Original Research Article

Trifluorobenzamidine prevents allergic rhinitis by regulating IgE, IL-4 and IL-5 in T-cells

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Abstract

Purpose: *To investigate the effect of trifluorobenzamidine (TBI) on a mouse model of ovalbumin (OVA)-induced allergic rhinitis.*

Methods: *Allergic rhinitis was established in mice via sensitization on days 1, 5 and 14 through intraperitoneal injection of OVA (100 µg) in PBS. On day 15, the mice were subjected to intranasal exposure to OVA (1.5 mg dissolved in PBS). Prior to 10 days of intranasal exposure to OVA, the mice were treated with TBI at doses of 5, 10 and 20 µg/kg. Cytokine levels were determined using enzyme-linked immunosorbent assay (ELISA) kits, while cyclooxygenase (COX)-2 and caspase-1 activity were assayed with western blotting.*

Results: *Treatment with TBI significantly (p < 0.05) reduced OVA-mediated increases in nasal rub scores, and decreased serum levels of IgE, TNF-α, thymic stromal lymphopoietin (TSLP), IL-1β and histamine in mice. It also significantly regulated spleen weight and IL-4 secretion (p < 0.05) in OVA-administered mice. TBI significantly downregulated the expressions of IL-5, IL-13, TNFα, TSLP, IL-1β and IL-6 (p < 0.05). Administration of TBI caused a marked reduction in OVA-mediated increase in caspase-1 activity in mice intranasal tissues, and also significantly reduced OVA-induced excessive production of MIP-2 and ICAM-1 (p < 0.05). Moreover, TBI prevented OVA-induced infiltration of eosinophils and mast cells into intranasal tissues (p < 0.05).*

Conclusion: *TBI reduces levels of IgE and various pro-inflammatory cytokines in OVA-administered mice. It also regulates Th1:Th2 ratio, inhibited activity of caspase-1, suppressed mast cell/eosinophil infiltration and reduced ICAM-1 and MIP-2 levels. Therefore, TBI possesses inhibitory potential against rhinitis allergy, and thus can potentially be developed as a new treatment strategy for asthma.*

Keywords: *Trifluorobenzamidine, Anti-inflammation, Allergic rhinitis, Cytokines, Caspase-1, Itching*

INTRODUCTION

Allergic rhinitis, the most common type of allergy, is associated with increasing prevalence [1]. In many patients, allergic rhinitis is also accompanied with conjunctivitis and atopic dermatitis [2]. The symptoms of allergic rhinitis, an inflammatory disease mediated by IgE, are discharge through the nose, sneezing and itching [3]. During allergic rhinitis, systemic inflammation occurs through recruitment of different immune cells [4]. Immediately after exposure to allergens, histamine and various cytokines and chemokines are secreted by the mast cells [4]. The

pathogenesis of allergic rhinitis is associated with disturbances in between Th1 (releasing IFN-γ) and Th2 (releasing IL-4, IL-5 and IL-13) [5].

During allergic rhinitis, Th2 and cytokines induce clinical characteristics of the disease, whereas others activate infiltration of mast cells and eosinophils [4,6]. The secretion of pro-inflammatory molecules such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6 and TSLP occurs in inflammatory cells [7]. These molecules promote infiltration of mast cells and eosinophils to intranasal tissues, besides enhancing the expressions of intercellular adhesion molecule-1 (ICAM-1) and macrophage inflammatory protein-2 (MIP-2) [7]. Effective treatment for allergic rhinitis is possible only through inhibition of the inflammatory process.

Natural as well as synthetic compounds containing heterocyclic moieties have shown promising biological effects against various diseases, and are used as skeletons in drug discovery programs [8]. The imidazole scaffold is used as the structural constituent of several potent bioactive molecules and therapeutic agents [9]. Heterocyclic compounds have shown promising effects as anti-microbial agents [10] and anti-tumor compounds [11]. They are also used in formulating drugs for treating neurological disorders; including Alzheimer's disease [12]. The present study investigated the effect of substituted trifluorobenzamidine (TBI) on a mouse model of OVA-induced allergic rhinitis.

