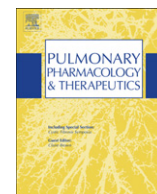


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A comparison of antiasthma drugs between acute and chronic ovalbumin-challenged guinea-pig models of asthma

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ABSTRACT

Pre-clinical evaluation of asthma therapies requires animal models of chronic airways inflammation, airway hyperresponsiveness (AHR) and lung remodelling that accurately predict drug effectiveness in human asthma. However, most animal models focus on acute allergen challenges where chronic inflammation and airway remodelling are absent. Chronic allergen challenge models have been developed in mice but few studies use guinea-pigs which may be more relevant to humans. We tested the hypothesis that a chronic rather than acute pulmonary inflammation model would best predict clinical outcome for asthma treatments. Guinea-pigs sensitized with ovalbumin (OVA) received single (acute) or nine OVA inhalation challenges at 48 h intervals (chronic). Airways function was recorded as specific airways conductance (sG_{aw}) in conscious animals for 12 h after OVA challenge. AHR to inhaled histamine, inflammatory cell influx and lung histology were determined 24 h after the single or 9th OVA exposure. The inhaled corticosteroid, fluticasone propionate (FP), the phosphodiesterase 4 inhibitor, roflumilast, and the inducible nitric oxide synthase (iNOS) inhibitor, GW274150, orally, were administered 24 and 0.5 h before and 6 h after the single or final chronic OVA exposure. Both models displayed early (EAR) and late (LAR) asthmatic responses to OVA challenge, as falls in sG_{aw} , AHR, as increased histamine-induced bronchoconstriction, and inflammatory cell influx. Tissue remodelling, seen as increased collagen and goblet cell hyperplasia, occurred after multiple OVA challenge. Treatment with FP and roflumilast inhibited the LAR, cell influx and AHR in both models, and the remodelling in the chronic model. GW274150 also inhibited the LAR, AHR and eosinophil influx in the acute model, but not, together with the remodelling, in the chronic model. In the clinical setting, inhaled corticosteroids and phosphodiesterase 4 inhibitors are relatively effective against most features of asthma whereas the iNOS inhibitor GW274150 was ineffective. Thus, while there remain certain differences between our data and clinical effectiveness of these antiasthma drugs, a chronic pulmonary inflammation guinea-pig model does appear to be a better pre-clinical predictor of potential asthma therapeutics than an acute model.

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1. Introduction

Asthma is a chronic inflammatory airways disorder, yet most pre-clinical models concentrate on acute inflammation following

allergen exposure [1]. There are a number of potentially important differences between acute and chronic inflammation. The acute inflammatory response is limited to the proximal airways and is not associated with tissue remodelling [2]. The cytokine profile of the lungs also varies with the number of allergen challenges [3]. These differences might explain why drug testing using current pre-clinical animal models does not always translate into the clinic, resulting in substantial losses in time and money.

Several studies in mice have used multiple exposures to allergen to induce chronic inflammation in which structural changes to the airways have been observed which are inhibited by corticosteroids and phosphodiesterase inhibitors [4–6]. However, there have been very few studies performed in guinea-pigs using chronic exposures

Non-standard abbreviations: AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; EAR, early asthmatic response; FP, fluticasone propionate; HDM, house dust mite; iNOS, inducible nitric oxide synthase; LAR, late asthmatic response; NO, nitric oxide; OVA, ovalbumin; PDE4, phosphodiesterase type 4.

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to allergen. The relative merits of mice and guinea-pigs for pre-clinical asthma studies are debatable, each having its own advantages. An advantage of guinea-pigs for asthma modelling is that its pharmacology more closely resembles that of humans [7,8]. For example, the airways are responsive to histamine and the early asthmatic response is mediated largely via mast cells releasing histamine rather than 5-hydroxytryptamine in rats and mice [9]. Previous studies have demonstrated airways remodelling and inflammation after multiple allergen challenges in guinea-pigs [8,10–14]. However, there were a number of limitations in these studies which our study hopes to address. Firstly, we can assess airways function, inflammatory cell influx and lung histology all in the same guinea-pig. Secondly, we can detect early (EAR) and late asthmatic responses (LAR) after allergen challenge and thirdly, our chronic exposure protocol only takes 32 days to complete. Thus, there is a need for a chronic lung inflammation model of asthma in guinea-pigs, in which remodelling, airways function changes and inflammation are measured simultaneously and responses to standard antiasthma drugs mimic those seen in humans.

This study compares drug responses in an established guinea-pig acute ovalbumin (OVA) model of asthma [15] with exposure to repeated OVA challenges. Inflammatory cell influx into the lungs, airways hyperreactivity (AHR), EAR, LAR and airway remodelling were assessed. The ability of the inhaled corticosteroid, fluticasone propionate (FP), the phosphodiesterase type 4 (PDE4) inhibitor, roflumilast, and the highly selective inducible nitric oxide synthase (iNOS) inhibitor, GW274150, to affect these parameters was assessed. FP is used clinically in asthma and inhibits the LAR, AHR, eosinophil influx [16] and airway remodelling, measured as angiogenesis [17]. Roflumilast has received European marketing approval for the treatment of severe chronic obstructive pulmonary disease and attenuates AHR [18] in humans with a modest reduction of the LAR [19], but not of EAR [18]. GW274150 is not used clinically. It has been shown to inhibit AHR, LAR and cellular infiltration in acute animal models of asthma [20] but was ineffective in clinical trials [21].

The study aimed to identify any differences in these three anti-inflammatory antiasthma drugs in single or multiple allergen challenged guinea-pigs. Multiple-challenge would provide a closer model to human asthmatics, even though they have been exposed to allergen for many years. We selected two anti-inflammatory drugs with proven effectiveness against human asthma, and GW274150 which was effective in acute animal models of pulmonary inflammation but not in human asthma.

2. Materials and methods

2.1. Ovalbumin sensitization and challenge

Male Dunkin-Hartley guinea-pigs (Harlan, UK) (200–250 g) were sensitized with i.p. OVA (100 µg) and Al(OH)₃ (100 mg) on days 1 and 5. On day 15, guinea-pigs following the acute protocol were challenged with inhaled OVA (0.01%, 1 h) or saline [15]. Guinea-pigs following the chronic protocol received a further 8 exposures of OVA (0.1%) or saline every 48 h. All but the last challenge was under mepyramine cover (30 mg/kg i.p.) to prevent fatal anaphylaxis (Fig. 1). All procedures were performed in compliance with the Animals (Scientific Procedures) Act 1986 and underwent ethical review by Cardiff University Biological Standards Committee. The ARRIVE Guidelines on Animal Research: Reporting *In Vivo* Experiments have been adhered to in the design and execution of the study.

2.2. Measurement of lung function

Specific airways conductance (sG_{aw}) was measured by whole-body plethysmography in conscious guinea-pigs. Guinea-pigs

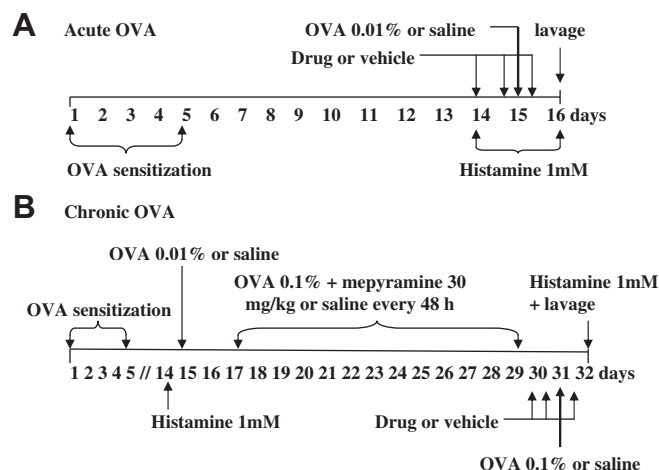


Fig. 1. Schematic diagram of the protocols for drug treatments and sensitization and challenge of guinea-pigs with OVA by a single acute exposure (A) and by repeated multiple exposures (B).

were placed in a restrainer with a face mask placed over the snout and slid into a sealed plethysmograph chamber. Two UP pressure transducers (Pioden Controls Ltd, Canterbury, Kent, UK) measured the changes in pressure associated with air flow across the pneumotachograph (Mercury FIL) (UP1) and box pressure (UP2), by means of a computerised data acquisition system (Biopac systems, Santa Barbara, CA, USA) using AcqKnowledge® software. Recordings were taken before (baseline) and at 0, 15, 30, 45 and 60 min, then hourly until 12 h and at 24 h after a single OVA exposure in acute experiments and at the same intervals after the final chronic OVA exposure. Before each experiment the animals were handled and familiarized with the equipment to reduce stress.

