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Influence of Roflumilast on Airway Reactivity and Apoptosis in Ovalbumin-Sensitized Guinea Pigs

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Abstract

Chronic inflammatory diseases, associated with airway obstruction and cough, are usually treated with bronchodilating and anti-inflammatory drugs. Inhibition of phosphodiesterases (PDE) leads to both of these effects and influences apoptosis of immune cells. In chronic obstructive pulmonary disease, roflumilast, a selective PDE4 inhibitor, has been recently approved for pharmacotherapy. The aim of this study was to evaluate the effect of long-term administration of roflumilast in experimental allergic inflammation in guinea pigs. Male adult guinea pigs were used in the study. There were four experimental groups sensitized with ovalbumin for 14 days and thereafter treated *per os*, by inhalation, and intraperitoneally for 7 days with roflumilast or vehicle. A control group was left without sensitization. Roflumilast reduced specific airway resistance after nebulization of histamine, as measured in a double-chamber whole-body plethysmograph. This effect was confirmed in *in vitro* organ bath, with significant decreases in tracheal and lung smooth muscle contractility after cumulative doses of histamine. Suppression of hematological and immunological markers of inflammation and enhanced apoptosis in animals treated with roflumilast points to the possibility of a beneficial effect of roflumilast in allergic inflammation.

Keywords

Apoptosis • Asthma • Inflammation • Phosphodiesterase-4 • Roflumilast

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1 Introduction

Allergic asthma is a chronic inflammatory disorder of airways, characterized by allergen-induced, IgE-mediated early and late bronchial obstructive reactions, development of acute and transient airway hyperresponsiveness, and

infiltration of inflammatory cells, particularly eosinophils and Th_2 lymphocytes, in the airways (Maarsingh et al. 2009). The accumulation of eosinophils and subsequent release of their potent mediators, including cytotoxic granule proteins, lipid mediators, cytokines, and chemokines are thought to contribute to airway inflammation, which underlies the asthma pathogenesis (Duncan et al. 2003). In addition, Th_2 cells produce the interleukins IL-4 and IL-13, which promote IgE production by B cells, whereas IL-5 acts to promote airway eosinophilia, and IL-9 and IL-13 contribute to airway hyperresponsiveness (Robinson 2005).

Apoptosis, a form of natural or physiological cell death different from necrosis, can remove somatic cells without causing an inflammatory response; so that one way to eliminate inflammatory cells from lesions would be the induction of apoptosis in immune cells (Raouf 2007).

A primary goal of asthma therapy is to achieve a control of clinical symptoms by improving lung function, reducing airway hyperresponsiveness, and eliminating inflammatory cells (Schalkwyk et al. 2005). Cyclic nucleotide hydrolyzing phosphodiesterases (PDEs) comprise a still-growing superfamily of isoenzymes with 11 members known at present. Among the cAMP-specific isoenzymes, PDE4 has received particular attention due to the fact that all of the inflammatory and immunomodulatory cells contain this isoform. Enhanced PDE4 function may play a role in the pathogenesis of asthma due to increased protein expression or activity (Spina 2008). Immunomodulatory function of inflammatory cells is broadly inhibited by selective PDE4 inhibitors. Thus, PDE4 inhibitors show a pronounced anti-inflammatory effect in various animal models, and as such, they have been proposed as a new therapeutic approach in a variety of inflammatory diseases, including asthma and chronic obstructive pulmonary disease (COPD) (Hatzelmann and Schudt 2001).

The mostly clinically tested PDE4 inhibitors, cilomilast and roflumilast, have a favorable side effect profile compared with the first generation of similar compounds or theophylline (Vignola 2004). Roflumilast and roflumilast N-oxide, a major metabolite in humans, are highly potent, competitive, and selective inhibitors of PDE4, having essentially no activity against PDE1, PDE2, PDE3, and PDE5 isoforms (Giembycz 2002). Roflumilast represents the first PDE4 inhibitor registered in the market for COPD therapy (Keravis and Lugnier 2011).

The aim of the present study was to compare the effects of different routes of roflumilast administration in an animal model of bronchial asthma, associated with eosinophil inflammation, on airway reactivity and apoptosis of inflammatory cells in bronchoalveolar lavage fluid (BALF).

