

Pharmacological characterization of the late phase reduction in lung functions and correlations with microvascular leakage and lung edema in allergen-challenged Brown Norway rats

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ABSTRACT

Late phase airflow obstruction and reduction in forced vital capacity are characteristic features of human asthma. Airway microvascular leakage and lung edema are also present in the inflammatory phase of asthma, but the impact of this vascular response on lung functions has not been precisely defined. This study was designed to evaluate the role of increased lung microvascular leakage and edema on the late phase changes in forced vital capacity (FVC) and peak expiratory flow (PEF) in allergen-challenged Brown Norway rats using pharmacological inhibitors of the allergic inflammatory response. Rats were sensitized and challenged with ovalbumin aerosol and forced expiratory lung functions (FVC, PEF) and wet and dry lung weights were measured 48 h after antigen challenge. Ovalbumin challenge reduced FVC (63% reduction) and PEF (33% reduction) and increased wet (65% increase) and dry (51% increase) lung weights. The antigen-induced reduction in FVC and PEF was completely inhibited by oral treatment with betamethasone and partially attenuated by inhibitors of arachidonic acid metabolism including indomethacin (cyclooxygenase inhibitor), 7-TM and MK-7246 (CRTH2 antagonists) and montelukast (CysLT₁ receptor antagonist). Antagonists of histamine H₁ receptors (mepyramine) and 5-HT receptors (methysergide) had no significant effects indicating that these pre-formed mast cell mediators were not involved. There was a highly significant ($P < 0.005$) correlation for the inhibition of FVC reduction and increase in wet and dry lung weights by these pharmacological agents. These results strongly support the hypothesis that lung microvascular leakage and the associated lung edema contribute to the reduction in forced expiratory lung functions in antigen-challenged Brown Norway rats and identify an important role for the cyclooxygenase and lipoxygenase products of arachidonic acid metabolism in these responses.

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1. Introduction

Airway microvascular leakage, mucosal edema and infiltration of fluid into the pulmonary parenchyma and airway lumen are key components of the inflammatory response in asthma [1]. The presence of protein-rich fluid in the pulmonary interstitium and airway lumen likely contributes to many of the pathophysiological abnormalities in asthmatic lungs that include decreased lung compliance, closure of peripheral airways, ventilation/perfusion mismatch, increased airway resistance and airway hyper-responsiveness [2,3]. However, detailed studies investigating the relationship between increased airway microvascular leakage and associated changes in lung functions have not been performed,

particularly during the late phase response. Furthermore, although airway microvascular leakage is present in both the early and late phase of allergic asthma, much of the research has been focused on mechanisms and mediators involved in the acute phase [4–6] and less is known about the important mediators in the late phase [7,8].

The aim of the present study was to investigate the role of airway microvascular leakage on the late phase reductions in forced expiratory lung functions in allergen-challenged Brown Norway rats using pharmacological antagonists and enzyme inhibitors of important mediators of the allergic inflammatory response. The allergen-challenged Brown Norway rat was used for this evaluation as it demonstrates a robust late phase pulmonary response to antigen challenge including reductions in forced vital capacity (FVC) and peak expiratory flow (PEF) [9,10] and the presence of increased microvascular leakage and lung edema [4,7,9,11]. The pharmacological agents used in this evaluation were: betamethasone, (corticosteroid), indomethacin (cyclooxygenase inhibitor), MK-

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7246 and 7-TM (chemoattractant receptor-homologous molecule expressed on Th2 cells [CRTH2] antagonist), montelukast (CysLT₁ receptor antagonist), mepyramine (histamine H₁-receptor antagonist) and methysergide (mixed 5-HT₁/5-HT₂ receptor antagonist).

2. Materials and methods

2.1. Animals

Male Brown Norway rats ranging in weight from 150 to 200 g were purchased from Charles River (Kingston, NY, USA). The rats were housed 3 rats/cage in a climate-controlled facility with a 12:12 light:dark cycle. They were given food and water ad libitum except food was withdrawn the night before study. Any animals needing medical attention were provided with immediate veterinary care. All experiments performed in this study were given full approval by the Institutional Animal Care and Use Committee of Merck Research Laboratories (Kenilworth, NJ, USA) which is a facility given accreditation by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

2.2. Sensitization and allergen challenge

Rats were sensitized to ovalbumin by intraperitoneal (i.p.) injection of a suspension of aluminum hydroxide (alum) gel admixed with ovalbumin suspended in 0.9% saline. Each rat received 1 ml of the suspension that contained 8 mg of the alum and 20 µg of the ovalbumin. Non-sensitized rats were injected with 1 ml of alum. Seven days after this injection, the rats were given a second booster injection of the same suspension mixture. The rats were fully sensitized to the ovalbumin by 14 days after the first sensitizing injection. At this time, the rats were placed into 21-L clear Plexiglas chamber and exposed to aerosolized ovalbumin (1%) in 0.9% saline for 30 min. Non-sensitized rats were exposed to aerosolized 0.9% saline for 30 min. The aerosols were generated using an ultrasonic nebulizer (DeVilbiss, Model Ultra-Neb 99) at a flow rate of 8 L/min. A vacuum source was connected to outlet ports in the Plexiglas chamber to balance the aerosol flow through the system.