Airways hyperresponsiveness (AHR) was determined from the bronchoconstrictor response to nebulised histamine (1 mM for 20 s) administered 24 h before the first OVA challenge and 24 h after the single OVA challenge or 24 h after the final chronic OVA challenge. sG_{aw} was measured before and at 0, 5 and 10 min after histamine.

2.3. Bronchoalveolar lavage (BAL)

Following the second histamine challenge and recording the bronchoconstrictor response (at least 20 min after histamine challenge), guinea-pigs were killed by overdose with sodium pentobarbitone (Euthatal i.p. 400 mg/kg). The trachea was then cannulated and the lungs removed before lavaging one half with saline (1 ml/100 g guinea-pig) twice. Since the baseline sG_{aw} had recovered before administration of Euthatal, the airways could be considered to be not constricted at the time of lavage. Total cells were counted in the pooled samples with a Neubauer haemocytometer and differential cell counts after Cytospin centrifugation of 100 µl of BAL fluid onto microscope slides and staining with 0.15% Leishman's solution in methanol.

2.4. Histology

Following lavage of one half-lung, the other half-lung was inflated with 10% buffered formaldehyde via the cannula and stored in 10% formaldehyde. 3–5 mm thick portions of the left superior lobe were sliced 1 mm below the bronchus and processed into wax blocks. 6 µm sections were fixed onto glass slides and stained with haematoxylin and eosin for general morphology, with picosirius

red for collagen or with Alcian blue/periodic acid Schiff (AB/PAS) for mucus.

2.5. Data analysis

Changes in sG_{aw} from baseline were expressed as a percentage of the baseline value preceding OVA or histamine challenge. As the maximum bronchoconstriction of the LAR occurred at different times in each animal, peak bronchoconstrictions between 7 and 12 h were also displayed along with the peak of the early phase (0–6 h). The area under curve (AUC) was calculated (%·hr) for the total 24 h time-course following OVA challenge and separately for the EAR (0–6 h) and the LAR (6–24 h). Total and differential inflammatory cell counts per ml of the recovered BAL fluid were recorded.

Collagen was quantified in the picosirius red-stained histology sections by drawing lines around the base of the epithelium (a) and around the outside of the lamina propria which stains predominantly red (b). Collagen was then calculated as $100 - [(a/b) \times 100]$. Epithelial area was measured in the AB/PAS stained sections and mucin-associated stained goblet cells were counted on the digital slide of the section. AB/PAS positive points were expressed per 10,000 epithelial pixels as the number of AB/PAS positive points/epithelial area $\times 10,000$. Thus, both collagen and mucin-associated stained goblet cells were expressed as a factor of the bronchiolar circumference. All values are the means $\pm$ SEM of n animals. Two groups were compared statistically by a two-tailed Student's t -test and three or more groups by one-way analysis of variance (ANOVA) followed by a Bonferroni post test. $P < 0.05$ was considered significant. Graphs were plotted with GraphPad Prism 5 (San Diego, CA) and results analysed with GraphPad Instat 3.

2.6. Drug administration

Fluticasone propionate (0.51 mg/ml nebulizer concentration) was administered by aerosol and roflumilast (1 mg/kg) and GW274150 (5 mg/kg, as the phosphate) were administered orally. They were administered 24 and 0.5 h before and 6 h after the acute OVA challenge or the last of the chronic OVA exposures.

2.7. Materials

Aluminium hydroxide, dimethyl sulfoxide, histamine diphosphate, mepyramine maleate, methyl cellulose and ovalbumin were obtained from Sigma–Aldrich, Pool, UK. Sodium pentobarbitone (Euthetal) was purchased from Merial, Harlow, UK. Fluticasone propionate, roflumilast and GW274150 were supplied by GlaxoSmithKline, Stevenage, Hertfordshire, UK. Fluticasone propionate was dissolved in saline:DMSO:ethanol (40:30:30). Roflumilast and GW274150 ([2-[(1-iminoethyl) amino]ethyl]-L-homocysteine) (as the phosphate) were dissolved in methyl cellulose (0.1% in sterile water). All other drugs and materials were dissolved in saline purchased from Baxter Healthcare, Newbury, UK.

3. Results

3.1. Early and late asthmatic responses

A single acute OVA challenge of OVA-sensitized guinea-pigs caused a significant bronchoconstriction, seen as a fall in sG_{aw} . This early asthmatic response (EAR) resolved to baseline after 5 h followed at 7 h by a second significant bronchoconstriction representing the late asthmatic response (LAR), which recovered to baseline by 8 h. The vehicles for FP (saline:DMSO:EtOH) and for roflumilast and GW274150 (methyl cellulose 0.1% in sterile water) had no effect on these responses (Fig. 2A, B). In OVA-sensitized

guinea-pigs receiving multiple OVA challenges, the final challenge also produced EAR and LAR, which were unaffected by treatment with these vehicles (Fig. 3A, B). Saline challenge had no significant effect on sG_{aw} (Data not shown).

Neither FP nor its vehicle affected the EAR in guinea-pigs receiving a single OVA challenge but FP significantly reduced the LAR by $83.4 \pm 10.9\%$ (Fig. 2A). Following the final chronic OVA exposure, an immediate bronchoconstriction was observed in FP-treated guinea-pigs, but the LAR was inhibited by $87.8 \pm 4.3\%$ by FP (Fig. 3A).

In guinea-pigs receiving a single OVA exposure, roflumilast significantly reduced the immediate bronchoconstriction by $45.0 \pm 6.6\%$ (Fig. 2B). The second bronchoconstriction seen in vehicle-treated guinea-pigs was significantly inhibited by $88.8 \pm 3.7\%$ after roflumilast treatment (Fig. 2B). In chronic OVA-challenged guinea-pigs, the early and late bronchoconstrictions after the final OVA exposure were significantly reduced by roflumilast treatment by $36.7 \pm 14.0\%$ and $85.0 \pm 2.5\%$, respectively (Fig. 3B).

In the acute OVA-challenged guinea-pigs treated with GW274150, the EAR was unchanged, but the LAR observed in the vehicle-treated group was significantly inhibited by $86.8 \pm 6.0\%$ (Fig. 2C). However, treatment with GW274150 did not inhibit either the EAR or LAR after the final OVA challenge in chronically OVA-exposed guinea-pigs (Fig. 3C).

3.2. Airway hyperresponsiveness

Before OVA challenge, histamine (1 mM) did not cause bronchoconstriction (Fig. 4). However, 24 h after single OVA challenge or the last chronic OVA challenge, guinea-pigs displayed significant bronchoconstriction to histamine indicating airways hyperresponsiveness (AHR). No bronchoconstriction occurred 24 h after acute or chronic saline challenges. After treatment with the vehicles for FP, roflumilast and GW274150 the bronchoconstrictor responses to histamine and therefore the AHR persisted. However, FP and roflumilast treatment abolished the bronchoconstrictions after acute and chronic OVA. GW274150 also blocked the AHR in the acute model (Fig. 4C) but not after chronic OVA (Fig. 4F).

3.3. Inflammatory cell influx

24 h after a single OVA exposure or the last chronic OVA exposure, total inflammatory cells, macrophages, eosinophils, lymphocytes and neutrophils in BAL fluid were significantly increased compared to naïve and saline-challenged animals (Fig. 5A). Multiple OVA exposures resulted in significantly more total BAL cells, macrophages and eosinophils compared to a single exposure (Fig. 5A).