2 Methods

The study protocol was approved by the Ethics Committee of Jessenius Faculty of Medicine, Comenius University in Martin, Slovakia. Male guinea pigs (TRIK tribe) aged 1–3 months, weighing 150–350 g were used in the study. The animals were kept in the animal house and obtained food and water *ad libitum*. They were divided into six groups consisting of eight animals each. One of the groups was left without sensitization and served as a non-sensitized healthy control group. In the other five groups the hyperresponsiveness was induced by exposure to ovalbumin antigen. The animals of one of the five sensitized groups were given physiological saline as a vehiculum only and served as an ovalbumin-sensitized control group. The other four groups were treated with roflumilast from the 14th day of sensitization for 7 days *per os* at a dose 1.0 mg/kg in saline (3.0 ml/kg), roflumilast by inhalation for 3 min (1.0 mg/ml in saline), roflumilast i.p. (1.0 mg/kg in saline; 3.0 ml/kg), and roflumilast combined with salbutamol by inhalation for 3 min (0.5 mg/ml of roflumilast + 0.5 mg/ml of salbutamol in saline), respectively.

2.1 Antigen-Induced Airway Hyperresponsiveness

Sensitization was performed for 21 days to cause tissue injury and subsequent structural changes accompanied with inflammation (Tagaya and

Tamaoki 2007). The ovalbumin allergen (1 % solution in *aqua pro injectione*) was administered on the 1st day of sensitization by two routes: 0.5 ml i.p. and another 0.5 ml s.c., and on the 3rd day 1.0 ml, i.p. Afterward, on the 14th and 21st day the ovalbumin solution was inhaled for 30 s in an inhalation chamber.

2.2 In Vivo Airway Reactivity Assessment

Specific airway resistance (RxV) was measured as a marker of in vivo airway reactivity The animals were placed in a double-chamber whole body plethysmograph and an aerosol of histamine in the concentration of 10^{-6} mol/l in saline was used for the evaluation of airway reactivity. As a control, inhalation of saline was used. Airways hyperresponsiveness was evaluated after 2 min of histamine or saline inhalation.

2.3 In Vitro Airway Reactivity Assessment

Smooth muscle reactivity of tissue strips from the lungs and trachea was recorded in a tissue bath in an organ chamber. The contractile response to cumulative doses of histamine (10^{-8}–10^{-3} mol/l) was used as a marker of *in vitro* airway smooth muscle reactivity (Mokry et al. 2008).

2.4 Evaluation of White Blood Cells Viability

After sacrificing the animals, BALF was collected by lavaging the lungs with pre-heated saline (2 × 10 ml/kg, 37 °C). Subsequently, BALF sample of 5.0 μl was mixed with tryptan blue solution (5.0 μl) to stain white blood cells. An automatic cell counter (Countess™, Invitrogen, Carlsbad, CA) was used to estimate the cell viability and the total cell count of in BALF.

2.5 Statistical Analysis

Data are shown as means ± SE. For statistical analysis, one-way ANOVA was used. Statistical significance was defined as $p < 0.05$.

3 Results

Roflumilast caused a significant decrease in RxV after histamine nebulization in the guinea pigs treated with it orally or by inhalation of a half dose roflumilast together with salbutamol, compared with the control OVA-sensitized animals (Fig. 1).

The *in vitro* contractile response of airway smooth muscle to cumulative doses of histamine was suppressed in lung and tracheal tissue strips; the effect was more pronounce in lung tissue.

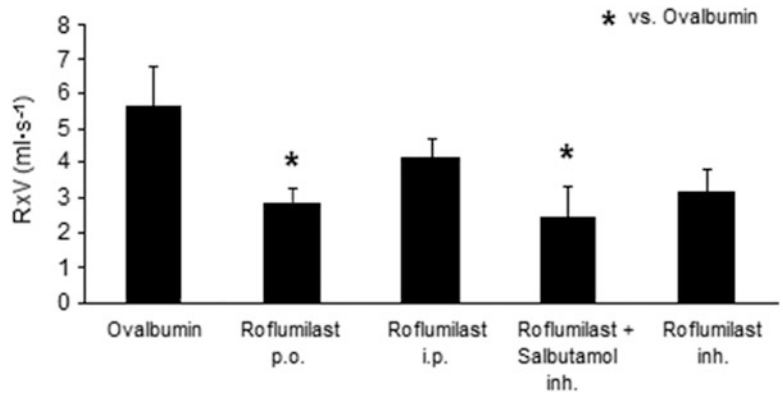

Fig. 1 Specific airway resistance after 7-day treatment with roflumilast by various routes of administration, *p.o* per os, *i.p.* intraperitoneally, *inh.* inhalation