2.3. Forced expiratory maneuvers

Forced vital capacity (FVC) and peak expiratory flow (PEF) were measured in anesthetized rats using a forced expiratory maneuvers system for rodents (Buxco Research Systems, NC, USA). The preparation of the animals and details of the operation of this system have been previously described in detail [9]. Briefly, rats were anesthetized (sodium pentobarbital, 50 mg/kg, i.p.), the trachea surgically isolated and a catheter was inserted through an incision in the tracheal wall and secured with thread. The rats were placed inside a whole body plethysmograph and the tracheal catheter connected to an outlet port that was situated at the front of the plethysmograph. A specially designed breathing valve was attached to the front of the plethysmograph on the outer connection of the tracheal port. A positive pressure and vacuum source was connected to the breathing valve to facilitate the inflation and deflation of the lungs during the forced expiratory maneuver. A differential pressure transducer (range = ±70 cmH₂O) was connected to an outlet port on the breathing valve to measure intrapulmonary pressure. The transducer was calibrated with a water manometer before the start of each experiment. Airflow was measured with a sensitive differential pressure transducer (range = ±2 cmH₂O) as the pressure drop across a wire mesh screen built into the plethysmograph wall. Volumes were measured by electrical differentiation of the airflow signal using FinePointe Software (Buxco

Research Systems, NC, USA). Volume calibrations were performed with a 5 ml syringe at the start of each experiment.

Following a 5 min equilibration period while the rats were entrained to artificial ventilation (volume = 3 ml, rate = 60 breaths/min), the lungs were rapidly inflated until an intrapulmonary pressure of +20 cmH₂O was reached followed immediately by rapid deflation of the lungs to a negative intratracheal pressure of −40 cmH₂O. From this maneuver, measurement of the FVC and PEF were obtained. Values reported in this report reflect the average FVC and PEF from 3 separate determinations made over a 5 min period.

2.4. Wet and dry lung weights

Wet and dry weights of rat lungs were measured using techniques that have been previously described in detail [9]. Briefly, rats were euthanized with sodium pentobarbital (125 mg/kg, i.p.) and the lungs and trachea were carefully dissected free from the thoracic cavity. The trachea was removed and the wet weight of the lungs was measured. To minimize variability in this assay, the extraction and dissection of the lungs and trachea was performed by the same individual for each experiment. The lungs were then placed in a 60 °C oven for 24 h to completely desiccate the tissue and the dry lung weight was measured. Pilot studies were performed to show that drying the lungs for 48 and 72 h at 60 °C produced no further change in dry lung weight.

2.5. Experimental studies

To determine the effect of drugs on the reduction in FVC and PEF and increase in wet and dry lung weights after antigen challenge, the sensitized rats received the test drugs, given orally for 4 consecutive days around the time of the antigen challenge. A cohort of both sensitized and non-sensitized rats received oral vehicle for 4 consecutive days and served as the vehicle controls. The rats were given drug or vehicle orally for 2 consecutive days before the aerosol ovalbumin or saline challenge, again 2 h before the aerosol challenge on the 3rd day and the final dose 24 h after the aerosol challenge on the 4th day. Lung functions and wet and dry lung weights were measured on the 5th day which is a time point (i.e., 48 h) after ovalbumin-antigen challenge that produces the maximum reduction in FVC and PEF [9].

2.6. Drugs

The following drugs were used in this study: mepyramine (Sigma St. Louis, MO), methysergide (Tocris Bioscience, Minneapolis, MN), montelukast (Merck Research Laboratories, Kenilworth, NJ, USA), indomethacin (Sigma St. Louis, MO), MK-7246 and 7-TM (Merck Research Laboratories, Kenilworth, NJ, USA) and betamethasone (Sigma St. Louis, MO). The doses of mepyramine, methysergide, montelukast, indomethacin and betamethasone used in the present study were based upon previously published data in rats [5,12–14]. MK-7246 [15–17] and 7-TM (compound 19 in Ref. [18]) are potent and selective CRTH2 receptor antagonists and the oral doses of these compounds used in these studies was based upon data generated at Merck Research Institute demonstrating significant inhibition of pulmonary eosinophilia induced by allergen-challenged in Brown Norway rats (unpublished observations).