EXPERIMENTAL

Mouse allergic rhinitis mice model

Five-week-old BALB/c mice (n = 70) were supplied by the Animal Center of Shandong University (Shandong, China). The mice were kept in sterilized cages at a temperature of 23 ± 2˚C and 60 % humidity in an environment with a 12-h light/12-h dark cycle, and given free access to water and feed. Approval for the animal study was obtained from the Animal Care and Use Committee, Municipal Hospital, China (approval no. SU/17/0047). All experimental procedures were conducted in accordance with the guidelines of National Institutes of Health [13]. The mice were randomly divided into seven groups (10 mice per group): negative control, model (1.5 mg OVA for sensitization), three TBI treatment groups (OVA + TBI at a dose of 5 μg/kg, OVA + TBI at a dose of 10 μg/kg, and OVA + TBI at a dose of 20 μg/kg); TBI group (TBI at a dose of 20 μg/kg) and dexamethasone group (OVA + DEX at a dose of 5 mg/kg). In the establishment of allergic rhinitis, mice

sensitization was done on days 1, 5 and 14 via intraperitoneal injection of OVA (100 μg) in PBS.

The mice were given intranasal exposure of OVA (1.5 mg dissolved in PBS) on day 15. Mice in negative control group were exposed to normal saline alone, while mice in treatment groups were intragastrically given TBI at doses of 5, 10 and 20 μg/kg before 10 days of exposure to OVA. Nasal rub score was calculated for 10 min on day 16 for each mouse. Cytokine levels were determined using ELISA kits, while COX-2 and caspase-1 activities were assayed with western blotting.

Determination of histamine and cytokine levels

Blood samples were taken from the mice and centrifuged at 2, 000 g for 15 min at 4˚C. The serum samples obtained were kept under liquid nitrogen prior to determination of cytokine levels. The serum levels of IgE, histamine, TNF- α, IL-1β, IL-6 and TSLP were measured using ELISA kits (Biology Engineering Institute, Nanjing, China) in line with the manufacturers' protocols.

Determination of cytokines in spleen and intranasal tissues

The mice were sacrificed on day 21 post-intranasal OVA administration under pentobarbital sodium anesthesia (35 mg/kg). The spleen and intranasal tissues of mice were excised and fixed in 4% paraformaldehyde for 24 h. Then, the tissues were embedded in paraffin, sliced into 5-μm thin sections and subsequently deparaffinized in xylene. This was followed with dehydration in serial dilutions of ethyl alcohol, washing with distilled water, and treatment with H_2O_2 (3 %) for 20 min. The levels of various inflammatory cytokines in mice spleen and intranasal tissues were determined using ELISA kits (Beyotime, Institute of Biotechnology) according to manufacturer's instructions.

Western blot analysis

The intranasal tissue samples were put ice-cold saline and homogenized for 40 min in radioimmunoprecipitation assay (RIPA) buffer. The lysates were centrifuged at 13,000 g for 15 min at 4˚C to obtain the supernatant fractions. The protein concentration of each supernatant was measured using bicinchoninic acid assay. Equal amounts of protein (30 μg/lane) were subjected to electrophoresis on 10 % SDS-polyacrylamide gel, followed by transfer to PVDF membrane. Non-specific binding of the blot was blocked by incubating the membranes with 5%

non-fat milk and Tween-20 (TBST) for 2 h at 37 ˚C. Thereafter, the membranes were incubated overnight at 4 ˚C with primary antibodies against caspase-1, after which the membrane was washed twice with TBST before incubation at room temperature with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (secondary antibody) for 2 h. Visualization and analysis of the protein blots were carried out with BeyoECL Moon (Beyotime Institute of Biotechnology) and Image Lab™ software, respectively.

Assay of caspase-1 activity

The intranasal tissues of mice kept in ice-cold saline were lysed by homogenization with RIPA assay buffer for 40 min. The supernatant obtained after centrifugation of the lysate at 15, 000 g for 20 minutes at 4˚C was used for determination of caspase-1 activity using Bio-Rad kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Absorbance (index of caspase-1 activity) was measured at 407 nm in a spectrophotometer.

Determination of infiltration of mast cells and eosinophils

The intranasal tissues excised from mice were fixed in 10 % formaldehyde and subsequently embedded in paraffin. The tissues were sliced into 2-μm thin sections, deparaffined in xylene and dehydrated in serial dilutions of ethyl alcohol. The infiltrations of mast cells and eosinophils were determined using alcian blue and safranin O staining (for mast cells), while hematoxylin and eosin (H&E) staining was used for eosinophils).

Statistical analysis

Data are presented as mean ± standard deviation. Statistically significant differences were determined using one-way analysis of variance (ANOVA), followed by Dunnett's post-hoc test. All statistical analyses were done using SPSS software (version 17.0; SPSS, Inc., Chicago, IL, USA). Differences were taken as statistically significant at $p < 0.05$.