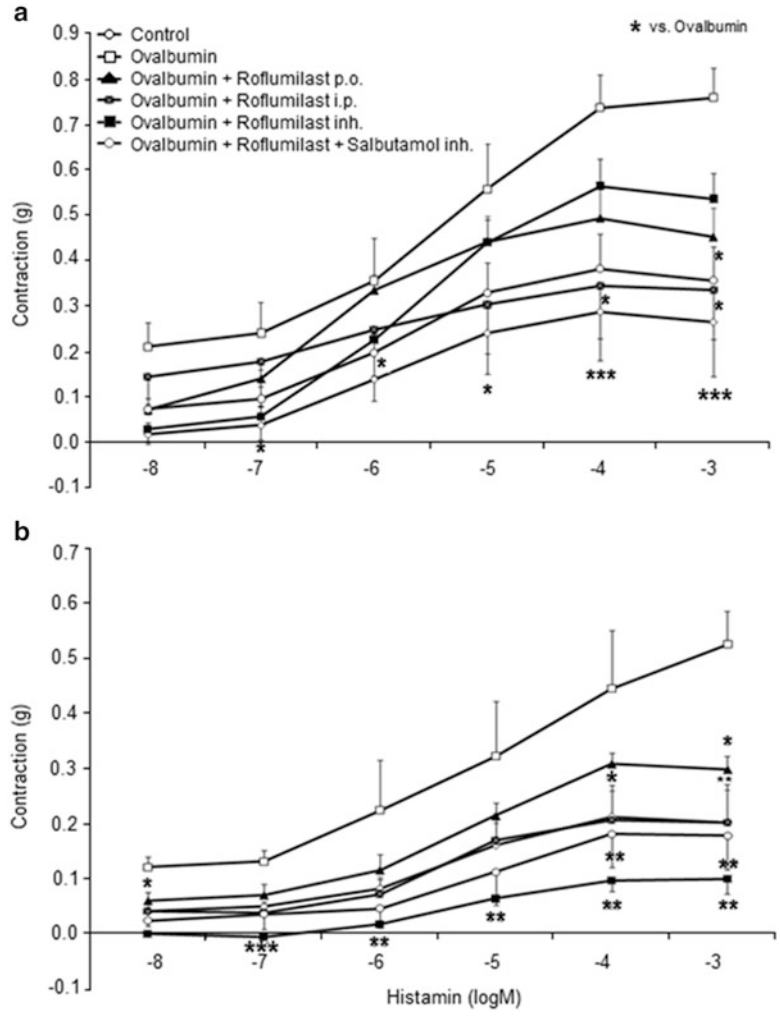

Fig. 2 Changes in tracheal (a) and lung (b) tissue reactivity in response to cumulative doses of histamine after 7-day treatment with roflumilast administered by various routes of administration; *p.o* per os, *i.p.* intraperitoneally, *inh.* inhalation

Roflumilast given alone by inhalation or in combination with salbutamol counteracted the histamine-induced suppression of tracheal and lung smooth muscle reactivity (Fig. 2a, b, respectively).

White blood cell viability in BALF in the ovalbumin-sensitized guinea pigs was significantly lower than that in the non-sensitized ones. In all roflumilast-treated groups, regardless of the route of administration, further suppression of blood cell viability was observed, with the most pronounced effect seen after i.p. roflumilast (Fig. 3a). However, the number of white blood cells in BALF significantly increased in the ovalbumin-sensitized guinea pigs, compared with the non-sensitized group. The increases were reverted by roflumilast given i.p. or by inhalation together with salbutamol (Fig. 3b).

