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Dose dependent effects of tadalafil and roflumilast on ovalbumin-induced airway hyperresponsiveness in guinea pigs

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ABSTRACT

Introduction: Chronic obstructive diseases of airways associated with cough and/or airway smooth muscle hyperresponsiveness are usually treated with bronchodilating and anti-inflammatory drugs. Recently, selective phosphodiesterase (PDE) 4 inhibitors have been introduced into the therapy of chronic obstructive pulmonary disease. Several studies have demonstrated their ability to influence the airway reactivity and eosinophilic inflammation by increasing the intracellular cAMP concentrations also in bronchial asthma. Furthermore, the expression of PDE5 in several immune cells suggests perspectives of PDE5 inhibitors in the therapy of inflammation, as well. **Purpose:** The aim of this study was to assess the dose-dependent effects of PDE4 and PDE5 inhibitors in allergic inflammation. Therefore, the effects of 7-days administration of PDE4 inhibitor roflumilast and PDE5 inhibitor tadalafil at two different doses in experimentally-induced allergic inflammation were evaluated. **Materials and Methods:** In the study, male adult guinea pigs were used. Control group was non-sensitized. Other animals were sensitized with ovalbumin over two weeks and thereafter treated intraperitoneally for 7 days with roflumilast or tadalafil (daily dose 0.5 mg/kg or 1.0 mg/kg b.w.), or with vehicle. **Results:** Both roflumilast and tadalafil reduced specific airway resistance after nebulization of histamine (marker of *in vivo* airway reactivity) at both doses used. The *in vitro* airway reactivity to cumulative doses of acetylcholine was significantly reduced for roflumilast at higher dose, predominantly in the lung tissue strips. Histamine-induced contractile responses were significantly influenced in both lung and tracheal tissue strips, predominantly at the higher doses. Tadalafil led to a decrease in contractile responses induced by both acetylcholine and histamine, with more significant effects in the lung tissue strips. These changes were associated with decreased numbers of circulating leukocytes and eosinophils and concentrations of interleukin (IL)-4, IL-5 and TNF- α in the lung homogenate. **Conclusions:** The selective PDE4 and PDE5 inhibitors alleviated allergic airway inflammation, with more significant effects at the higher doses.

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Phosphodiesterase; PDE inhibition; airways; bronchial asthma; hyperresponsiveness; smooth muscle; inflammation; roflumilast; tadalafil

Introduction

Bronchial asthma and chronic obstructive pulmonary disease (COPD) are both known as inflammatory airway diseases associated with cough and bronchoconstriction. In the bronchial asthma, an eosinophilic inflammation is usually accompanied by a bronchial hyperresponsiveness (BHR). Most of inflammatory cells participating in the inflammation express special enzymes – phosphodiesterases (PDEs). PDEs represent a superfamily of phosphohydrolases, which are able to catalyze a degradation of cyclic 3',5' adenosine and/or guanosine monophosphate (cAMP and cGMP). As there are several different PDE subtypes, their origin and localization is considered to be a major regulatory

mechanism in controlling the concentration of cyclic nucleotides in the respective cells and tissues.

Theophylline, a commonly used drug for the therapy of COPD and bronchial asthma, inhibits most of the PDE subtypes in different tissues and organs, i.e. its effects are non-selective regarding the PDE inhibition.^[1] Furthermore, it possesses other mechanisms of action, including an activation of histone-deacetylase and antagonism of adenosine receptors.^[2,3] Simultaneous influencing of several PDEs, a broad spectrum of mechanisms of action, and a relatively narrow therapeutic index based on its pharmacokinetic properties result in serious adverse effects of theophylline and other methylxanthines.

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Therefore, keeping favorable effects of PDE inhibitors and minimizing their adverse effects leads researchers to focus on agents with more selective action (e.g. selective PDE inhibitors).

PDEs are currently classified into 11 super-families, with an estimation of more than 100 of different mRNA products.^[1,4] As the PDE isoforms are associated with specific body functions and pathological condition, the selective PDE inhibitors could influence them without (or with only limited) effects on other organs or systems, and without an occurrence of considerable adverse effects.^[5]

There are several selective PDE inhibitors which have been successfully used in experimental and clinical studies. Due to their involvement in the respiratory diseases, the inhibitors of PDE3, PDE4, PDE5, and PDE7 are of particular attention of researchers.^[6] A broad experimental and clinical evidence has confirmed that the inhibition of PDE4 (e.g. by roflumilast) could effectively suppress the neutrophil-mediated inflammation in COPD.^[7,8] More recently, the efficacy of roflumilast has been proven also in allergic asthma,^[4,9–11] and therefore the use of roflumilast in asthma and asthma-COPD overlap has been considered.^[12–14]

PDE5 inhibitors are often used in the therapy of erectile dysfunction, pulmonary hypertension, and other diseases, where relaxation of smooth muscle is of benefit.^[2] However, their potential role in the allergic inflammation has been suggested, as an expression of PDE5 has been confirmed in several immune cells.^[1,6]

Boswell-Smith et al.^[15] have demonstrated that increased intracellular levels of cAMP and cGMP caused by the inhibition of PDE4 and PDE5, respectively, led to suppressed immunological responses and relaxation of smooth muscle. Since bronchial asthma and COPD are associated with increased levels of exhaled nitric oxide (NO), chronic inflammation and increased airway smooth muscle tone, the NO/soluble guanylyl cyclase/cGMP pathway could be involved in these obstructive diseases.^[16] We have hypothesized that the administration of selective PDE4, or PDE5 inhibitors could improve the symptomatology of allergic asthma associated with eosinophilic inflammation and airway hyperresponsiveness.

