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Effect of verapamil on bronchial goblet cells of asthma: An experimental study on sensitized animals☆

Mohammad Reza Khakzad [a,1], Majid Mirsadraee [b,*], Amirhooshang Mohammadpour [c], Kamram Ghafarzadegan [d], Raziye Hadi [a], Mahdiye Saghari [a,1], Mojtaba Meshkat [a]

[a] Zakaria Research Center, Medical School of Islamic Azad University – Mashhad Branch, Mashhad, Iran
[b] Department of Internal Medicine, Aria Hospital, Islamic Azad University – Mashhad Branch, No. 80, 15 Kosar St., Kosar Ave., Vakilabad Blvd., Mashhad 91786, Iran
[c] Pharmacodynamics and Toxicology Department, Pharmaceutical Research Center, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran
[d] Research Center of Moayed Pathobiology Laboratory and Department of Razavi Hospital, Mashhad, Iran

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ABSTRACT

Introduction: Goblet cell hyperplasia (GCH) and mucus hypersecretion in the airway is recognized as an important contributor to morbidity and mortality in asthma and COPD. Verapamil is a calcium channel blocker that binds to the alpha-subunit of L-type calcium channels and inhibits the mucin gene via the calmodulin and CaM kinase pathway. The objective of this study was to determine the in vivo effect of verapamil on GCH and eosinophilic inflammation in sensitized mice.

Methods: Male BALB/c mice were sensitized to ovalbumin using the standard method. Two groups of animals were received verapamil via an intramuscular injection: 1-low dose (0.5 mg/kg/day for two weeks), 2-high dose (1.5 mg/kg/day for two weeks). Serum and bronchoalveolar lavage fluid (BALF) was collected and analyzed for inflammatory cells, interferon-γ and IL-4. The left lung was sent for histopathological evaluation, especially for periodic acid-Schiff (PAS), to identify goblet cells in the epithelium. The degree of inflammatory cell infiltration, including eosinophils, mucus plugging, and smooth muscle thickness of the airways were classified on a semi quantitative scale.

Results: Inflammatory cell infiltration in peribronchial and perivascular areas was observed in all sensitized groups. Eosinophils percentage in the BALF significantly decreased in verapamil-treated mice compared with sensitized mice (from 19.8% in asthmatic to 5.4% for low dose and 4.4% for high dose). The ratio of airway goblet cells per epithelial cells were significantly lower in verapamil-treated mice versus sensitized mice ($1.57 \pm 1.30\%$ for low dose; $1.50 \pm 0.93\%$ for high dose versus $12.93 \pm 7.55\%$, $P < 0.05$, respectively). Mucus production of goblet cells decreased significantly in verapamil-treated mice versus sensitized mice (mean score was 1.45 ± 0.30 for low dose; 0.81 ± 1.00 for high dose versus 2.85 ± 0.86 in the sensitized control group, $P < 0.05$, respectively). The concentration of serum and BALF-IFN-γ in verapamil-treated mice markedly increased by the verapamil treatment when compared to sensitized mice (15.1 ± 0.43 versus 4.7 ± 0.96, $P < 0.05$ and 91.8 ± 47.7 versus 14.8 ± 4.6, $P < 0.01$, respectively).

Conclusion: Verapamil is a useful drug with therapeutic targeting on GCH and a potential way to limit mucous production and improve bronchial inflammation.

1. Introduction

Goblet cells are found throughout the conducting airway [1] and their primary function is secreting mucins onto the internal surface of the respiratory tract [2]. Mucins are high molecular weight glycoproteins that consist of a peptide backbone and numerous oligosaccharide side chains that rapidly secrete to protect the epithelial lining from the effects of dust, fumes, microorganisms and other debris delivered by inhalation of approximately 12,000 breaths per day [3]. Mucus hypersecretion has been shown as a clinical feature in respiratory disease, including fatal asthma [4] and COPD

Abbreviations: ELISA, enzyme linked immunosorbent assay; LT, leukotriene; PBS, phosphate-buffered saline; OVA, ovalbumin; Alum, aluminum hydroxide; BALF, bronchoalveolar lavage fluid; COPD, chronic obstructive pulmonary disease; GCH, goblet cell hyperplasia; I.P., intraperitoneal; I.M., intramuscular; INF-γ, interferon-γ; IL-4, interleukin-4.

☆ Place of research: Zakaria Research Center, the Medical School of Islamic Azad University – Mashhad Branch, Mashhad, Iran.

* Corresponding author. Tel.: +98 915 111 5860; fax: +98 511 881 6965.
E-mail addresses: majidmirsadraee@mshdiau.ac.ir, majidmirsadraee@yahoo.com (M. Mirsadraee).

[1] The first and last authors contributed equally to this work.

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[5]. In patients with these types of lung diseases, the goblet cells were 3 times higher [6] especially in peripheral airways [7].

Calcium-activated chloride channels (mCLCA3 in mice and hCLCA1 in humans) is a key signaling molecule involved in the development of goblet cell hyperplasia (GCH) [8]; however, the mechanism by which the cytokines induce GCH in the airway is not well understood. Some investigations have showed that the expression of CLCA is likely to be regulated by Th2 cell-derived cytokines such as IL-13, leading to airway epithelial goblet cell metaplasia, subepithelial fibrosis or hyperplasia of smooth muscle and this effect could be prevented by corticosteroids [9]. The nuclear factor of activated T-cells also can bind to the interleukin (IL)-4 and IL-13 promoters via calcium-activated transcription factors [10].