RESULTS

TBI reversed OVA-mediated increases in rhinitis allergy biomarkers in serum

The count of nasal rubs formed in mice due to OVA-administration was reduced significantly ($p < 0.05$) on treatment with TBI (Figure 1 A). The reduction in nasal rub count by TBI was dose-dependent. The OVA-mediated increase in nasal

rub count was reduced completely in mice treated with TBI at a dose of 20 μg/kg. Moreover, TBI significantly suppressed OVA-mediated increases in IgE, histamine, TNF-α, TSLP and IL-1β in the serum of rats ($p < 0.05$; Figure 1 B - F).Treatment of mice with DEX (positive control) also reduced the OVA-mediated secretion of rhinitis allergy biomarkers in serum (Figure 1). However, TBI had no effect on the levels of rhinitis allergy biomarkers in normal control mice.
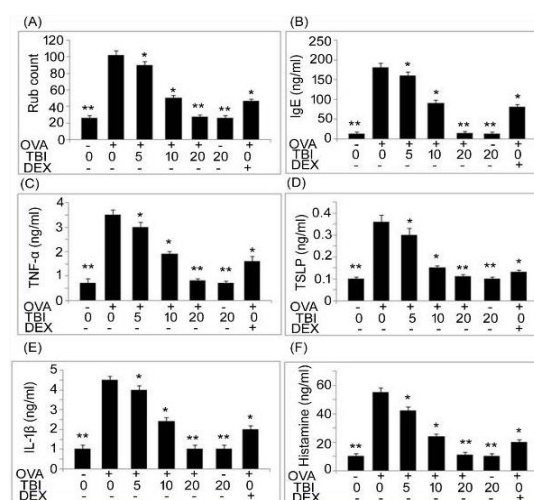
Figure 1: Effect of TBI on OVA-mediated rhinitis allergy biomarkers in mice. Rhinitis allergic mice were treated with TBI (5, 10 and 20 μg/kg) or DEX (5 mg/kg) on day 10 prior to OVA administration. (A) Nasal rubs formed after 10 min of OVA administration. Serum levels of IgE (B), TNF-α (C), TSLP (D), IL-1β (E)and histamine(F), as assayed using ELISA kits; *$p < 0.05$, **$p < 0.05$ vs. OVA-group
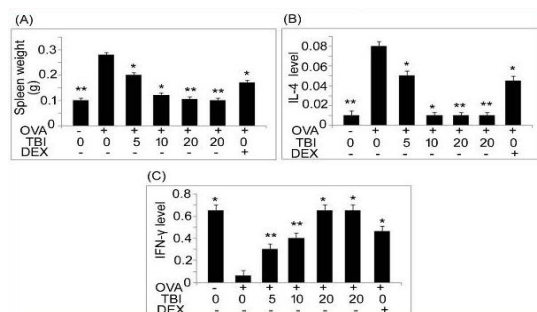
TBI alleviated OVA-mediated imbalance in Th1:Th2 ratio in mice spleen

Spleen weight and IL-4 level in OVA-administered mice were significantly increased ($p < 0.05$), relative to the control group (Figure 2 A and B). However, TBI treatment reversed the OVA-induced increases in spleen weight and IL-4 secretion in a dose-based manner. The OVA-mediated increases in IL-4 level and increased spleen weight were lowered to normal levels in mice group treated with TBI at a dose of 20 μg/kg. Moreover, TBI significantly increased the levels of IFN-γ which were suppressed by OVA treatment ($p < 0.05$; Figure 2 C).

## TBI reduced OVA-mediated increase in rhinitis allergy biomarkers in intranasal tissues

The OVA-administration led to significant ($p < 0.05$) up-regulations of IL-5 and IL-13 in mice intranasal tissues, relative to normal control

(Figure 3 A and B). However, treatment of OVA-administered mice with TBI significantly reduced IL-5 and IL-13 levels in a dose-dependent manner ($p < 0.05$). Although down-regulations ofIL-5 and IL-13 by TBI in OVA-administered mice were significant at all the three tested doses, the effect was maximum at the dose of 20 µg/kg. In OVA-administered mice, TBI treatment significantly reduced the expressions of TNFα, TSLP, IL-1β and IL-6 ($p < 0.05$; Figure 3 C - F). Dexamethasone (DEX) treatment also significantly ($p < 0.05$) downregulated the expression of the proinflammatory cytokines in OVA-administered mice (Figure 3).
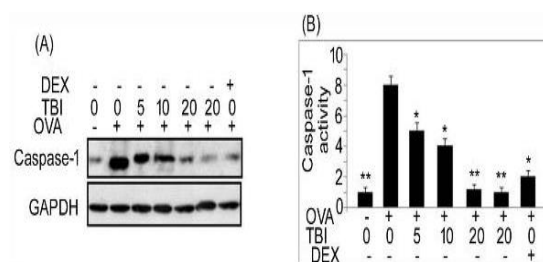