Total inflammatory cell numbers and eosinophils after acute and chronic OVA challenge were significantly reduced by FP compared to vehicle-administered guinea-pigs (Fig. 5B). Roflumilast significantly decreased the total cell counts and eosinophils compared to vehicle, after the acute OVA challenge, and in the chronic OVA-challenged group decreased the total cells, macrophages, eosinophils and neutrophils (Fig. 5C).

GW274150 treatment significantly reduced the total number of cells and neutrophils compared to vehicle in both acute and chronic OVA-challenged guinea-pigs. In GW274150-treated guinea-pigs, the total cell count was significantly greater after multiple OVA challenges compared to a single OVA challenge. In acute OVA-challenged guinea-pigs, GW274150 treatment significantly increased macrophages. In chronically OVA-challenged guinea-pigs, GW274150 had no significant effect on eosinophil number compared to vehicle (Fig. 5D).

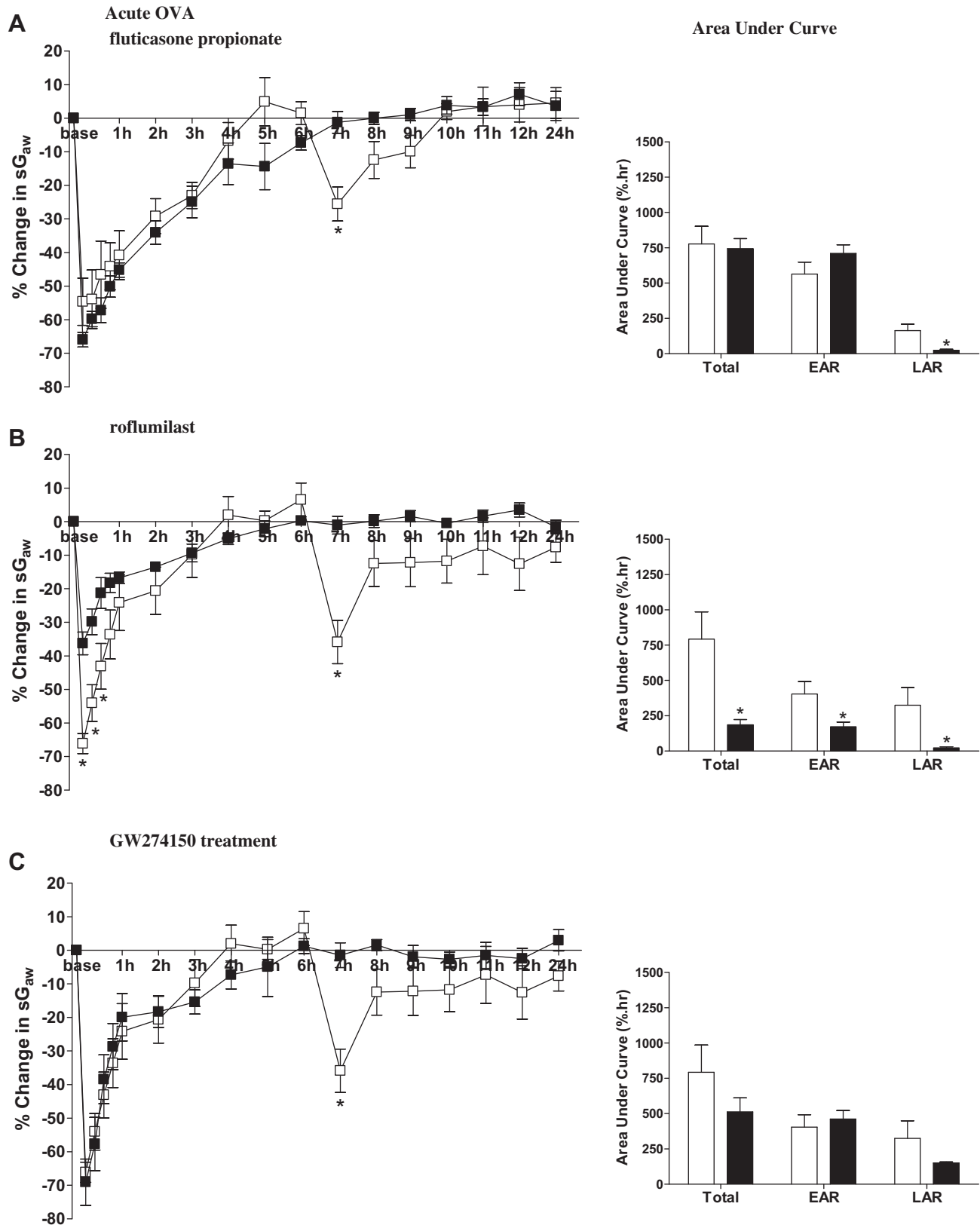


Fig. 2. Mean time-courses for changes in sG_{aw} in OVA-sensitized guinea-pigs treated with vehicle ($\square$) or drug ($\blacksquare$) following a single **acute** inhalation challenge (60 min) with OVA (0.01%). Animals were treated with A. fluticasone propionate (FP, 0.51 mg/ml) by inhalation (15 min) or its vehicle (saline:DMSO:ethanol; 40:30:30), B. roflumilast (1 mg/kg) or C. GW274150 (5 mg/kg) orally or their vehicle (methyl cellulose, 0.1% in sterile water). Drugs or vehicles were administered 24 and 0.5 h before and 6 h after OVA challenge. Mean changes in sG_{aw} are expressed as mean $\pm$ SEM percentage change from baseline where a negative value represents a bronchoconstriction. Panels on the right are the areas under the curve (AUC, %hr) measured only from negative sG_{aw} values for the entire 24 h period (Total), the EAR from 0 to 6 h and the LAR from 6 to 24 h *Significant difference between vehicle and drug treatment by Student's two-tailed t -test ($P < 0.05$; $n = 6$).

