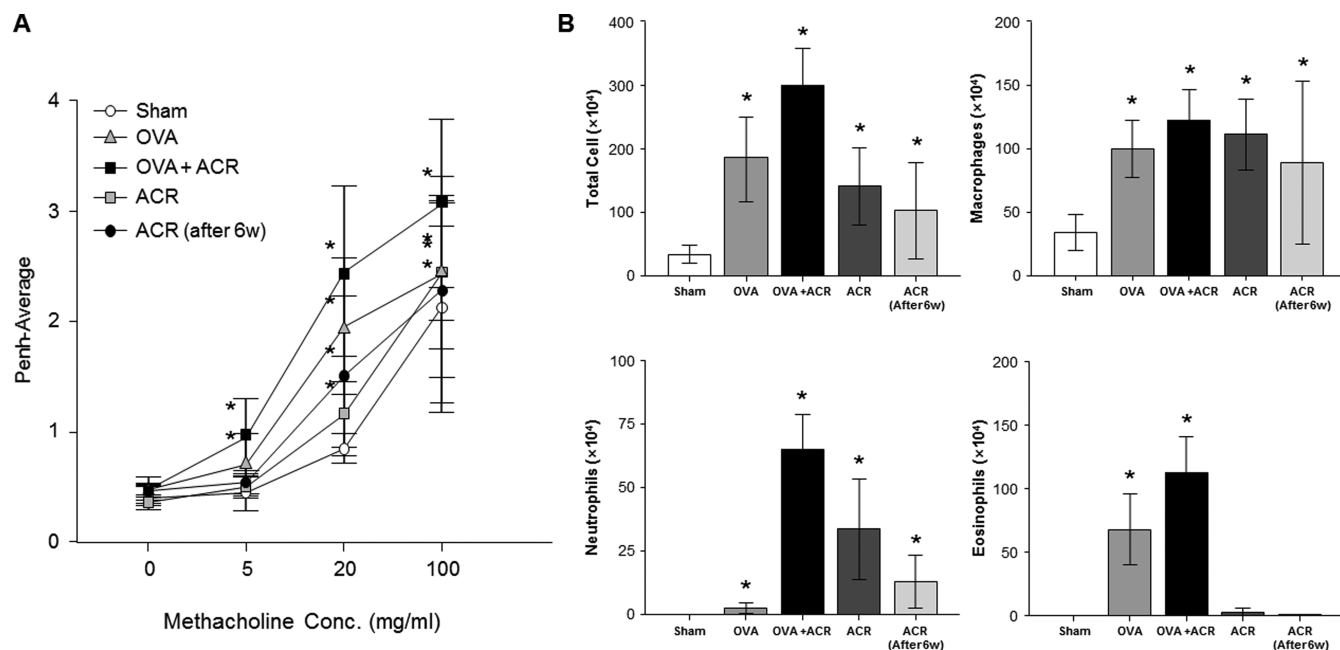


Figure 2. Cell differentials and airway hyper-responsiveness following acrolein exposure. (A) Mice exposed to acrolein (5 ppm, for 10 min) or OVA exhibited increased bronchial hyper-responsiveness compared to the control group ($P < 0.05$, Figure 2). Airway hyper-responsiveness was more increased in the OVA + acrolein exposure group than in the OVA group and persisted in the acrolein exposure group for 8 weeks. (B) Mice exposed to acrolein (5 ppm, for 10 min) or OVA group increased airway inflammatory cell infiltration compared to control group ($P < 0.05$, Figure 2). Airway inflammations were more increased in OVA + acrolein exposure group than in OVA group and persisted in acrolein exposure group for 8 weeks. $* < 0.05$ compared with control.

Western Blotting. Western blot and protein extraction from lung tissue was used as previously described.¹⁰ The membranes were with CLDN4 (1:200, Abcam), CLDN5 (1:200, Abcam), VEGF (1:1000, Thermo Fisher, Waltham, MA, USA), nuclear factor (erythroid-derived 2)-like 2 (Nrf2) (1:1000, Abcam), or Kelch-like ECH-associated protein 1 (Keap1) (1:1000, Abcam) overnight at 4 °C.

