

EFFECTS OF ROFLUMILAST, SELECTIVE PDE4 INHIBITOR, ON AIRWAY REACTIVITY IN OVALBUMIN-SENSITIZED GUINEA PIGS

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Abstract

Background: Roflumilast as a phosphodiesterase 4 inhibitor has shown to increase lung functions and decrease the number of exacerbations in chronic obstructive lung disease. In this study, its ability to decrease the airway hyperresponsiveness in a model of eosinophil inflammation was evaluated. **Methods:** Healthy adult male guinea pigs were divided into groups as follows: the first group was considered as a healthy control group (without sensitization and therapy), animals in the second group were sensitized with ovalbumin, but left without further treatment, and the animals in the third group were sensitized with ovalbumin and treated with roflumilast perorally for 7 consecutive days. *In vivo* airway reactivity was evaluated using double-chamber whole body plethysmograph and measuring the specific airway resistance after nebulization of histamine aerosol. *In vitro* experiments were performed with tissue strips of trachea and lungs in organ bath, where their contractile responses to cumulative doses of acetylcholine and histamine were registered. The numbers of inflammatory cells in blood and bronchoalveolar lavage fluid were measured using standard staining. **Results:** Guinea pigs with roflumilast treatment showed decreased *in vivo* and *in vitro* airway reactivity associated with suppressed recruitment of inflammatory cells (especially eosinophils) in blood and bronchoalveolar lavage fluid. **Conclusion:** Roflumilast has demonstrated the therapeutic potential in the model of ovalbumin induced eosinophil inflammation typically present in patients with bronchial asthma.

Keywords: roflumilast, ovalbumin, phosphodiesterase inhibitors, airway reactivity, eosinophil inflammation, bronchial asthma

INTRODUCTION

Obstructive lung diseases are affecting millions of people, and these numbers are suspected to increase with the coming years. It is estimated that more than 200 million people have chronic obstructive pulmonary disease (COPD) and more than 300 million have bronchial asthma worldwide (1,2). According to World Health Organization authorities, COPD will be the third most common cause of death worldwide in 2030. As we can see from these numbers, the obstructive diseases of respiratory system are becoming a huge problem to human health by restricting quality of human lives.

Patients with obstructive lung diseases have airway inflammation, airflow obstruction and airway hyperresponsiveness, together manifesting with various clinical symptoms. The symptoms can sometimes be so severe that it can impair the quality of daily life in an average human being. In asthmatic patients, the airway hyperresponsiveness together with airway inflammation leads to chest tightness and wheezing due to airways obstruction (3). Pathologies like infiltration of mast cells, lymphocytes and eosinophils in the bronchial epithelium (4) and thickening of the sub-epithelial reticular layers (5) lead to presentation of the above mentioned symptoms even in mild and newly diagnosed asthmatic patients (6).

There are several pharmacological groups of drugs used for the therapy of chronic bronchial asthma, including long-acting β_2 -agonist and inhaled corticosteroids. Other drugs like leukotriene receptor antagonists and theophylline are also recommended (7), but these drugs doesn't improve lung function as much as corticosteroids and β_2 -agonist do. The treatment with β_2 -agonist and corticosteroids depends on patients' clinical symptoms and lung function test (8,9). However, even though many patients are controlled clinically well with these drugs, research shows that airway hyperresponsiveness and airway inflamma-

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tion persist (10,11). Persistence of this pathology may lead to airway remodeling and worsening of long-term outcome (12,13). Therefore, further targets have been studied, including phosphodiesterase (PDE) inhibition as one of the mechanisms of action observed by theophylline. By inhibiting PDE (14, 15), cAMP levels are increasing intracellularly in both airway smooth muscle and inflammatory cells. This leads to reduced airway hyperresponsiveness and airway inflammation and hence lung functions could increase with better long-term outcome. One of the recently introduced groups of drugs – the selective PDE4 inhibitors – has demonstrated some promising effects in patients with COPD (16,17). The only clinically approved selective PDE4 inhibitor is roflumilast. As a 2nd generation representative of PDE4 inhibitors, its adverse effects profile is more convenient compared to rolipram. Even though COPD and asthma are different diseases, they both are obstructive diseases of lungs, thus having a chronic inflammatory course. Therefore, roflumilast could also increase lung functions in asthmatic patients. The aim of this study was to evaluate the efficacy of roflumilast on airway reactivity and cellular involvement in an experimental model of ovalbumin-induced eosinophil inflammation in guinea pigs.

