Toluene diisocyanate exposure induces airway inflammation of bronchial epithelial cells via the activation of transient receptor potential melastatin 8

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Toluene diisocyanate (TDI) is the most important cause of occupational asthma (OA), and various pathogenic mechanisms have been suggested. Of these mechanisms, neurogenic inflammation is an important inducer of airway inflammation. Transient receptor potential melastatin 8 (TRPM8) is a well-established cold-sensing cation channel that is expressed in both neuronal cells and bronchial epithelial cells. A recent genome-wide association study of TDI-exposed workers found a significant association between the phenotype of TDI-induced OA and the single-nucleotide polymorphism rs10803666, which has been mapped to the TRPM8 gene. We hypothesized that TRPM8 located in airway epithelial cells may be involved in the pathogenic mechanisms of TDI-induced OA and investigated its role. Bronchial epithelial cells were treated with TDI in a dose- and time-dependent manner. The expression levels of TRPM8 mRNA and protein were determined by quantitative real-time polymerase chain reaction and western blotting. TDI-induced morphological changes in the cells were evaluated by immunocytochemistry. Alterations in the transcripts of inflammatory cytokines were examined in accordance with TRPM8 activation by TDI. TRPM8 expression at both the mRNA and protein levels was enhanced by TDI in airway epithelial cells. TRPM8 activation by TDI led to significant increases in the mRNA of interleukin (IL)-4, IL-13, IL-25 and IL-33. The increased expression of the cytokine genes by TDI was partly attenuated after treatment with a TRPM8 antagonist. TDI exposure induces increased expression of TRPM8 mRNA in airway epithelial cells coupled with enhanced expression of inflammatory cytokines, suggesting a novel role of TRPM8 in the pathogenesis of TDI-induced OA.

INTRODUCTION

Toluene diisocyanate (TDI)-induced occupational asthma (OA) is the most common type of OA worldwide. However, its pathogenesis is more complicated than other types of OA. Several studies have addressed the inflammatory process of isocyanate-induced OA, describing heterogenic immunological pathways, such as Th1-controlled inflammation and Th2-triggered allergic processes, as well as non-immunological pathways, such as epithelial injury, airway remodeling, oxidative stress generation and neurogenic inflammation. However, these mechanisms remain incompletely understood, and an ideal diagnostic marker has not been identified. Because TDI-induced OA has a poor prognosis with persistent asthmatic symptoms and a progressive decline in lung function, efforts have been made to identify individual risk factors for the development of this condition.

The risk of TDI-induced OA is associated with a polymorphism of the neurokinin 2 receptor, a receptor for the neuropeptides that are released from activated airway sensory neurons, suggesting that neurogenic inflammation is involved in the development of TDI-induced OA. In addition, a genome-wide association study of TDI-exposed workers revealed that alpha T-catenin single-nucleotide polymorphisms (SNPs) were significantly associated with the phenotype of TDI-induced OA. Moreover, two SNPs of TRPM8 (rs10803666 and rs12434022, which have been mapped to the TRPM8 gene and are located on 2q37 and 14q31) showed significant associations using a recessive analysis model.
The TRPM8 is a nonselective calcium-permeable cation channel that is activated by cold temperatures of <25 °C and several chemicals, including the cooling agents menthol and icilin. TRPM8 is expressed on a subset of sensory neurons from the dorsal root ganglion and trigeminal ganglia, as well as in a number of non-neuronal areas. Recent studies have shown that activation of the TRPM8 variant in lung epithelial cells by cold air leads to increased expression of proinflammatory cytokine genes, such as interleukin (IL)-6 and IL-8. Moreover, TRPM8-mediated mucus hypersecretion has been demonstrated in patients with chronic obstructive pulmonary disease, suggesting that TRPM8 is potentially involved in chronic airway inflammation.

Epithelial cells form a continuous lining along the airways, providing a protective barrier between the external and internal environments. In addition to forming a barrier, the epithelium has the ability to generate a wide range of mediators that can modulate inflammatory responses, helping to either maintain homeostasis or enhance inflammation. The asthmatic airway epithelium is a major source of other cytokines and chemokines, and increased susceptibility to injury and altered repair are important for both airway remodelling and the mucosal metaplastic responses in patients with chronic asthma.

The goal of the present study was to explore the hypothesis that TDI exposure can cause changes in airway epithelial cells by activating TRPM8 and that this TDI-induced TRPM8 activation induces airway inflammation.