4 Discussion

Airway responsiveness is usually described as the ability of airways to narrow after exposure to constricting agents, usually some inhaled allergens or antigens. Airway hyperresponsiveness is a hallmark of asthma. Histamine and metacholine are routinely used to assess the contractile airway response in bronchial asthma and COPD. Furthermore, fluctuations in airway hyperresponsiveness correlate with the severity

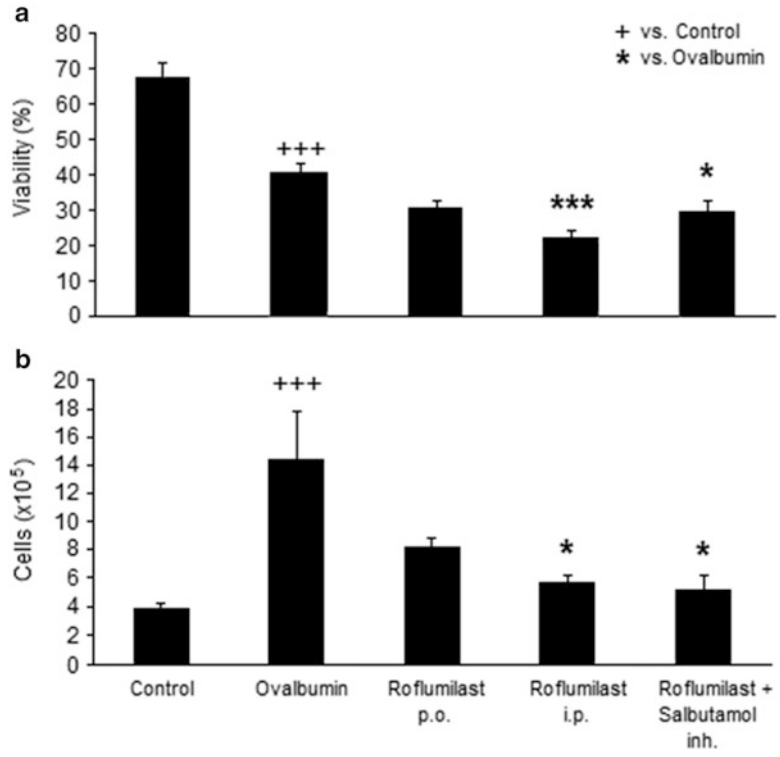

Fig. 3 Changes in white blood cells viability (a) and in the number of white blood cells in BALF (b) in control healthy and ovalbumin-sensitized animals after 7-day treatment with roflumilast by various routes of administration; *p.o* per os, *i.p.* intraperitoneally, *inh* inhalation

of disease and the requirement for drugs needed to control symptoms (O'Byrne and Inman 2003). Histamine is an inducer of bronchoconstriction, exerting its effect through a direct interaction with smooth muscle cells (Louw et al. 2007).

Roflumilast is a selective inhibitor of phosphodiesterase 4 (PDE4) with anti-inflammatory and immunomodulating activities demonstrated previously in several asthma and COPD models (Hatzelmann et al. 2010). In the present study, roflumilast was administered for 7 days to animals either orally, intraperitoneally, by inhalational alone or in combination with salbutamol. Reduction in specific airway resistance after nebulization of histamine was observed in animals treated with roflumilast orally and by inhalational together with salbutamol. Similar results were obtained in a clinical study conducted by Louw et al. (2007), who confirmed a decrease in airway hyperresponsiveness after a single oral dose of 1,000 μg of roflumilast in patients with bronchial asthma. Therefore, the PDE4 inhibitor roflumilast may reduce airway hyperresponsiveness both in experimental animal models and in patients with bronchial asthma. The majority of studies on PDE4 inhibitors, as therapeutic tools, were done in patients with COPD, where there is a different kind of inflammation involved, consisting predominantly of neutrophils. Studies on the role of roflumilast and on the PDE4 involvement in eosinophil inflammation are thus essential.

The existing clinical data demonstrate that the efficacy of roflumilast is relatively well confirmed. However, several other issues remain to be clarified, including the safety profile and systematic anti-inflammatory effects of roflumilast. A major safety issue is the emetogenic potential of roflumilast rarely reported after oral administration (Antoniu 2011). Based on these facts, we chose multiple application ways to test the ability of roflumilast to influence airway reactivity and viability of inflammatory white blood cells. Inhibition of PDE4 has been previously confirmed as a suitable target to decrease airway inflammation and contractility of airway smooth muscle. In a study of Mokry et al. (2008), however, citalopram, a first generation PDE4

inhibitor, was used with multiple other mechanisms of action, e.g., inhibition of serotonin reuptake in central nervous system.

Initially, PDE4 inhibition was expected to induce bronchodilation and reduction of the underlying eosinophil inflammation in bronchial asthma models, which was confirmed in preclinical tests (Antoniu 2011). In the present study, a significant reduction in contractility of airway smooth muscle in each group of animals treated with roflumilast, compared with untreated ones, was observed, regardless of the route of administration. The most significant decrease in smooth muscle reactivity was recorded in lung tissue strips of obtained from animals treated with roflumilast by inhalational alone or combined with salbutamol. Thus, inhalative administration could be considered a suitable way of roflumilast administration. This suggestion needs further testing.

