



Effect of inhaled roflumilast on the prevention and resolution of allergen-induced late phase airflow obstruction in Brown Norway rats

Richard W. Chapman, Aileen House, Howard Jones, Jennifer Richard, Chander Celly, Dan Prelusky, Pauline Ting, John C. Hunter, James Lamca, Jonathan E. Phillips*

Schering-Plough Research Institute, Pulmonary and Peripheral Neurobiology (M/S 1700), 2015 Galloping Hill Road, Kenilworth, NJ 07033, United States

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Abstract

Orally active phosphodiesterase 4 (PDE4) inhibitors have been developed for the treatment of asthma and chronic obstructive pulmonary disorders (COPD) although their full development has been limited by adverse side effects. Administration of PDE4 inhibitors by inhalation may improve their therapeutic index, but limited information exists on the efficacy of inhaled PDE4 inhibitors to improve lung function. In this study in ovalbumin-sensitized Brown Norway rats, roflumilast was given either intratracheally or by nose-only inhalation and changes in lung function (forced vital capacity, FVC; peak expiratory flow, PEF) and inflammatory cell influx (total cells, eosinophils and neutrophils) into the bronchoalveolar lavage (BAL) fluid were evaluated 24 h after allergen challenge. Intratracheal roflumilast, given 5 h before antigen challenge, inhibited the antigen-induced reductions in FVC (ED_{50} = 140 μ g/kg, i.t.) and total cells appearing in the bronchoalveolar lavage fluid (ED_{50} = 50 μ g/kg, i.t.). By the nose-only inhalation route, roflumilast reduced the bronchoalveolar lavage fluid total cells (ED_{50} = 10 μ g/kg, estimated pulmonary deposition). Intratracheal roflumilast (600 μ g/kg, i.t.) was also given to rats 24 h after the antigen challenge and reversed the antigen-induced reductions of FVC by 38% at 1 h, 54% at 5 h and 71% by 16 h. Intratracheal roflumilast also reduced the number of inflammatory cells in the bronchoalveolar lavage fluid and reduced the interstitial airway edema caused by the antigen challenge. These results support the development of inhaled PDE4 inhibitors for the treatment of asthma and COPD, particularly for the improvement of lung function.

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1. Introduction

Phosphodiesterase 4 (PDE4) is expressed by a variety of different inflammatory cells that are associated with the acute and chronic lung inflammation in diseases such as asthma and chronic obstructive lung disease (COPD) (Spina, 2003; Chung, 2006). PDE4 is also found in many different effector cells that control lung functions such as airway smooth muscle, airway epithelium, vascular endothelium and airway sensory nerves (Torphy, 1998; Spina, 2003). Inhibitors of PDE4 suppress lung inflammation, reduce airway hyperresponsiveness and improve lung function in animal models of asthma and COPD (Bundshuh et al., 2001; Billah et al., 2002; Kumar et al.,

2003; Kuss et al., 2003) and several orally active PDE4 inhibitors, notably cilomilast and roflumilast, have been progressed into clinical trials (Rabe et al., 2005; Lipworth 2005; Bousquet et al., 2006; Giembycz, 2006; Rennard et al., 2006). However, the full clinical development of orally active PDE4 inhibitors has been limited by their mechanism-based side effects that include nausea and emesis in humans and vasculitis in the gastrointestinal tract, mesenteric blood vessels and heart of rodents (Larson et al., 1996; Dietsch et al., 2006) and cardiac tissue of primates (Losco et al., 2004).

In an attempt to improve the therapeutic index with PDE4 inhibitors, several companies have taken the approach of giving the compounds by inhalation (Kuss et al., 2003). This method of delivery deposits locally high concentrations of the drug directly to the target organ and minimizes systemic absorption through the oral route. Some of these inhaled products have entered into clinical trials, but there is a paucity of preclinical

* Corresponding author. Tel.: +1 908 740 5532; fax: +1 908 740 7175.