2.7. Statistics

Statistical analysis was performed using a two-way analysis of variance using Fisher's PLSD to determine significant differences between the treatment groups. A *P*-value < 0.05 was considered as

significantly different. The results are expressed as the mean $\pm$ SEM.

3. Results

3.1. Effects of inhaled antigen

Allergen challenge to sensitized rats significantly ($p < 0.05$) reduced both FVC and PEF to values of 63 and 33% less than the values seen in the non-sensitized controls (Fig. 1). Both wet and dry lung weights were of greater magnitude in the sensitized, challenged rats averaging 65% and 51% more than that seen in the non-sensitized animals (Fig. 1). The wet:dry lung weight ratio was 5.45 ± 0.23 in sensitized challenged rats which was significantly ($P < 0.05$) greater than that (4.69 ± 0.043) in non-sensitized saline challenged rats.

3.2. Effect of drugs on responses to antigen challenge

Treatment of sensitized rats with betamethasone (1 mg/kg/day) completely blocked the reductions in FVC and PEF and the increase in wet and dry lung weights due to the antigen challenge (Fig. 2). Partial inhibition of both the FVC reduction and the increase in wet lung weight was seen after 4 consecutive daily oral doses of indomethacin, (10 mg/kg/day), 7-TM and MK-7246 (30 mg/kg/day) and montelukast (10 mg/kg/day) (Fig. 3). The inhibition of PEF and dry lung weights by these drugs was of similar magnitude to that seen

with FVC and wet lung weight, respectively (Table 1). On the other hand, mepyramine (10 mg/kg/day) and methysergide (30 mg/kg/day) had no significant effect on both the reduction in FVC and the increase in wet lung weights due to antigen challenge (Fig. 4) or on the decrease in PEF and the increase in dry lung weight (Table 1).

There was a highly significant ($P < 0.005$) correlation between reductions in FVC and the increases in wet and dry lung weights when the results were analyzed as the percent inhibition due to the drugs (Fig. 5). The correlation coefficient for FVC and wet lung weight was $r^2 = 0.784$ and for FVC and dry lung weight was $r^2 = 0.825$. There was also a highly significant positive correlation between the inhibition of reductions in PEF and increases in wet and dry lung weights with these drugs with correlation coefficients of $r^2 = 0.843$ and $r^2 = 0.919$ for wet and dry lung weights, respectively.

4. Discussion

The results of this study demonstrate a reduction in FVC and PEF and an increase in wet and dry lung weights in antigen-challenged Brown Norway rats. These results confirm previous findings in Brown Norway rats of a robust late phase response to antigen challenge with a reduction in FVC and PEF and an increase in lung edema associated with increased microvascular leakage [4,7,9–11]. To investigate whether these vascular responses to the antigen challenge contributed to the associated reductions in lung function, experiments were performed with a variety of pharmacological