There are several studies demonstrating a dose dependent action of roflumilast on allergic inflammation in experimental conditions.^[17–19] As the effects of roflumilast and tadalafil have not yet been compared

in a dose-relationship, in this study the effects of selective PDE4 inhibitor roflumilast, and selective PDE5 inhibitor tadalafil were analyzed in two different doses in a guinea pig model of airway hyperresponsiveness associated with eosinophilic inflammation evoked by ovalbumin.

Materials and methods

The study protocol was approved by the local Ethics Committee of Jessenius Faculty of Medicine in Martin, Comenius University in Bratislava, Slovakia and by the National Veterinary Board, Slovakia. All experiments were performed in line with guidelines for protection of animals used for scientific-educational purposes.

Healthy adult male guinea pigs ($n = 48$, 250–350 g) of TRIK tribe (supplied by Department of Toxicology and Laboratory Animal Breeding Station, Dobra Voda, Slovakia) were used for the study. They were kept in a faculty animal house where they underwent 7-days quarantine and acclimation. During their keeping in the animal house, they had adequate food and water *ad libitum*.

Animals were randomly divided into 6 groups ($n = 8$ in each group). One of the groups was left without a sensitization and served as a non-sensitized healthy control group (Control group, *naïve*). In the remaining five groups, the airway hyperresponsiveness was induced by repeated exposures to antigen (ovalbumin). The guinea pigs in one of the sensitized groups were given vehicle (*aqua pro injectione*) only, and served as an ovalbumin-sensitized non-treated group (Ovalbumin group). The sensitized animals in the latter four sensitized groups were treated with selective PDE4 inhibitor roflumilast (DND Pharm-Technology Co., Inc., China), with selective PDE5 inhibitor tadalafil (DND Pharm-Technology Co., Inc., China), each at the doses of 0.5 mg/kg or 1.0 mg/kg b.w. (dissolved in *aqua pro injectione* to reach the respective injection volume 3 mL/kg). All the tested drugs were administered intraperitoneally once daily for 7 consecutive days.

Ovalbumin-induced airway hyperresponsiveness

The sensitization of animals by ovalbumin, which causes airway reactivity changes on the immunological basis, was performed during 14 days.^[20] Ovalbumin

(Sigma Aldrich, Germany, 1% concentration in *aqua pro injectione*) was administered on the 1st day of sensitization intraperitoneally (0.5 mL) and subcutaneously (0.5 mL), and on the 3rd day intraperitoneally (1.0 mL). On the 14th day and the 21st day, 1% ovalbumin dissolved in saline was inhaled for 1 minute using an inhalation chamber (challenge). The measurement of an *in vivo* airway reactivity to a bronchoconstriction mediator histamine (Sigma Aldrich, Germany) followed in 5 hours after the ovalbumin challenge and *in vitro* measurements were performed on the following day (within 24 hours after the challenge) after sacrificing the animals. In the treated groups, tested drugs (vehicle – *aqua pro injectione*, roflumilast, and tadalafil) were administered at a doses of 0.5 mg/kg, or 1.0 mg/kg b.w. intraperitoneally (dissolved in *aqua pro injectione* to reach the respective injection volume 3 mL/kg) for seven consecutive days, starting from 15th day. The 7th dose was administered 30 minutes before the *in vivo* assessment and before killing the animal on the last day. Only animals with a minimum of 20% increase in the specific airway resistance after the ovalbumin challenge on the 14th day were included in the further testing.

Assessment of *in vivo* airway reactivity

The specific airway resistance (sRaw), a sensitive marker of airway reactivity in *in vivo* conditions, was measured using a double-chamber whole-body plethysmograph for small laboratory animals (HSE type 855, Hugo Sachs Elektronik, Germany). The method previously described by Pennock et al.^[21] is based on the measurement of the time delay between the pneumotachometer signals from thoracic and nasal chambers, whereas the volume changes in the nasal and in the thoracic chambers were evaluated separately. The specific airway resistance calculated from a phase displacement between the two chambers was evaluated immediately after an inhalation (2 min) of bronchoconstricting agent (histamine at the concentration of 10^{-6} mol/L in saline) on the 14th day and on the 21st day of sensitization 5 hours after the challenge with ovalbumin. For comparison, the reactivity after a nebulization of saline was used. During the intervals, fresh air was insufflated into the nasal chamber. The results were evaluated using the software HSE Pulmodyn Pennock (Hugo Sachs Elektronik, Germany). Because the specific airway resistance can be influenced not only by the contraction of airway smooth muscles but also by a mucosal edema and by changes in the secretion of

epithelium, to avoid any pitfalls with interpretation of these data the tissue samples from lungs and trachea were analyzed also in *in vitro* conditions.