Immunohistochemical Analysis. Staining methods in mouse lung tissue were conducted as previously described.¹⁰ We used antibody CLDN4 (1:1000, Abcam) or CLDN5 (1:200, Abcam). Stained tissue was quantified using ImageJ (National Institutes of Health, Bethesda, MD, USA).

ELISA for Carbonyl Products of ROS. Measurement of carbonyl using OxiSelect competitive ELISA kit (Cell Biolabs, San Diego, CA, USA) was performed according to the manufacturer's protocol. Cutoff values of 10 $\mu\text{g/mL}$ were used based on manufacturer's recommendations.

ELISA for Measurement of Th2 Cytokine Interleukin (IL)-5 in BALF. The Th2 cytokine IL-5 were measured in BALF by IL-5 ELISA kit (Creative Diagnostics, NY, USA). ELISA was performed according to the manufacturer's protocol. The detection limit of IL-5 assay is 8 pg/mL.

Statistical Analysis. The data are analyzed as means $\pm$ standard deviation or standard error of the mean by using SPSS (ver. 20.0; SPSS Inc.; Chicago, IL, USA). Group differences analyzed using a two-sample *t* test, Mann–Whitney test, or Pearson's χ^2 test.

RESULTS

AHR and Inflammatory Cell Infiltration Following Acrolein Exposure. Mice exposed to acrolein (5 ppm for 10 min) or ovalbumin (OVA) exhibited increased airway inflammatory cell infiltration and AHR compared to sham group ($P < 0.05$) (Figure 2). Airway inflammation and AHR were further increased in OVA + acrolein exposure group compared to OVA group and persisted in the acrolein exposure group for 8 weeks.

Tight Junction (TJ) Protein Expression Following Acrolein Exposure. Claudin (CLDN)4, CLDN5, and CLDN17 protein levels, as assessed by Western blotting, were increased in OVA or acrolein exposure groups compared with sham group ($P < 0.05$) (Figure 3A). CLDN4, CLDN5, and CLDN17 proteins were further increased in the OVA + acrolein exposure group compared with OVA group ($P < 0.05$) (Figure 3A). CLDN4, CLDN5, and CLDN17 protein levels expression, as assessed by immunohistochemical staining, was increased in OVA and acrolein exposure groups compared with sham group ($P < 0.05$) (Figure 3B). CLDN4, CLDN5, and CLDN17 protein were further increased in OVA + acrolein exposure group compared with OVA group ($P < 0.05$, Figure 3B).

