Title: Tiotropium bromide has a more potent effect than corticosteroid in the acute neutrophilic asthma mouse model

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Background: Neutrophilic asthma (NeuA) is usually corticosteroid resistant. Tiotropium bromide (TIO) is a bronchodilator that is used as an add-on therapy to the inhaled corticosteroid and long-acting β2 agonist in asthma. However, the role of TIO in NeuA is not fully known. We conducted this study to evaluate the effect of TIO on NeuA comparing with that of corticosteroids.

Methods: C57BL/6 female mice were sensitized with ovalbumin and lipopolysaccharide for inducing neutrophilic inflammation. Dexamethasone (DEX) was administered on days 14, 17, 20, and 23. TIO was inhaled on days 21, 21, and 23. On day 24, the mice were sacrificed. Airway hyper-responsiveness (AHR), the level of cytokine in the bronchoalveolar lavage (BAL) and lung homogenates, and the lung tissue histopathology were compared between the groups.

Results: Neutrophil counts, T helper 2 cells (Th2)/Th17 cytokines, and pro-inflammatory cytokine in BAL fluid were elevated in the NeuA group. TIO group showed lower total cells, neutrophil counts, and eosinophil counts in BAL fluid compared to those of DEX group (p < 0.001, p < 0.05, and p < 0.001, each). Airway resistance was attenuated in the TIO group, which was elevated in the NeuA group (p < 0.001). Total protein, interleukin (IL)-5, and IL-17A in the BAL fluid were lower in the TIO group than those of the NeuA group (p < 0.05, each).

Conclusions: TIO showed more potent effects than DEX on improving airway inflammation and attenuating airway resistance in NeuA.

Abstract word count: 239 words

Key Words: Neutrophilic asthma, Tiotropium bromide, Corticosteroid
**Introduction**

Asthma is characterized by a chronic airway inflammation with heterogeneous features\(^1\), \(^2\). Severe asthma accounts for 5%-15% of all asthma cases. Impacts of severe asthma on the quality of life of patients, socioeconomic burden, and healthcare-related burden are enormous\(^3\), \(^4\). Its pathophysiology varies, including complex airway inflammation, non-eosinophilic inflammation, severe airway remodeling, and fixed airflow limitations. One of the proposed mechanisms of severe asthma is the increased neutrophilic inflammation of the airway\(^5\), which is the main process of neutrophilic asthma (NeuA).

NeuA is a major category of non-eosinophilic asthma. It shows a low type 2 immune response and a non-atopic phenotype\(^6\). Usually, NeuA is corticosteroid insensitive\(^7\), \(^8\). The T helper 1 (T\(_{\text{H}1}\)) and T helper 17 (T\(_{\text{H}17}\)) processes of NeuA are considered to be the main processes of inflammation\(^9\), \(^10\). Therefore, the treatment of severe NeuA is not limited to corticosteroid use.

Tiotropium bromide (TIO) is a long-acting muscarinic antagonist\(^11\). It improves asthma by modulating neuronal acetylcholine and decreasing airway smooth muscle thickening\(^12\). It is usually used as an add-on therapy to the combination of inhaled corticosteroid and long-acting \(\beta_2\) agonist in the Global Initiative for Asthma guideline as step 4 or step 5 treatment. TIO inhibits the M\(_3\) receptor in the smooth muscle of the airway, which leads to bronchodilation. In an *in vitro* study, it showed anti-inflammatory effects by controlling pro-inflammatory cytokine such as interleukin (IL)-8 or IL-17A\(^13\). However, the action of TIO in NeuA remains unclear. This study was conducted to evaluate the effects of TIO in NeuA mouse model.
Material and Methods

Mouse experimental model of this study

We developed a NeuA mouse model in a previous study. Six-week-old female C57BL mice (Orient Bio., Seongnam, Korea) were sensitized with ovalbumin (OVA) (Sigma-Aldrich, St. Louis, MO, USA). OVA (100μg) and aluminum hydroxide (2mg) (Sigma-Aldrich) were dissolved in the saline and injected into the peritoneum to sensitize the T_H2 inflammation on days 0 and 7. After sensitization, OVA (50μg) and lipopolysaccharide (LPS) (2 mg/kg) were administered intranasally. Intranasal OVA was administered on days 14, 15, 21, 22, and 23. Intranasal LPS was administered to shift the eosinophilic inflammation to neutrophilic inflammation on days 18, 21, and 23. The mice were sacrificed on days 24, 14, 15 (Figure 1).

