Phosphatidylinositol 3-Kinase Mediates β-Catenin Dysfunction of Airway Epithelium in a Toluene Diisocyanate-Induced Murine Asthma Model

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ABSTRACT

Cell-cell junctions are critical for the maintenance of cellular as well as tissue polarity and integrity. Yet the role of phosphatidylinositol 3-kinase (PI3K) in dysregulation of airway epithelial adherens junctions in toluene diisocyanate (TDI)-induced asthma has not been addressed. Male BALB/c mice were first dermally sensitized and then challenged with TDI by means of compressed air nebulization. The mice were treated intratracheally with PI3K inhibitor LY294002. Levels of phospho-Akt in airway epithelium and whole lung tissues were markedly increased in TDI group compared with control mice, which decreased after administration of LY294002. The dilated intercellular spaces of airway epithelium induced by TDI were partially recovered by LY294002. Both the protein expression and distribution of adherens junction proteins E-cadherin and β-catenin were altered by TDI. Treatment with LY294002 rescued the distribution of E-cadherin and β-catenin at cell-cell membranes, restored total β-catenin pool, but had no effect on protein level of E-cadherin. At the same time, LY294002 also inhibited phosphorylation of ERK, glycogen synthase kinase 3β and tyrosine 654 of β-catenin induced by TDI.

In summary, our results showed that the PI3K pathway mediates β-catenin dysregulation in a TDI-induced murine asthma model, which may be associated with increased tyrosine phosphorylation of β-catenin.

Key words: asthma; toluene diisocyanate; adherens junctions; β-catenin; PI3K

Airway epithelial cells (ECs) are central mediators in the pathogenesis of asthma (Heijink et al., 2014). They lie at the interface between the host and the environment and represent the first line of defense against alien microorganisms, gases, and allergens (Xiao et al., 2011). As a barrier to the external environment, ECs not only play a critical role in maintaining physiological homeostasis but also act as an essential regulator of inflammatory, immune, and regenerative responses to allergens, viruses, and environmental pollutants that contribute to asthma pathogenesis (Lambrecht and Hammad, 2012). They display a highly regulated and impermeable barrier through the formation of tight junctions (TJs) and adherens junctions (AJs), which consist of E-cadherin, β-catenin, and α-catenin (Nawijn et al., 2011). β-Catenin links E-cadherin at the plasma membrane to α-catenin (Takeichi, 1995). The E-cadherin–β-catenin–α-catenin complex forms a dynamic, rather than stable, link to the cytoskeleton. Without diminishing the importance of other cellular junctions, cadherin-mediated AJs are particularly important in controlling the specificity, formation, and maintenance of intercellular adhesion (Lewis et al., 1994). In addition, they direct coordinated cellular organization and movements within epithelia and transmit information from the environment to the interior of cells (Brembeck et al., 2006). Furthermore, AJs are required for the assembly of TJs (Tunggal et al., 2005), which seal the
paracellular space. Loss of AJs results in disruption of cell-cell and cell-matrix contacts, ineffective EC polarization and differentiation, and premature apoptosis (Hermiston and Gordon, 1995), which are associated with a wide variety of human malignancies (Blanco et al., 2002; Cheng et al., 2001; Tetsu and McCormick, 1999) as well as fibrotic disorders (Tan et al., 2010; Zheng et al., 2009).