E-mail address: jonathan.phillips@spcorp.com (J.E. Phillips).

data with inhaled PDE4 inhibitors, particularly for their effects on lung function. AWD 12-281 is an inhaled PDE4 inhibitor that was efficacious for the suppression of cellular lung inflammation in the allergen-challenged Brown Norway rat and this compound was also active against the acute phase bronchoconstriction induced by allergen challenge in sensitized guinea pigs (Kuss et al., 2003).

Roflumilast is a potent and selective PDE4 inhibitor that is orally active in a variety of animal models of asthma and COPD (Kumar et al., 2003; Bundshuh et al., 2001). In this study in ovalbumin-sensitized Brown Norway rats, roflumilast was given by the intratracheal and by nose-only inhalation routes of administration to assess its effects on the late phase reductions in lung function and inflammatory cell influx into the bronchoalveolar lavage fluid induced by antigen challenge. Two different dosing paradigms were used that involved administration of roflumilast before the antigen challenge and a second paradigm that involved administration of roflumilast 24 h after the antigen challenge which is a time at which the reductions in lung function and lung inflammation are already established.

2. Materials and methods

2.1. Sensitization and challenge with antigen

Brown Norway rats, ranging in weight from 200 to 300 g were obtained from Charles River Laboratory (Wilmington, MA). The rats were sensitized by an intra-peritoneal injection of 1 ml alum-precipitated antigen containing 20 µg of ovalbumin (grade III; Sigma chemical Co., St Louis, MO) and 8 mg of alum suspended in 0.9% sodium chloride solution. A booster injection of this alum–ovalbumin mixture was given 7 days later. Non-sensitized animals were injected with alum only. Seven days after the second injection, animals were exposed to aerosolized ovalbumin (1%) for 30 min which was performed by placing the rats into a closed plexiglass chamber (21 l) and filling the chamber with aerosolized ovalbumin which was generated by an ultrasonic nebulizer (DeVilbiss, Somerset, PA, USA; Model Ultra-Neb 99) and circulated through the chamber at a flow rate of approximately 8 l/min. The experiments performed in this study had been given the prior approval from the Animal Care and Use Committee of Schering-Plough Research Institute, which is a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

2.2. Measurement of lung function

The rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and a tracheal catheter was surgically implanted. The rats were placed inside a whole body plethysmograph and the tracheal port was connected to an outlet port at the front of the plethysmograph which in turn was connected to a specially designed breathing valve that facilitated the forced inspiratory and expiratory maneuvers. Pulmonary airflow was measured with the aid of a differential pressure transducer (± 2 cmH₂O, Validyne Corp. California, USA) which measured the pressure drop across

a wire mesh screen inserted in a 1-inch hole at the top of the plethysmograph. Volume was measured by electronic integration of the airflow signal and a volume calibration was performed before each experiment using a 3-ml syringe. Airway opening pressure was measured directly from the breathing valve using a differential pressure transducer (± 70 cmH₂O, Motorola Inc., California, USA). The plethysmograph, breathing valve and software used to operate the system are part of a commercially available forced expiratory maneuvers system for rodents (Buxco Electronics, Troy, NY, USA).

The lungs were inflated to total lung capacity defined by an increase in airway inflation pressure to 20 cmH₂O. This was followed by a rapid deflation (over 0.2 s) of the lungs to a deflation pressure of -40 cmH₂O. The details of this forced expiratory maneuver have been described in detail elsewhere (Celly et al., 2006). From this procedure, forced vital capacity (FVC) and peak expiratory flow (PEF) were measured. This procedure was performed in triplicate and the results expressed as the mean of these three values.