Protocol of drug challenge

The experimental drugs used TIO and dexamethasone (DEX). TIO (25 μg/ml) was administered by inhalation for 3 minutes per mouse in the inhalation chamber. This was done on days 21, 22, and 23 (Figure 1). DEX (4 mg/kg) was injected into the peritoneum on days 14, 17, 20, and 23(Figure 1). Mice were randomly allocated and grouped into (1) the control, (2) OVA + LPS (O+L), (3) OVA + LPS + DEX (O+L+D), and (4) OVA + LPS + TIO (O+L+T).

Airway resistance

Airway resistance was measured by the resistance of the respiratory system (Rrs) in the FlexiVent system (SCIREQ, Montreal, QC, Canada). Baseline Rrs in mice was measured by exposure to nebulized phosphate-buffered saline (PBS) for 3 minutes. The Rrs values were re-assessed after exposure to increasing concentrations of methacholine (Sigma-Aldrich) using an Aerosonic ultrasonic nebulizer (DeVilbiss, Somerset, PA, USA). The average values
of the Rrs were obtained, which were measured during each 3 minutes sequence for each concentration of methacholine\textsuperscript{17}.

**Broncho-alveolar lavage**

Broncho-alveolar lavage (BAL) was performed via the cannulated trachea with a silicon tube. We instilled 0.8 ml of cold sterile PBS into the lung and withdrew the BAL fluid. The BAL fluid was subjected to cytopsin for 10 minutes, and then the supernatant was stored at -80°C for further analyses. Fifty μL aliquots of BAL fluid were centrifuged at 43 g for 5 minutes and stained with Diff-Quick (Sysmax, Kobe, Japan). Total cell counts were obtained using a hemocytometer LUNA automated cell counter. The percentages of macrophages, eosinophils, lymphocytes, and neutrophils were measured by counting 500 leukocytes in the BAL fluid slides under a light microscope\textsuperscript{18}.

**Measurement of inflammatory cytokines**

We measured the levels of inflammatory cytokines in BAL fluid, such as IL-4, IL-5, IL-17A, and IL-22. The levels of IL-1β, IL-6, tumor necrosis factor (TNF)-α, and interferon (IFN)-γ in the lung homogenates were also measured. An enzyme-linked immunosorbent assay kits were used for measurement (R&D Systems, Minneapolis, MN, USA).

**Lung tissue histopathology and inflammation score**

The lungs of the mice were fixed in 4% paraformaldehyde for 24 hours after being sacrificed. The sections were embedded in paraffin and cut into 4 μm thickness slides by a microtome. Hematoxylin and eosin (H&E) staining was performed for microscopic examination. We assessed the inflammation of the peri-bronchial area using a quantitative method that was used in a previous study\textsuperscript{19}.
Statistical analyses

One-way and two-way analysis of variance (ANOVA) were used to evaluate the effect of medication on various measurements in BAL fluid, such as cell counts, total protein, and inflammatory cytokines. The differences in total cell counts and their differences between the medications were compared using repeated-measures ANOVA. Post-hoc analyses using Dunnett’s and Turkey’s multiple comparison tests were used to compare multiple groups. Statistical significance was set at \( p \)-value < 0.05. All analyses were performed using GraphPad Prism 5.01 (GraphPad Software, San Diego, CA, USA).

Ethics approval

This study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the School of Medicine, The Catholic University of Korea (approval no. CUMC-2020-0079-04).

Results

Tiotropium bromide attenuates the airway resistance significantly in neutrophilic asthma

The O+L group, which was consistent with the NeuA group, showed a significant increase in airway resistance. DEX, which is the conventional treatment for asthma, did not attenuate the airway resistance of the respiratory system in the NeuA mouse model. Unlike DEX, the TIO treated group (O+L+T) showed significant improvement in airway resistance compared to the O+L group. Interestingly, the O+L+T group showed a similar level of airway resistance compared to that of the control group (Figure 2).