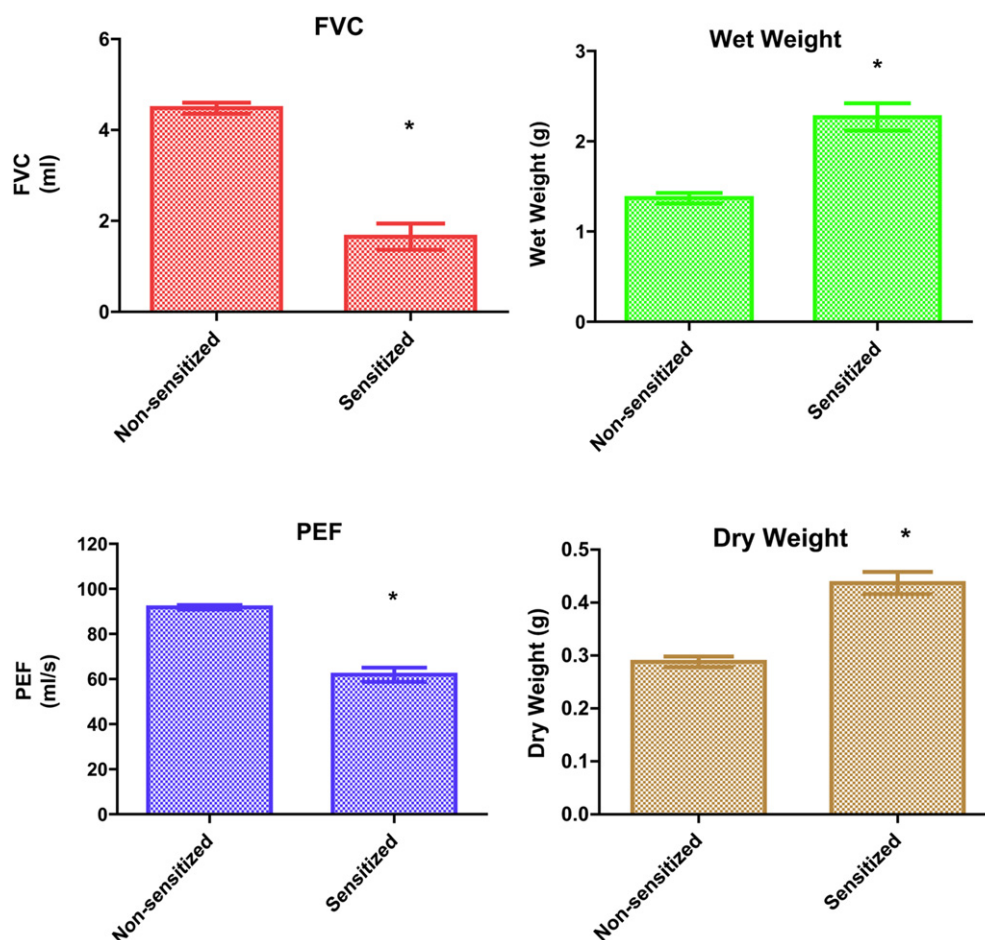


Fig. 1. Reduction in FVC and PEF and increase in lung wet and dry lung weights in ovalbumin-challenged sensitized rats. Values mean $\pm$ SEM ($N = 8$ per group). * $P < 0.05$ compared to non-sensitized rats.

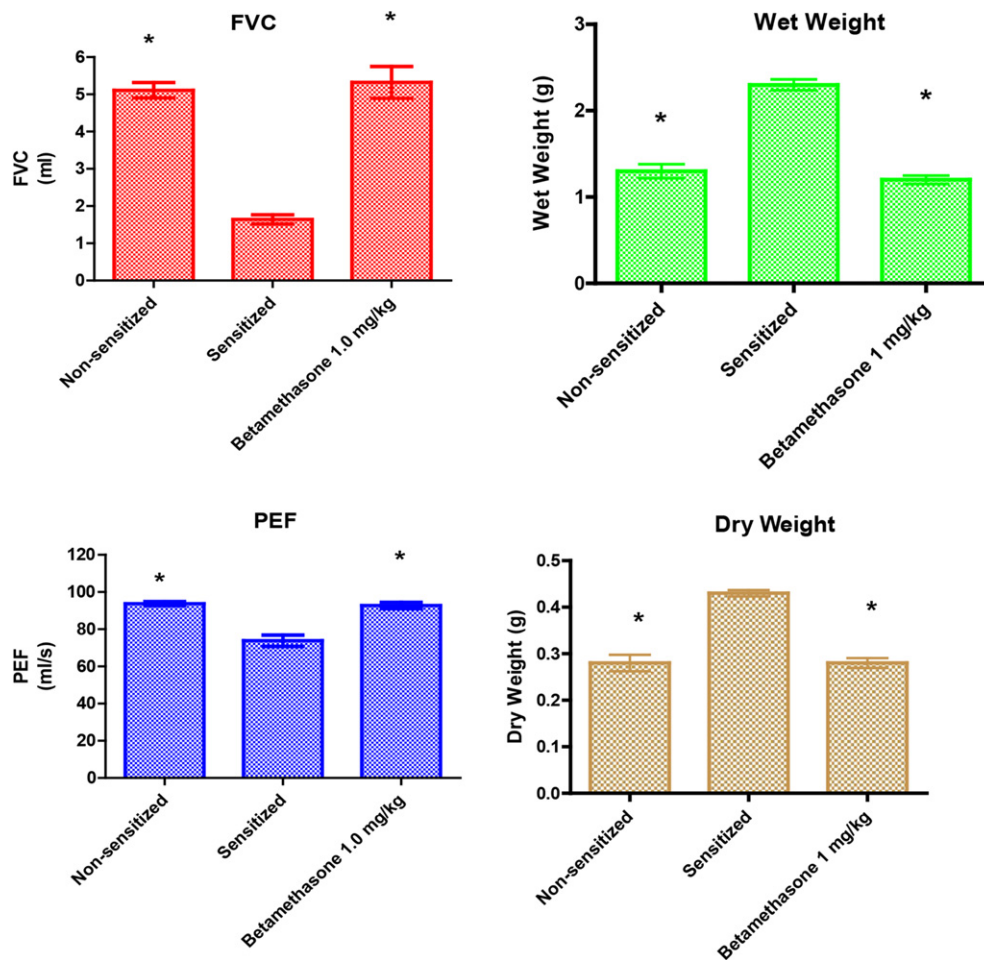


Fig. 2. Blockade of antigen-induced reduction in FVC and PEF and increase in lung wet and dry lung weights with oral betamethasone (1 mg/kg/day). Betamethasone or vehicle were given orally to sensitized rats for 2 consecutive days before the antigen challenge, again 2 h before the antigen challenge on the 3rd day and the final dose was given 24 h after antigen challenge on the 4th day. Lung functions and wet and dry lung weights were measured on the 5th day (i.e. 48 h after antigen challenge. Values mean $\pm$ SEM ($N = 8$ per group). * $P < 0.05$ compared to sensitized, vehicle-treated rats.

