Transient Receptor Potential Ion Channels Mediate Adherens Junctions Dysfunction in a Toluene Diisocyanate-Induced Murine Asthma Model

Lihong Yao,*1 Shuyu Chen,†1 Haixiong Tang,‡1 Peikai Huang,§ Shushan Wei,§ Zhenyu Liang,* Xin Chen,¶ Hongyu Wang,‖ Ailin Tao,† Rongchang Chen,*2 and Qingling Zhang§,2

*State Key Laboratory of Respiratory Diseases, Guangzhou Institute of Respiratory Health, The First Affiliated Hospital of Guangzhou Medical University; †Guangdong Provincial Key Laboratory of Allergy & Clinical Immunology, State Key Laboratory of Respiratory Disease, The Second Affiliated Hospital of Guangzhou Medical University, Guangzhou Medical University, Guangzhou 510180, China; ‡Department of Respiratory Medicine, Minzu Hospital of Guangxi Zhuang Autonomous Region, Guangxi Medical University, Nanning 530001, China; §State Key Laboratory of Respiratory Diseases, Department of Allergy and Clinical Immunology, Guangzhou Institute of Respiratory Health, The First Affiliated Hospital of Guangzhou Medical University, Guangzhou Medical University, Guangzhou 510180, China; ¶Department of Respiratory Medicine, Zhujiang Hospital, Southern Medical University, Guangzhou 510280, China; ‖Division of Respirology, Department of Medicine, McMaster University, Firestone Institute for Respiratory Health (FIRH), The Research Institute of St. Joe’s Hamilton (RISH), St. Joseph’s Healthcare, Hamilton, ON L8N 4A6, Canada

1These authors contributed equally to this study.
2To whom correspondence should be addressed. E-mail: qingling@gird.cn and E-mail: chenrcstatekeylab@gmail.com.

ABSTRACT

Disruption of epithelial cell-cell junctions is essential for the initiation and perpetuation of airway inflammation in asthma. We've previously reported compromised epithelial barrier integrity in a toluene disocyanate (TDI)-induced occupational asthma model. This study is aimed to explore the role of transient receptor potential vanilloid 4 (TRPV4) and transient receptor potential ankyrin 1 (TRPA1) in the dysfunction of adherens junctions in TDI-induced asthma. Mice were sensitized and challenged with TDI for a chemical-induced asthma model. Selective blockers of TRPV4 glycogen synthase kinase (GSK)2193874, 5 and 10 mg/kg) and TRPA1 (HC030031, 10 and 20 mg/kg) were intraperitoneally given to the mice. Immunohistochemistry revealed different expression pattern of TRPV4 and TRPA1 in lung. TDI exposure increased TRPV4 expression in the airway, which can be suppressed by GSK2193874, while treatment with neither TDI alone nor TDI together with HC030031 led to changes of TRPA1 expression in the lung. Blocking either TRPV4 or TRPA1 blunted TDI-induced airway hyperreactivity, airway neutrophilia and eosinophilia, as well as Th2 responses in a dose-dependent manner. At the same time, membrane levels of E-cadherin and β-catenin were significantly decreased after TDI inhalation, which were inhibited by GSK2193874 or HC030031. Moreover, GSK2193874 and HC030031 also suppressed serine phosphorylation of glycogen synthase kinase 3β, tyrosine phosphorylation of β-catenin, as well as activation and nuclear transport of β-catenin in mice sensitized and challenged with TDI. Our study suggested that both TRPV4 and TRPA1 contribute critically to E-cadherin and β-catenin dysfunction in TDI-induced asthma, proposing novel therapeutic targets for asthma.