Evaluation of *in vitro* airway reactivity

After the ovalbumin challenge and eventual treatment, the animals were killed by an overdose of anesthetics and subsequent exsanguination; trachea and lungs were immediately excised. The measurement of *in vitro* airway reactivity was realized in the organ baths, multi-chamber tissue bath systems (Experimetria-ISO-09-TSZ8, Hungary). Tracheal strips (approximately 15 mm) were cut on the opposite side of the smooth muscle. Lung tissue strips ($2 \times 2 \times 15$ mm) were cut from the margin of upper lobes of the left lungs. The strips were mounted between two hooks and placed into the organ chambers containing Krebs-Henseleit buffer (NaCl 110.00 mmol/L, KCl 4.80 mmol/L, CaCl_2 2.35 mmol/L, Mg_2SO_4 1.20 mmol/L, KH_2PO_4 1.20 mmol/L, NaHCO_3 25.00 mmol/L, and glucose 10.00 mmol/L; in glass distilled water). The chambers were maintained at 36.5 ± 0.5 °C and aerated continuously with a mixture of 95% O_2 and 5% CO_2 to maintain pH 7.5 ± 0.1 . One of the hooks was connected to a force transducer (EXP, Experimetria, Hungary) and an amplifier (EXP CLSG-4, Experimetria, Hungary), and the tension changes were recorded online using a special computer software (SPEL Advanced Iso Sys v3.2, Experimetria, Hungary). The tissue strips were initially set to 4 grams of tension for 30 min (loading phase). Then, in each strip the tension was readjusted to a baseline value of 2 grams for another 30 min (adaptation phase). During both periods, the tissue strips were washed at 10 min intervals with the pre-warmed Krebs-Henseleit buffer. The contractile responses to cumulative doses of histamine and acetylcholine (10^{-8} – 10^{-3} mol/L, Sigma-Aldrich, Germany, dissolved in *aqua pro injectione*) were used as a marker of *in vitro* airway smooth muscle reactivity after the adaptation phase had been finished. Data of the tracheal and lung tissue reactivity are shown in grams (g) of the smooth muscle tension.^[22]

Hematological assay

Samples of blood were taken from the heart immediately after an overdosing of animals with anesthetics during an exsanguination phase. Bronchoalveolar lavage (BAL) of the right lung was performed twice

using instillation and immediate suction of pre-heated saline (37 °C) at the dose of 0.01 mL/g b.w. The total leukocyte count in the blood was determined in a Bürker's chamber after staining by Türk. Differential leukocytes counts in the blood and BAL fluid were evaluated microscopically after staining by May-Grünwald/Giemsa-Romanowski and relative counts of cells were expressed in percents (%).

Detection of markers of inflammation and oxidative stress

A standard ELISA method with commercially available kits for guinea pigs (Antibodies-online Inc., USA, Cusabio Life Sciences, USCN Life Sciences Inc., Blue Gene Biotech) was used to determine the concentrations of pro-inflammatory mediators: interleukin (IL)-4, IL-5, and tumor necrosis factor alpha (TNF- α) in the lung homogenates.

Statistical analysis

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

Results

As a marker of *in vivo* airway responsiveness, the specific airway resistance measured in the whole body double chamber plethysmograph was used. The sensitization and challenge of guinea pigs with ovalbumin led to a significant increase in the specific airway resistance on the 14th and on the 21st day (treated with vehicle). The administration of roflumilast and tadalafil at the daily doses of 0.5 mg/kg as well as 1.0 mg/kg b.w. caused a significant decrease in the specific airway resistance after the histamine nebulization, with the most significant decrease observed in the group treated with tadalafil at the higher dose (Fig. 1).

The *in vitro* testing of the tracheal and lung smooth muscle tissue strips contractility to cumulative doses of acetylcholine and histamine demonstrated a significant increase in the reactivity after the sensitization compared to the Control group (*naïve* animals). The administration of roflumilast led to a suppression of contractile responses evoked by acetylcholine and histamine in the tracheal and lung tissue only at the dose of

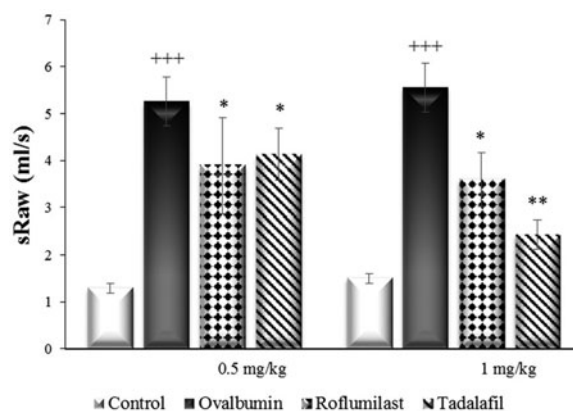


Figure 1. Specific airway resistance measured after inhalation of histamine aerosol (10^{-6} mol/L; 2 minutes) measured before sensitization, after sensitization and after subsequent 7-days treatment with vehicle, roflumilast, or tadalafil (both at the dose of 0.5 mg/kg b.w. or 1.0 mg/kg b.w. intraperitoneally). (Data are expressed as means $\pm$ SEM; for statistical comparisons: +++ $p < 0.001$ vs Before sensitization; * $p < 0.05$ vs After sensitization; ** $p < 0.01$ vs After sensitization).