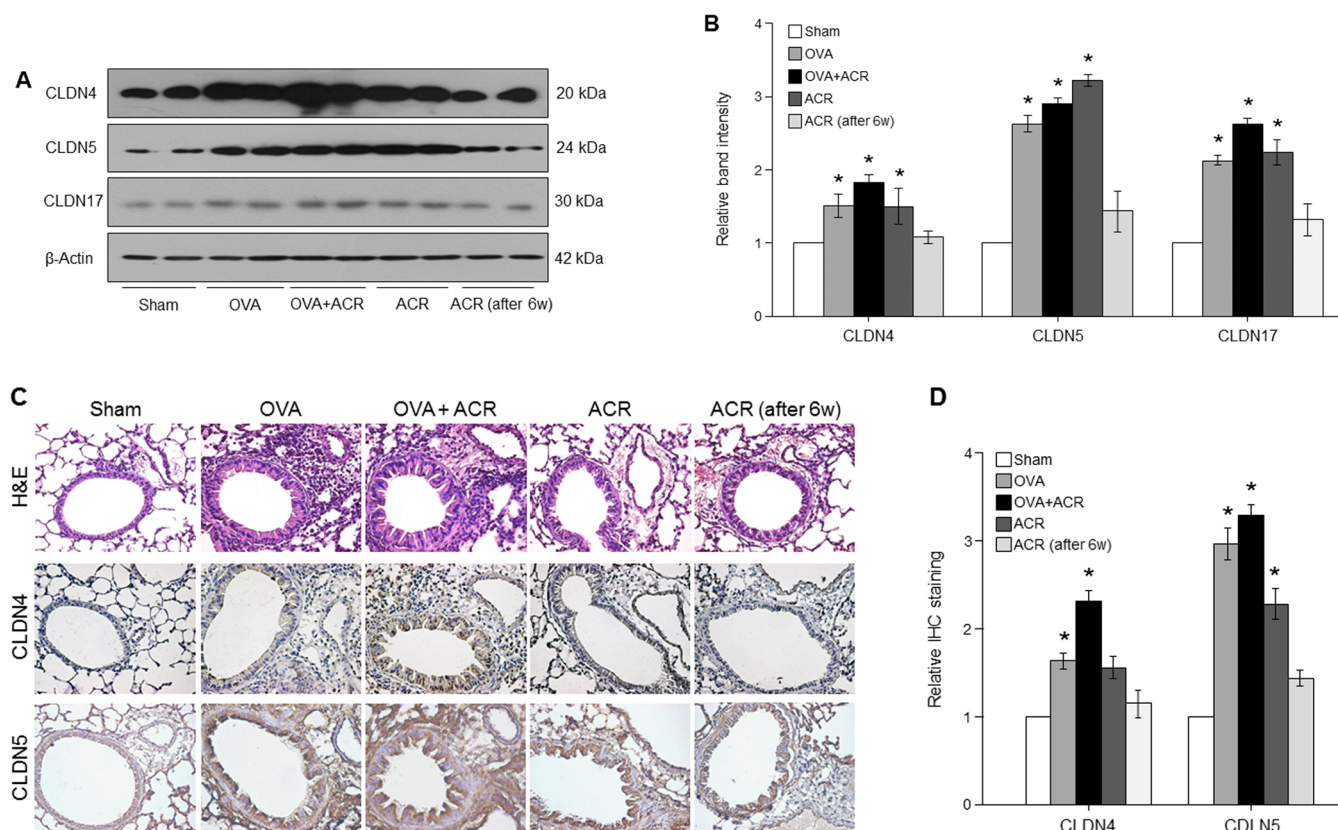


Figure 3. Tight junctional proteins change following acrolein exposure. CLDN4, CLDN5, and CLDN17 protein levels by Western blotting (A) and immunohistochemical staining (C) was increased in OVA group and acrolein exposure groups compared with control group ($P < 0.05$, Figure 3). CLDN4, CLDN5, and CLDN17 protein levels were further increased in OVA + acrolein exposure group compared with OVA group ($P < 0.05$, B, D). * <0.05 compared with controls.

Transcription Factors such as VEGF, Nrf2, and Keap1 Following Acrolein Exposure. The protein levels of VEGF and Nrf2 were significantly increased in OVA and acrolein exposure groups compared with sham group, whereas Keap1 was significantly increased in OVA + acrolein group compared with sham group ($P < 0.05$) (Figure 4). The expression of VEGF and Nrf2 persisted in the acrolein exposure group for 8 weeks.

ROS and IL-5 Levels Following Acrolein Exposure. ROS and protein carbonyl levels were increased in the lungs of mice from the OVA and acrolein exposure groups compared with control group and were further increased in OVA + acrolein exposure group ($P < 0.05$) (Figure 5). IL-5 levels were increased in the lungs of mice from OVA and acrolein exposure groups compared with sham group and were further increased in OVA + acrolein exposure group ($P < 0.05$, Figure 6).

Persistent Expression Markers Following Acrolein Exposure. CLDN4, CLDN5, VEGF, and Nrf2 were expressed in mice lung tissues exposed to a single high concentration of acrolein for 8 weeks ($P < 0.05$) (Figure 6).