Tiotropium bromide reduces the airway inflammation of neutrophilic asthma

Neutrophilic inflammation was induced in the O+L group compared to that in the control group after OVA and LPS administration. Overall airway inflammation in the BAL fluid was significantly decreased in the O+L+T group. However, no significant changes were
observed in the O+L+D group. In particular, neutrophil and eosinophil counts significantly decreased after treatment with TIO. Unlike in the O+L+T group, neutrophils did not decrease after DEX treatment (Figure 3).

Total protein levels were elevated in the O+L group. The DEX group did not show significant changes compared with the O+L group. However, the total protein levels in the TIO group were significantly improved compared to that in the NeuA group. Inflammation scores by H&E staining were significant improved after treatment with DEX or TIO. The O+L+T group showed lower inflammation scores than the O+L+D group, but there was no statistically significant difference between the two groups.

**Tiotropium bromide has more potent effects than corticosteroids on improving TH2/TH17 inflammation**

After inducing neutrophilic inflammation, IL-5, IL-17A, and IL-22 in the BAL fluid were elevated in the NeuA group compared to the control group. IL-5 and IL-17A levels in the BAL fluid decreased significantly after treatment with TIO, which was not observed after treatment with DEX. IL-22 levels in the BAL fluid showed a similar tendency, but there was no clinical significance (Figure 4). In the analyses of lung homogenates, the levels of IL-1β, IL-6, TNF-α, and IFN-γ were elevated after induction by OVA and LPS. The levels of cytokines in the lung homogenate decreased after being treatment with TIO or with DEX. The TIO group showed more improvement than the DEX group; however, the difference was not statistically significant (Figure 5). TIO showed more potent effects on improving neutrophilic inflammation than DEX. Moreover, TH2 and TH17-related inflammatory cytokines improved in the TIO group compared to those in the DEX group.

**Discussion**

NeuA is characterized by more trapped air, lower lung functions, thicker airway inflammation, and more events of exacerbation than non-neutrophilic asthma9, 20. NeuA is
often less responsive to the classic asthma medications, such as corticosteroids\textsuperscript{21-23}. In a previous study, it was found that T\textsubscript{H}17 and IL-17A play a key role in neutrophilic airway inflammation\textsuperscript{9}. Increased neutrophils promote smooth muscle cell proliferation, pro-inflammatory mediators, and neutrophil recruitment to the airways\textsuperscript{24}. These are also possible causes of heterogeneity in airway inflammation, which is difficult to treat.

Many studies have shown that TIO can play potential roles in improving neutrophilic inflammation. TIO improves smooth muscle cell proliferation and remodeling by inhibiting the M\textsubscript{3} receptor in the airway. This leads to an improvement in bronchoconstriction. Acetylcholine, which is also controlled by TIO, modulates the airway inflammatory response that is not fully controlled by T\textsubscript{H}2 cells\textsuperscript{25}. In contrast to the TIO, there are several reasons that corticosteroid is not effective for controlling the neutrophilic inflammation in the NeuA. NeuA is mainly mediated by T\textsubscript{H}17 cells, Which are not controlled by corticosteroids. In a previous study, NeuA was shown to have corticosteroid-insensitivity, which was mediated by histone deacetylase 2 inactivity\textsuperscript{9}. Therefore, we conducted this study to evaluate the effect of TIO alone on NeuA. We revealed some aspects of TIO as an appropriate drug for controlling NeuA.

First, TIO showed a significant improvement in the inflammation in NeuA. Neutrophil counts significantly improved after TIO treatment. Total protein, IL-17A, and IL-5 in the BAL fluid, which were related to T\textsubscript{H}1 and T\textsubscript{H}17 responses, were decreased after TIO treatment in the NeuA group. In this study, we demonstrated the effect of TIO on neutrophilic inflammation by controlling T\textsubscript{H}1 and T\textsubscript{H}17 cells. Second, TIO showed more improvement in inflammatory cytokines than DEX. TIO showed clinically relevant improvement compared to that of DEX, which did not show any statistically significant difference. Third, airway resistance after TIO treatment was attenuated in the control group. This means that the TIO showed improved the airway flow in NeuA which is the main target of asthma care. Based on these results, we can assume the possibility of early administration of TIO in NeuA. Fourth, we administrated TIO via inhalation method, which was the proper way of delivery as
originally designed. In many previous studies, TIO was usually administered by intratracheal or intraperitoneal method, which is not the right way of using TIO. Therefore, we developed a proper TIO inhalation mouse model for further evaluation.