MATERIALS AND METHODS

The study protocol was approved by local Ethics Committee at Jessenius School of Medicine, Comenius University, Martin, Slovakia. 24 healthy adult male guinea pigs (250–350 g) were used for the study. They were kept in an animal house and had food and water *ad libitum*. In two groups of animals (n=8 in each), airway hyperresponsiveness was induced with antigen (ovalbumin, Sigma Aldrich, Germany) and the last group was used as naïve controls without sensitization (n=8). From the two sensitized groups, the first one was left without treatment – only vehicle (1.5 % methylcellulose solution at the dose of 3.0 mL/kg) was used. All animals in the second sensitized group were treated with PDE4 inhibitor roflumilast (DND Pharm-Technologies Co., China) 1.0 mg/kg b.w. perorally dissolved in 1.5 % methylcellulose solution (3.0 mL/kg) for 7 consecutive days.

Antigen-induced airway hyperresponsiveness

Animals in groups 2 and 3 were sensitized with ovalbumin in following schedule:

the 1st day - 0.5 mL of 1.0 % ovalbumin subcutaneously and 0.5 mL of 1.0 % ovalbumin intraperitoneally, the 3rd day - 1.0 mL of 1.0 % ovalbumin intraperitoneally, and the 14th day - 1.0 % ovalbumin inhaled for 35 seconds. Animals in group 3 were treated with roflumilast administered perorally at the single daily dose of 1.0 mg/kg in a vehicle composed of 3.0 mL/kg 1.5 % methylcellulose for seven days (from the 15th to 21st day). The airway reactivity was measured *in vivo* 5 hours after last ovalbumin administration and *in vitro* after sacrificing the animals (18). Only animals with minimum 20% increase in specific airway resistance after ovalbumin challenge on the 14th day compared to measured values before sensitization were included in the further testing.

Evaluation of in vivo airway reactivity

In vivo reactivity of airways was measured in double-chamber whole body plethysmograph (Hugo Sachs, Germany). Specific airway resistance as a marker of *in vivo* airway reactivity was measured after inhalation of histamine aerosol for 2 minutes at the concentration of 10⁻⁶ mol/L. Reactivity after nebulization of saline was performed for comparison. There were breaks for minimum of 5 minutes between these two exposures. Fresh air was administered to the head chamber during the break (18).

Evaluation of in vitro airway reactivity

Trachea and lungs were excised immediately after sacrificing the animals. Trachea strip at the size of 20 mm and strip of left lung tissue at the size of 2 x 2 x 15 cm were obtained. They were then placed separately in organ chambers containing Krebs-Henseleit's buffer (NaCl 110.00 mmol/L, KCl 4.80 mmol/L, CaCl₂ 2.35 mmol/L, MgSO₄ 1.20 mmol/L, KHPO₄ 1.20 mmol/L, NaHCO₃ 25.00 mmol/L, and glucose 10.00 mmol/L in glass-distilled water).