DISCUSSION

We found that a high concentration of acrolein persistently increased lung inflammation in an asthmatic mice model. Six weeks following acrolein exposure, CLDN4, CLDN5, VEGF, and Nrf2 were expressed in lung tissues of mice, suggesting that a single chemical exposure can cause RADS and that TJ

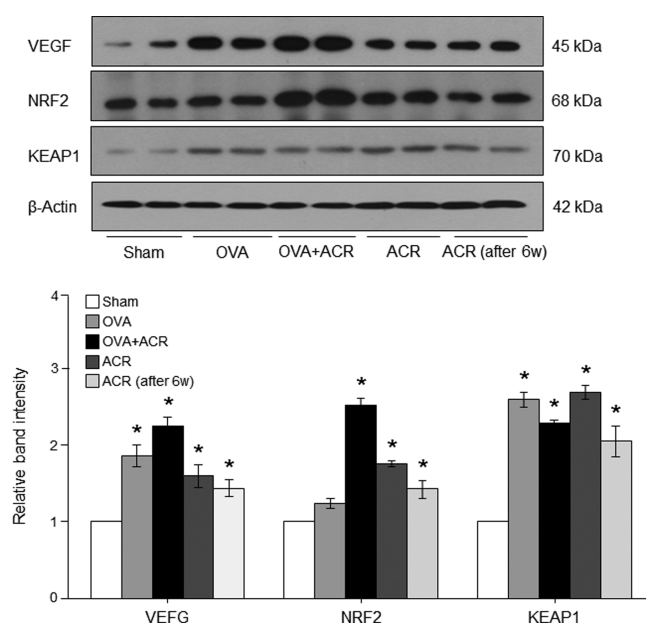


Figure 4. Endothelial angiogenesis factor and antioxidant transcription factors following acrolein exposure. The protein levels of VEGF and Nrf2 were significantly increased in OVA and acrolein exposure groups compared with control group, whereas Keap1 protein was significantly increased in OVA + acrolein group compared with control group ($P < 0.05$, Figure 4). The expression of VEGF and Nrf2 persisted in the acrolein exposure group for 8 weeks. * <0.05 compared with controls.

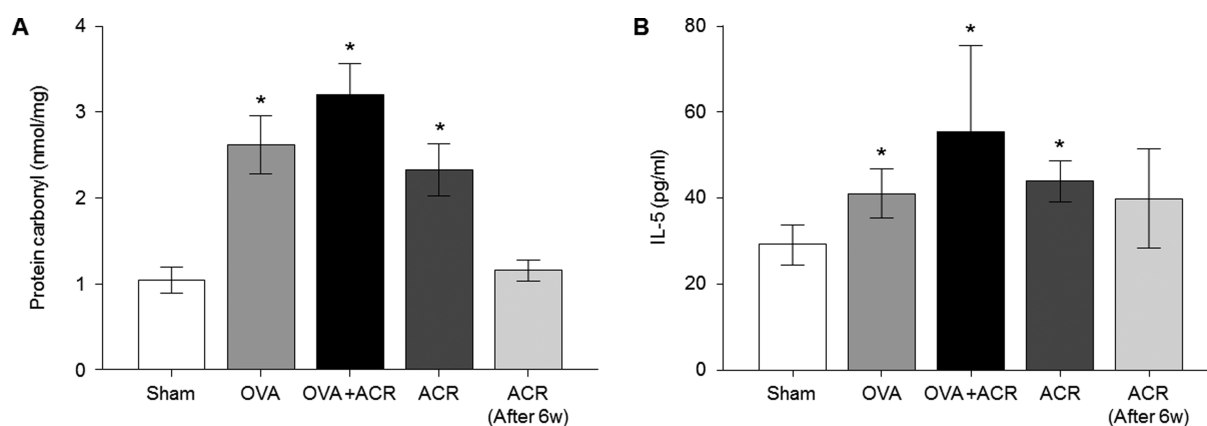


Figure 5. Reactive oxygen species and IL-5 following acrolein exposure. ROS protein carbonyl levels in the lungs were increased in the lungs of mice from the OVA and acrolein exposure groups compared with control group and more were further increased in OVA + acrolein exposure group. IL-5 levels in the lungs were increased in OVA or acrolein exposure group compared with control group and more increased in OVA + acrolein exposure group. * <0.05 compared with controls.