As airway epithelial barrier function has important implications for asthma, a growing number of studies investigated the mechanisms of epithelial barrier dysfunction in the airways of asthma patients (Hackett et al., 2013; Post et al., 2013). Phosphatidylinositol 3-kinases (PI3Ks) are a family of proteins that catalyze the phosphorylation of the 3-OH position of phosphoinositides and generate lipids that control a wide variety of intracellular signaling pathways. Previous investigators have found that PI3K is involved in AJs regulation in mammalian cells (Hordijk et al., 1997). Sander et al. (1998) subsequently confirmed that E-cadherin-mediated cell-cell adhesion is dependent on PI3K activity, by using pharmacological inhibitors of PI3Ks, named LY294002 and wortmannin, and the dominant-negative mutant delta p85 which carries a deletion in the binding site for the p110 catalytic subunit. PI3K was also found to orchestrate AJs integrity by regulating the expression of cytoskeletal-associated E-cadherin and β-catenin at the site of cell-cell contact (Laprise et al., 2002). In 2010, Cain RJ et al. showed that p110α, a PI3K subunit, can regulate endothelial AJs via Pyk2 and Rac1. Depletion of p110α, rather than other p110 isoforms, decreases tumor necrosis factor -induced endothelial permeability, Tyr phosphorylation of VE-cadherin, and leukocyte trans-endothelial migration (Cain et al., 2010). Subsequently, Elloul S et al. reported that PI3K/AKT signaling mediates redistribution of the adhesion protein Arafdin and therefore controls cancer cell migration (Elloul et al., 2014). However, the role of PI3K in AJs dysfunction of asthma is currently not evaluated.

We have previously reported that toluene diisocyanate (TDI) can downregulate expression of TJ proteins and increase the permeability of bronchial epithelium in vitro (Zhao et al., 2009) and demonstrated that TDI could induce E-cadherin rearrangement through ERK pathway (Song et al., 2013a,b). Yet the exact mechanism through which TDI impairs AJs still needs to be explored. In this study, we tested the hypothesis that PI3K-dependent signaling pathways is involved in AJs disassembly of airway epithelium in a TDI-induced murine asthma model.

**MATERIALS AND METHODS**

For detailed methods including animal protocols and experimental procedures, please see the online Supplementary material.

**Animals and reagents.** Six- to eight-week-old specific-pathogen-free male BALB/c mice were purchased from Southern Medical University. The mice were housed in an SPF house with 12-h dark/light cycles and fed with sterile water and irradiated food *ad libitum*. All studies were conducted in accordance with the committee of Southern Medical University on the use and care of animals. The protocols were approved by the Animal Subjects Committee of Nanfang Hospital (NFYY-2013-085). TDI (toluene-2,4-diisocyanate, ≥98.0%), methacholine and acetone were obtained from Sigma-Aldrich (Shanghai, China). The PI3K inhibitor LY294002 was purchased from Enzo life sciences (Farmingdale, New York). The vehicle (AOO) used to dissolve TDI consists of a mixture of 2 volumes of acetone and 3 volumes of olive oil for dermal sensitization and 1 volume of acetone and 4 volumes of olive oil for airway challenge. ELISA kits for detection of IFN-γ, IL-4, IL-5, and IL-13 were purchased from BoShiDe biological engineering company (Wuhan, China); and ELISA kit for IgE was from R&D Systems.

Statistical analysis. Statistical analysis was performed using SPSS version 16.0. Data were expressed as mean ± standard error (SE) and comparisons among groups were analyzed by 1-way analysis of variance accompanied by Bonferroni post hoc test for multiple comparisons. *P < .05* was considered statistically significant.

**RESULTS**

**Effect of LY294002 on Akt Phosphorylation in Lung Tissues**

Akt is a direct downstream substrate of PI3K. Upon activation, PI3K converts the plasma membrane lipid phosphoinositide-4,5-bisphosphate (PI(4,5)P2) to PI(3,4,5)P3, which leads to recruitment of Akt to the plasma membrane through its association with the newly generated PI(3,4,5)P3. Akt is subsequently activated through phosphorylation by the intracellular kinases PDK1 (3-phosphoinositide-dependent protein kinase 1) and rictor-mTORC2 (mammalian target of rapamycin complex 2) at positions Thr 308 and Ser473, respectively (Franke, 2008; Sarbassov et al., 2005). So we assessed the level of p-AKT (Ser473) to detect PI3K enzyme activity in lung tissues as other studies (Kosmidou et al., 2001; Miyahara et al., 2007). Protein levels of p-Akt in airway epithelium and its total expression in lung were significantly upregulated after airway challenge with TDI and then inhibited by LY294002 (Fig. 1).