**MATERIALS AND METHODS**

**Chemicals**

Toluene-2, 4-diisocyanate, N-(4-tet-butylphenyl)-4-(3-chloropyridin-2-yl) piperazine-1-carboxamide (BCTC), menthol and dexamethasone were purchased from Sigma Chemical. (St. Louis, MO, USA). Rabbit anti-TRPM8 (C-term) polyclonal antibody was purchased from Abcam (Cambridge, UK). Oligonucleotides were purchased from Sigma Chemical. (St. Louis, MO, USA). Rabbit IgG secondary antibody (Molecular Probes, Eugene, OR, USA) was obtained as described above. cDNA corresponding to human interleukin IL-4, −6, −8, −13, −25 and −33 was amplified by PCR from 1 μl of the cDNA synthesis reaction using SYBR Premix Ex Taq (TaKaRa Biotechnology) and the following primers:

- **IL-4:** sense, 5′-CAACAAGTTCATCCCTTGCTTG-3′; antisense, 5′-TCT GGACCCTGCAAAGTGGTG-3′.
- **IL-6:** sense, 5′-GTTCGTCGATGTTGGAACC-3′; antisense, 5′-AACCCTAAAGACAGGATGATT-3′.
- **IL-8:** sense, 5′-CTGGCCGTGGCTCTCTTG-3′; antisense, 5′-TTA GCACCTTGGCAGAAAATCTG-3′.
- **IL-13:** sense, 5′-CCTCATGGCCCTTTTTGTTGAC-3′; antisense, 5′-TCTGTTCTGGGTGATTGTA-3′.
- **IL-25:** sense, 5′-CCAGGGCTGGGATTCTGG-3′; antisense, 5′-TGGCTGTAGGTTGGTTGCC-3′.
- **IL-33:** sense, 5′-AAAGAGAAGTGGCTCCCATG-3′; antisense, 5′-AAGGCAAAGCCTCACCAGT-3′.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was simultaneously amplified as an internal standard. Experiments were reproduced a minimum of three times with different passages of cells.

**Immunocytochemistry**

BEAS-2B cells were cultured on a 22-mm round cover slip (BD Biosciences, Bedford, MA, USA) until 75% confluence and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min. The cells were then washed three times with PBS, and nonspecific binding was blocked using a solution of 5% bovine serum albumin in PBS. The cells were then washed twice with PBS, and nonspecific binding was blocked using a solution of 5% bovine serum albumin in PBS. The cells were rinsed three times with PBS and incubated at room temperature for 2 h with a rabbit polyclonal IgG antibody fraction specific to human TRPM8 (Abcam), which was diluted 1:250 in the blocking solution. The cells were washed and treated for 1 h at room temperature with an Alexa-Fluor 488 conjugated goat anti-rabbit IgG secondary antibody (Molecular Probes, Eugene, OR, USA) at a dilution of 1:500 in the blocking solution. The nuclei were counterstained blue using 4’, 6-diamidino-2-phenylindole (DAPI) at 300 ng dilution in PBS. Controls consisted of untreated cells or cells treated with either primary or secondary antibodies alone. Images were collected using a ZEISS LSM710 laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany) equipped with filters to visualize green fluorescent protein and DAPI. The immunoreactivity of TRPM8 was detected as green fluorescence.

**Statistical analysis**

Statistical analysis was performed using ANOVA and Dunnnett’s multiple-comparison post-test, with a significance level of P < 0.05. The paired t-test with a significance level of P < 0.05 was also used.
RESULTS
Identification of TRPM8 transcript and protein in lung epithelial cells
We investigated whether TRPM8 is expressed on BEAS-2B cells. Expression of TRPM8 was identified using RT-PCR. Figure 1 shows the amplified PCR products generated after 35 cycles and the TRPM8 protein expression using western blot analysis. TRPM8 activation by menthol for 3 h led to significant increases in both TRPM8 mRNA and protein expression. Each bar corresponds to the mean normalized product intensity and s.d. An asterisk represents statistically significant increases in mRNA and protein expression relative to untreated cells (paired t-test, *P<0.05, **P<0.01, ***P<0.001).

Effect of TDI on TRPM8 transcripts and protein in lung epithelial cells
To determine whether TDI can activate TRPM8, the TRPM8 transcript and protein were measured after treatment with TDI (0.25–1 mM), and the TRPM8 agonist menthol (2–6 mM) was used as a positive control. TDI tended to increase the TRPM8 transcript, but this change did not reach statistical significance. However, TDI led to a robust increase in the protein level, which was significantly higher than that achieved with menthol treatment (P<0.01) (Figure 2).