The chambers were maintained at 37.0 ± 0.5 °C, and aerated with 95 % oxygen and 5 % CO₂ to maintain pH 7.5 ± 0.1 . The strips were mounted between two hooks, one above and one below, pulling them on either side. On one of the hooks a force transducer (EXP, Experimetria, Hungary) with amplifier (EXP CLSG-4, Experimetria, Hungary) was attached, so the changes in tension were measured. Then in loading phase, which lasted for 30 min, the tension was set at 4.0 grams. Afterwards for the adaptation phase for 30 min, the tension was set at 2.0 grams. Every 10 min, the Krebs-Henseleit's buffer was changed with fresh one. Cumulative doses of acetylcholine (10^{-8} to 10^{-3} mol/L, Sigma-Aldrich, Germany) and histamine (10^{-8} to 10^{-3} mol/L, Sigma-Aldrich, Germany) were administered and continuous contractions were recorded by the special computer software (SPEL Advanced Iso Sys v3.2, Experimetria, Hungary). Data of the tracheal and lung tissue reactivity are shown in grams (g) of the smooth muscle tension.

Cells in the blood and bronchoalveolar lavage fluid

Samples of blood were taken immediately after sacrificing the animals. Bronchoalveolar lavage (BAL) of right lung was performed twice using administration of pre-heated saline (37 °C) at the dose of 0.01 mL/g b.w. The total white blood cells (WBC) count in blood was determined in Bürker's chamber after staining by Türk by blinded investigator. Differential leukocytes count in blood and BAL fluid was evaluated microscopically after panchromatic staining by May-Grünwald/Giemsa-Romanowski and relative counts of neutrophils, eosinophils, monocytes, and lymphocytes (in blood) and mononuclears, neutrophils and eosinophils (in BAL fluid) were determined (in %) by blinded investigator.

Statistical analysis

Data are shown as means $\pm$ SE. For statistical analysis, one-way ANOVA with post-hoc LSD test was used. A $p < 0.05$ was considered as statistically significant.

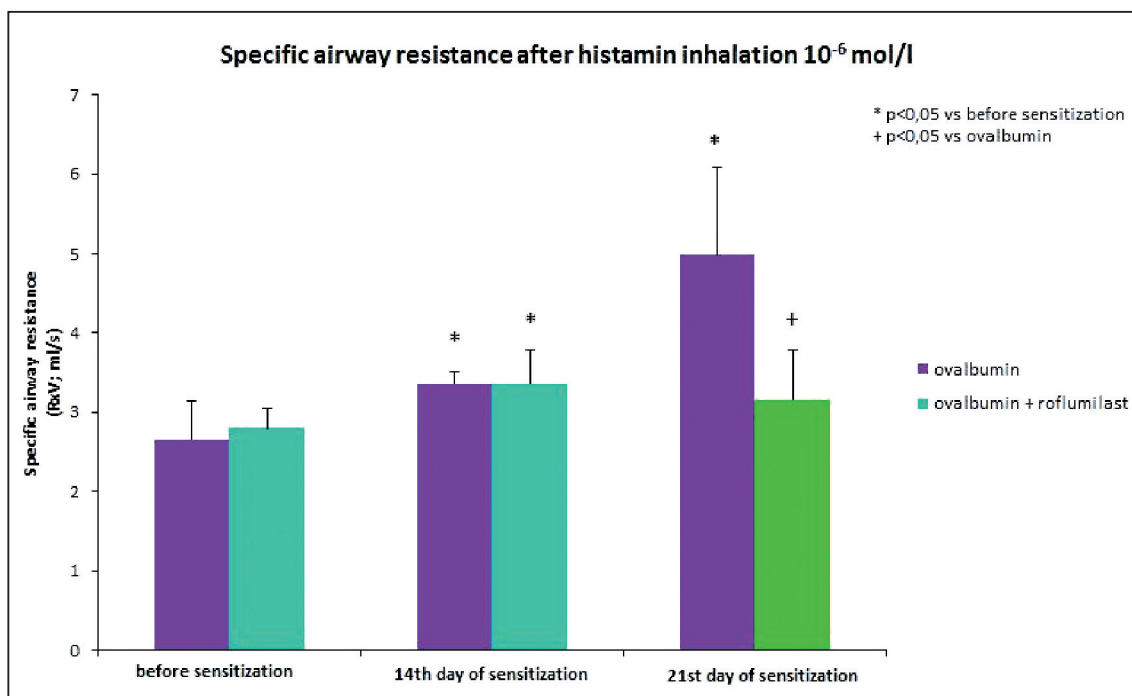


Fig. 1 Specific airway resistance after inhalation of histamine (10^{-6} mol/L) in ovalbumin sensitized guinea pigs. First column represent before sensitization, second column after 14 days and third column after 21 days and after administration of roflumilast in group 3 (green-colored).