**Effect of LY294002 on AHR**

Airway reactivity was assessed 24 h after the third challenge. As expected, TDI-induced asthmatic mice exhibited a significant increase in airway hyperresponsiveness (AHR). Although mice treated with LY294002 showed a decline of AHR when provoked by methacholine (6.25, 12.5, and 25 mg/ml, *P < .05*) (Fig. 2A), indicating an antagonistic role of LY294002 for TDI-induced AHR.

**Effect of LY294002 on Levels of IL-4, IL-5, IL-13, and IgE**

IL-4, IL-5, and IL-13 in BAL fluid and IgE in the serum were much higher in TDI group when compared with control (*P < .05*). LY294002 dramatically suppressed the TDI-induced release of IL-4, IL-13, and IgE (*P < .05*), yet had no effect on IL-5 (Fig. 2B and 2C). IFN-γ was not detected.

**Effect of LY294002 on Airway Inflammation and Goblet Cell Metaplasia Induced by TDI**

Numbers of total and differential inflammatory cells were counted in BAL fluid. In agreement with total cell counts, higher amounts of neutrophils and eosinophils were found after sensitization and challenge with TDI (*P < .05*), which were significantly decreased by LY294002 (Fig. 2A, 3A, 3D, and 3E). Similarly, histologic analysis revealed typical pathologic features of TDI-induced airway disease. Numerous inflammatory cells infiltrated around the bronchus, and the lining airway epithelium was thickened and proliferated in TDI group. Mice treated with LY294002 showed marked reductions in both thickening of the epithelial layer and extravasation of inflammatory cells in peribronchial and perivascular regions (Fig. 3B and 3F). At the same time, the evident goblet cell metaplasia induced by TDI was also ameliorated after treatment with LY294002 (Fig. 3C and 3G).
FIG. 1. Western blot and immunohistochemistry analysis of p-Akt in lung. A, Protein level of p-Akt in lung tissues was significantly upregulated after airway challenge with toluene diisocyanate (TDI) and then inhibited by LY294002. B, Densitometric analysis of Western blot. \( n = 6 \), \* \( P < .05 \) versus control group; \# \( P < .05 \) versus TDI + DMSO group. Immunohistochemistry (C) and integrated optical density (D) \( (n = 8 \) image field of 8 slices from 4 mice per group) showed abundant cytoplasm expression of p-Akt in airway epithelium after TDI inhalation, which was decreased by LY294002. Original magnification was \( \times 400 \). There were no significant differences between TDI group and TDI + DMSO group.

FIG. 2. TDI-induced airway hyperresponsiveness (AHR), IgE and IL-4, IL-5, IL-13. A, Lung resistance (R\(_L\)) was assessed 24 h after the last airway challenge to determine airway reactivity. B, Total serum IgE levels. C, IL-4, IL-5, and IL-13 in BAL fluids. \( n = 6-10 \) per group. \* \( P < .05 \) versus control; \# \( P < .05 \) versus TDI + DMSO group. There were no significant differences between TDI group and TDI + DMSO group.
Effect of LY294002 on Epithelial Barrier Function

Electron microscopy was used to assess epithelial barrier function. As can be seen in Figure 4A, the cell-cell contact between adjacent bronchial epithelia was almost intact in AOO-inhaled mice, and was obviously disrupted in TDI-challenged mice. The widened intercellular space induced by TDI was partly recovered by LY294002.