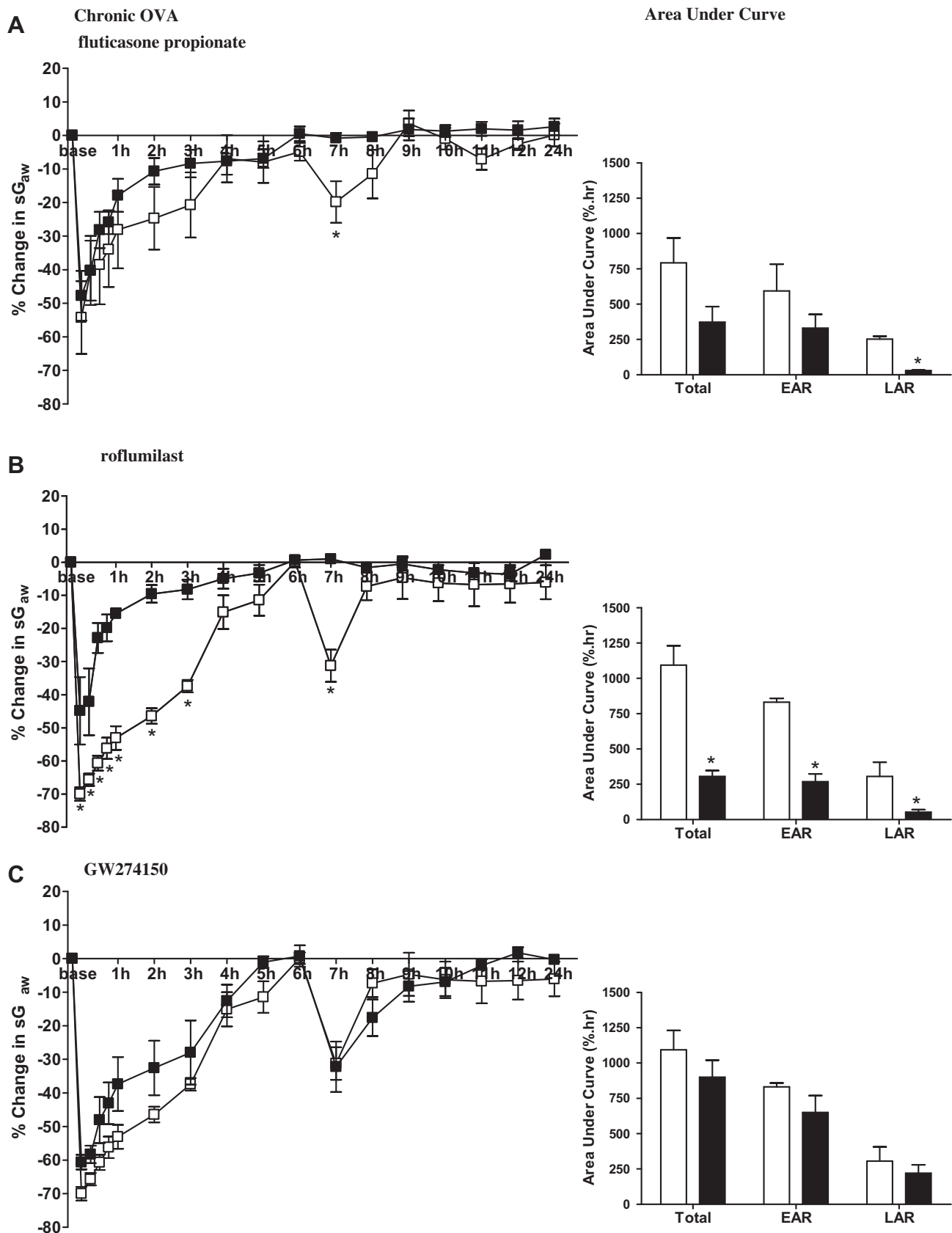


Fig. 3. Mean time-courses for changes in sG_{aw} in OVA-sensitized guinea-pigs treated with vehicle ($\square$) or drug ($\blacksquare$) following the last of 9 chronic inhalation challenges (60 min) with OVA 48 h apart. The first challenge was with 0.01% OVA and thereafter with 0.1% OVA, under mepyramine (30 mg/kg) cover for challenges 2–8. Animals were treated with A. fluticasone propionate (FP, 0.51 mg/ml) by inhalation (15 min) or its vehicle (saline:DMSO:ethanol; 40:30:30), B. roflumilast (1 mg/kg) or C. GW274150 (5 mg/kg) orally or their vehicle (methyl cellulose, 0.1% in sterile water). Drugs or vehicles were administered 24 and 0.5 h before and 6 h after the final OVA challenge. Mean changes in sG_{aw} are expressed as mean $\pm$ SEM percentage change from baseline where a negative value represents a bronchoconstriction. Panels on the right are the areas under the curve (AUC, %hr) measured only from negative sG_{aw} values for the entire 24 h period total, the EAR from 0 to 6 h and the LAR from 6 to 24 h *Significant difference between vehicle and drug treatment by Student's two-tailed t -test ($P < 0.05$; $n = 6$).

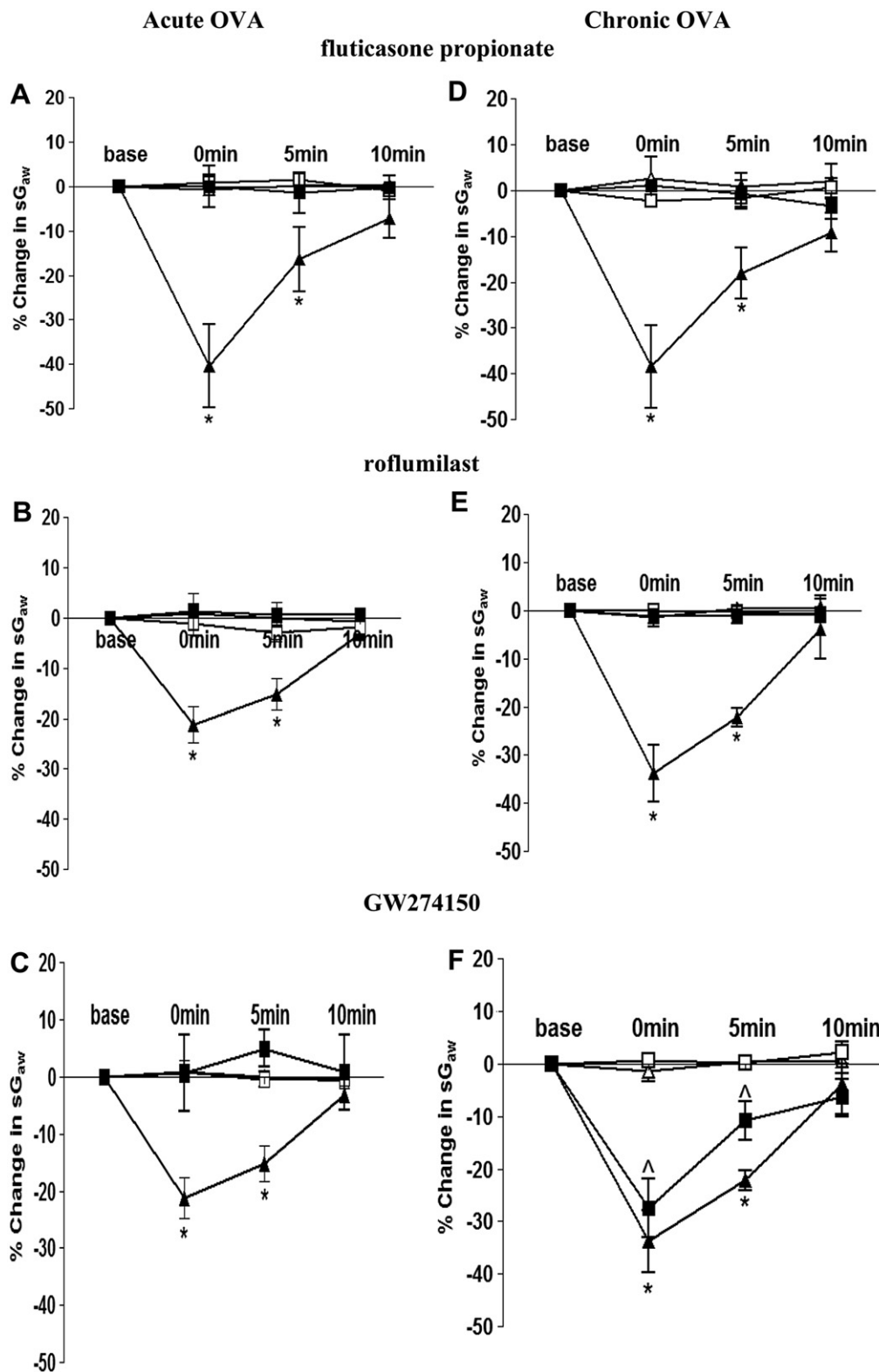


Fig. 4. Bronchoconstrictor responses to a nose-only histamine exposure (1 mMol for 20 s) 24 h before (open symbols) and 24 h after (solid symbols) inhalation challenge of OVA-sensitized guinea-pigs with OVA (0.01% or 0.1%, 60 min) or saline. Animals received either a single acute inhalation of OVA or saline (**left panels**) or 9 chronic inhalations (**right panels**) every 48 h and the response to histamine recorded 24 h after the final challenge. OVA-challenged animals were treated with drug (■□) or vehicle (▲△). These were FP (0.51 mg/ml) by inhalation (15 min) or its vehicle saline:DMSO:ethanol (40:30:30) (A and D), roflumilast (Rof, 1 mg/kg) (B and E) or GW274150F (GW, 5 mg/kg) (C and F) orally or their vehicle (methyl cellulose (0.1% in sterile water). Drugs or vehicles were administered 24 and 0.5 h before and 6 h after OVA challenge. sG_{aw} values were recorded at 0, 5 and 10 min after histamine challenge and expressed as the mean percentage change $\pm$ SEM from baseline where a negative value represents a bronchoconstriction. * Significantly different from vehicle-treated OVA challenge, ^ significantly different from GW274150 pre-challenge sG_{aw} values by Student's two-tailed t -test ($P < 0.05$; $n = 6$).