Expression of TRPM8 protein in lung epithelial cells
The expression of TRPM8 protein in BEAS-2B cells was evaluated by immunocytochemical analysis using a polyclonal antibody for human TRPM8 (Figure 3). Without any treatment, TRPM8 protein was not detected. However, after treatment with TDI or menthol, time- and dose-dependent intense green staining was detected. The staining pattern suggested localization of the TRPM8 to the plasma membrane (peripheral staining) and endoplasmic reticulum (perinuclear staining).
Inhibition of TRPM8 transcript and protein by BCTC in lung epithelial cells

To test the hypothesis that TDI selectively activates TRPM8 on bronchial epithelial cells, BEAS-2B cells were treated with TDI after pretreatment with BCTC, an antagonist of TRPM8, at 50–100 μM. Increased TRPM transcripts by TDI were attenuated by BCTC (Figure 4a). Likewise, 50–100 μM BCTC blocked the TDI-induced increase in TRPM8 protein expression (Figure 4b).

Effect of TRPM8 activation on cytokine gene expression in lung epithelial cells

The coupling of TRPM8 activation in BEAS-2B cells with enhanced expression of proinflammatory cytokines (IL-6 and IL-8), epithelial-driven cytokines (IL-25 and IL-33) and Th2 cytokines (IL-4 and IL-13) was evaluated following treatment with TDI. No alteration was observed in IL-6 and IL-8 transcripts after TDI exposure. Instead, co-treatment with TDI and BCTC resulted in significantly lower IL-6 and IL-8 transcripts compared with the control (Figure 5). The degree of decrement in IL-6 by BCTC was similar to that by dexamethasone. The TRPM8 activation by TDI led to a significant increase in the transcripts for IL-4, IL-13 and IL-25 (Figures 6 and 7). The transcript for IL-33 tended to increase with 1 mM of TDI, but this change did not reach statistical significance. TDI-induced increases in cytokines gene expression were partly attenuated after co-treatment with BCTC and dexamethasone (Figures 6 and 7).
DISCUSSION

In the current study, we demonstrated that TRPM8 is activated by TDI and that activation of this receptor is involved in airway inflammation involving lung epithelial cells.

TDI, which is a reactive compound widely used in the manufacturing of polyurethane foams and coatings, can cause respiratory symptoms, such as coughing, rhinitis, dyspnea and chest tightness, in exposed workers.\(^4\) It can act as an irritant as well as a strong sensitizer; as a sensitizer, driven by TDI-specific antibodies, it promotes airway inflammation via the immunological pathway. As an irritant material, TDI leads to stimulation of trigeminal nerve endings in airways, thereby inducing the local release of tachykinin neuropeptides, such as substance P, neurokinin A and calcitonin-gene-related peptide. These findings suggest that TRP receptors may be involved in neurogenic inflammation of the airways in patients with TDI-induced OA.\(^15\) Some studies have investigated the role of TRP receptors in TDI-OA. Taylor-Clark \textit{et al.}\(^16\) showed that TRPA1 is a molecular transducer through which TDI causes sensory nerve activation and respiratory reflexes in an animal model. Devos \textit{et al.}\(^17\) recently demonstrated that TDI can directly activate TRPA1 and that both TRPA1 and mast cells in airways are critical for the induction of airway hyper-responsiveness in an animal model of TDI-induced OA.

A previous genome-wide association study of TDI-exposed workers showed that two SNPs (rs10803666 and rs12434022) of TRPM8 had significant associations with the phenotype of TDI-induced OA with higher odds ratios, indicating strong correlations.\(^7\) TRPM8 is expressed on a subpopulation of airway vagal afferent nerves, pulmonary arterial smooth muscle cells and lung epithelial cells. Sabnis \textit{et al.}\(^10\) showed that a TRPM8 variant manipulated for stable overexpression of TRPM8 was expressed primarily within endoplasmic reticulum membranes of lung epithelial cells. In the present study,
we demonstrated that TRPM8 was spontaneously expressed in human bronchial epithelial cell lines without the use of a TRPM8 variant and that TDI itself can cause TRPM8 activation comparative to its agonist, menthol. These findings suggest that TDI exposure can induce TRPM8 activation in the airway epithelial cells of TDI-exposed workers.