Airway smooth muscle hyperplasia is also another important feature of asthma remodeling, which at times cannot be reversed by the current treatment of asthma [11]. Changing the concentration of free Ca2+ in the cytosol ([Ca2+]i) of the airway smooth muscle by altered calcium homeostasis [12] or sarco/endoplasmic reticulum calcium-adenosine triphosphatase [13] are two reported mechanisms that may induce airway smooth muscle hyperplasia in asthmatic subjects. Although the calcium channel blockers were not placed in the list of potent therapy for asthma [14], hypothetically we believe that these drugs may decrease GCH and airway smooth muscle hyperplasia and could show beneficial effects in the treatment of chronic asthma and remodeling. Verapamil is a calcium channel blocker that binds to calcium channels and blocks the inward movement of calcium into cells through "slow channels" [15]. The objective of this study was to determine the effect of verapamil (as a member of calcium channel blockers) on GCH, mucus secretion and the T helper 2 system mediator on sensitized mice as a model for asthma. Furthermore, we tried to compare the effect of verapamil with beclomethasone in GCH.

2. Materials and methods

2.1. Animals

Thirty male BALB/c mice aged 6–8 weeks were used in this experimental study. The mice were divided into five groups (6 mice per group). One group was reserved as the non-sensitized non-treated control group (negative control). This group passed all steps, but was nebulized with phosphate-buffered saline (PBS) as the placebo. The other groups were sensitized with ovalbumin (OVA) and divided into four groups: non-treated sensitized mice (positive control) and three treatment groups with low and high doses of verapamil and beclomethasone.

2.2. Sensitization protocol

The animals were sensitized with an intraperitoneal injection of aluminum hydroxide and ovalbumin, and then given an inhalation of ovalbumin for 15 courses over 75 days as like as our previous study [16] and guidance of European Respiratory Society task force [17]. Control mice were simultaneously nebulized by PBS without preliminary aluminum hydroxide intraperitoneal injection. Samples included bronchoalveolar lavage fluid (BALF) and lung tissues were collected 48 h after the final intranasal OVA/PBS challenge or drug administration.

2.3. Treatment with verapamil and beclomethasone

Verapamil (Lekoptin 5 mg/ml, Novartis, UK) was diluted in sterile normal saline in two final concentrations, 0.5 mg/kg/day (as the low dose) and 1.5 mg/kg/day (as the high dose). These two concentrations of verapamil were used in two groups of animals (groups 3 and 4). The drug was injected intramuscularly once a day for 14 days after completing the sensitizing process. Group 5 received 150 μg/kg of beclomethasone (APO-Beclomethasone Nasal Spray Toronto, Canada) which was prepared by being dissolved in sterile PBS and administered every day for 14 days by nebulizing into the animal box.

2.4. Obtaining samples from serum and bronchoalveolar lavage fluid

After 48 h of sensitization and treatment in the respective groups, anesthesia with intraperitoneal Ketamine (Xylazine 44 mg/kg) was administered and samples from blood and BALF were collected.

Blood samples, obtained by cardiac puncture, were allowed to clot at room temperature, and then were centrifuged at 550 × g for 30 min. Aliquots of serum were stored at −70 °C until analyzed for cytokine assay.

Immediately after blood collection, thoracic cavities were carefully opened. The trachea was exposed, and a catheter was inserted into the upper part of the trachea for bronchoalveolar lavage. Bronchoalveolar lavage was performed by three injections and aspiration (total 1.5–0.5 ml in each lavage) of ice-cold PBS containing 0.1% bovine serum albumin and 0.05 mM EDTA-2Na (85–90% of the volume injected was recovered). The BALF from each animal was pooled in a plastic tube, cooled on ice and centrifuged (150 × g) at 4 °C for 10 min. Cell pellets were resuspended in the same buffer (0.5 ml). Then the total number of nucleated cells was counted by a hemocytometer chamber. For differential cell count a smear was prepared and stained with May Grunwald–Giemsa. Based on standard morphological criteria at least 300 cells were studied. The supernatant of the BALF was stored at −70 °C to determine the cytokine level.

2.5. Cytokine levels

The amounts of cytokines in the supernatant of the BALF and serum were measured using the enzyme linked immunosorbent assay (ELISA) (U-Cy Tech biosciences, 5 pg/ml sensitivity for IL-4, and 2 pg/ml sensitivity for interferon-γ (IFN-γ)). According to the manufacturer's recommendations, each sample was activated before it was measured.

2.6. Histopathological study

After bronchoalveolar lavage was performed, both lungs were perfused with 5 ml of 10% buffered formalin (pH 7.4) via the trachea. Lung tissues were excised, embedded in paraffin, and then cut at 3 μm thickness. Then the tissue sections were stained with hematoxylin and eosin (H&E) for general histopathology, Masson's trichrome stain for the evaluation of muscular hypertrophy and collagen deposition and periodic acid-Schiff (PAS) solution, which enabled us to evaluate the hyperplasia goblet cells, the mucus-producing cells and mucus within the airway epithelium. The ratio of PAS-positive cells/total cells in a 50 × 50 μm area of microscopy was also used to determine the goblet cell hyperplasia quantitatively.

For each mouse, five airway sections that were randomly distributed throughout the left lung were analyzed, and their average scores were calculated.

The degree of peribronchiolar and perivascular inflammation was evaluated by a subjective scale. Briefly, the scoring system for cell infiltration was as follows: 0 – no cells; 1 – a few cells; 2 – a