RESULTS

The model of airway hyperresponsiveness used in our study showed significant increase in *in vivo* airway reactivity in (specific airway resistance after inhalation of histamine aerosol) already after 14 days of sensitization with ovalbumin. However, the most significant increase in this parameter was observed in sensitized non-treated animals after 21 days (Fig. 1). Peroral administration of roflumilast for seven days decreased the airway reactivity in the ovalbumin-sensitized guinea pigs.

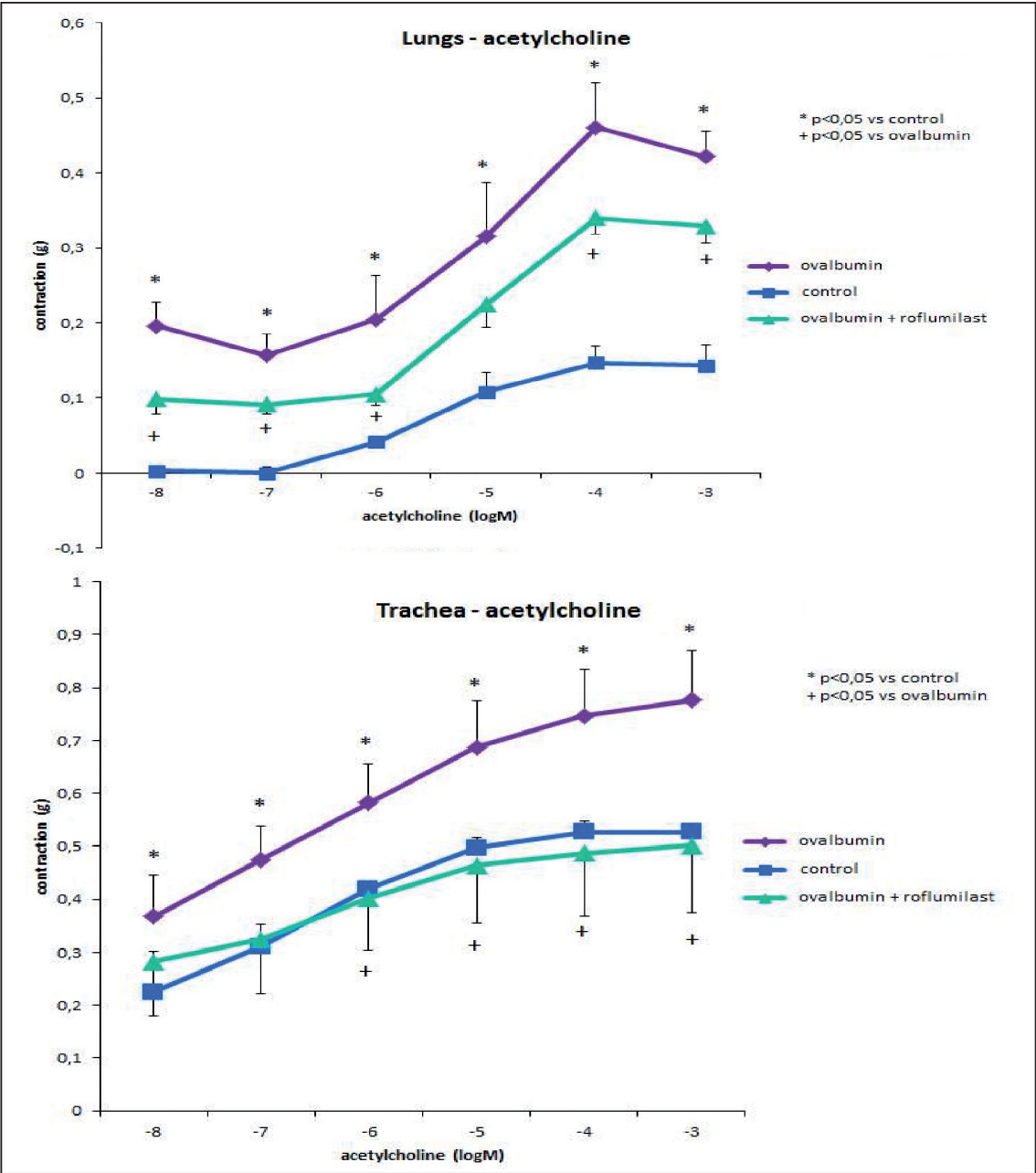


Fig. 2 *In vitro* airway reactivity to cumulative doses of acetylcholine in lungs and trachea.

The administration of cumulative doses of acetylcholine and histamine to organ baths with lung and trachea led to stepwise-increased contraction in both tissues. We demonstrated significant increase of lung tissue and trachea reactivity in sensitized animals compared to healthy control to cumulative doses of acetylcholine. Furthermore, depression of reactivity to cumulative doses of acetylcholine in both trachea and lungs was observed after seven days treatment with roflumilast (Fig. 2).

Similar changes of *in vitro* airway contractility changes were observed when histamine was added to organ baths in cumulative manner (Fig. 3); however, these changes reached the level of statistical significance only in higher concentrations of histamine.