Effect of LY294002 on AJ Proteins E-Cadherin and β-Catenin

Immunohistochemistry and Western blot were performed to evaluate the specific AJ proteins E-cadherin and β-catenin. Both lie especially at the lateral side and apical-lateral border in airway epithelium of control mice (Fig. 4A and 4B; Fig. 5D and 5E), although the alveoli had much less immunostaining of AJ proteins. The alveolar cells have relatively abundant expression of β-catenin, whereas E-cadherin displayed very faint expression in the alveolar epithelium but strong staining in alveolar macrophages (Fig. 5C and 5F). We can see that alveolar expression of E-cadherin and β-catenin did not differ among all groups (Fig. 5G and 5H). Yet the immunoreactivity of E-cadherin and β-catenin was significantly decreased at the EC-cell contact and diffused in the cytoplasm and nucleus of airway epithelium in TDI group, which was partly rescued by LY294002. In line with these, the protein levels of E-cadherin and β-catenin were markedly decreased after TDI inhalation, which is mainly attributed to their compromised expression at cell-cell membranes in airway epithelium. Treatment with LY294002 reversed the downregulation of β-catenin, yet had no effect on total pulmonary expression of E-cadherin (Fig. 4B).

Effect of LY294002 on Expression of Phospho-ERK, Phospho-GSK3β, and Phospho-β-Catenin

When compared with AOO-inhaled mice, protein levels of phospho-ERK, phospho-glycogen synthase kinase (GSK) 3β (Ser9), and phospho-β-catenin (Tyr654) were distinctly upregulated after TDI challenge. And they were significantly inhibited by
LY294002 (Fig. 6A and 6B). Similar results were seen in immunohistochemistry-stained sections. There was much more abundant expression of p-ERK and p-GSK3β (Ser9) in TDI-exposed airway epithelium, which were decreased after treatment with LY294002 (Fig. 6C–6E).

Effect of LY294002 on None-Sensitized Mice Challenged With TDI
To explore whether PI3K also participates in irritant-mediated (cytotoxic) responses in TDI-induced asthma, we challenged mice with TDI without dermal sensitization and found that the none-sensitized mice challenged 3 times with TDI shared similar pathophysiological features (though milder) with mice sensitized and challenged 3 times with TDI: AHR, infiltration of inflammatory cells especially neutrophils and eosinophils around the bronchus, thickened airway epithelium, raised levels of IL-4, IL-5, and IL-13 in BAL fluids and higher total serum IgE, and LY treatment had similar effects (Fig. 7). It is worth noting that the third TDI challenge was 6 days after the first challenge, which is enough for generating an immune response according to the work of Vanirbeek et al. (2004). So the none-sensitized mice challenged 3 times with TDI are actually TDI sensitized and TDI challenged. In this case, distinguishing irritant-induced and immune-mediated responses with this protocol becomes difficult. Intriguingly, though the none-sensitized mice challenged 2 times with TDI displayed much less inflammatory infiltration into the airways (no evident AHR, elevated Th2-related cytokines in BALF nor serum IgE were observed in this group), with a few neutrophils and eosinophils rarely seen, we found that β-catenin is activated, with much immunoreactivity diffused in the cytoplasm and nucleus (Supplementary Fig. S2–S5). As Western blot showed no changes in total β-catenin expression and immunohistochemistry demonstrated decreased membrane expression and increased cytoplasmic expression, we infer that β-catenin of cell membrane is breached with increased phosphorylation at Tyr654 in AOO-sensitized mice challenged 2 times with TDI. Treatment with LY294002 attenuated this response, suggesting that PI3K also mediates irritant-induced β-catenin dysfunction.