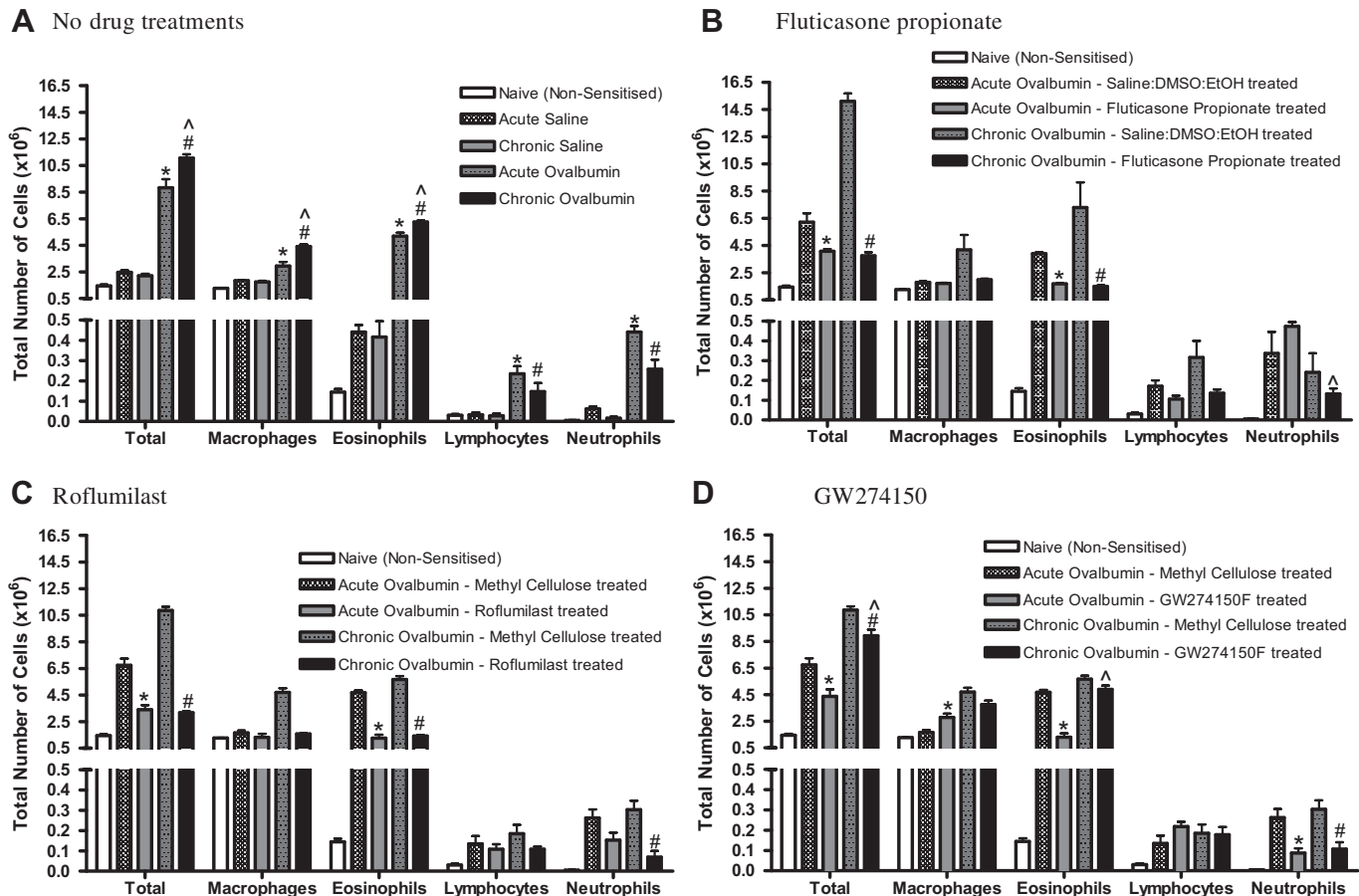


Fig. 5. Total cells, macrophages, eosinophils, lymphocytes and neutrophils found in the bronchoalveolar fluid of A. naïve (non-sensitized) and OVA-sensitized guinea-pigs challenged with single inhalation exposures of saline (acute saline) or OVA (0.01%, acute ovalbumin) or 9 exposures of saline (chronic saline) or OVA (0.1%, chronic ovalbumin). Acute or chronic OVA-challenged guinea-pigs were treated with B. fluticasone propionate (0.51 mg/ml) or its vehicle (saline:DMSO:ethanol; 40:30:30) by inhalation (15 min); C. roflumilast (1 mg/kg) or D. GW274150 (5 mg/kg) orally or their vehicle methyl cellulose (0.1% in sterile water). Drugs or vehicles were administered 24 and 0.5 h before and 6 h after OVA challenge. Results are expressed as mean $\pm$ SEM cell number ($\times 10^6$) per ml of BALF, the lower part of the axis is expanded in order to show lymphocyte and neutrophil numbers. In A. *Significantly different from acute saline-treated; #significantly different from chronic saline-treated; significantly different from acute OVA-treated. In B, C. and D. *Significantly different from acute OVA vehicle-treated; #Significantly different from chronic OVA vehicle-treated; significantly different from acute OVA drug-treated. One-way analysis of variance followed by a Bonferroni post-test ($P < 0.05$; $n = 6$).

3.4. Airway histology

Fig. 6 shows representative sections through bronchioles stained with haematoxylin and eosin for each of the challenge groups. Acute OVA challenge with or without drug treatments did not alter the appearance of the bronchioles. Chronic OVA challenge clearly increased smooth muscle mass and epithelial thickness, compared with acute OVA in a bronchiole of equivalent cross-sectional area. Fig. 7 shows the chronic OVA-challenged groups treated with FP, roflumilast and GW274150 or their vehicles. FP and roflumilast treatment can be seen in these typical sections to reduce the amount of smooth muscle, whereas GW274150 does not. Staining with picrosirius red and Alcian blue/periodic acid Schiff, respectively, were used to quantify bronchiolar collagen and goblet cells. No significant difference was observed in bronchiolar collagen between single saline or single OVA challenges (Fig. 8A). However, multiple challenges of OVA significantly increased the percentage of bronchiolar collagen from $19.7 \pm 3.5\%$ in multiple saline-challenged guinea-pigs to $58.1 \pm 5.0\%$ after chronic OVA (Fig. 8A). FP and roflumilast treatment reduced the percentage of bronchiolar collagen in chronically OVA-challenged guinea-pigs compared to their saline:DMSO:EtOH ($55.3 \pm 8.1\%$) or methyl cellulose ($46.7 \pm 2.9\%$) vehicle treatments to 27.9 ± 6.4 and

$23.2 \pm 6.0\%$, respectively but GW274150 had no effect ($53.3 \pm 2.6\%$) (Fig. 8A).

No significant differences in epithelial mucin levels were observed between acute saline and acute OVA challenge, measured as the number of mucin-associated goblet cells per 10,000 epithelial pixels. Chronic OVA challenge increased mucin-associated goblet cells from 1.9 ± 0.2 in chronic saline-treated animals to 9.4 ± 1.9 after chronic OVA. Treatment of chronic OVA-challenged guinea-pigs with FP (3.3 ± 0.3) and roflumilast (3.6 ± 0.3) significantly reduced mucin-associated goblet cells compared to their respective saline:DMSO:EtOH (8.2 ± 0.8) and methyl cellulose (9.1 ± 1.7) vehicle treatments. However, GW274150 was ineffective (8.8 ± 1.0) (Fig. 8B).