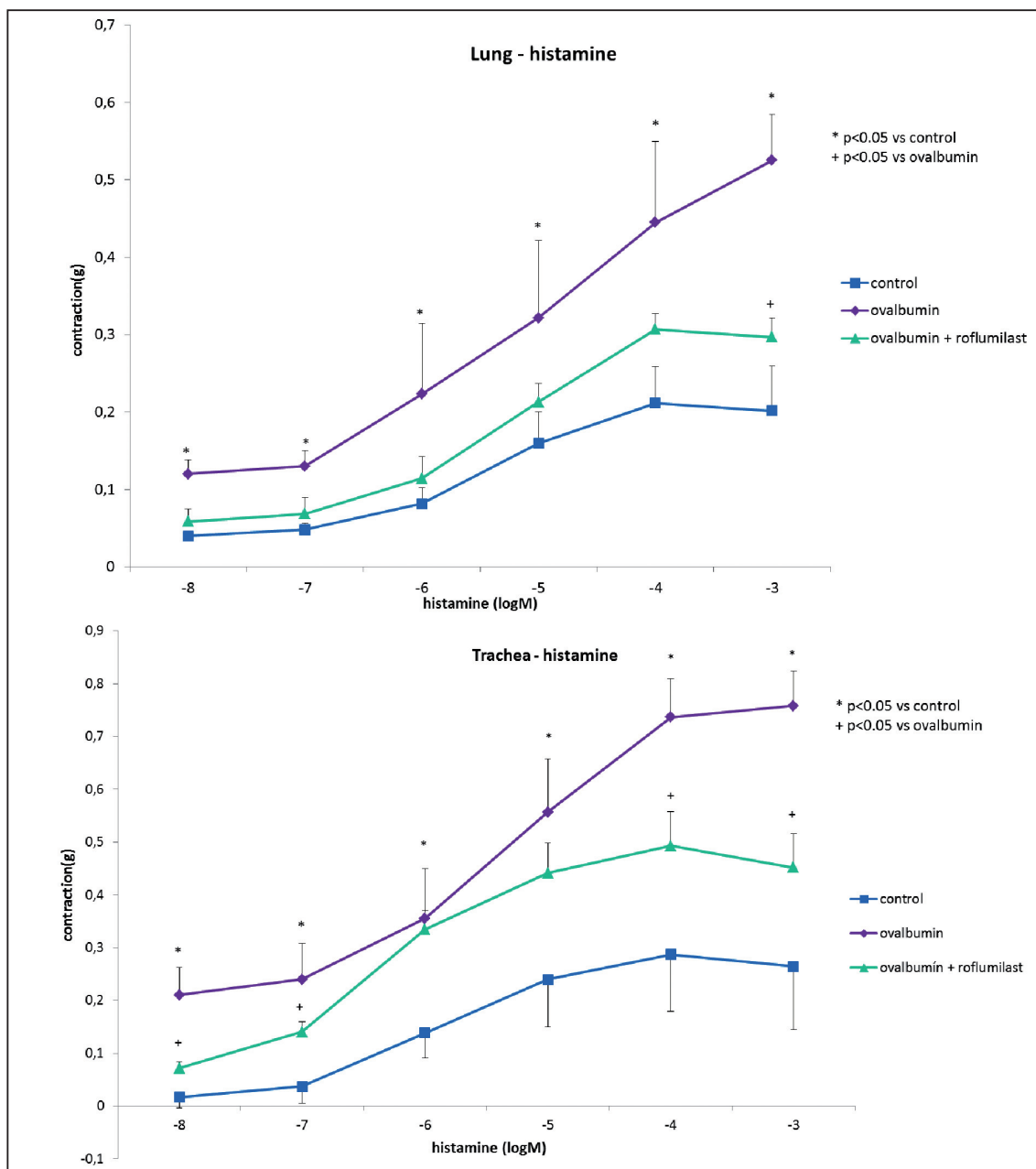


Fig. 3 *In vitro* airway reactivity to cumulative doses of histamine in lungs and trachea.

By evaluating the absolute number and differential counts of white blood cells in blood we found that ovalbumin sensitization in our experimental model led to significant increase of total white blood cells count as well as significant increase of percentual number of eosinophils (Tab. 1). Treatment with roflumilast led to suppression of total white blood cells count as well as differential count of eosinophils.

Table 1 Changes of total and differential white blood cells count in blood (WBC – white blood cells; *p < 0.05 vs. control, +p < 0.05 vs. ovalbumin)

average SE	control	ovalbumin	ovalbumin + roflumilast
WBC (x10 ⁹ .l ⁻¹)	2.1 0.1	3.4* 0.4	2.7+ 0.3
Neutrophils (%)	31.9 1.9	37.6 2.8	52.5 3.7
Lymfocytes (%)	66.1 1.9	58.9 2.8	45.5 3.5
Monocytes (%)	1.2 0.1	1.0 0.1	1.2 0.1
Eosinophils (%)	0.8 0.2	2.5* 0.4	0.7+ 0.1

In BAL fluid, ovalbumin-sensitization led to increase in differential count of eosinophils accompanied by decrease of mononuclears count. Treatment with roflumilast led to restoring these changes, i.e. statistically significant decrease of eosinophils and decrease of mononuclears compared to non-treated sensitized guinea pigs (Tab. 2).

Table 2 Changes of differential WBC count in BAL fluid after sensitization of guinea pigs (*p < 0.05 vs. control, +p < 0.05 vs. ovalbumin)

average SE	control	ovalbumin	ovalbumin + roflumilast
Mononuclears (%)	90.2 1.9	67.2* 4.8	86.8+ 1.5
Neutrophils (%)	6.6 1.3	5.9 1.6	5.7 1.8
Eosinophils (%)	3.2 0.7	27.0* 4.5	7.5+ 1.3

DISCUSSION

PDE hydrolyses the phosphodiester bond between the phosphate group and the pentose sugar and decreases the level of cAMP. By inhibiting this enzyme, cAMP levels increase with subsequent bronchial smooth muscle relaxation and suppression of inflammation by reducing the release of inflammatory mediators (19,20). It means that having higher levels of cAMP in cells leads to decreased airway hyperresponsiveness (19). Our study with the second generation PDE4 inhibitor roflumilast indirectly confirms this observation, as it led

to significant suppression of both *in vivo* and *in vitro* airway reactivity and eosinophils recruitment in blood and BAL fluid.