©The Author(s) 2018. Published by Oxford University Press on behalf of the Society of Toxicology. All rights reserved. For permissions, please e-mail: journals.permissions@oup.com
As the first continuous line of defense against foreign environmental insults, the airway epithelial cells not only play a central role in orchestrating pulmonary homeostasis, but also underlie inflammatory responses to inhaled allergens, viruses, and environmental pollutants that contributes critically to asthma pathogenesis (Lambrecht and Hammad, 2014; Xiao et al., 2011). Intact epithelial integrity is maintained through the formation of tight junctions (TJs), adherens junctions (AJs), and hemidesmosomes, which are composed of a number of junctional molecules including occludin, claudins, zona occludens-1, 2, 3, and E-cadherin, β-catenin (Nawijn et al., 2011). Among them, the E-cadherin-mediated AJs are crucially important for the formation of epithelial cell-cell contacts (Harris and Tepass, 2010). At the plasma membrane, E-cadherin is tightly bound to β-catenin, creating an adhesive junctional complex connecting to the actin and microtubule cytoskeleton. Disruption of the E-cadherin-β-catenin complex would lead to impaired epithelial cell-cell adherence and barrier dysfunction (Nelson and Nusse, 2004), as well as disordered intracellular signaling pathways and transcriptional regulation, which are associated with a broad spectrum of pathological processes, including tumor metastasis, fibrogenesis, inflammation, etc (Valenta et al., 2012).

Defects of epithelial barrier function have long been reported in both asthma patients and mouse models (Hackett et al., 2013; Yao et al., 2015). Previously, we’ve demonstrated compromised epithelial integrity in the airway of a toluene diisocyanate (TDI)-induced occupational asthma model, with loss of cell membrane E-cadherin and β-catenin as well as activation of β-catenin-mediated signaling (Yao et al., 2015, 2016). Blocking β-catenin signaling attenuates TDI-induced airway inflammation and remodeling (Yao et al., 2017), suggesting that β-catenin is an important regulator and potential therapeutic target in asthma (Kumawat et al., 2014). However, the mechanisms involved in E-cadherin and β-catenin dysfunction are still largely unknown.

Transient receptor potential ion channels (TRP channels) are a large group of cation channels that can sense temperature, pain, as well as intracellular and extracellular chemical stimuli and play important roles in many pathophysiological processes. TRP channels are not only the principal mediators of pain and play important roles in many pathophysiological processes. TRP channels are not only the principal mediators of pain and play important roles in many pathophysiological processes.

TRP channels are not only the principal mediators of pain and play important roles in many pathophysiological processes. TRPV1 (Taylor-Clark et al., 2009), TRPV3 (Finger et al., 2015), TRPV4 (Valentijn et al., 2014), TRPM2 (Kim et al., 2009), TRPM7 (Nilius et al., 2006), TRPM8 (Finger et al., 2013) have also been shown to participate in barrier functions. TRPM8 agonists promote the recovery of disrupted epidermal permeability barrier function (Denda et al., 2010a,b).

Hence, the role of TRP channels in regulating epithelial barrier homeostasis becomes conflicting and obscure. Interestingly, TDI has been demonstrated to activate TRPA1 rather than TRPV1 (Taylor-Clark et al., 2009). In vitro studies show that TDI is able to induce an elevation of Ca2+ concentration in human lung epithelial cells as well as neurons and T cells (Chung et al., 2010), implying that calcium permeable channels might be involved in TDI-induced responses. As inhibitors of TRPV4 and TRPA1 have been both proved to be safe and effective in other disease models, in this study, we intend to apply specific antagonists to study the roles of 2 of the family members TRPV4 and TRPA1 in TDI-induced asthma model.

**MATERIALS AND METHODS**

**Animals.** All animal care and experimental procedures studies complied with the guidelines of the Committee of Guangzhou Medical University on the use and care of animals and were approved by the Animal Subjects Committee of Guangzhou Medical University. A 6- to 8-week-old male C57BL/6 mice (20–22 g) were purchased from Southern Medical University. The mice were housed in a specific pathogen-free facility with 12-h dark/light cycles [temperature 23 °C ± 2 °C, humidity range 40%–70%, 12-h light/dark cycle (lighting: 7:00-19:00)] and with sterile water and irradiated food available ad libitum.