Figure 2: Effect of TBI on OVA-mediated imbalance in Th1:Th2 ratio in mice spleen. Rhinitis allergic mice were treated with TBI (5, 10 and 20 µg/kg) or DEX (5 mg/kg) on day 10 prior to OVA administration. (A) Spleen weight of after 10 days of OVA administration. Levels of IL-4 (B) and IFN-γ (C) in spleen, as assayed using ELISA; *$p < 0.05$, **$p < 0.05$ vs. OVA-group
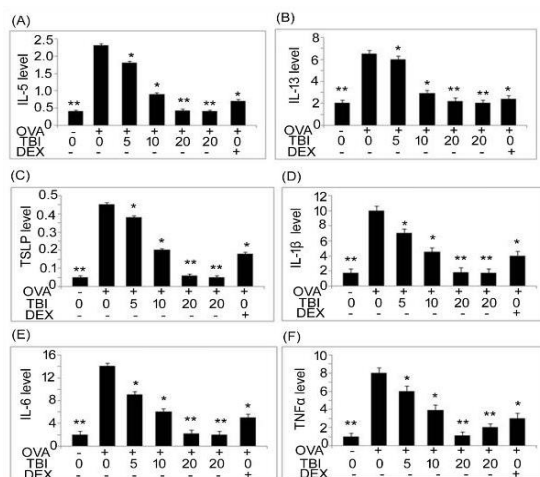
Figure 3: Effect of TBI on OVA-mediated upregulations of rhinitis allergy biomarkers. Rhinitis allergic mice were treated with TBI (5, 10 and 20 µg/kg) or DEX (5 mg/kg) on day 10 prior to OVA administration. Levels of IL-5 (A), IL-13 (B), TSLP (C), IL-1β (D),IL-6(E)and TNF-α (F) in intranasal tissues of mice, as determined using ELISA kits; *$p < 0.05$, **$p < 0.05$ vs. OVA-group

TBI reversed OVA-mediated caspase-1 activation in intranasal tissues

Western blotting showed that OVA-administration led to a marked enhancement of caspase-1 activity in mice intranasal tissues, relative to normal control (Figure 4 A). However, TBI treatment caused a marked reduction in OVA-mediated enhancement of caspase-1 activity. The TBI-induced reduction in caspase-1 activity in OVA-administered mice was evident from a TBI dose of 5 µg/kg, and was maximum at a TBI dose of 20 µg/kg. The reduction in caspase-1 activity in OVA-administered mice by TBI was also confirmed from ELISA assay (Figure 4 B).
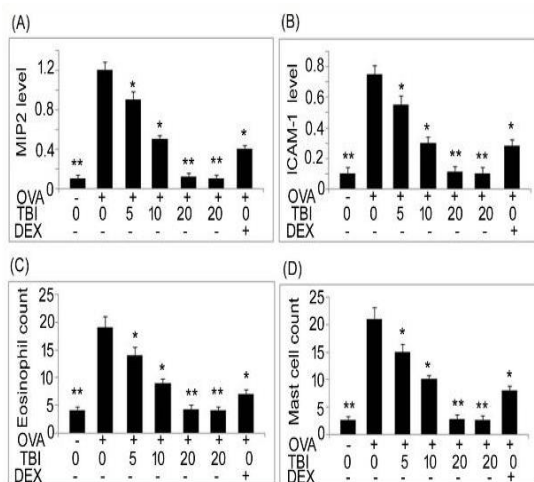

Figure 4: Effect of TBI on caspase-1 activity in intranasal tissues of mice. Rhinitis allergic mice were treated with TBI (5, 10 and 20 µg/kg) or DEX (5 mg/kg) on day 10 prior to OVA administration. Changes in caspase-1 activity caused by TBI in OVA-administered mice, as determined using Western blotting (A), and changes in caspase-1 activity caused by TBI in OVA-administered mice, as determined using ELISA (B). *$P < 0.05$, **$p < 0.05$ vs. OVA-group

TBI alleviated OVA-mediated infiltrations of eosinophils and mast cells into mice intranasal tissues

In mice, OVA-administration caused significant ($p < 0.05$) increases in ICAM-1 and MIP-2 levels in intranasal tissues (Figure 5 A and B). However, treatment with TBI significantly reduced the OVA-induced excessive productions of MIP-2 and ICAM-1 ($p < 0.05$). The suppression of OVA-mediated increases in ICAM-1 and MIP-2was significant at a TBI dose of 5 µg/kg, and became maximum at a TBI dose of 20 µg/kg. Moreover, TBI significantly prevented OVA-induced infiltration of eosinophils and mast cells ($p < 0.05$) into the intranasal tissues (Figure 5 C and D).