DISCUSSION
It’s the first time to imply a critical role for PI3K pathway in dysregulation of β-catenin in TDI-induced asthma. Although considerable controversy remains regarding its pathogenesis, TDI-induced asthma is characterized by hyperresponsiveness and inflammation of the airway (Ban et al., 2006). In this study, we developed a murine model of TDI-induced asthma. These mice share the following typical pathophysiologic features with clinical TDI-induced asthma patients: AHR, infiltration of inflammatory cells especially neutrophils and eosinophils around the bronchus, thickened airway epithelium, goblet cell hyperplasia, raised levels of IL-4, IL-5, and IL-13 in BAL fluids, and higher total serum IgE. LY294002, a PI3K inhibitor, has been shown to inhibit the kinase activity by competing with the ATP-binding site with no effect on other protein kinases such as protein kinase A, protein kinase C, mitogen-activated protein kinase, PtdIns 4-kinase, and diacylglycerol kinase (Vlahos et al., 1994). This study also verified that LY294002 inhibited PI3K activity by blocking Ser473 phosphorylation of Akt in lung tissues. Treatment with LY294002 attenuated TDI-induced AHR, inflammatory aggregation, goblet cell metaplasia, and decreased levels of IL-4, IL-13, and IgE. Although there was no significant difference of IL-5 between LY294002 group and TDI group, which is inconsistent with the results in ovalbumin-induced asthma (Kwak et al., 2003). This could be explained as that PI3K pathway may have different functions in different models.
FIG. 5. Immunohistochemical staining of E-cadherin and β-catenin in lung. A and D, E-cadherin (A) and β-catenin (D) immunoreactivity in airways. B and E, Amplified view of E-cadherin (B) and β-catenin (E) in the airway. C and F, E-cadherin (C) and β-catenin (F) immunostaining in the alveolar region. Note the weak staining of E-cadherin in alveolar epithelia. G and H, Integrated optical density of E-cadherin and β-catenin in alveolar showed no significant differences between groups (n = 8 image field of 8 slices from 4 mice per group). Original magnification was ×1000.
Our previous studies (Song et al., 2013a, b; Zhao et al., 2009) have shown that TDI could increase airway epithelial permeability, impair TJs, and induce E-cadherin rearrangement, both of which are important for maintaining proper functions of airway epithelium. Unlike previous study, here we observed decreased expression of E-cadherin, which may be related to different means of airway challenge. Downregulation of β-catenin and dilated interepithelial spaces of the airway were also observed. Pretreatment with LY294002 restored E-cadherin distribution, ameliorated β-catenin disruption and the broadened intercellular spaces. We previously demonstrated that ERK activation is involved in the aberrant distribution of E-cadherin in bronchial epithelia. Here, we also found increased level of p-ERK in airway epithelium after TDI inhalation, and administration of LY294002 could inhibit activation of ERK, along with recovered immunoreactivity of E-cadherin, indicating PI3K may regulate aberrant distribution of E-cadherin through ERK pathway. Though the expression of E-cadherin was not recovered after LY294002 treatment, but according to the work of Heijink et al. (2010) and one of our previous studies (Song et al., 2013b), E-cadherin delocalization would affect epithelial barrier function. So it is possible that treatment with LY294002 improves cell-cell contacts by redistribution of E-cadherin. At the same time, we observed that inhibition of PI3K also attenuated dysfunction of β-catenin in AOO-sensitized mice challenged 2 times with TDI (see Supplementary Fig. S2–S6). These data suggest that PI3K contributes to both immune- and irritant-induced breakdown of β-catenin in airway epithelium of TDI-induced asthma, which involves both immune- and irritant-mediated (cytotoxic) responses (Lange et al., 1999; Lummus et al., 2011). However, the underlining mechanisms still remain to be evaluated.

β-Catenin is a prominent example of a protein that acts dually both as an AJ adhesion protein and an essential mediator of the Wnt signal transduction pathway (Bienz, 2005; Nelson and Nusse, 2004). β-Catenin functions in cell adhesion at the plasma membrane, by linking E-cadherin to α-catenin. Cytoplasmic levels of β-catenin are tightly controlled by a destruction complex including the adenoma polyposis coli gene product, the scaffold molecules axin (conductin homolog; also