1.0 mg/kg b.w. (Fig. 2). The administration of tadalafil caused significant changes of contractile response to cumulative doses of acetylcholine and histamine in tracheal tissue only at the concentration of 1.0 mg/kg. In the lung tissue strips, however, significant suppression of contractile responses to histamine was observed at both tested doses. (Fig. 3).

A significant increase of total leukocyte count in the blood and of differential counts of eosinophils in the blood and BAL fluid after sensitization in the non-treated Ovalbumin group confirms a generation of eosinophilic inflammation evoked by our sensitization protocol (Table 1). The intraperitoneal administration of both roflumilast and tadalafil for 7 consecutive days led to a significant decrease in differential counts of eosinophils in the blood only at the dose of 1.0 mg/kg. However, the lower dose (0.5 mg/kg b.w.) was accompanied by a significant decrease in the number of eosinophils in the BAL fluid observed at both tested doses. These changes were associated with a significant increase in the number of monocytes-macrophages in the BAL fluid (Table 1).

An analysis of concentrations of IL-4, IL-5, and TNF- α in the lung tissue homogenates revealed a significant elevation of these parameters in the sensitized non-treated Ovalbumin group. Administration of roflumilast suppressed significantly the levels of IL-4 and IL-5. Tadalafil, contrary, led to a decrease in IL-5 levels only, observed in the groups treated with both 0.5 mg/kg and 1.0 mg/kg. In our experiments,