Regents. TDI (toluene-2, 4-diisocyanate, ≥98.0%) was obtained from Sigma-Aldrich (Shanghai, China) and stored in a shady, cool, and dry place after reception. The vehicle used to dissolve TDI consisted of a mixture of 2 volumes of acetone and 3 volumes of olive oil for sensitization, and 1 volume of acetone and 4 volumes of olive oil for sensitization, and 1 volume of acetone and 4 volumes of olive oil for sensitization. Acetone and olive oil were kept in separate containers. Immediately before use, TDI was added to the prepared vehicle and gently mixed. All preparations and solutions were protected from light. Enzyme-linked immunosorbent assay (ELISA) kits for IgE and IL-4, IL-5, IL-13, IL-6, and IL-18 were bought from eBioscience (San Diego, California). Primary antibodies against β-catenin and E-cadherin were from Abcam, rabbit anti-TRPV4 antibody from Invitrogen, rabbit anti-TRPA1 antibodies from Abclonal (Wuhan, China), rabbit anti-nonphospho-β-catenin (Ser33/37/Thr41), antiphospho-β-catenin (Tyr654), antiglycogen synthase kinase 3β (GSK3β), antiphospho-GSK3β (Ser9) antibodies from Cell Signaling Technology.

**TDI-induced asthma model.** TDI-induced asthma model was prepared as described elsewhere (Yao et al., 2015). All mice were randomized to the paralleled 6 groups: (1) vehicle-sensitized, vehicle-challenged, dimethyl sulfoxide (DMSO)-treated mice (control group); (2) TDI-sensitized, TDI-challenged, DMSO-treated mice (TDI group); (3) TDI-sensitized, TDI-challenged, 5 mg/kg GSK2193874-treated mice (TDI-GSK 5 mg/kg group); (4) TDI-sensitized, TDI-challenged, 10 mg/kg GSK2193874-treated mice (TDI-GSK 10 mg/kg group); (5) TDI-sensitized, TDI-challenged, 10 mg/kg HC030031-treated mice (TDI-HC 10 mg/kg group); (6) TDI-sensitized, TDI-challenged, 20 mg/kg HC030031-treated mice (TDI-HC 20 mg/kg group). Briefly, on days 1 and 8, the mice were derrmally treated with 0.3% TDI on the dorsum of both ears (20 μl per ear) (De Vooght et al., 2009). On days 15,
18 and 21, the mice were placed in a horizontal rectangle chamber (50 × 30 × 25 cm, 20 mice placed in per chamber) and challenged for 3 h each via the airways with 3% TDI by means of compressed air (filtered) nebulization (NE-C28; Omрон; with generated particle size <5 μm). Control mice were treated by the same procedures with the same amount of vehicle. The TRPV4 inhibitor, GSK2193874 (Selleck, Shanghai, China), and TRPA1 inhibitor HC030031 (Selleck) were dissolved in DMSO and diluted with PBS (pH 7.4) and then given to the mice via peritoneal injection once daily, beginning immediately after the first challenge to the last day of challenge for a consecutive of 7 days. Control mice received injection of the same volume of solvent by comparison.

Airway hyperresponsiveness assessment. Twenty-four hours after the last airway challenge, measurements of lung resistance (Rc) were performed on aesthetised and mechanically ventilated (Buxco Electronics, Troy, New York) mice in response to increasing doses of nebulized methacholine (6.25, 12.5, 25, and 50 mg/ml). Measurements of Rc were performed every 5 min following each nebulization step until a plateau phase was reached. Results were expressed as percentage of baseline Rc value (value at 0 mg/ml methacholine) for each graded concentration of methacholine.

Measurement of serum IgE. Mice were sacrificed with overdose of pentobarbital (100 mg/kg, i.p.) one day after the last airway challenge. Blood samples were taken from the retroorbital plexus/sinus, allowed to rest at room temperature for 1 h, then centrifuged (3000 × g, 20 min), and supernatants were harvested for detection of total IgE antibody with a proprietary sandwich ELISA with 2 monoclonal antibodies according to the manufacturer’s instructions (eBioscience, San Diego).

Bronchoalveolar lavage. After blood was taken, the lungs were lavaged in situ, twice with 0.8-ml sterile saline (0.9% NaCl, pre-warmed), and the recovered fluids were pooled. Total cells in bronchoalveolar lavage fluid (BALF) were counted, and a cytospin sample was prepared and stained with hematoxylin and eosin (H&E) for blinded assessment of differential cell percentages in BALF. Then the remaining fluids were centrifuged (1000 × g, 10 min), and supernatants were stored for further detection of IL-4, IL-5, IL-13, IL-6, and IL-18 using commercially available ELISA kits (eBioscience).