To determine whether TRPM8 activation by TDI is associated with airway inflammation in TDI-induced OA, we examined the expression of several cytokine genes, including proinflammatory cytokines, such as IL-6 and IL-8; Th2 cytokines, including IL-4 and IL-13; and epithelial-driven cytokines, such as IL-25 and IL-33. Mattoli et al. demonstrated that IL-6 was released from TDI-exposed airway epithelial cells and that it consecutively induced T-cell activation and proliferation. By releasing IL-8, a potent activating and chemotactic factor of neutrophils, diisocyanate-exposed epithelial cells enhance the infiltration of neutrophils and affect smooth muscle cells. This indicates that IL-6 and IL-8 are directly involved in airway inflammation and bronchial remodeling in patients with TDI-induced OA. When we co-treated BEAS-2B cells with TDI and a TRPM8 antagonist, we found significant reductions in both the IL-6 and IL-8 transcripts, and the degree of decrement was similar to that with dexamethasone. These results suggest that ongoing inflammation of airway epithelial cells by TDI exposure is suppressed by TRPM8 antagonists and that TRPM8 can be a

Figure 6 TRPM8-mediated alteration in (a) IL-4 and (b) IL-13 transcription in BEAS-2B cells. Each bar represents the mean values normalized to untreated controls for the cell populations and standard deviations. Values significantly different from untreated and TDI (1 mM)-treated cells are indicated by * and #, respectively (paired t-test, *, #, P<0.05; **, ##, P<0.01). TDI, toluene diisocyanate.

Figure 7 TRPM8-mediated alteration in (a) IL-25 and (b) IL-33 transcription in BEAS-2B cells. Each bar represents the mean values normalized to untreated controls for the cell populations and standard deviations. Values significantly different from untreated and TDI (1 mM)-treated cells are indicated by * and #, respectively (paired t-test, *, #, P<0.05; **, ##, P<0.01; ###, P<0.001). TDI, toluene diisocyanate.
key contributor to airway inflammation in patients with TDI-induced OA.

Several studies have shown infiltration of inflammatory cells in the airway mucosa, especially eosinophils, CD4+ cells and mast cells, in both animal models and human airways affected by TDI-induced OA. T cells and eosinophils have been shown to release various cytokines, including IL-4 and IL-5, to induce immune responses. In the present study, we found that TRPM8 activation by TDI was coupled with enhanced expression of IL-4 and IL-13 mRNA from bronchial epithelial cells. Moreover, treatment with a TRPM8 antagonist attenuated the expression of IL-4 and IL-13 mRNA levels, suggesting that bronchial epithelial cells have a role in the induction of Th2-type inflammation in TDI-induced OA. However, the mechanism by which TRPM8 activation by TDI induces Th2 inflammation in bronchial epithelial cells remains unknown.

Considering that TDI is redox-active and capable of inducing oxidative stress that includes inflammation and cell death, and considering that production of reactive oxygen species by TRPM8 agonists is dependent on TRPM8-mediated calcium entry, we postulate that TDI induces the production of reactive oxygen species in airway epithelial cells followed by TRPM8 activation and Th2-cytokine release from airway epithelial cells.

Airway epithelial cells constitutively express IL-25. On exposure to allergens with proteolytic activities or viruses, the release of IL-25 mRNA and IL-25 protein from bronchial epithelial cells increases. IL-25 reportedly induces type 2 innate lymphoid cells and CD4+ T cells to produce IL-4, IL-5 and IL-13 and has a critical role in airway eosinophilia, mucus overproduction and airway remodeling.

In the present study, we found that IL-25 mRNA increased along with TRPM8 activation by TDI and observed a positive correlation between IL-25 mRNA and IL-4 or IL-13 mRNA levels. IL-33 is an inducer of Th2 adaptive immunity. It is released by damaged epithelial cells during injury or necrosis caused by various triggers, such as viruses, allergens and smoke. Although the change was not statistically significant, IL-33 mRNA tended to be increased in accordance with TRPM8 activation by TDI and attenuated with the TRPM8 antagonist in this study. This is the first study to show an association between increases in epithelial-driven cytokines and TRPM8 activation by TDI in an in vitro model, which is a novel function of TRPM8 in the pathogenic mechanism of TDI-induced OA. However, further studies are needed to reveal the pathway for release of IL-25 or IL-33 by airway epithelial cells.

In conclusion, our results demonstrate that TDI exposure can induce TRPM8 activation in bronchial epithelial cells. TRPM8 activation by TDI enhances airway inflammation similar to that induced by Th2 cytokines (IL-4 and IL-13) and epithelial-driven cytokines (IL-25 and IL-33), and this was partly attenuated with a TRPM8 antagonist. The role of TRPM8 in inflammatory responses involving bronchial epithelial cells will serve as a foundation for further development of new therapeutic targets for patients with TDI-induced OA.

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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