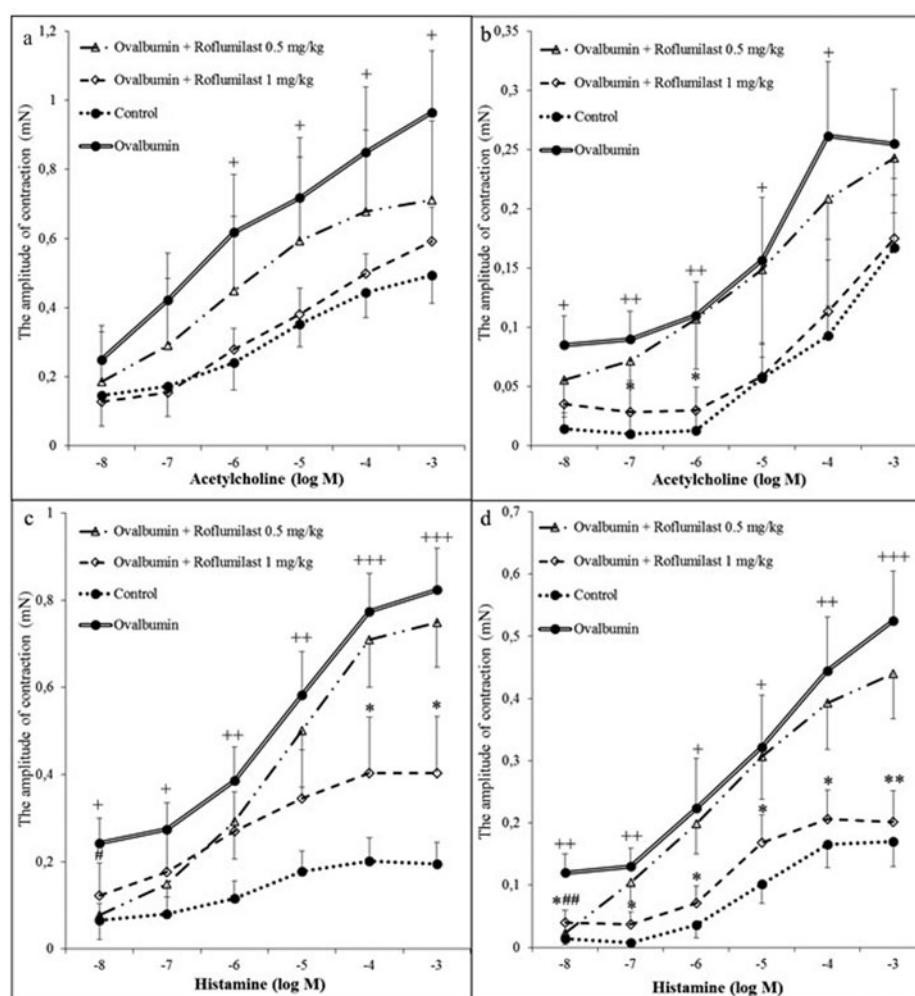


Figure 2. *In vitro* airway reactivity of tracheal (a, c) and lung (b, d) tissue strips to acetylcholine (a, b) and histamine (c, d) in naïve (Control) and ovalbumin-sensitized guinea pigs after 7-days pre-treatment with vehicle (Control, Ovalbumin), or roflumilast (at the dose of 0.5 and 1.0 mg/kg b.w. intraperitoneally). (Data are expressed as means $\pm$ SEM; for statistical between-group comparisons: $^+p < 0.05$ Ovalbumin vs Control; $^{++}p < 0.01$ Ovalbumin vs Control; $^{+++}p < 0.001$ Ovalbumin vs Control; $^*p < 0.05$ Ovalbumin+Roflumilast 1.0 mg/kg vs Ovalbumin; $^{**}p < 0.01$ Ovalbumin+Roflumilast 1.0 mg/kg vs Ovalbumin; $^{\#}p < 0.05$ Ovalbumin+Roflumilast 0.5 mg/kg vs Ovalbumin; $^{\#\#}p < 0.01$ Ovalbumin+Roflumilast 0.5 mg/kg vs Ovalbumin).

only tendency to lower TNF- α levels in roflumilast treated group was demonstrated, with no changes in the tadalafil treated groups of guinea pigs (Table 2).

Discussion

The allergic bronchial asthma is usually associated with an eosinophilic inflammation. In our model we used the allergen (1% ovalbumin), which caused a tissue injury and subsequent structural changes accompanied with inflammation.^[23,24] Several selective inhibitors of PDE have been studied for their ability to influence the airway inflammation and hyperresponsiveness of the airways.^[25] The second generation of PDE4 inhibitors (e.g. piclamilast, cilomilast, and roflumilast) has been confirmed to

possess bronchodilating, anti-inflammatory, and antitussive effects in both experimental and clinical conditions, and roflumilast has been recently introduced into the therapy of moderate to severe COPD. Nevertheless, these promising results emphasize the necessity of testing the inhibitors of other PDE isoforms in the respiratory diseases with an inflammatory component like bronchial asthma.^[8,10,26,27]

In our experiments, the influence of the selective PDE4 inhibitor roflumilast on airway reactivity in two different doses was evaluated. We found that roflumilast significantly decreased the specific airway resistance, a marker of *in vivo* airway reactivity, at both doses (0.5 and 1.0 mg/kg b.w.), and reduced *in vitro* airway reactivity in both tracheal and lung tissue strips in the ovalbumin-sensitized guinea pigs only in the higher