2.3. Bronchoalveolar lavage and lung histology

The rats were sacrificed at the end of each study and a tracheal catheter was inserted. Bronchoalveolar lavage fluid was collected by lavaging the lungs with 2 aliquots of 5 ml of 0.9% sodium chloride solution. Total recovery volume per rat was approximately 8 ml. The total cell count in the bronchoalveolar lavage was performed using a hemocytometer. For the differential white cell count, cytopspins were prepared with 200 µl of bronchoalveolar lavage fluid using a cytocentrifuge (Shandon Inc., Pittsburgh, PA, USA) at 250 revolutions per min for 10 min. The air-dried cytopspins were stained with Leukostat stain (Fisher Scientific, Pittsburgh, PA, USA). Cells were identified as either eosinophils, neutrophils or mononuclear cells by standard morphology and 200 cells counted under 400 $\times$ magnification. For the histological evaluation of lung tissue, the lungs were fixed in formalin and embedded in paraffin wax. Sections of lung tissue were cut at 5 µm thickness, mounted on glass slides and stained with hematoxylin and eosin (H $\times$ E) to assess lung histopathology including the presence of interstitial airway edema (Celly et al., 2006).

2.4. Intratracheal and nose-only inhalation procedures

Intratracheal administration of roflumilast was performed by placing 3 mg of the micronized drug, admixed with lactose into a fine-tipped dry powder microspray needle (Penn Century, Philadelphia, PA, USA). The microspray needle was inserted into the trachea of lightly anesthetized rats (5% isoflurane at a flow rate of 1 ml/min, supplemented with oxygen) to a position just above the carina and injected into the trachea and lungs with 3 ml of air. We have found that approximately 20% of the injected material deposits into the trachea and lungs with this intratracheal technique.

Roflumilast was also given by nose-only inhalation to conscious rats using a system that has been previously described in detail but adapted for the delivery of drugs by dry

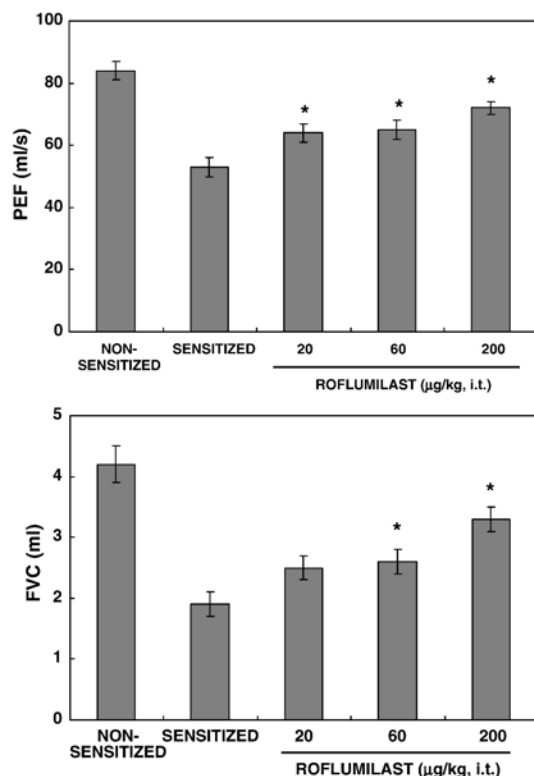


Fig. 1 Improvement in peak expiratory flow (PEF) and forced vital capacity (FVC) by intratracheal roflumilast. Compound or lactose vehicle were given 5 h before the antigen challenge and lung functions were measured 24 h after antigen. Values represent the mean $\pm$ S.E.M ($n = 8$ per treatment group). * $P < 0.05$ compared to the sensitized group.

powder inhalation (Chapman et al., 1998). Briefly, the system contains an inhalation chamber with 12 animal exposure ports, a Wright dust feed (BGI, Inc., Waltham, MA) that