Histopathology. The left lung lobes were harvested and inflated/ fixed with 4% neutral formalin. Fixed tissues were embedded in paraffin for sectioning. Prepared lung sections (4 μm) were then subjected to H&E staining for airway inflammation and cellular infiltration analysis. Airway inflammation and cellular infiltrate were scored by an blinded observer, and were semi-quantified as previously described (Yao et al., 2016). Epithelial denudation was measured by assessing the percentage of the denuded area in the entire circumference of the bronchus (Berair et al., 2017; Nishigaya et al., 1991).

For airway goblet cell metaplasia, lung sections were stained with periodic acid-Schiff base (PAS), and semiquantitative scoring was used to compare groups. PAS-positive epithelial cells of the total epithelial cells were counted to obtain a percentage and compare groups. For airway smooth muscle density, we performed immunohistochemistry of α-SMA. The staining was analyzed by Image-Pro Plus software. Scoring was performed at a magnification of 200× by examining at least 40 image fields of 20 slices from 6 mice per group. Sections were assigned a random code to blind the examiner to the identity of each specimen.

Western blot and immunohistochemistry. The right lung lobes were dissected from the mice, then snapfrozen in liquid nitrogen and stored at -80°C. One part of the tissue samples were ground to a fine powder under liquid nitrogen and lysed in buffer containing protease inhibitor, calcineurin inhibitors and PMSF. Another part of the samples were homogenized for membrane and nuclear protein isolation using a subcellular protein fractionation kit (No. 87790, Thermo Fisher Scientific) following the manufacturer’s recommendations. All extracted proteins were boiled with 5× sample buffer for 10 min, and then loaded on SDS-PAGE for immunoblotting. Na/K ATPase (Abcam, ab76020), β-actin (Abclonal) and Histone H3 (Abclonal) were used as membrane, cytosolic, and nuclear markers, respectively. Immunoreactive bands were exposed to Odyssey CLx Imager for image capture. Data analysis was performed with Odyssey Software.

For immunohistochemistry of TRPV4, TRPA1, E-cadherin and β-catenin, deparaffinized sections were submerged in either citrate buffer (pH = 6.0) or EDTA (pH = 9.0) for antigen retrieval. Samples were treated with H2O2 for 15 min to block endogenous peroxidase, and then incubated at 4°C overnight in recommended dilutions of anti-TRPV4 (Invitrogen, No. PA5-41066), anti-TRPA1 (Abclonal, No. A8568), anti-α-SMA (Abcam, No. ab32757), anti-β-catenin (Cell Signaling Technology, No. 3195) and anti-β-catenin (Cell Signaling Technology, No. 8480) antibodies. After washing with PBS, slices were incubated with a secondary antibody for 20 min at room temperature. Signals were visualized with diaminobenzidine (DAB).

Statistical analysis. Statistical analysis was performed using SPSS version 20.0 (SPSS Inc., Chicago, Illinois). Data were expressed as Mean ± SE and were normally distributed. Comparisons among groups were analyzed by 1-way ANOVA, followed by Bonferroni post hoc test for multiple comparisons. A p < .05 was considered statistically significant.

RESULTS

TRPV4 and TRPA1 Antagonists Had No Effects on Airway Reactivity, Inflammation, and Epithelial Morphology in Naive Mice

To evaluate the effects of TRPV4 and TRPA1 antagonists alone on naive mice, C57Bl/6 mice were injected with GSK2193874, HC030031 or vehicle control once daily for a consecutive of 7 days. Results showed that GSK2193874 and HC030031 did not impact airway reactivity, inflammation, epithelial morphology at concentrations used in the TDI-exposed mice (Figure 1). Levels of IL-4, IL-5, and IL-13 in BALF of these mice were below the detection limit.