We observed similar effects in tracheal tissue strips. However, the intraperitoneal administration here showed a more significant suppression of *in vitro* airway reactivity. That roflumilast reduced airway smooth muscle reactivity in lung strips from the allergen sensitized animals more than in control non-sensitized animals speaks for its direct bronchodilating effect and for the involvement of PDE4 not only in the regulation of inflammation, but also of smooth muscle contraction and relaxation.

The differences we observed between the reactivity of lung and tracheal smooth muscles are due likely to increased content of vascular smooth muscle in lung tissue compared with tracheal tissue. In a previous study by Bundschuh et al. (2001) roflumilast inhibited the ovalbumin-evoked contractions of tracheal strips prepared from ovalbumin-sensitized guinea pigs. Roflumilast administered intravenously displayed bronchodilatory activity and it dose-dependently attenuated allergen-induced bronchoconstriction in guinea pigs. In a study by Chapman et al. (2007) a robust effect of inhaled roflumilast was observed, leading to improvement of lung function in the allergen-challenged Brown Norway rats. The protective effects of inhaled roflumilast on lung function appeared to be superior to its effect when given orally. These results support the hypothesis that inhaled PDE4 inhibitors could be efficacious in inflammatory lung diseases, particularly due to improvement of lung functions.

As mentioned above, roflumilast is an anti-inflammatory drug leading to the elevation of intracellular cAMP and subsequently down-regulation of a variety of inflammatory cells activities, predominantly neutrophils and eosinophils. In an animal model of asthma, PDE4 inhibitors reduced inflammatory cell infiltration, mainly eosinophils (Bundschuh et al. 2001). Novel therapies aimed at enhancing apoptosis and phagocytic removal of immune cells might become a reality for patients with bronchial asthma (Walsh 2008). In the present study, we demonstrate that roflumilast affected the viability of inflammatory cells in BALF. The highest viability was observed in the healthy non-sensitized group, while in the OVA-sensitized group the cellular viability was significantly reduced. On the other hand, the number of inflammatory cells in BALF significantly increased in OVA-sensitized group compared with the non-sensitized one.

We found that roflumilast led to a significant reduction of both the number of total inflammatory cells and living inflammatory cells. However, the viability of inflammatory white blood cells was reduced compared with the healthy non-sensitized group. These results suggest that roflumilast induced apoptosis of inflammatory cells in the sensitized animals as a way of keeping in check inflammatory changes induced by OVA sensitization. Roflumilast-induced apoptosis was the strongest in animals treated intraperitoneally or by inhalation with salbutamol. In these groups, a concentration of viable (living) inflammatory cells in BALF was even lower than in the non-sensitized group.

IL-5 appears to be a specific survival factor for eosinophils, at least within the human system. Not surprisingly then, eosinophilia and high IL-5 expression have often been associated with one another, especially in chronic allergic disorders such as bronchial asthma. Moreover, it appears that the severity of asthma negatively

correlates with the amount of eosinophil apoptosis in the airways. A study by Simon (2001) suggests that delayed apoptosis represents one important mechanism leading to tissue eosinophilia. PDE inhibition has been shown to accelerate spontaneous apoptosis in both eosinophils and neutrophils, as well as partially to block IL-5-mediated delayed eosinophil apoptosis. Results from human subjects have also demonstrated significant pulmonary anti-inflammatory effects of roflumilast. Roflumilast treatment has been associated with an approximately 40 % reduction in sputum leukocyte number *versus* placebo (McIvor 2008). The animal studies with roflumilast demonstrate that it reduced the accumulation of inflammatory cells in BALF following a short-term exposure of guinea pigs, mice, or rats to allergen (Rabe 2011).

In conclusion, our experimental data suggest the potential of roflumilast to improve lung function and to exert anti-inflammatory, bronchodilating, and pro-apoptotic effects in ovalbumin-induced airway hyperresponsiveness, predominantly after intraperitoneal and inhalative administration. As a combination of the processes above outlined could be useful in therapy of patients with bronchial asthma, further studies are necessary to verify these results in clinical conditions.

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Conflicts of Interest The authors declare no conflicts of interest in relation to this article.

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