generates dry powder aerosols of micronized roflumilast admixed lactose carrier, a respirable dust cyclone (BGI, Inc., Waltham, MA) to remove large particle ($> 10 \mu\text{m}$) material, and a 7 stage cascade impactor (In-Tox Products, Moriarty, NM) to measure particle size distribution of the dry powder. An absolute filter was connected to a vacuum source (flow rate 1 l/min) which in turn was attached to a sampling port on the inhalation chamber. The concentration of dry powder aerosol deposited on the filter was measured over the entire 30 min exposure period. The rats were restrained in polycarbonate tubes (type CHT-250, CH Technologies, Westwood, NJ) designed for the anatomy of rats and exposed, by nose-only inhalation, to the contents of the inhalation chamber. The exposure duration was fixed at 30 min per rat and the dose of inhaled roflumilast was varied with different ratios, where a 1:10 and 1:100 ratio represents 10% and 1% w/w of roflumilast mixed with lactose, respectively. Estimated pulmonary deposition (in the trachea, bronchi and lung parenchyma) was determined from the particle size distribution (Raabe et al. 1988; Hsieh et al. 1999). These values were shown to correlate with the amount of drug that could be extracted from the lungs immediately after the exposure period from sentinel rats ($n = 2$, data not shown).

2.5. Compounds

Roflumilast and albuterol were synthesized at Schering-Plough Research Institute. Betamethasone was purchased from Sigma Chemical Company (St Louis, MO). The compounds were given either by intratracheal administration or by nose-only inhalation. The vehicle for both of these studies was micronized lactose. Compounds were also given by oral gavage and the vehicle for these oral studies was 0.4% methylcellulose.

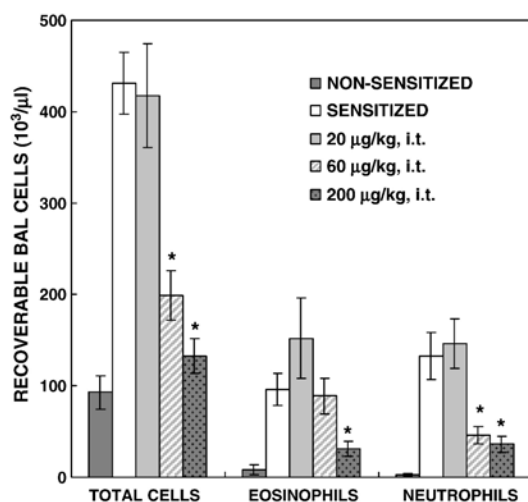


Fig. 2 Reduction in total cells, eosinophils and neutrophils in the bronchoalveolar lavage fluid by intratracheal roflumilast. Compound or lactose vehicle were given 5 h before the antigen challenge and bronchoalveolar lavage fluid cells were measured 24 h after antigen. Values represent the mean $\pm$ S.E.M ($n = 8$ per treatment group). * $P < 0.05$ compared to the sensitized group.

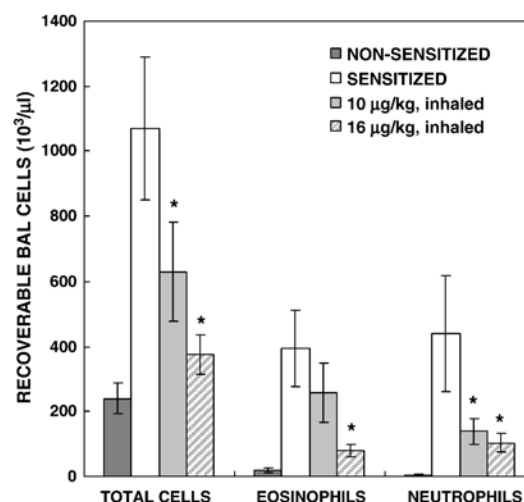


Fig. 3 Reduction in total cells, eosinophils and neutrophils in the bronchoalveolar lavage fluid by inhaled roflumilast delivered by the Battelle nose-only inhalation system. Compound or lactose vehicle were given 5 h before the antigen challenge and lung inflammation was measured 24 h after antigen. Values represent the mean $\pm$ S.E.M ($n = 8$ per treatment group). * $P < 0.05$ compared to the sensitized group.