DISCUSSION

Rhinitis allergy is characterized by nasal mucosa inflammation caused by the interaction of allergens with IgE [14].

Figure 5: Effect of TBI on OVA-mediated inflammatory cell infiltration into intranasal tissues. Rhinitis allergy mice were treated with TBI (5, 10 and 20 µg/kg) or DEX (5 mg/kg) on day 10 prior to OVA administration. (A) MIP-2 and (B) ICAM-1 levels, as assayed in intranasal tissues using ELISA. (C) Eosinophil infiltration, and (D) mast cell infiltration, as examined using H&E and A&S stains, respectively. *$P < 0.05$, **$p < 0.05$ vs. OVA-group

During early stage of rhinitis allergy, mast cells stimulate inflammatory cytokine secretion which is associated with the secretion of inflammation mediators, including histamine [14]. The main mediator of rhinitis allergy which promotes release of mucous is histamine [14]. Moreover, there is imbalance between Th cells and inflammation-related cytokines such as IL-4, IL-5 and IL-13secreted by the Th2 cells [15]. Interferon gamma (IFNγ) exhibits anti-inflammatory role, thereby regulating inflammation during rhinitis allergy [16]. Allergic conditions lead to excessive secretion of IL-4, IL-5 and IL-13 by mast cells, Th2 cells and eosinophils [17].

The present study found marked elevations in serum and intranasal levels of rhinitis allergy biomarkers in mice after OVA-administration. The level of IFNγ, an anti-inflammatory factor was suppressed in mice administered OVA. These findings are consistent with earlier studies on allergy [17]. However, it was found that TBI reduced OVA-mediated increases in levels of IL-4, IL-5 and IL-13, and increased IFNγ levels. Thus, TBI acted as an anti-inflammatory molecule by regulating the levels of these cytokines in rhinitis allergy mice model. The interaction of pro-inflammatory factors (IL-1β and TNF-α) with other downstream cytokines plays major role in inducing allergic inflammation [17].

It has been reported that IL-1β and TNF-α promote secretion of TSLP by different immune cells and tissues during allergy [18]. Thymic stromal lymphopoietin (TSLP), another major factor involved in allergic inflammation, is produced at high levels during allergies, and its secretion correlates with infiltration of inflammatory cells [18]. Interleukin-6 is involved in the regulation of lung inflammation and maintenance of biological homeostasis [17].

Allergic inflammation leads to activation of caspase-1 which subsequently enhances the expressions of IL-1β and TSLP [7]. It has been reported that caspase-1 inactivation inhibited inflammatory processes in rhinitis allergy mice [19]. Caspase-1 activation and enhanced IL-1β level promote eosinophil infiltration into intranasal tissues [20]. In the present study, TBI treatment caused pronounced reductions in levels of IL-1β, TSLP and TNF-α in OVA-administered mice. The activation of caspase-1 in intranasal tissues of mice by OVA-administration was suppressed by treatment with TBI. The infiltration of mast cells and eosinophils into the intranasal tissues is involved in the pathogenesis of rhinitis allergy [14]. The presence of eosinophils in intranasal tissues is considered as evidence of inflammation in rhinitis allergy [21].

During allergy, MIP-2 and ICAM-1 are responsible for induction of infiltration by inflammatory cells [22]. Moreover, these two molecules cause damage to inflammatory tissues by activating various processes [23]. The MIP-2 level is up-regulated in tissues during inflammation, a process which enhances eosinophil infiltration [23]. In the present study, TBI treatment significantly suppressed the count of mast cells and eosinophils in intranasal tissues of OVA-administered mice. However, treatment of OVA-administered mice with TBI markedly suppressed the levels of MIP-2 and ICAM-1 in intranasal tissues.

CONCLUSION

The findings of this study reveal that TBI suppresses the serum levels of allergic indicators, IgE and various inflammatory cytokines, in OVA-administered mice. It also regulates Th1/Th2 balance, inhibits caspase-1 activity, suppresses mast cell/eosinophil infiltration, and reduced ICAM-1 andMIP-2 levels in OVA-administered mice. Therefore, it may be reasonably concluded that TBI possesses inhibitory potential against rhinitis allergy, and should be developed as a new drug for treatment of asthma.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Yongbo Zhang- conceived and designed the study; Zhuo Wu, Yihui Yang, Lu Ding- collected and analyzed the data; Yongbo Zhang, Zhuo Wu, Yihui Yang, Lu Ding -wrote the manuscript. Yongbo Zhang- Approved final version of the manuscript. All authors read and approved the manuscript for publication.

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