TRPV4/TRPA1 Inhibition Leads to Decreased Airway Hyperreactivity and Airway Inflammation in TDI Asthma

Allergic airway inflammation was generated by exposing BALB/c mice to TDI. As expected, TDI sensitization and challenge induced increased airway hyperreactivity to methacholine, obvious peribronchial inflammation and epithelial injury, airway goblet cell hyperplasia and smooth muscle hypertrophy, copious amounts of neutrophils, and eosinophils accumulating in the airway lumen, higher levels of IL-4, IL-5, IL-13, IL-6, AND IL-18 in BALF and increased total serum IgE (Figs. 2 and 3). Almost all these responses were inhibited by treatment with TRPV4 or
TRPA1 antagonists, in a dose-dependent manner, except for the raised serum IgE, indicating critical roles for TRPV4 and TRPA1 in the initiation of TDI-induced allergic airway inflammation.

TRPV4 and TRPA1 Expression in the Lung of TDI-exposed Asthma

Pulmonary expression of TRPV4 and TRPA1 in TDI sensitized and challenged mice were analyzed. As for TRPV4, immunohistochemistry showed that there's relatively weak staining in the lungs of control mice. TDI exposure led to increased immunoreactivity of TRPV4, especially in the airway epithelia, and the infiltrating inflammatory cells also displayed some positive staining (Figs. 4A and 4B). In line with this, western blot revealed upregulated TRPV4 expression in the whole lung after TDI exposure (Figure 4C). Treatment with GSK2193874 at the doses of 5 mg/kg and 10 mg/kg markedly suppressed pulmonary TRPV4 expression.

As for TRPA1, there is relatively very faint staining in bronchial and alveolar regions of the lung in control mice. And it looks that TDI sensitization and challenge did not alter TRPA1 expression pattern in the lung. Treatment with TRPA1 inhibitor HC030031 also showed no effects on pulmonary TRPA1 expression (Figs. 4D–F).

TRPV4/TRPA1 Inhibition Rescued Membrane Location of E-cadherin and β-Catenin

Immunohistochemistry and Western blot were performed to evaluate the specific adheren junctional proteins E-cadherin and β-catenin. Though western blot analysis showed no alteration of E-cadherin and β-catenin expression levels in the whole lung (data not shown), immunohistochemical staining did reveal remarkable changes in airway epithelia. As can be seen in Figure 5, in control mice, both E-cadherin and β-catenin locate especially at the lateral side and apicolateral border of the airway epithelial cells, with the alveoli having much less immunostaining of the 2 that was not affected by TDI (data not shown). Yet, after TDI inhalation, the immunoreactivity of E-cadherin and β-catenin was significantly disrupted at the epithelial cell–cell contacts and scattered in the cytoplasm and nucleus. Treatment with GSK2193874 or HC030031 partly restored
Figure 2. Blocking TRPV4 with GSK2193874 or TRPA1 with HC030031 attenuated TDI-induced airway inflammation and hyperreactivity. A, Representative H&E- and PAS-stained lung sections of different groups. The arrows in the upper panel arrows indicate epithelial denudation. The round circles indicate inflammatory infiltrates around the airway. The arrows in the lower panel indicate positive PAS staining in epithelia. The lower panel showing immunohistochemical staining of α-SMA indicates smooth muscle density. Original magnification was 200×. Scale bar = 100 μm. B, Semiquantification of peribronchial and perivascular inflammation, as well as epithelial denudation was performed (n = 8–10). C, PAS-positive epithelial cells of the total epithelial cells were counted to obtain a percentage and compare groups (n = 6–8). D, Integrated optical density of α-SMA in the bronchial region was analyzed by Image-Pro Plus software. Results were expressed as fold changes to control (n = 6–8). E, Total inflammatory cell numbers in BALF (n = 8–10). F, Numbers of differential inflammatory cells in BALF (n = 8–10). G-H, AHR was measured by lung resistance (Rl). Results were shown as percentage of baseline value (n = 4–5). *p < .05 compared with control; #p < .05 compared with TDI group. There were no significant differences between GSK2193874 and HC030031 treatment groups.
the TDI-induced rearrangement of E-cadherin and β-catenin, with the effects more obvious at higher doses. Western blot analysis of subcellular fractions verified decreased levels of E-cadherin and β-catenin in membrane extracts triggered by TDI, accompanied by increased phosphorylation of β-catenin at the site of Tyr654 (Figure 6), which orchestrates liberation of β-catenin from the E-cadherin-β-catenin complex at the membrane (Nelson and Nusse, 2004). And these were partly restored by blocking GSK2193874 or HC030031 (Figure 6). While the protein levels of E-cadherin and β-catenin in whole lung homogenates were not altered by TDI together with or without GSK2193874 or HC030031 treatment (data not shown), which may be the result of a counteraction of lowered membrane levels and raised cytoplasmic and nuclear levels.