Two different dosing paradigms were used in these experiments. In studies involving the administration of the drugs before the antigen challenge, compounds or vehicle were given as a single dose 5 h before the aerosolized ovalbumin challenge. Pilot studies with intratracheal roflumilast had found that pretreatment times of 1 and 3 h before the antigen challenge were less effective for the inhibition of antigen-induced reductions in lung function compared to the 5 h pretreatment time. In all studies that involved administration of the drug before the antigen challenge, measurements of lung function and cellular lung inflammation were measured 24 h after the antigen challenge. In studies involving the administration of drugs after the antigen challenge, the compounds or vehicle were given 24 h after the aerosolized ovalbumin challenge and measurements were made at either 1, 5 or 16 h after the drug or vehicle administration.

2.6. Statistical analysis

Values are represented as the mean $\pm$ S.E.M. Statistically significant effects were assessed using Analysis of Variance with post hoc analysis performed by Fisher's Protected Least Significant Difference technique.

3. Results

3.1. Administration before the antigen challenge

Intratracheal roflumilast (20–200 $\mu\text{g/kg}$, i.t.), given as a single dose 5 h before antigen, inhibited the reductions in FVC and PEF that were induced by the antigen challenge (Fig. 1). The ED_{50} of intratracheal roflumilast to inhibit the reduction in FVC was 140 $\mu\text{g/kg}$, i.t. Pretreatment of sensitized rats with intratracheal roflumilast (20–200 $\mu\text{g/kg}$, i.t.) also reduced the number of inflammatory cells in the bronchoalveolar lavage fluid measured 24 h after the antigen challenge (Fig. 2). Effects were seen on both eosinophils and neutrophils. The ED_{50} of intratracheal roflumilast to inhibit the increase in total cells was 50 $\mu\text{g/kg}$, i.t. In the Battelle nose-only inhalation system, inhaled roflumilast inhibited the increase in total cells, eosinophils and neutrophils induced by the antigen challenge (Fig. 3) with an estimated ED_{50} of 10 $\mu\text{g/kg}$ for the respired dose of roflumilast (deposited in the trachea, bronchi and pulmonary parenchyma) to inhibit the number of leukocytes in the bronchoalveolar lavage fluid.

Table 1
Time-dependent reversal of antigen-induced reductions in lung function by intratracheal roflumilast

Time after roflumilast ^a (h)	Percent reversal ^b	
	FVC (ml)	PEF (ml/s)
1	38 $\pm$ 25	5 $\pm$ 31
5	54 $\pm$ 11 ^c	40 $\pm$ 15 ^c
16	71 $\pm$ 13 ^c	81 $\pm$ 11 ^c

^a Roflumilast (600 $\mu\text{g/kg}$, i.t.) given 24 h after antigen challenge.

^b Mean $\pm$ S.E.M. ($n = 8$ per timepoint).

^c $P < 0.05$ compared to lactose (i.t.) treated sensitized, ovalbumin-challenged controls.