agents targeted to some the important allergic and inflammatory mediators of the allergic response in rats and correlations were made between the magnitude of the inhibition of airway microvascular leakage and inhibition of reductions in FVC and PEF. There was a strong positive correlation between inhibition of FVC and PEF and inhibition of the increase in lung and dry lung weights with these drugs with complete blockade of these response with betamethasone (corticosteroid), partial blockade with indomethacin (cyclooxygenase inhibitor), 7-TM, MK-7246 (CRTH2 antagonists) and montelukast (CysLT₁ receptor antagonist) and no significant effects with mepyramine (histamine H₁-receptor antagonist) and methysergide (mixed 5-HT₁/5-HT₂ receptor antagonist). These results strongly suggest that the airway microvascular leakage occurring during the late phase of an allergen challenge contribute to the reductions in lung function and identify an important role for products of arachidonic acid metabolism in these late phase allergic responses.

A variety of techniques have been used to measure the presence of edema and microvascular leakage in the lungs [7–9,19–22]. In the present study, a gravimetric method [9] was used to measure the changes in wet and dry lung weights and the wet:dry lung weight ratio after antigen challenge. Our previous experience with this technique demonstrated an increase in wet:dry lung weight ratio 24 h after antigen challenge in Brown Norway rats [9]. In the

present study, measurements were made 48 h after the antigen challenge which is a time point that demonstrates histological evidence of interstitial lung edema, large numbers of inflammatory cells in the lungs and evidence of airway remodeling [9]. We found large increases in both the wet lung weight and the wet:dry lung weight ratio indicating increased water content in the lungs 48 h after the antigen challenge. These findings are supported by observations made in studies using magnetic resonance imaging to measure the presence of lung edema in the lungs of Brown Norway rats after antigen challenge [7,11,22]. There was also a large increase in the dry lung weight which most probably reflects the presence of large macromolecules (collagen, fibrin), vascular proteins and inflammatory cells as they migrate from blood vessels into the lungs. Similar conclusions have been reached in studies measuring wet and dry lung weights in allergen-challenged rats [8].

To investigate the role of increased lung microvascular leakage and lung edema to the reductions in FVC and PEF, experiments were performed with 6 different classes of drugs targeted to some of the important mediators of the allergic response in rats [4,13,23–25]. Previous studies have found that oral and inhaled corticosteroids inhibit the reductions in FVC and PEF and the increased lung edema due to antigen challenge in Brown Norway rats [9–11,22,26,27], so it came as no surprise that oral betamethasone given for 4 consecutive days completely blocked the reductions in