4. Discussion

Many of the advances in the understanding of the pathobiology of asthma have come from animal models of provoked airway inflammation [22]. Animal models represent the primary system available for predicting *in vivo* processes of human asthma and currently short-term acute models of asthma are commonly used [9]. These have significant limitations and ignore the chronic nature of asthma [23]. Several developmental drug targets (PGD₂

antagonists, VLA-4 mAb, PDE4 inhibitors) have demonstrated efficacy in acute allergen challenge animal models but not in clinical asthma raising concern about the predictability of allergen challenge models [24]. Here, we demonstrate the benefits of using multiple OVA challenges compared to a solitary OVA challenge in a guinea-pig asthma model.

4.1. Airways responses to OVA challenge

Early and late bronchoconstrictions followed both acute and chronic OVA challenge and is consistent with our previous acute studies [15]. The multiple OVA challenge model had a similar decrease in sG_{aw} during the EAR and LAR as the acute OVA model. It is worth emphasizing that the final multiple OVA exposure was at ten times the dose used for the single exposure. The higher dose of OVA was accompanied by mepyramine treatment to protect against fatal anaphylaxis, which was then withdrawn for the final OVA exposure to permit identification of the EAR. Thus, there was a substantial development of tolerance to the allergen but this did not change the qualitative nature of the responses to the allergen. Airway hyperresponsiveness (AHR) was observed in both models as an increase in the bronchoconstriction by inhaled histamine, which was greater after multiple challenges probably due to the added contribution of airway remodelling.

Influx of inflammatory cells into the airways, measured in BAL fluid, was greater after multiple challenges, corroborating previous findings [14]. Macrophages, lymphocytes and eosinophils increased, eosinophils becoming the predominant cell type. Increases in airway eosinophils are consistent with human findings as patients with allergen-induced late-phase reactions demonstrate significant increases in BAL eosinophils [25]. Although neutrophils were also increased in OVA-challenged guinea-pigs they are not likely to have a prominent role in the LAR as a response can be observed after depletion of neutrophils [26]. Toward and Broadley [27] showed that the peak neutrophil influx occurred during the EAR with negligible influx at 24 h.

Airways histology revealed that the bronchioles of multiple OVA-challenged guinea-pig bronchioles showed thicker airway smooth muscle and epithelium than from acutely challenged animals. Kenyon and Last [28] showed a similar doubling of collagen staining with Sirius red in mice challenged with OVA three times a week for 6–8 weeks. Bazan-Perkins et al. [14] also showed increased fibrosis in the guinea-pig lung following nine OVA challenges. Increased bronchiolar collagen indicated airways fibrosis and increased mucin-associated goblet cells showed goblet cell hyperplasia. These two indices of airway remodelling in the multiple-challenge model indicate its superior clinical relevance to asthma than the acute OVA model. The vehicles for the drug interventions had no effect on functional responses, cell influx or lung histology.

4.2. Fluticasone propionate

The inhaled corticosteroid, fluticasone propionate, had no inhibitory effect on the EAR in acute and multiple OVA-challenged guinea-pigs. However, in agreement with the literature in both animals [29] and humans [16], FP significantly inhibited the LAR. FP also inhibited the AHR and influx of leukocytes mainly as a result of the significant decrease in eosinophils. Since eosinophils are associated with AHR [30] and the LAR [31], their inhibition by corticosteroids and of gene transcription of inflammatory mediators [32] may explain the inhibition of AHR and LAR.

FP treatment visually reduced the elevated levels of bronchiolar smooth muscle after chronic OVA exposure and significantly inhibited collagen and mucin-associated goblet cells. This agrees

with observations in guinea-pigs with budesonide [10] and with FP in mice [4]. In human asthmatics, corticosteroids reduce mucous levels [33] and FP reduced sputum levels of procollagen type I C-terminal peptide (PICP), a marker of collagen type I deposition [34]. However, inhaled corticosteroids generally show poor effectiveness against the structural remodelling in chronic asthmatics, with some effect in mild asthma [35].

4.3. Roflumilast

The PDE4 inhibitor, roflumilast, attenuated the EAR and virtually abolished the LAR in both acute and multiple OVA-challenged guinea-pigs. Roflumilast prevents the breakdown of cyclic adenosine monophosphate (cAMP) into 5' adenosine monophosphate causing cAMP accumulation and airways smooth muscle relaxation [36]. The ensuing bronchodilatation should attenuate the EAR, consistent with findings in asthmatics treated with roflumilast [19]. However, the inhibition of the EAR is more likely due to stabilization of mast cells, whose degranulation is responsible for the EAR [19,37]. Roflumilast inhibited the AHR to histamine and eosinophil influx, confirming previous clinical [18] and animal studies [38]. Inhibition of eosinophils could explain why no LAR or AHR was observed in roflumilast-treated animals. Clinical studies show rather less effectiveness against the LAR than seen in our chronic model [19]. Roflumilast inhibited the raised neutrophils in the chronic OVA-challenged guinea-pigs which agrees with previous studies in mice [39], where it also inhibited intraepithelial eosinophils [6]. This effect is likely due to inhibition of TNF- α , which can cause influx of neutrophils [38].

Roflumilast visibly inhibited the increased smooth muscle and significantly reduced collagen in the lamina propria and mucin-containing epithelial goblet cells of multiple OVA-challenged guinea-pigs. This confirms a reduction in collagen and hypertrophy of the tracheal epithelium in chronically OVA-exposed mice by roflumilast [6] and of matrix protein deposition in human asthmatics [40] and explains why roflumilast improves breathing in asthmatics. Increased mucus in the airways of asthmatics also causes airway occlusion due to goblet cell hyperplasia, through upregulation of MUC5AC genes during asthmatic episodes [33]. Roflumilast inhibits this upregulation in human epithelial cells [41]. It is perhaps somewhat surprising that both FP and roflumilast should reverse the remodelling after only three doses administered before and after the final OVA exposure. In human asthmatics, it is notoriously difficult to reverse established remodelling with corticosteroids [35,42]. In mouse models of chronic OVA exposure, the remodelling has been shown to persist for several weeks [1,28], so how could FP and roflumilast reverse it so quickly? Since our model is established in only 18 days, it probably does not have the extent or duration of remodelling seen in chronic asthma. It does not therefore fully represent long-term chronic asthma and is relatively more easily reversed by corticosteroids than in humans.

4.4. The iNOS inhibitor, GW274150

The dose of GW274150 used here is known to inhibit iNOS in mice, rats and guinea-pigs [20]. An oral dose in humans of 90 mg (i.e. approximately 20% of the dose used here) given daily for 14 days prior to an allergen challenge was shown to inhibit exhaled NO for at least 9 h [21]. We can therefore assume that the levels of the inhibitor and of iNOS inhibition will persist beyond the LAR in our experiments. GW274150 had no effect on the EAR in both the acute and multiple-challenge models. NO activates guanylyl cyclase to generate cyclic guanosine monophosphate (cGMP) resulting in smooth muscle relaxation [43]. NO is also pro-inflammatory by producing reactive nitrogen species such as peroxynitrite [43].