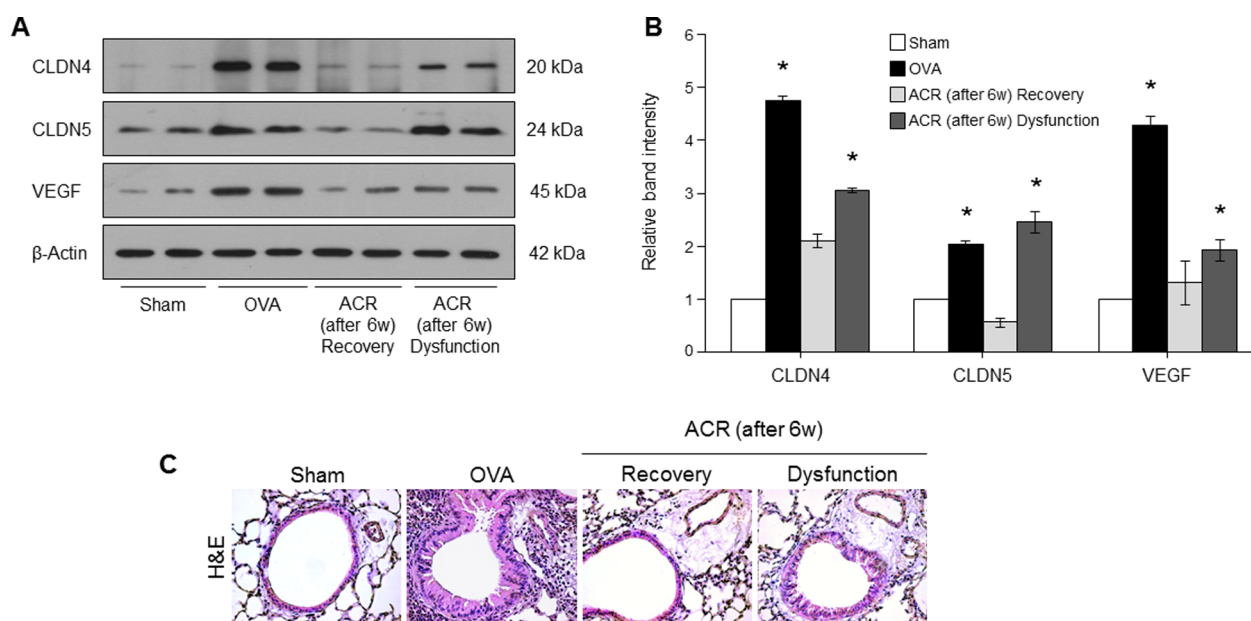


Figure 6. Persistent expression markers following acrolein exposure. Six weeks following acrolein exposure, CLDN4, CLDN5, and VEGF protein levels by Western blotting (A, B) and immunohistochemical staining (C) were expressed in the lung tissues. * <0.05 compared with controls.

proteins (as well as VEGF and Nrf2) may be involved (Figure 7).

A diagnosis of irritant-induced asthma¹¹ suggests the following three conditions: (a) the criteria used to define RADS;¹ (b) chronic or repeated exposure to a moderate concentration of chemicals within permissible exposure levels and development of asthma with a latency period, but without evidence of immunoglobulin E-mediated signal pathway; or (c) new-onset asthma in a highly susceptible subject (e.g., with pre-existing nonspecific AHR) associated with inhalation to identified irritate substances, even at concentrations within permissible levels.

Previous studies^{12,13,14} developed a mice model of RADS using a 15 min single exposure to 50–200 ppm of chlorine. The acute inflammation with macrophages and neutrophils resolved within 48 h, but AHR persisted for at least 28 days, indicating mice would be useful for mechanistic and intervention observation. An earlier report by McGovern et al. (2010)¹⁵ suggested that intraperitoneal dimethylthiourea given 1 h pre/

post-exposure to chlorine protected against irritant-induced asthmatic mice, while Demnati et al. (1998)¹⁵ suggested that corticosteroids attenuate neutrophilic inflammation to chlorine in rats. In this study, the authors developed a mice model of RADS with short time (10 min) acrolein inhalation (5 ppm). The inflammatory responses with macrophages and neutrophils and AHR persisted for at least 56 days, indicating that this may be a reference model of RADS for future studying of the mechanism of disease and intervention.