TRPV4/TRPA1 Is Required for β-Catenin Activation and Mediates Its Translocation to the Nucleus

Activation and nuclear translocation of β-catenin would result in the transcription and expression of a list of genes associated with airway remodeling and inflammation in asthma (Yao et al., 2017). As previously reported, TDI phosphorylates GSK3β at Ser9, activates β-catenin (non-phosphorylated) and increases its nuclear transmigration, all of which were suppressed by GSK2193874 and HC030031 in a dose-dependent manner (Figure 6).

DISCUSSION

The TRP channel superfamily encompasses a group of transmembrane proteins that can respond to a considerable diversity of endogenous and exogenous stimuli of both physical and chemical nature (Grace et al., 2014). As multiple signal integrators, TRP channels exhibit a general preference for calcium ions, which triggers opening of voltage gated ion channels and hence a vast series of intracellular events and eventually results in specific tissue responses (Benemei et al., 2015). They are widely expressed throughout the body including the respiratory tracts and lungs. In this study, we first evaluated pulmonary expression of 2 of the TRP family members TRPV4 and TRPA1, and found that they are differently expressed. TRPV4 showed most abundant expression in the bronchial epithelia, less in smooth muscles, alveolar epithelia and the infiltrating inflammatory cells; while TRPA1 had relatively very faint staining in lung parenchymal cells. Interestingly, TDI sensitization and inhalation markedly upregulated TRPV4 expression in the airway, with the most prominent changes in bronchial epithelia, yet TRPA1 expression in the lung remained unchanged after TDI treatment. Despite their different expression, TRPV4 and TRPA1 play some similar roles in pulmonary pathophysiological processes. Studies revealed that ex vivo activation of TRPV4 leads to contraction of bronchial smooth muscle cells, the major cause of airway hyperreactivity (AHR) (McAlexander et al., 2014). Genetic ablation of TRPV4 decreased antigen induced airway responses to aerosolised 5-HT (Birrell et al., 2016), significantly alleviated functional, histological, and inflammatory hallmarks of acid-induced acute lung injury (Yin et al., 2016), and protected mice from house dust mite (Dermatophagoides farinae)-induced airway remodeling (Gombedza et al., 2017). Likewise, TRPA1 has also been demonstrated to participate in nonallergic AHR, which may be attributed to a neuroimmune interaction involving sensory neurons and mast cell activation (Hox et al., 2013). Moreover, TRPA1 deficiency inhibited ovalbumin-induced leukocyte infiltration in the airways, reduced cytokine and mucus production, and almost completely abolished airway hyperreactivity to contractile stimuli (Caceres et al., 2009). These studies suggest that both TRPV4 and TRPA1 perform to contribute critically to AHR and asthmatic airway inflammation. In line with these findings, in the present study, we found that blockade of TRPV4 with GSK2193874 or TRPA1 with HC030031 dramatically inhibited the release of Th2 cytokines and IL-6, IL-18 in BALF. A–C, Levels of Th2 related cytokines IL-4, IL-5, and IL-13 in BALF were quantified by ELISA (n = 6–8). D, E, BALF levels of IL-6 and IL-8 (n = 6–8). F, TDI sensitization and challenge leads to increased total serum IgE titers, yet treatment with either GSK2193874 or HC030031 had no significant effects on IgE as assessed by ELISA (n = 6–8). * p < .05 compared with control; # p < .05 compared with TDI group. There were no significant differences between GSK2193874 and HC030031 treatment groups.
Figure 4. Pulmonary expression of TRPV4 and TRPA1 in TDI-induced asthma. A, Representative immunohistochemical staining of TRPV4 in the bronchial and alveolar regions of control, TDI-exposed and GSK2193874-treated lungs. The round circles indicate inflammatory infiltrates around the airway. Original magnification was 400×. Scale bar = 50 μm. B, Amplified view of TRPV4 expression in different cell types of TDI-sensitized and challenged mice without GSK2193874 treatment. Scale bar = 20 μm. C, Western blot analysis of TRPV4 in whole lung homogenates and subsequent densitometric analysis of the blots (n = 4–6). D, Representative immunohistochemical staining of TRPA1 in the bronchial and alveolar regions of control, TDI-exposed and HC030031-treated lungs. The round circles indicate inflammatory infiltrates around the airway. Original magnification was 400×. Scale bar = 50 μm. E, Amplified view of TRPA1 expression in different cell types of TDI-sensitized and challenged mice without HC030031 treatment. Scale bar = 20 μm. F, Western blot analysis of TRPV4 in whole lung homogenates and subsequent densitometric analysis of the blots (n = 4–6). *p < .05 compared with control; #p < .05 compared with TDI group. There were no significant differences between GSK2193874 and HC030031 treatment groups.
attenuated TDI-induced airway hyperresponsiveness (AHR), bronchial neutrophil and eosinophil infiltration, as well as pulmonary Th2 responses (no significant differences were found between the effects of GSK2193874 and those of HC030031), implying that TRPV4 and TRPA1 take pivotal roles in the pathogenesis of TDI asthma. Though there’s currently no direct evidence suggesting that TDI can bind to TRPV4, which mainly senses temperature changes, mechanical stress and arachidonic acid metabolites (Grace et al., 2014), TRPV4 may be activated by the diverse series of signaling pathways following TDI exposure. TRPA1, on the other hand, can directly recognize TDI, as well as endogenous byproducts derived from peroxidation (Benemei et al., 2015), mediating the initiation and perpetuation of airway inflammatory responses. Yet, how TRPV4 and TRPA1 modulate TDI-related asthmatic responses still remains further investigation.