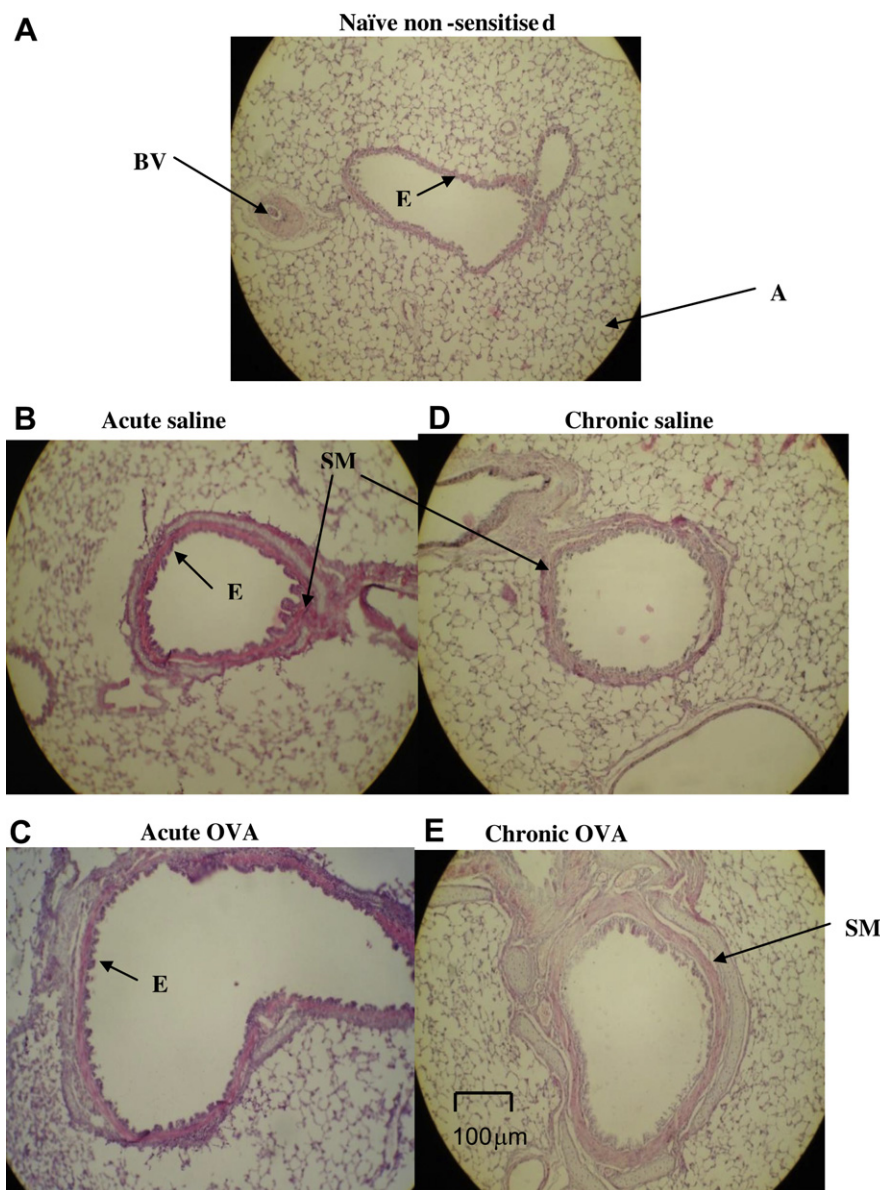


Fig. 6. A. Representative sections of bronchioles from guinea-pig lungs stained with haematoxylin and eosin taken from naïve animals (A) or 24 h after a single saline (acute saline) (B) or OVA (0.01%, 60 min) challenge (acute OVA) (C) or 24 h after the ninth challenge with saline (chronic saline) (D) or OVA (0.1%, chronic OVA) (E). Increased thickness of the smooth muscle (SM) is shown in the section from chronic OVA-treated guinea-pigs. A, alveolae; E, epithelium, BV, blood vessel. Scale bar = 100 µm.

Inhibition of iNOS by GW274150 might therefore exert anti-inflammatory actions. Indeed, GW274150 significantly reduced total leukocytes, neutrophils and eosinophils in the BAL fluid of acute OVA-challenged guinea-pigs, the latter probably being associated with the concomitant reduction in LAR and AHR. GW274150 treatment appeared to increase macrophages, which could be a result of NO reducing macrophage chemoattraction and function [43]. In the multiple OVA-challenged guinea-pigs, GW274150 treatment reduced total cells and neutrophils, but not eosinophils, which could explain why the iNOS inhibitor failed to affect the LAR or AHR in our chronic model. In previous animal [20] and human [21] studies, GW274150 had no effect on total or differential cell numbers or LAR.

Although GW274150 caused a significant reduction in the AHR in acute OVA-challenged guinea-pigs, other studies in mice have shown that knocking out iNOS results in an increase in inflammation, fibrosis and AHR [44]. Thus, our results show that the protection against AHR by GW274150 in acute asthma is lost in

chronic asthma. This suggests that either NO is involved in AHR in mild asthma but not chronic asthma or that GW274150 attenuates AHR in the acute model by a mechanism unrelated to iNOS inhibition.

GW274150 had little if any effect on the structural changes in the airways caused by multiple OVA challenge. It is therefore not surprising that GW274150 has proved ineffective in clinical trials with human asthmatics. In contrast to the present results, the iNOS inhibitor 1400 W, attenuated airways remodelling and contractile function of isolated lung strips after chronic OVA challenge of guinea-pigs [12]. However, that study was carried out in guinea-pig lung strips and may not reflect the situation *in vivo*.

In summary, our study has demonstrated differences between acute (single allergen challenge) and chronic (multiple allergen challenges) guinea-pig models of pulmonary inflammation in terms of responses to therapeutic agents targeted at the treatment of asthma. FP and roflumilast, which have proven clinical efficacy, were effective in both acute and chronic models of