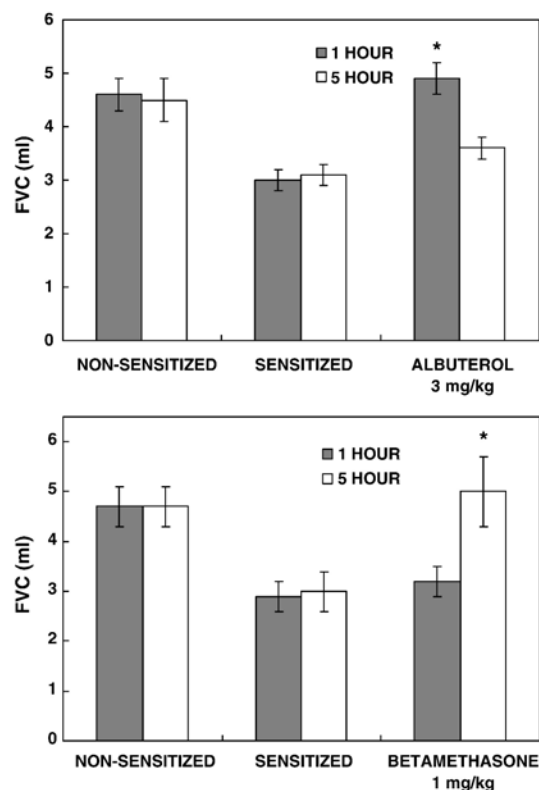


Fig. 4 Improvement in forced vital capacity (FVC) by oral albuterol and betamethasone given 24 h after antigen challenge. Lung function was measured either 1 or 5 h after the drug or vehicle. Values represent the mean $\pm$ S.E.M. ($n = 8$ per treatment group). * $P < 0.05$ compared to the sensitized group.

3.2. Administration after the antigen challenge

Intratracheal roflumilast (600 $\mu\text{g/kg}$, i.t.), given as a single dose 24 h after the antigen challenge, reversed the reductions of FVC and PEF induced by the antigen challenge in a time-dependent

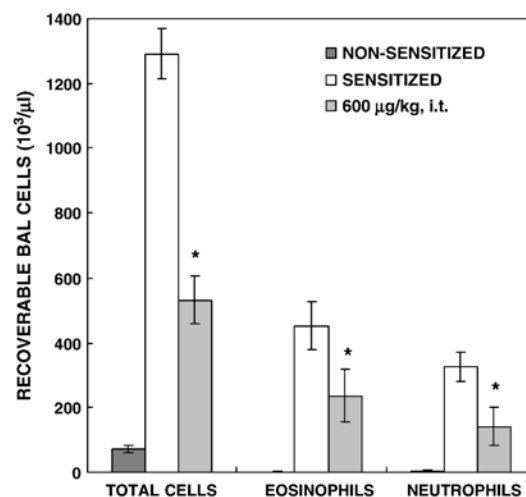


Fig. 5 Reduction in total cells, eosinophils and neutrophils in the bronchoalveolar lavage fluid by intratracheal roflumilast given 24 h after antigen challenge. Number of bronchoalveolar lavage inflammatory cells measured 5 h after the drug or vehicle. Values represent the mean $\pm$ S.E.M. ($n = 8$ per treatment group). * $P < 0.05$ compared to the sensitized group.