As the gatekeeper of airway mucosa and allergic sensitization, E-cadherin orchestrates the critical immunological decision between tissue homeostasis versus inflammatory responses through regulation of cell-cell contacts, proliferation, differentiation, and production of growth factors and proinflammatory mediators. Proper localization of E-cadherin is essential for epithelial barrier integrity (Heijink et al., 2010), and E-cadherin disruption would induce dendritic cell maturation, inhibit regulatory T cells to be retained in the airway mucosa and additionally serve to facilitate transmigration of effector

---

Figure 5. Effects of GSK2193874 or HC030031 on E-cadherin and β-catenin distribution in the bronchial epithelia. A, Representative immunohistochemical staining of E-cadherin in the airways of control, TDI-exposed and GSK2193874- or HC030031- treated mice. Original magnification was 1000×. B, Representative immunohistochemical staining of β-catenin in the airways of control and TDI-exposed mice. The arrows in control images showing solid lines between adjacent epithelial cells indicate intact E-cadherin or β-catenin expression in the epithelia in control mice. The arrows in TDI images indicated impaired expression on the membrane and diffused expression in the cytoplasm and nucleus of E-cadherin or β-catenin in TDI-exposed mice. Scale bar = 20 μm. Original magnification was 1000×.
intraepithelial T cells into the airway lumen (Nawijn et al., 2011). It has already been well demonstrated in our previous work that epithelial E-cadherin expression was disrupted in TDI-induced asthma model (Yao et al., 2015). This was confirmed again in this study, showing decreased membrane location of E-cadherin in lungs of TDI-treated mice compared with control. Evidence proved that both TRPV4 and TRPA1 can affect epithelial or endothelial barrier function. Activation of TRPV4 would increase endothelial permeability in isolated lung (Alvarez et al., 2006; Wu et al., 2009), and downregulate E-cadherin expression in breast cancer cells (Lee et al., 2017); while TRPA1 mediates reversible TJ permeability increase in Madin-Darby canine kidney
cells (Kanda et al., 2018). These suggest that TRPV4 and TRPA1 contribute to epithelial and endothelial barrier dysfunction. In our study, though treatment with GSK2193874 or HC030031 did not alter total E-cadherin expression in the lung (data not shown), the decreased level of E-cadherin in cell-cell contacts (membrane) induced by TDI was significantly restored, which is in consistent with the findings in other models (Chun and Prince, 2009; Denda et al., 2010a,b), indicating that TRPV4 and TRPA1 are engaged in TDI-induced E-cadherin mediated AJs disruption and barrier integrity. It is worth noting TDI-induced inflammation is dominated by airway neutrophil aggregation, while neutrophils possess a marked capacity for cleaving E-cadherin and interfering with its cell-cell adhesion function (Boxio et al., 2016). Coincidentally, decreased numbers of neutrophils were observed after treatment with GSK2193874 and HC030031, which may partly account for TRPs regulating E-cadherin.