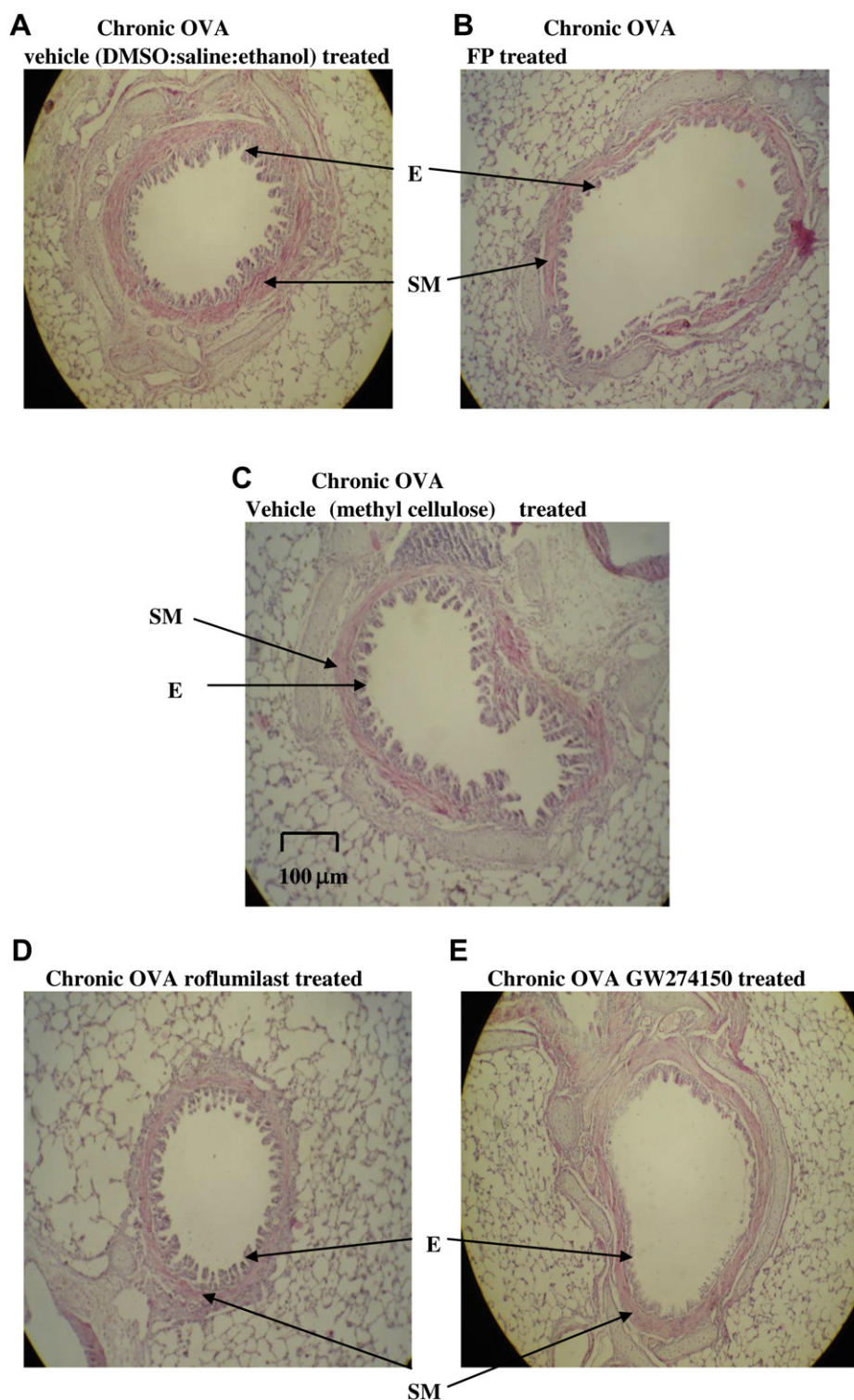


Fig. 7. Representative sections of bronchioles from guinea-pig lungs stained with haematoxylin and eosin taken after 9 challenges with OVA every 48 h. Guinea-pigs treated with vehicle (saline:DMSO:ethanol; 40:30:30) by inhalation (15 min) show increased smooth muscle (SM) (A) which is reduced by treatment with fluticasone propionate (FP, 0.51 mg/kg) delivered in this vehicle (B). Similarly; in guinea-pigs treated orally with vehicle (methyl cellulose 0.1% in sterile water) there were increases in smooth muscle (SM) (C), which were reduced by treatment with roflumilast (1 mg/kg p.o in this vehicle) (D) but not by GW274150F (5 mg/kg p.o in this vehicle) (E). Drugs or vehicles were administered 24 and 0.5 h before and 6 h after OVA challenge. E, epithelium. Scale bar = 100 µm.

pulmonary inflammation. However, an important difference was demonstrated for GW274150, the highly selective iNOS inhibitor that has been shown to be effective in models of acute inflammation [20] but was ineffective in the clinical treatment of asthma [19]. As in previous studies, GW274150 was effective in

our acute guinea-pig model of pulmonary inflammation, but significantly, its efficacy was lost in the chronic model. While others have developed similar chronic models in mice [4–6], we suggest that guinea-pigs can offer advantages over mice in that their pharmacology and anatomy more closely match those of

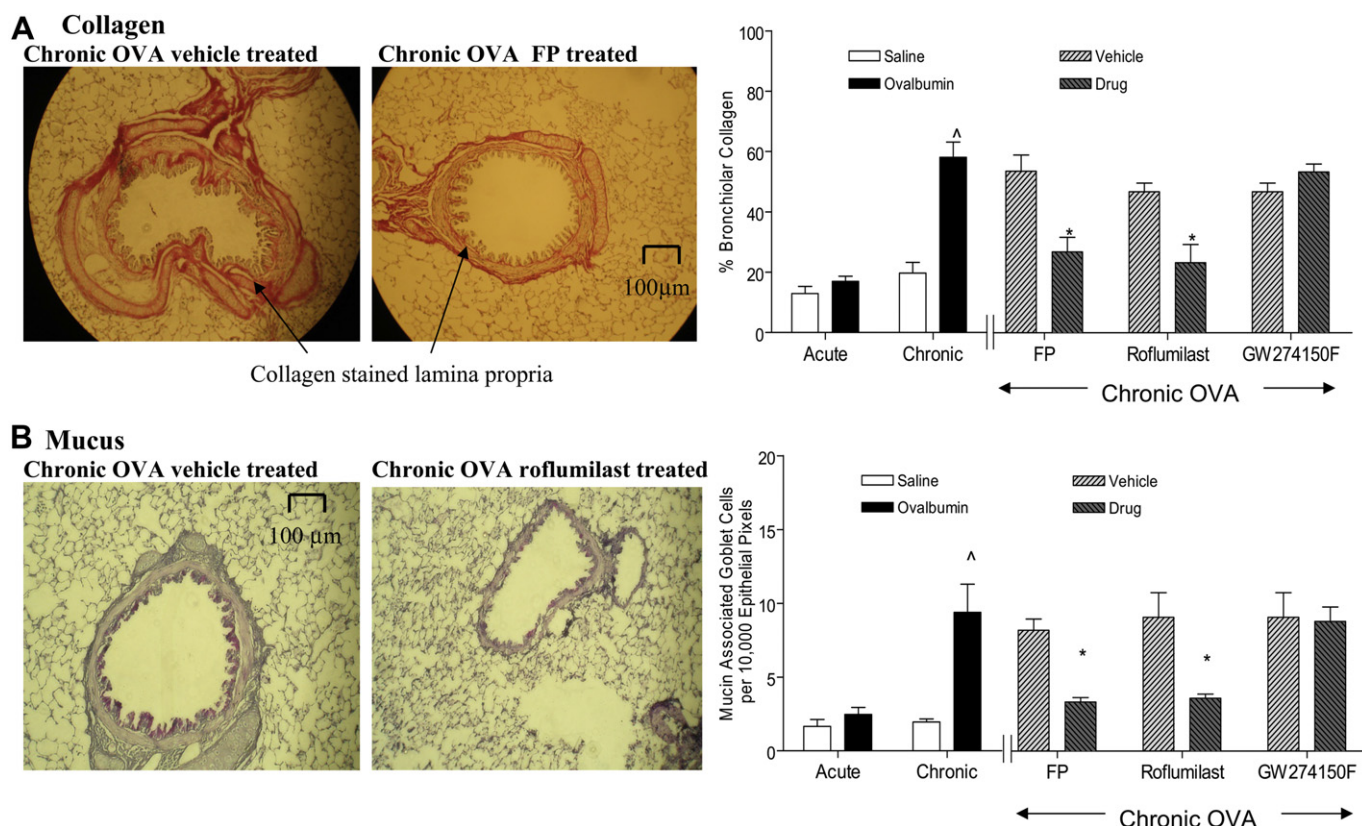


Fig. 8. Bronchiolar histology of sections of bronchioles from guinea-pig lungs stained A. with picosirius red to identify collagen or B. with Alcian blue/periodic acid Schiff (AB/PAS) to identify mucus-containing epithelial goblet cells. Representative sections with collagen staining are shown for chronic OVA-challenged guinea-pigs treated with vehicle by inhalation (saline:DMSO:ethanol) and with inhaled fluticasone propionate 0.51 mg/ml 24 and 0.5 h before OVA challenge and 6 h post-OVA challenge. Representative sections with mucus staining are shown for chronic OVA-challenged guinea-pigs treated with vehicle (methyl cellulose, p.o.) and with roflumilast (1 mg/kg p.o.). Scale bar = 100 μ m. The histograms show the mean $\pm$ SEM area of collagen staining and number of mucin-associated goblet cells per 10,000 epithelial pixels in bronchiolar sections. Lungs were taken from guinea-pigs challenged (60 min) with single (0.01%, acute) or 9 inhalation exposures (0.1% chronic) to saline (open histograms) or OVA (solid histograms). In the chronic OVA-challenged guinea-pigs, the effects of drug treatments (dark diagonal hatching) with fluticasone propionate (FP, 0.51 mg/ml, 15 min inhalation), roflumilast (1 mg/kg) or GW274150 (5 mg/kg) orally or their vehicles (light diagonal hatching) were examined. ^Δ Significant difference between OVA and saline challenge, and * Significant difference between drug and vehicle by Student's two-tailed *t*-test. ($P < 0.05$, $n = 4$).

humans [7,8] and measurement of airways function is easier. Others have also used more complex antigens with greater relevance to asthma, such as house dust mite (HDM) extract, to develop chronic models in mice [45]. However, it is questionable whether the inflammatory effects of HDM can be attributed entirely to an allergic response since an active component, Der p1, causes inflammation without sensitization [46] possibly due to protease activity or lipopolysaccharide contamination. Whilst our chronic model provided most of the features of human asthma and its responsiveness to antiasthma drugs, it did fall short in some respects, such as the rather more easily reversed remodelling by corticosteroids and roflumilast than encountered clinically. However, the chronic model was superior to the acute model for pre-clinical drug evaluation and has the advantage that it can be set up in a relatively short period of time. More lengthy chronic exposure regimes would obviously be preferable to mimic the poorly reversed well established remodelling seen in human asthma but in drug development programs these would be less acceptable and more costly to use routinely.

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