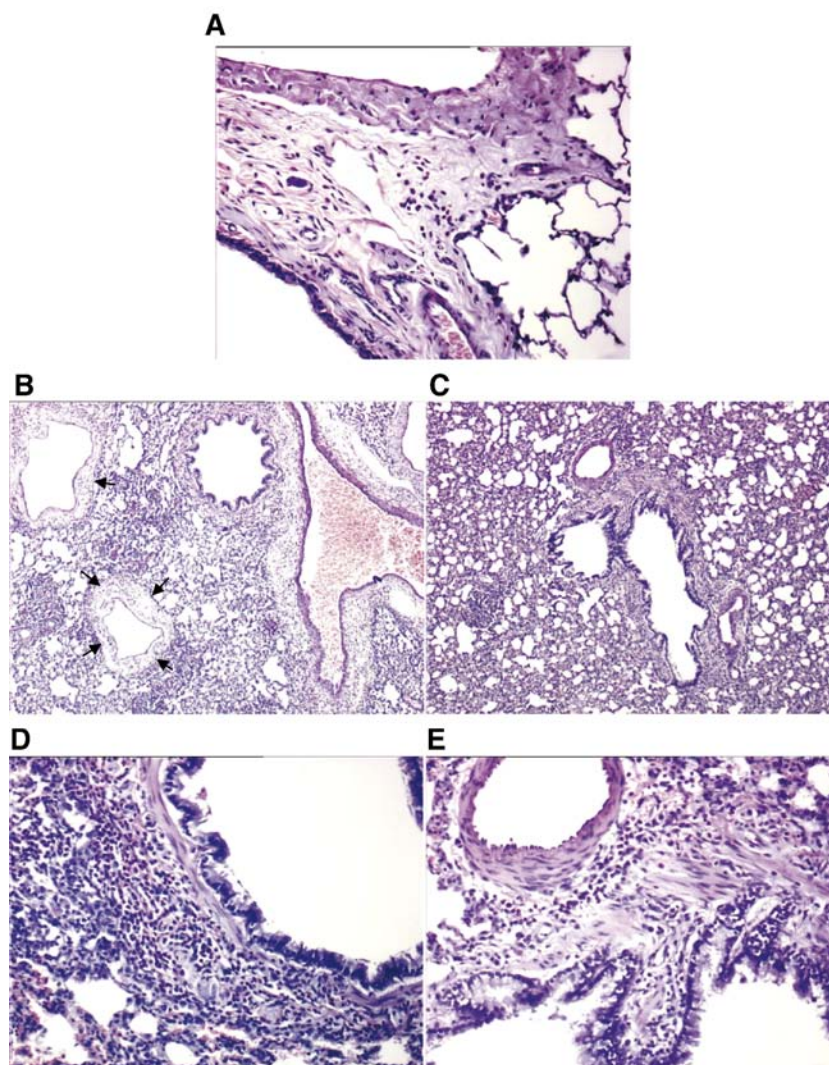


Fig. 6 Light micrograph of Brown Norway rat lungs collected from different treatment groups and stained with H × E. A. A lung section from a non-sensitized animal showing well-inflated alveoli, an intact airway with ciliated epithelium, a blood vessel with no evidence of perivascular edema and an absence of inflammatory cells in the lung tissue (25×). B. A low magnification lung section from an antigen-challenged animal showing evidence of fluid accumulation around the perivascular space (arrows) of two blood vessels and infolding of the airway epithelium (6.25×). C. A low magnification lung section from an antigen-challenged animal 5 h after roflumilast (600 µg, i.t.) showing absence of fluid accumulation around the blood vessel (6.25×). D. A lung section from an antigen-challenged animal showing massive recruitment of eosinophils around the airway (25×). E. A lung section from an antigen-challenged animal showing partial resolution of the tissue eosinophils around the airway (25×).

manner (Table 1). At 1 h after the administration of the intratracheal roflumilast, the reversal of FVC was 38% that increased to 54% by 5 h and to 71% by 16 h. A lower dose of roflumilast (200 µg/kg, i.t.) was inactive (13% reversal at 5 h). Oral albuterol (3 mg/kg), a short acting beta-adrenergic bronchodilator, completely reversed the antigen-induced reductions in FVC at 1 h but was less effective by 5 h (Fig. 4). On the other hand, the oral betamethasone (1 mg/kg) completely reversed the antigen-induced reductions in FVC at 5 h, but was inactive at 1 h (Fig. 4).

To assess its effects on inflammatory cell influx into the bronchoalveolar lavage fluid and histopathological changes in the lungs, intratracheal roflumilast (600 µg/kg, i.t.) was given 24 h after the antigen challenge and studied 5 h after administration of the drug. In this study, roflumilast partially reversed the increase in total cells, eosinophils and neutrophils

that were induced by the antigen challenge (Fig. 5). Histological evaluation of the lung tissue at this time found evidence of antigen-induced perivascular edema that was markedly suppressed in rats treated with intratracheal roflumilast (600 µg/kg, i.t.) (Fig. 6). No fluid accumulation was observed around the airways. There was also evidence of tissue eosinophilia in the antigen-challenged rats that was partially suppressed by roflumilast administration (Fig. 6).

4. Discussion

Roflumilast is an orally active PDE4 inhibitor that has shown promising results in clinical trials in patients with asthma and COPD (Rabe et al., 2005; Lipworth 2005; Bousquet et al., 2006). This compound is a potent and selective inhibitor of the

PDE4 isoenzyme and is orally active in a variety of animal models of inflammatory lung disease, including the allergen-challenged Brown Norway rat (Hatzelmann and Schudt, 2001; Bundshuh et al., 2001). Previous studies with this compound have used the oral route of administration, but in this study, roflumilast was given by the intratracheal and nose-only inhalation routes of administration to assess its effect on the late phase obstruction, cellular lung inflammation and histopathological changes in the lungs of allergen-challenged Brown Norway rats. When it was given before the antigen challenge, roflumilast inhibited both the late phase reductions in lung function and the increase in inflammatory cell influx in the bronchoalveolar lavage fluid. When roflumilast was given after the allergen challenge, it partially reversed the reductions in lung function, the inflammatory cell influx in the bronchoalveolar lavage fluid and the presence of interstitial airway edema that was induced by the antigen challenge.