There were a few limitations to this study. First, we did not observe a dose-dependent relationship between TIO and NeuA. However, the decided level of TIO treatment was calculated using a minimal animal equivalent dose of human drug. Therefore, we showed minimal effects of TIO in the NeuA mouse model. Second, we did not discover a possible pathway for controlling neutrophilic inflammation using TIO. We simply showed the effect of TIO on NeuA. We are planning to conduct an upcoming study to evaluate the possible mechanism of TIO.

In this study, we demonstrated the therapeutic effects of TIO in NeuA. These results show the possibility of TIO as the primary medication in NeuA. Further studies on the $T_{H1}/T_{H17}$ inflammation cascade of TIO should be conducted in the future.

**Author’s contributions**


**Conflict of interest**

All authors do not have any conflict of interest.

**Funding**

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**Figure legends**

Figure 1. Schematic flow of acute neutrophilic asthma mouse model study.

Mice were sensitized via OVA on days 0 and 7. OVA was challenged on days 14, 15, 21, 22, and 23 via intranasally. Neutrophilic inflammation was induced by LPS which was administered on days 18, 21, and 23 via intranasally. Dexamethasone was challenged on days 14, 17, 20, and 23 via intraperitoneally. Tiotropium bromide was inhaled on days 21, 22, and 23. OVA, ovalbumin, LPS, lipopolysaccharides

C57BL/6, Female, 6W

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Sensitization

Challenge

Sacrifice
Figure 2. Airway resistance of acute neutrophilic asthma according to the medication.

Airway resistance by forced oscillation technique. It was measured in response to the increasing dose of methacholine. Tests have proceeded in four groups, such as control, O+L, O+L+D, and O+L+T. The level of airway resistance was elevated in O+L group and O+L+D group did not show the improvement of airway hyperresponsiveness. O+L+T group showed significant attenuated airway resistance compared to that of O+L group and that of O+L+D group. O; ovalbumin, L; lipopolysaccharides, D; dexamethasone, T; Tiotropium bromide.

OVA-LPS vs. all (*p < 0.05, **p < 0.01, ***p < 0.001)
OVA-LPS-DEX vs. OVA-LPS-TIO (†p < 0.05, ‡p < 0.01, §§p < 0.001)
Figure 3. Lung inflammation status of neutrophilic asthma according to the challenged drugs, such as dexamethasone or Tiotropium bromide.

(a), (b) Compared to the O+L group, dexamethasone group (O+L+D) did not show significant improvement of the level of total cells, neutrophils, eosinophils, and total protein in bronchoalveolar lavage (BAL) fluid. On the contrary, Tiotropium bromide group (O+L+T) showed significant improvement both in total cells including neutrophils of BAL fluid and in the total protein level of BAL fluid. (c), (d) Inflammation score which was calculated by hematoxylin and eosin staining of lung tissue histopathology showed improvement both in O+L+D group and in O+L+T group. O; ovalbumin, L; lipopolysaccharides, D; dexamethasone, T; Tiotropium bromide
Figure 4. Inflammatory cytokines related to neutrophilic inflammation such as interleukin (IL)-5 and IL-17A in bronchoalveolar lavage (BAL) fluid were attenuated by Tiotropium bromide. There was no significant decrease of cytokines, such as IL-5 and IL-17A, after being administered dexamethasone. Compared to those of the dexamethasone group (O+L+D), Tiotropium bromide group (O+L+T) showed a significant decrease of IL-5 and IL-17A in BAL fluid. Both IL-22 and IL-4 did not differ from the neutrophilic asthma group (O+L) after dexamethasone or Tiotropium bromide was applied. O; ovalbumin, L; lipopolysaccharides, D; dexamethasone, T; Tiotropium bromide

O+L vs. all (*p<0.05, **p<0.01, ***p<0.001)
Figure 5. Inflammatory cytokines in lung tissue homogenate showed a declining tendency after Tiotropium bromide (TIO) without clinical significance. The T helper cytokines from lung homogenates such as interleukin (IL)-1β or IL-6 and proinflammatory cytokines such as tumor necrosis factor (TNF)-α, and interferon (IFN)-γ shows the tendency on the decline after treated TIO but there was no clinical significance compared to the neutrophilic asthma group. O; ovalbumin, L; lipopolysaccharides, D; dexamethasone, T; Tiotropium bromide