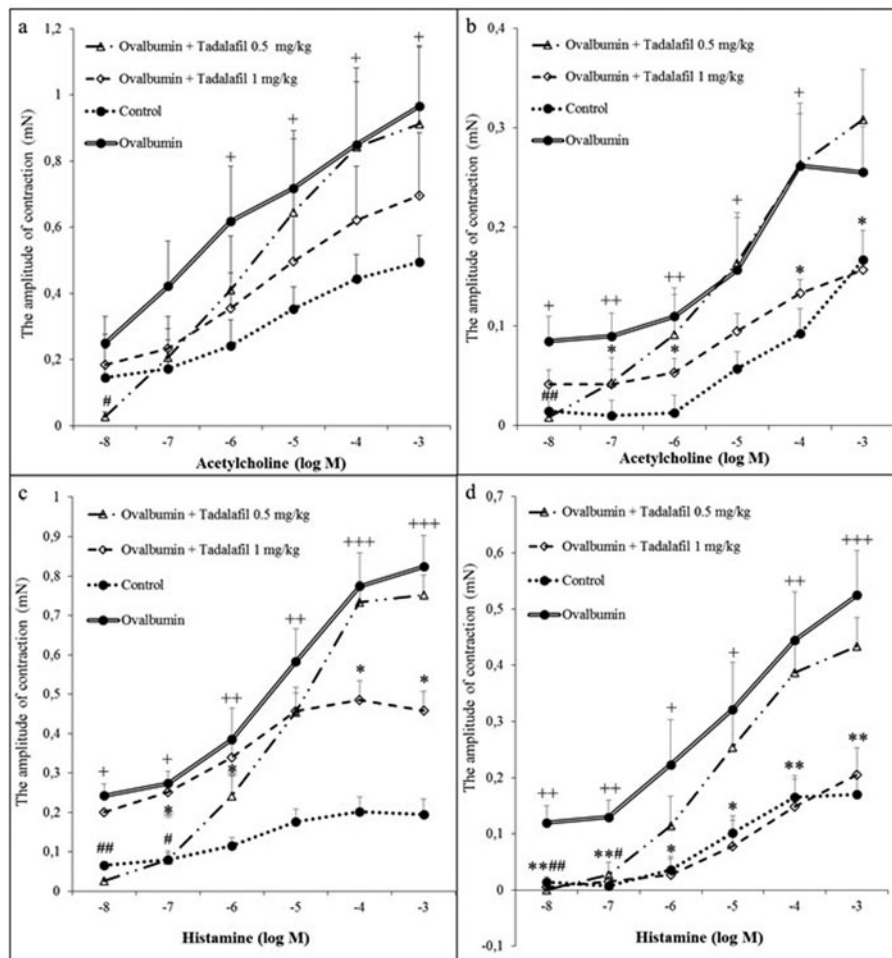


Figure 3. *In vitro* airway reactivity of tracheal (a, c) and lung (b, d) tissue strips to acetylcholine (a, b) and histamine (c, d) in naive (Control) and ovalbumin-sensitized guinea pigs after 7-days pre-treatment with vehicle (Control, Ovalbumin), or tadalafil (at the dose of 0.5 and 1.0 mg/kg b.w. intraperitoneally). (Data are expressed as means $\pm$ SEM; for statistical between-group comparisons: + p < 0.05 Ovalbumin vs Control; ++ p < 0.01 Ovalbumin vs Control; +++ p < 0.001 Ovalbumin vs Control; * p < 0.05 Ovalbumin+Tadalafil 1.0 mg/kg vs Ovalbumin; ** p < 0.01 Ovalbumin+Tadalafil 1.0 mg/kg vs Ovalbumin; # p < 0.05 Ovalbumin+Tadalafil 0.5 mg/kg vs Ovalbumin; ## p < 0.01 Ovalbumin+Tadalafil 0.5 mg/kg vs Ovalbumin).

Table 1. Absolute and relative counts of leukocytes in the blood and bronchoalveolar lavage (BAL) fluid in naive (Control), ovalbumin-sensitized, and ovalbumin-sensitized and treated guinea pigs with roflumilast or tadalafil at the doses of 0.5 mg/kg or 1.0 mg/kg b.w. (Leu: absolute number leukocytes; Lym: lymphocytes; Neu: neutrophils; Eos: eosinophils; Mon: monocytes; Mon-Mac: monocytes-macrophages; BAL – bronchoalveolar lavage fluid; data are expressed as means $\pm$ SEM; for statistical between-group comparisons: ++ p < 0.01 vs Control, +++ p < 0.001 vs Control, * p < 0.05 vs Ovalbumin, ** p < 0.01 vs Ovalbumin, *** p < 0.001 vs Ovalbumin).

	BLOOD					BAL		
	Leu ($\times 10^9/l$)	Lym (%)	Neu (%)	Mon (%)	Eoz (%)	Mon (%)	Neu (%)	Eoz (%)
Control	2.1	64.2	33.9	1.16	0.7	95.36	3.4	1.24
	± 0.11	± 1.83	± 1.80	± 0.17	± 0.25	± 0.82	± 0.49	± 0.38
Ovalbumin	3.44	59.77	36.89	0.91	2.24	60.64	6.71	32.64
	± 0.46 ++	± 3.49	± 3.56	± 0.10	± 0.31 ++	± 2.62 +++	± 1.94	± 3.27 +++
Roflumilast	2.13	62.81	31.13	0.88	3.05	77.48	4.20	18.32
0.5 mg/kg	± 0.18 **	± 0.81	± 1.69 *	± 0.14	± 0.92	± 3.87 ***	± 0.57	± 3.36 **
Roflumilast	2.73	64.92	30.53	1.16	0.66	86.79	5.71	7.5
1.0 mg/kg	± 0.32	± 3.53	± 3.65 *	± 0.10	± 0.15 ***	± 1.50 ***	± 1.77	± 1.29 ***
Tadalafil	2.65	56.72	40.82	0.95	1.52	86.35	4.22	9.43
0.5 mg/kg	± 0.40	± 4.10	± 3.90	± 0.21	± 0.37	± 1.99 ***	± 0.81	± 1.78 ***
Tadalafil	2.25	60.33	38.23	0.81	0.63	75.17	10.23	14.60
1.0 mg/kg	± 0.34 *	± 2.13	± 2.13	± 0.11	± 0.22 ***	± 5.17 **	± 2.26	± 3.45 *

Table 2. Concentrations of IL-4, IL-5, and TNF α in lung homogenate in naïve (Control), ovalbumin-sensitized, and ovalbumin-sensitized and treated guinea pigs with roflumilast or tadalafil at the doses of 0.5 mg/kg or 1.0 mg/kg b.w. (data are expressed as means $\pm$ SEM; for statistical between-group comparisons: + $p < 0.05$ vs Control, ++ $p < 0.01$ vs Control, +++ $p < 0.001$ vs Control, * $p < 0.05$ vs Ovalbumin, ** $p < 0.01$ vs Ovalbumin; *** $p < 0.001$ vs Ovalbumin).

	IL-4 (pg/ml)	IL-5 (pg/ml)	TNF α (pg/ml)
Control	152.04 ± 9.91	305.81 ± 53.29	64.50 ± 6.76
Ovalbumin	178.45 ± 13.20 +	854.29 ± 41.92 +++	131.88 ± 26.24 ++
Roflumilast	148.35 ± 3.15 *	582.98 ± 35.24 *	114.38 ± 0.92
0.5 mg/kg	139.05 ± 13.01 **	457.64 ± 33.81 **	106.26 ± 0.92
Roflumilast	180.37 ± 5.69	356.72 ± 34.10 ***	157.77 ± 25.84
1.0 mg/kg	160.48 ± 4.69	271.23 ± 51.24 ***	183.69 ± 12.54
Tadalafil			
0.5 mg/kg			
Tadalafil			
1.0 mg/kg			