Late phase airflow obstruction is occasionally seen in a subpopulation of human asthmatics (Stokes Peebles et al., 1998) but routinely occurs in the allergen-challenged Brown Norway rat (Lin et al., 2002; Celly et al., 2006). Although late phase airflow obstruction occurs between 4 and 12 h after antigen challenge in humans, the peak changes in lung mechanics in Brown Norway rats occur between 24 and 72 h after the allergen challenge (Celly et al., 2006). The mechanisms that cause this late phase airflow obstruction in rats involve inflammatory processes, as complete inhibition of this pulmonary response is seen with corticosteroids (Beckmann et al., 2001; Tigani et al., 2003; Celly et al., 2006). Contributing factors probably include the presence of interstitial airway edema, mucus secretion into the airway lumen and release of inflammatory mediators from eosinophils and neutrophils. Each of these factors increases after allergen challenge in Brown Norway rats and the temporal change in each of these parameters is similar to that seen with the reductions in lung function (Beckmann et al., 2001; Tigani et al., 2002; Celly et al., 2006). It is also possible that airway smooth muscle contraction contributes to this response as a significant improvement in lung function was found in our study following treatment with albuterol, a short acting beta-adrenergic bronchodilator.

When roflumilast was given before the antigen challenge by the intratracheal or nose-only routes of administration, it potently inhibited the reduction in lung function and the inflammatory cell influx into the bronchoalveolar lavage fluid. Oral roflumilast is also effective for the inhibition of late phase airflow obstruction and inflammatory cell influx into the lungs of allergen-challenged Brown Norway rats, but a much higher dose is required to produce these effects by the oral route compared to topical delivery to the lungs (Celly et al., 2005). It is likely that inhaled roflumilast would inhibit late phase airflow obstruction in humans as oral roflumilast blocks late phase responses in human asthmatics following antigen challenge (van Schalkwyk et al., 2005).

An important finding in this study was the marked improvement in lung function when intratracheal roflumilast was given after the antigen challenge. The improvement in lung function increased over time with small effects seen at 1 h,

significant reversal of FVC and PEF seen by 5 h with near maximal effects occurring by 16 h after drug administration. In this regard, roflumilast did not act like a beta-adrenergic bronchodilator as we found that oral albuterol improved lung function within 1 h of its administration but was inactive at 5 h. On the other hand, oral betamethasone significantly improved lung functions by 5 h but was inactive at 1 h which is similar to the data obtained with intratracheal roflumilast. These results would support the hypothesis that the improvement in lung function by inhaled roflumilast is related to its anti-inflammatory properties. In support of this concept, there was partial resolution of the perivascular edema and suppression of the influx of inflammatory cells into the bronchoalveolar lavage fluid. It is important to note that in a paradigm where oral roflumilast was given to rats 24 h after the antigen challenge, there was no improvement in lung function 5 h after the administration of the drug (Celly et al., 2005). These results strongly suggest that PDE4 inhibitors are more effective for the improvement of lung function when they are given directly to the lungs compared to their activity by the oral route of administration.

In conclusion, the results of this study demonstrate a robust effect of inhaled roflumilast to improve lung function in the allergen-challenged Brown Norway rat. Importantly, the protective effects of inhaled roflumilast on lung function appear to be superior to its effect when given orally. These results support the hypothesis that inhaled PDE4 inhibitors will be efficacious in inflammatory lung diseases, particularly for the improvement of lung function.

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