As we have seen in *in vivo* experiment, the airway resistance markedly decreased in the group of guinea pigs treated with roflumilast compared to the group of sensitized non-treated animals. Both of these groups were sensitized with ovalbumin to mimic allergic (eosinophil) inflammation. As we are aware that specific airway resistance measurement using the double chamber whole body plethysmograph does not exclude involvement of nasal airflow changes possibly evoked by sensitization, we have performed also *in vitro* studies with tracheal and lung tissue strips. *In vitro* experiment showed decreased contractile force both in the lung tissue and tracheal tissue in the roflumilast treated guinea pigs. Previously, the anti-inflammatory and bronchodilating potential of citalopram (18) and rolipram (21) were confirmed. However, both citalopram and rolipram at the doses required for these effects have unacceptable adverse effects limiting their clinical use in bronchial asthma therapy. Similar problems face the non-selective PDE inhibitors (methylxanthine derivatives, e.g. theophylline), whose bronchodilating effect can be reached only in higher plasma concentrations, which are very close to the level of serious adverse effects (22,23). Nevertheless, they have similar potency to inhibit eosinophil inflammation and thus to attract the attention in studying the second generation of selective PDE4 inhibitors also in allergic inflammation. This was confirmed also by other authors, who described immunomodulation effect of selective PDE3, PDE4 and PDE7 inhibitors (20,24).

Our results suggest that roflumilast has a positive effect on decreasing the airway hyperresponsiveness and suppressing the accumulation of eosinophils in our model of allergen-induced inflammation. Therefore, besides COPD it could be considered as a suitable therapeutic tool also in treatment of bronchial asthma (25,26). However, to confirm its clinical effectiveness in bronchial asthma, further studies are necessary. Furthermore, studies with combining different PDE-inhibitors may decrease the airway hyperresponsiveness even more (21).

It is known that methylxanthine derivatives like theophylline are able to inhibit PDEs non-selectively. Their bronchodilating effects in chronic airway diseases associated with inflammation are clinically used, despite the risk of adverse effects. However, the anti-inflammatory, immunomodulation and recently demonstrated antitussive effects are also of importance, where PDE inhibition participates (22,23). Thus, the combination of PDE4 inhibitor with one of the others (e.g. PDE3, PDE5, or PDE7 inhibitor) may show some good results. There are several studies testing dual PDE inhibitors, e.g. PDE3/PDE4 (27) and PDE4/PDE7 (21). These combinations have shown another perspective to decrease the airway hyperresponsiveness. This finding was confirmed also in previous experiments, where PDE4 inhibitor rolipram and PDE7 inhibitor BRL50492 were more effective in bronchodilation and anti-inflammatory action when administered both simultaneously in half doses compared to single administration of each of them (21,28,29).

Based on our results we can conclude, that the ovalbumin sensitization used in our model led to a significant increase of airway reactivity (both *in vivo* and *in vitro*), with concomitant increase of eosinophils in blood and BAL fluid. Therefore, this model could be used as an experimental model of eosinophil inflammation. Treatment with roflumilast at the single daily dose of 1.0 mg/kg perorally for seven days led to significant decrease of *in vivo* and *in vitro* airway reactivity, and to decreased absolute and percentual counts of eosinophils in blood and in BAL fluid compared to non-treated sensitized animals, suggesting anti-inflammatory properties of this drug potentially useful in allergic inflammation. Therefore, roflumilast seems to be effective not only in COPD, but after careful experimental and clinical testing it could be introduced also in the therapeutic arsenal of bronchial asthma.

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