doses. This suggests a direct smooth muscle relaxing action of roflumilast at the higher doses, with other potential mechanisms involved in the suppression of specific airway resistance after administration of the lower doses (reduced edema of mucous membrane and secretion). A previously tested PDE4 inhibitor citalopram significantly suppressed the airway reactivity only in *in vitro* conditions but not *in vivo*,^[22] whereas rolipram demonstrated potent *in vivo* and *in vitro* activity in the ovalbumin-sensitized guinea pigs.^[28] However, the doses of rolipram and citalopram were 1.0 mg/kg b.w. only. The suppression of airway hyperreactivity in guinea pigs sensitized with ovalbumin (with no effect on late asthmatic responses) was observed also in another study with rolipram at the same dose.^[24]

There are several selective PDE4 inhibitors that have demonstrated a potent anti-inflammatory and immunomodulation effects with markedly reduced adverse effects comparing to non-selective PDE inhibitors (e.g. rolipram, citalopram, cilomilast, roflumilast, or piclamilast).^[29] In our recently published study we have confirmed potent anti-inflammatory, antitussive and bronchodilating action of another selective PDE4 inhibitor YM976.^[28] Toward and Broadley^[24] observed a decrease in the eosinophil count in BAL fluid after the therapy with rolipram. Similar results were observed in our study, where roflumilast at both doses led to the lower number of eosinophils in the BAL fluid. In blood, the eosinophils count was reduced only after the therapy with higher doses of roflumilast. However, both total white blood cell count and relative neutrophil count declined after

both doses used in our experiments. This confirms the efficacy of a PDE4 inhibition in the suppression of neutrophil activation and suggests a potent anti-inflammatory action also in the allergen-induced eosinophilic inflammation. Our results are in accordance with a recently published study by Kim et al.^[30], where the authors found a significant decline in the eosinophils and total white blood cells counts in mice sensitized by ovalbumin after administration of roflumilast at the dose of 1.0 mg/kg b.w. Another *in vivo* study demonstrated the changes in the mobilization of neutrophils and eosinophils and their activation, probably due to an increase in intracellular cAMP levels after roflumilast therapy. This prevented the cells to release their content, and ameliorated the antigen-evoked contractions of the tracheal rings.^[31]

Wollin et al.^[17,18] found in their two studies that roflumilast inhibited a leukotriene (LTB₄) production and cytokine and chemokine release in a dose dependent manner (ID₅₀ for conductance and dynamic lung compliance was 0.33 mg/kg, and 0.25 mg/kg, respectively; ID₅₀ for LTB₄ release was 0.05 mg/kg). The specific airway resistance was found to be decreased by 50% (ID₅₀) at the roflumilast doses of 1.5 mg/kg. This supports a potential benefit of roflumilast in the therapy of bronchial asthma. As the changes in the leukocyte numbers in the blood and BAL fluid are tightly associated with a secretion of several cytokines (the mast cells have been indicated as a main source of cytokines, including IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, and TNF- α ; 32), we evaluated the levels of IL-4, IL-5, and TNF- α in lung homogenates. The secretion of IL-4 (and IL-13) stimulates B-lymphocytes to an increased production of allergen-specific IgE. The IgE is bound to receptors on mast cells and granulocytes and in case of a contact with allergens, a rapid degranulation and release of various mediators occurs. Furthermore, IL-4 is considered to be a crucial mediator responsible for differentiation of T-cells to Th2 subtype.^[33] IL-5 acts as an eosinophil-protecting and maturation factor and plays an important role in the stimulation of eosinophils in the allergic inflammation. TNF- α released from the mast cells enhances an expression of adhesion molecules (E-selectin, ICAM-1) on the endothelial cells which enable an infiltration of leukocytes into the site of inflammation. It offers an important signal for maturation and migration of dendritic cells, thus supporting the immune response. TNF- α activates alveolar macrophages and neutrophils, and

together with TGF- β released from the mast cells participates in the fibrotic changes of *bronchi* in chronic asthma.^[34] In our study, roflumilast at both doses significantly reduced the secretion of IL-4 and IL-5 in the lung tissue. Roflumilast also demonstrated a tendency to decrease the concentration of TNF- α ; however, without reaching the level of statistical significance. The inhibition of PDE4 leads to a suppression of inflammatory mediators expression *via* increased intracellular concentrations of cAMP, with simultaneous suppression of TNF- α and granulocytes/monocytes colony stimulating factor (GM-CSF) secretion.^[6] The airway inflammation leads to the tissue remodeling due to an over-expression of matrix metalloproteases (MMP), which is induced also by TNF- α . Inhibitors of PDE4 are potent blockers of pro-MMP secretion induced by TNF- α , and therefore, may be of benefit in the diseases associated with abnormal tissue destruction and remodeling.^[35] In a murine model of asthma, the selective inhibition of PDE4 with roflumilast led to a significant decrease of IL-13 and IL-4,^[30] that is in concordance with our data obtained from a guinea pig lung tissue. In other murine model of asthma, inhaled administration of roflumilast was effective in suppression of IL-4, IL-5, TNF- α , IgE specific for ovalbumin, and white blood cell count in the BAL fluid. However, its combination with a corticosteroid fluticasone propionate was even more potent, suggesting the perspectives of the combined use of PDE4 inhibitors with other anti-inflammatory or bronchodilating drugs.^[36] Patel et al.^[37] have recently demonstrated that selective PDE4 inhibition (cilomilast, piclamilast, and rolipram), but not PDE3 inhibition (cilostamid, cilostazol, and milrinone), enhanced the anti-inflammatory action of long-acting β_2 -agonists (formoterol). This effect is mainly related to a decreased production of TNF- α and subsequently of IL-8 (CXCL-8), due to increased intracellular levels of cAMP in the smooth muscle cells after activation of β_2 -receptors and induction of mitogen-activated protein (MAP) kinase phosphatase (MKP-1).^[37]