Loss of E-cadherin would promote liberation of beta-catenin from cell-cell contacts, and subsequently activation of the canonical beta-catenin signaling, which is central for TDI-induced AHR and bronchial inflammation (Sineva and Pospelov, 2014; Yao et al., 2017). In line with our previously reported studies, disrupted membrane expression and activation of beta-catenin were found in TDI-exposed lungs. These were inhibited by GSK2193874 and HC030031, accompanied by ameliorated tyrosine phosphorylation of beta-catenin at Tyr654, which functions to facilitate uncoupling of beta-catenin from E-cadherin at the cell membrane (Nelson and Nusse, 2004). Accordingly, we also observed blunted activation of beta-catenin in TDI-exposed mice after TRPV4 or TRPA1 inhibition, as well as diminished cytoplasmic and nuclear retention. As a principal regulator of beta-catenin signaling, GSK3beta strictly modifies beta-catenin activity through stabilization of beta-catenin destruction complex. Serine phosphorylation of GSK3beta interrupts the degradation ability of the complex, and therefore leads to beta-catenin activation (McCubrey et al., 2014), which may in turn contributes to the breakdown of membrane E-cadherin and beta-catenin (Conacci-Sorrell et al., 2003). So in agreement with the changes of beta-catenin, decreased levels of phosphorylated GSK3beta (Ser9) were detected in TDI sensitized and challenged mice after treatment with TRPV4 and TRPA1 antagonists. Together, these results suggested that TRPV4 and TRPA1 can modulate the dual functions of beta-catenin.

In summary, our data demonstrated that TRPV4 and TRPA1 contribute to TDI-induced lung epithelial E-cadherin and beta-catenin dysfunction, therefore contributing to TDI-induced asthmatic airway inflammation. Our findings present novel therapeutic targets for the prevention and treatment of asthma.

**FUNDING**

This study was supported by National Postdoctoral Program for Innovative Talents (Grant No. BX201700060), National Natural Science Foundation of China (Grant Nos. 81800022 and 81871266), Project funded by China Postdoctoral Science Foundation (Grant No. 2018M633094), Natural Science Foundation of Guangdong Province (Grant No. 2018A0303102044), Young Scientist Foundation of Guangxi Medical University (Grant No. GXMUYSF201636), the Precision Medicine Research of The National Key Research and Development Plan of China (Grant No. 2016YFC0905800), Scientific and Technological Project of Guangzhou (Grant Nos. 20160402008 and 2018040200042), National Key R&D Program of China (Grant No. 2017YFC1310601), Guangzhou Healthcare Collaborative Innovation Major Project (Grant No. 201604020012), State Key Laboratory of Respiratory Disease Independent Project (Grant No. SKLRD2016Z012), Guangzhou Medical University Project for Specially Appointed Professors (Kian Fan Chung) for Construction of a High-level University, and Scientific Research Starting Foundation for Postdoctoral Fellows of Guangzhou.

**AUTHOR CONTRIBUTIONS**

C.R., Z.Q., Y.L. and T.H. designed the experiment and wrote the manuscript. Y.L., C.S. and T.H. performed the experiments and analyzed the data. H.P. and W.S. helped with the experiment. L.Z., C.X., W.H. and T.A. helped with data analysis. All the authors agreed that the final approval of the version to be published and ensured questions relating to the accuracy or integrity of any part of the work were appropriately investigated and resolved.

**CONFLICT OF INTEREST**

The authors declare that there are no conflicts of interest.

**REFERENCES**