The authors compared airway inflammation and responsiveness in both the acrolein alone and OVA sensitization + acrolein groups to determine whether allergen sensitization causes RADS. Animals in the OVA sensitization + acrolein group exhibited increased airway inflammation and airway responsiveness compared with acrolein alone exposure group, suggesting that allergen sensitization is an important factor for RADS development following chemical exposure.

Short-term inhalation to acrolein was found to suppress IL-1 α , -17E, and -33 in epithelial cells in response to allergens,

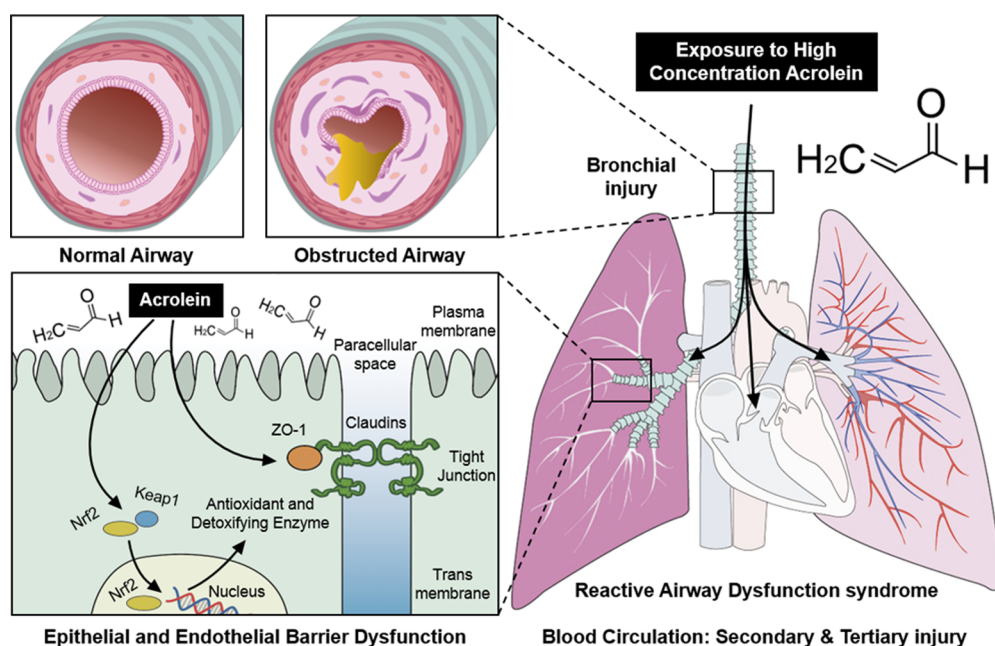


Figure 7. Summary of acrolein change to tight junction in airway systems.

and inhibition signaling was shown by several diet-derived thiol-reactive electrophiles that have attributed health benefits due to their anti-inflammatory properties.¹⁶ OVA treatment increased in lungs of several mRNA Th2 cytokines, and these increases were attenuated following acrolein.^{17,18} Acrolein exposure following lipopolysaccharides treatment decreased lipopolysaccharides induced production of several cytokines of TNF- α , IL-12, and IFN- γ , etc.

In this study, in contrast with previous studies, IL-5 was highly increased in OVA + acrolein exposure group compared with OVA group, indicating that acrolein can cause an increase in Th2 cytokines. Further studies will be needed to clarify the effect and mechanism of acrolein on asthma.