FIG. 6. Expression of p-ERK, p-glycogen synthase kinase (GSK) 3β, and p-β-catenin in lung tissue. Protein levels of p-ERK, p-GSK3β (Ser9), and p-β-catenin (Tyr654) determined by Western blot (A) were distinctly upregulated after TDI challenge and were all significantly inhibited by LY294002. Here ERK, GSK3β, and β-catenin were used as loading controls respectively. B, Densitometric analysis of Western blot. Immunohistochemistry and subsequent semiquantitative analysis (C) revealed more abundant expression of p-ERK (C) and p-GSK3β (D) in TDI-exposed airway epithelium, which were decreased after treatment with LY294002. n = 6, *P < .05 versus control; #P < .05 versus TDI + DMSO group. Original magnification was ×400. There were no significant differences between TDI group and TDI + DMSO group.
known as axin2), GSK3β and casein kinase (Brembeck et al., 2006). In the absence of Wnt signaling, the level of β-catenin (cytoplasmic) is kept low through degradation of β-catenin that is in excess of binding sites, such as cadherins at the plasma membrane (Nelson and Nusse, 2004). Activation of Wnt signaling leads to inhibition of GSK3β activity, resulting in cytoplasmic accumulation and nuclear translocation of β-catenin, which binds the TCF/LEF family of transcription factors to form a complex at the cell membrane (Behrens et al., 1993; Brembeck et al., 2006; Taddei et al., 2002). In this study, increasing phosphorylation of β-catenin (Tyr654) and fadig of E-cadherin and β-catenin at the cell membrane were seen after inhalation of TDI. Though alveolar cells were also found to have relatively abundant expression of β-catenin, we infer that TDI-induced phosphorylation of β-catenin at Tyr654 mainly occurs in airway epithelium as immunohistochemistry revealed very faint expression of E-cadherin in the alveolar. Administration of LY294002 inhibited phosphorylation of β-catenin at Tyr654 and partly recovered cytomembrane β-catenin, whereas had no effect on total pool of E-cadherin. Intriguingly, there’s a striking increase of total β-catenin expression in LY294002-treated mice that was even higher than control. Though LY294002 alleviated airway epithelial hyperplasia induced by TDI, epithelium was still thickened with abundant β-catenin expression in mice treated with LY294002, which, together with restored β-catenin at cell membrane, may be responsible for the robust elevation of total β-catenin pool. The results indicate that activation of
PI3K signaling could phosphorylate β-catenin at Tyr654 and therefore leads to β-catenin disassembling from E-cadherin at the cell membrane. By which way PI3K signaling regulates tyrosine phosphorylation of β-catenin is still unknown yet.

Intriguingly, neutrophils were found to induce tyrosine phosphorylation of β-catenin and hyperpermeability in coro-
nary venular endothelial cells (Tinsley et al., 1999). Hence, infiltrated neutrophils into the airway may increase tyrosine phosphorylation of β-catenin, leading to disassociation of β-catenin from E-cadherin at the membrane. Studies have demonstrated that PI3K family is crucially involved in chemoattractant-induced neutrophil migration (Hirsch et al., 2000; Li et al., 2000; Sasaki et al., 2000). Here, TDI-induced neutrophil recruitment in BAL fluids and peribronchial regions was attenuated by inhibition of PI3K, which is in agreement with the changes of β-catenin, proposing that PI3K pathway may impair cytomembrane β-catenin function by attracting neutrophil migration into the airways and increasing tyrosine phosphorylated β-catenin of TDI-inhaled asthmatic mice. In addition, neutrophils may disrupt AJs by release of various proteases (Allport et al., 1997). Inhibition of neutrophil serine proteases in human lung A549 cell line significantly prevented the detachment of A549 cells and the degradation of the TJs as well as E-cadherin and β-catenin (Tanga et al., 2012). Our study shows that PI3K may contribute to β-catenin dysfunction of airway epithelium by attracting neutrophil migration into the airways of TDI-inhaled asthmatic mice.

In conclusion, our results support that PI3K mediates β-cate-
nin dysfunction of airway epithilium, which may be associated with increased tyrosine phosphorylation of β-catenin in a TDI-induced murine asthma model.