The PDE5 inhibitor tadalafil led in this study to a decrease of both *in vivo* and *in vitro* airway reactivity, as well. However, a significant suppression of contractile responses to the cumulative doses of acetylcholine and histamine was found in ovalbumin-sensitized animals only in the lung tissue and preferentially at the higher dose. This is in concordance with previous results with sildenafil (a short acting PDE5 inhibitor) used in the

guinea pigs exposed to ovalbumin,^[38] and in a model of tracheal hyperresponsiveness in antigen-challenged rats.^[39] The difference observed in the responses of the tracheal and lung tissue strips could be explained by a different distribution of PDEs in these tissues – a stronger influence on *in vitro* airway reactivity in the lung tissue strips is associated with a higher expression of PDE5 in the vascular smooth muscle, which is present in a higher proportion in the lung tissue compared to the tracheal tissue.^[40]

In our study, the long-acting selective PDE5 inhibitor tadalafil confirmed its effectiveness in the model of allergic inflammation, as its higher dose (1.0 mg/kg) led to the reduced total leukocyte count and relative count of eosinophils in the blood. Furthermore, both doses of tadalafil suppressed eosinophil counts in BAL fluid. These findings are in accordance with several previous studies.^[38,41,42] However, the concentrations of IL-4 and TNF- α in lung homogenate were not influenced significantly and had a tendency to increase with the dose of tadalafil (TNF- α). This can be explained by a dynamics of TNF- α release, which precedes further IL-4 secretion. Furthermore, the difference between the action of roflumilast and tadalafil can be explained by different doses necessary to reach the same effect, as confirmed by a previous study of Takeda et al.^[19]. They found that both roflumilast and tadalafil are (after a single dose) potent modulators of allergen-induced alterations in tracheal smooth muscle responsiveness in mice, with higher dose of tadalafil necessary. Furthermore, they confirmed a potential of their co-administration at lower (suboptimal) doses (0.1 mg/kg of roflumilast and 2 mg/kg of tadalafil). In contrary to that study, we used the 7-days lasting administration of these drugs, with potentially stronger action both on airway hyperresponsiveness and inflammation. The doses of roflumilast and tadalafil used in our experiments were chosen in order to compare the results of roflumilast and tadalafil at the same doses in guinea pigs. We are aware of the fact that the pharmacological effects were not maximal based on the previous study by Sawamura et al. in rats^[43]; however, the effects of tadalafil in that study were evaluated on vascular smooth muscle (pulmonary hypertension).

Contrary to the IL-4 concentrations, IL-5 levels were suppressed significantly, with more significant suppression observed in sensitized guinea pigs treated with the dose of 1.0 mg/kg b.w. This finding could be of

importance in the therapy of severe allergic asthma, as an inhibition of IL-5 by monoclonal antibodies (e.g. mepolizumab, reslizumab) has been recently introduced into the therapy of patients with severe, refractory asthma with eosinophilia.^[11]

We are aware of the fact that the plasma levels of tested drugs should be measured and the level of PDE inhibition should be confirmed in order to make any final conclusions about the involvement of PDE and its inhibition in effects observed in our study. However, we have demonstrated the potential of these drugs to influence *in vivo* and *in vitro* airway reactivity as well as markers of inflammation.

Our data suggest that both of the tested agents (roflumilast and tadalafil) could be used in diseases associated with allergic inflammation, which was in this study mimicked by the ovalbumin sensitization. Furthermore, the patients may benefit from their synergistic effect – this concept of dual PDE inhibition has been previously described in the literature and new dual PDE inhibitors (e.g. PDE3/4, PDE4/5, and PDE4/7), or co-administration of two selective PDE inhibitors are intensively studied for their anti-inflammatory action.^[28,44,45]

Conclusions

Selective inhibitors of PDE4 roflumilast and of PDE5 tadalafil led to the suppression of airway reactivity and markers of inflammation in the model of ovalbumin-induced eosinophilic inflammation associated with airway hyperresponsiveness in a dose dependent manner. The results suggest a potential benefit of both PDE inhibitors in the therapy of allergic bronchial asthma or asthma-COPD overlap. However, further studies are needed to confirm this hypothesis.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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