Mechanism of single and high chemicals induced asthma remain poorly understood. An initial report of RADS¹ described damage of epithelium and bronchial wall inflammatory changes with lymphocyte and plasma cell infiltration. Postulated mechanisms include inflammatory effects mediated by neuropeptides.^{19,20} ROS may result in the production of IL-8, chemokines, and monocyte chemoattractant protein-1, which lead to neutrophilic inflammation. Irritant exposure may contribute to coughing and pathophysiologic changes seen in RADS.²¹

In this study, mice exposed to acrolein or OVA exhibited increased airway inflammatory cell infiltration such as neutrophils, eosinophils, and monocytes compared to the sham group. Airway inflammation was further increased in OVA + acrolein exposure group compared to the OVA group and persisted in the acrolein exposure group for 8 weeks. These results suggest that acrolein recruits various inflammatory cells including neutrophils and persists in the airway for a minimum of 8 weeks.

CLDNs are structural barriers protein of TJs with functions for regulation of electrolyte and cell membrane permeability.^{22,23} CLDN1, -3, -4, -5, -7, -8, and -18 are expressed in human bronchi.^{24–27} CLDN5 expression tends to be inversely correlated with the severity of lung inflammation and injury. CLDN5 expression is increased at higher levels in injury-

resistant mice strains compared with injury-sensitive mice strains subjected to acrolein-induced acute lung injury.²⁸ In this study, CLDN4, CLDN5, and CLDN17 were examined to determine whether TJ proteins are involved in RADS. CLDN4, CLDN5, and CLDN17 were increased in the OVA + acrolein exposure group compared with OVA group, suggesting that a single chemical exposure can alter TJ proteins and that TJ proteins are involved in the development of RADS.

Acrolein is an irritant and plays a causal role in airway inflammation and injury due to smoking.²⁹ Cigarette smoke and acrolein activate the same intracellular mechanism that induced the release of pro-inflammatory cytokines and ROS proposed to be responsible for the inflammatory processes of respiratory diseases.²⁹

In this study, a ROS marker protein carbonyl levels were increased in the lungs from OVA and acrolein exposure groups compared with the control group and were further increased in the OVA + acrolein exposure group, suggesting that acrolein increases ROS causing airway inflammation. Also the protein levels of Nrf2 and Keap1 were significantly increased in OVA and acrolein exposure groups compared with sham group and persist for 8 weeks, suggesting that acrolein produces antioxidant enzyme following acrolein exposure and may be involved in RADS.

VEGF is a pro-angiogenic factor expressed in lungs, where it plays a key in homeostasis maintenance of alveolar compartment. Cigarette smoke inhalation in rodents elicits lung inflammation responses accompanied by an increase in VEGF expression in the BALF.³⁰ Acrolein and possibly ROS contained in cigarette smoke induced VEGF release from respiratory cells through p38 mitogen-activated protein kinase pathway.³¹

In this study, the VEGF level was significantly increased in OVA and acrolein exposure groups compared with control group, and VEGF persisted in the acrolein exposure group for 8 weeks, suggesting that acrolein may be involved in RADS.

In conclusion, our data revealed that claudins, VEGF, Nrf2, Keap1, and nonspecific airway hyper-reactivity are increased in

mice co-exposed to allergens and acrolein and persist for 8 weeks in a mouse model, suggesting that airway inflammation and airway hyper-responsiveness may persist following acrolein exposure.

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Author Contributions

A.S.J. designed the experiment and wrote the manuscript. B.G.K. performed the animal model experiments and analyzed the data. P.H.L., S.H.L., and J.S.H. collected the samples and assisted in interpreting the data.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

RADS, reactive airways dysfunction syndrome; OVA, ovalbumin; VEGF, vascular endothelial growth factor; ROS, reactive oxygen species; IP, intraperitoneal; D-PBS, Dulbecco's phosphate-buffered saline; AHR, airway hyper-responsiveness; BAL, bronchoalveolar lavage; IL, interleukin; Penh, enhanced pause; TBS, tris-buffered saline; ELISA, enzyme-linked immunosorbent assay; TJ, tight junction; CLDN, claudin; Nrf2, nuclear factor erythroid-derived 2-related factor 2; Keap1, Kelch-like ECH-associated protein 1

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