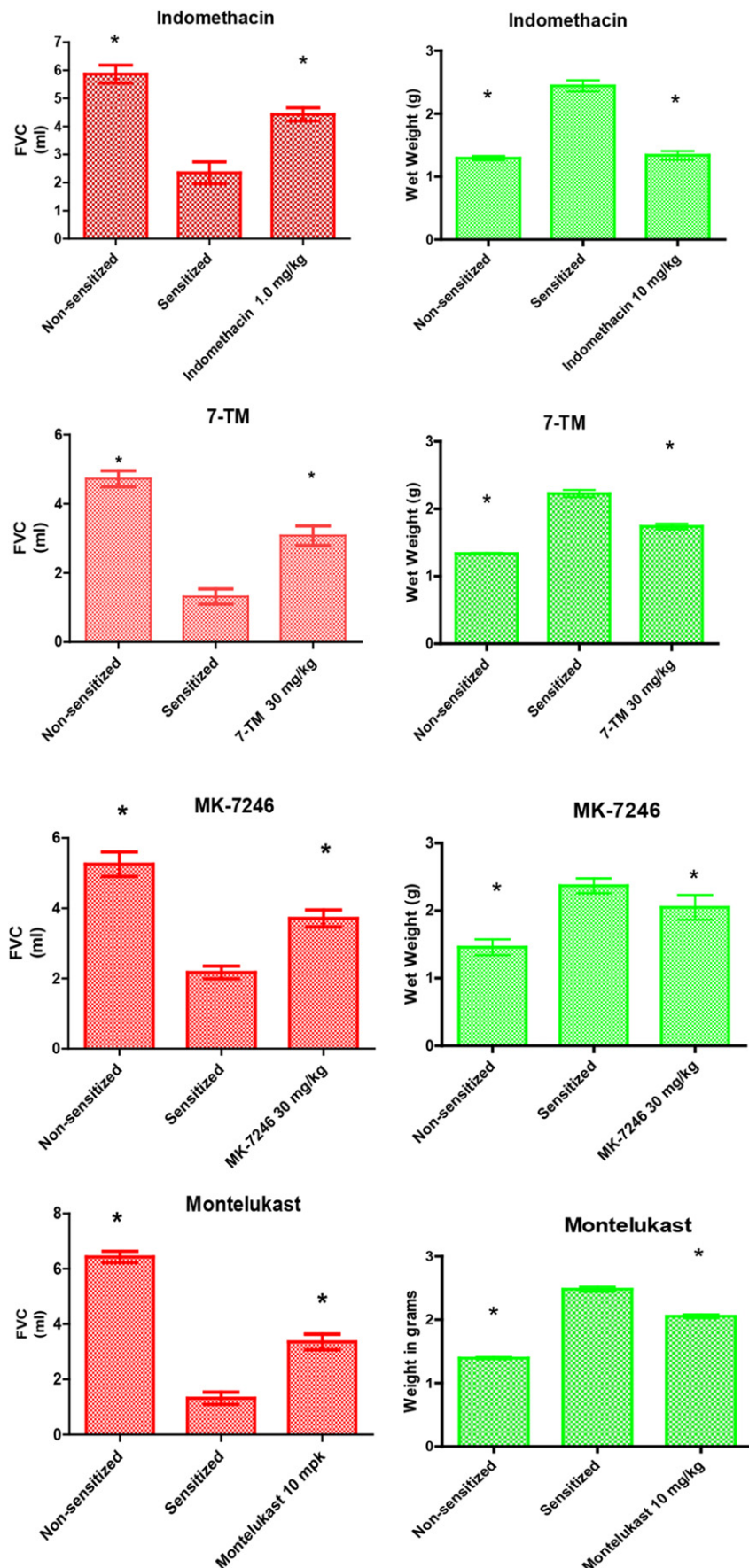


Fig. 3. Effect of oral indomethacin, 7-TM, MK-7246 and montelukast on antigen-induced reduction in FVC and increase in wet lung weight in ovalbumin-sensitized rats. The dosing paradigm used for these drugs was identical to that described in Fig. 2. Values mean $\pm$ SEM ($N = 8$ per group). * $P < 0.05$ compared to sensitized, vehicle-treated rats.

Table 1

Inhibition (%) of the reduction in FVC and PEF and the increase in wet and dry lung weights by 7 different pharmacological agents. Values represent the mean and $\pm$ SEM where noted.

Compound	% inhibition			
	FVC ($\pm$ SEM)	PEF ($\pm$ SEM)	Wet Wt	Dry Wt
Betamethasone	105 ($\pm$ 10)	94 ($\pm$ 9)	100	100
Indomethacin	61 ($\pm$ 7)	74 ($\pm$ 8)	96	84
7-TM	52 ($\pm$ 8)	76 ($\pm$ 6)	57	56
MK-7246	37 ($\pm$ 15)	39 ($\pm$ 22)	35	31
Montelukast	32 ($\pm$ 6)	29 ($\pm$ 3)	36	25
Methysergide	29 ($\pm$ 13)	30 ($\pm$ 10)	5	0
Mepyramine	0 ($\pm$ 0)	0 ($\pm$ 0)	0	0

lung function and the increase in wet and dry lung weight induced by the antigen challenge in this study. Prostaglandins and leukotrienes are important mediators of the allergic response in most species, including rats [4,25,28,29] and humans [30–32] and in our study, both indomethacin, a COX1/COX2 inhibitor and montelukast, a CysLT₁ receptor antagonist, were active against both the reductions in lung function and the airway microvascular leakage indicating an important role for products of the cyclooxygenase and lipoxygenase pathways in these responses.

Prostaglandin D₂ is a major prostaglandin produced by mast cells and is an important mediator of allergic asthma [31,32]. Two G-protein coupled receptors (DP₁ and DP₂ (CRTH2) receptors) mediate the important actions of PGD₂ with vasodilation primarily associated with activation of the DP₁ receptor and recruitment and activation of inflammatory cells e.g. Th2 cells, eosinophils, basophils, as the primary mode of action from stimulation of the CRTH2 receptor [32]. In our studies, 7-TM (identified as compound 19 in Ref. [18]) and MK-7246 [15–17], two potent and selective CRTH2 receptor antagonists inhibited both the reduction in lung functions and the airway microvascular leakage induced by the antigen challenge. These results identify an important role for PGD₂ acting via the CRTH2 receptor in these late phase responses. It is likely that the action of CRTH2 receptor activation ultimately involves release of vasoactive pulmonary mediators from CRTH2 receptor bearing inflammatory cells, but additional experiments will be required to identify the important mediators and mechanisms involved in these responses. It is important to note that the findings in our study in rats are supported by data in allergic sheep showing inhibition of the late phase bronchoconstriction by MK-7246 [15] indicating the important role for PGD₂ in the late phase airflow obstruction occurring after antigen challenge. In our experiments, it appears that 7-TM was slightly more efficacious than MK-7246 to

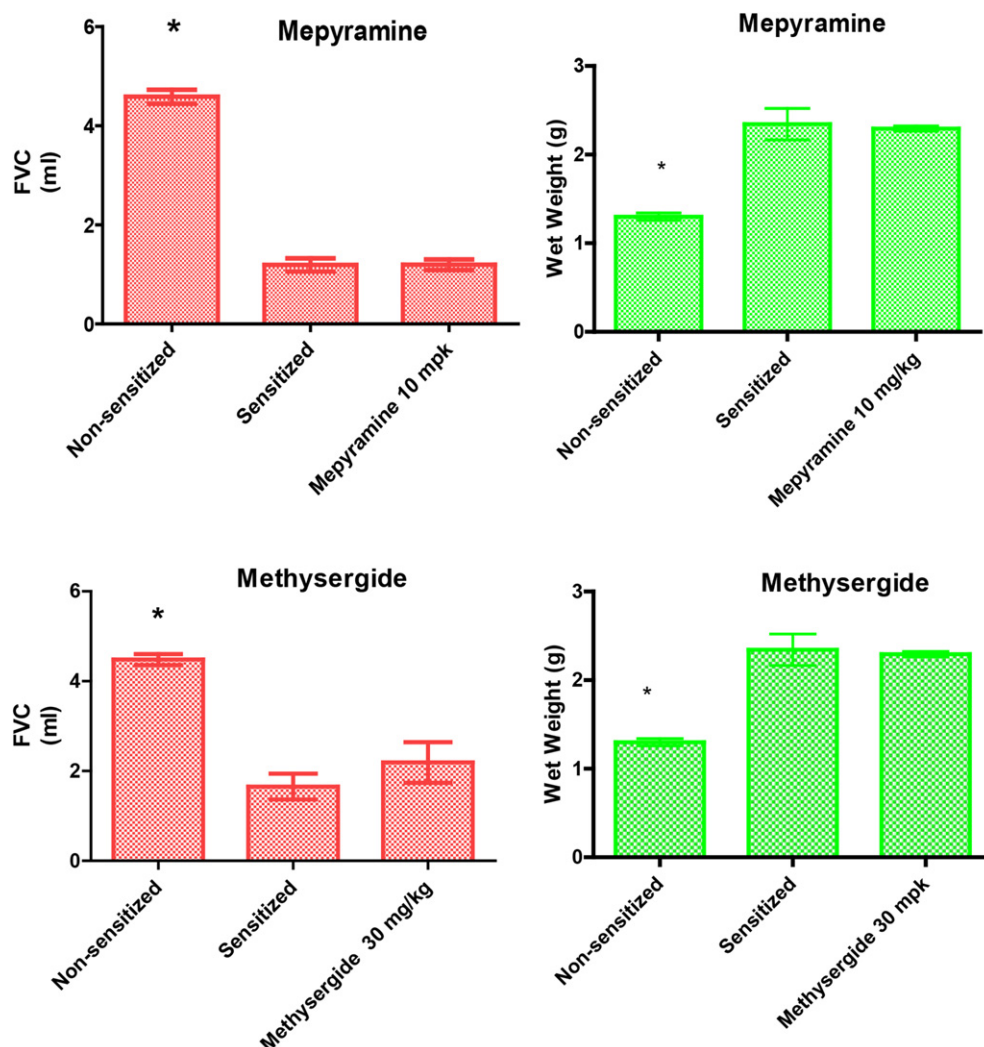


Fig. 4. Effect of oral mepyramine and methysergide on antigen-induced reduction in FVC and increase in wet lung weight in ovalbumin-sensitized rats. The dosing paradigm used for these drugs was identical to that described in Fig. 2. Values mean $\pm$ SEM ($N = 8$ per group). * $P < 0.05$ compared to sensitized, vehicle-treated rats.

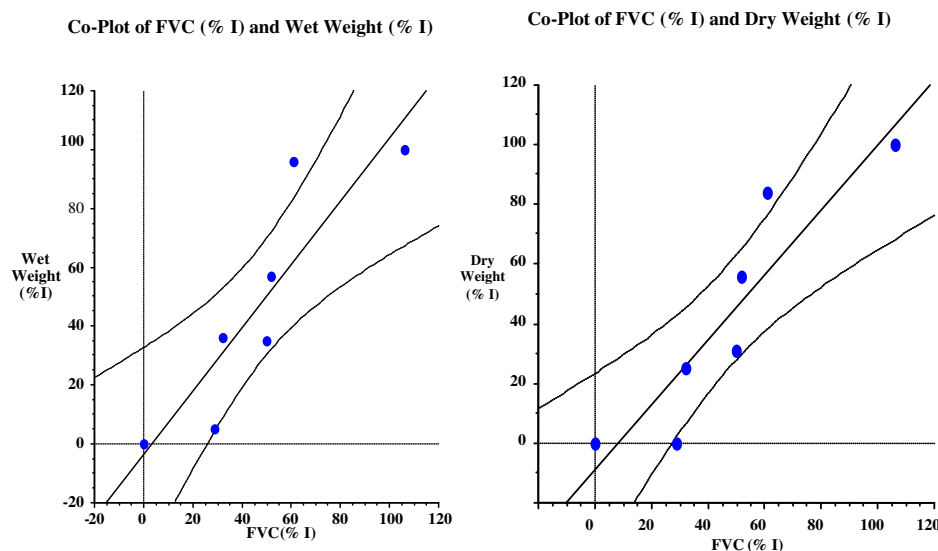


Fig. 5. Inhibition (%) of the antigen-induced reduction in FVC and increases in wet and dry lung weights produced by the 7 different drugs used in this study. There was a highly significant ($P < 0.005$) correlation between the FVC and wet and dry lung weights (correlation coefficients (r^2) of 0.784 and 0.825, respectively) after drug treatment.

inhibit the late phase responses in rats. Additional studies will be required to dissect out mechanisms for this difference as both compounds are potent and selective CRTH2 receptor antagonists [16,17,33] with good oral bioavailability for in vivo studies [15,18].

We also investigated the role of the pre-formed mast cell mediators, histamine and 5-HT on the late phase responses based upon previously published data showing that these mast cell mediators are involved in both acute phase [4,23,24] and late phase [34] reactions to antigen challenge. However, our results find no statistically significant effects with mepyramine, a histamine H_1 -receptor antagonist and methysergide, a mixed 5-HT $_1$ /5-HT $_2$ antagonist on the antigen-induced late phase reduction in lung functions and the airway microvascular leakage indicating that these mast cell mediators play no major role in these responses. Serotonin is a potent bronchoconstrictor in rodents [23,35,36] and there may be some residual effects of this mediator to reduce lung function during the late phase response as there was a small inhibitor effect of methysergide on the reductions in FVC and PEF. However, methysergide had no effect on the airway microvascular leakage which leaves us to conclude that serotonin has very little role on the late phase responses measured in our studies in rats.

There were highly significant correlations between the reduction in lung functions and the airway microvascular leakage when the data were expressed as the inhibition due to the drugs (Fig. 5). These results identify an important link between vascular leakage in the airways and reductions in forced expiratory lung functions. The mechanisms involved in this response are likely to involve decreased lung compliance which has been previously demonstrated in allergen-challenged rats [9]. Under conditions of reduced lung compliance, exposure of the airways to a positive intrapulmonary pressure of +20 cmH $_2$ O will result in a lower volume of lung inflation and when this is followed by a negative intrapulmonary pressure -40 cmH $_2$ O, the total volume of air evacuated from the lungs (FVC) will be reduced. Respiratory system resistance is also increased in the late phase of allergen challenge in rats [9] and this, along with reduced elastic recoil from a lower level of lung inflation, will factor into the reduced PEF during the late phase response. It is important to note that the relative reduction of FVC (63% reduction) was greater than the relative reduction in PEF (33% reduction) after antigen challenge which suggests that the major

impact of the fluid movement into the lungs is on reduced lung compliance and lower lung volumes.

In conclusion, the results from this study in allergen-challenged Brown Norway rats has identified an important role for the airway microvascular leakage and lung edema contributing to the late phase reductions in forced expiratory lung functions. Our results suggest that drugs that inhibit the late phase airway microvascular leakage, such as corticosteroids, CysLT $_1$ receptor antagonists and CRTH2 receptor antagonists, are likely to have significant benefit for the improvement in lung functions in asthmatics.

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