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Inhibition of Inflammation and Remodeling by Roflumilast and Dexamethasone in Murine Chronic Asthma

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Abbreviations: AHR, airway hyper-reactivity; PDE, phosphodiesterase; Penh, enhanced Pause; TGF-β1, transforming growth factor-β1; NF-κB, nuclear factor-κB

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Abstract

Phosphodiesterase (PDE) inhibitors have potential as alternatives or adjuncts to glucocorticoid therapy in asthma. We compared roflumilast (a selective PDE4 inhibitor) with pentoxifylline (a non-selective inhibitor) and dexamethasone in ameliorating the lesions of chronic asthma in a mouse model. BALB/c mice sensitized to ovalbumin were chronically challenged with aerosolized antigen for 6 weeks. During weeks 5 and 6, groups of animals were treated with roflumilast or dexamethasone by daily gavage, or with pentoxifylline by daily intraperitoneal injection. Airway hyper-reactivity (AHR) was evaluated by whole body plethysmography and airway lesions by histomorphometry and immunohistochemistry. Compared to vehicle alone, treatment with roflumilast or dexamethasone significantly reduced accumulation of eosinophils and chronic inflammatory cells, subepithelial collagenization and thickening of the airway epithelium. Dexamethasone also reduced goblet cell hyperplasia/metaplasia, subepithelial accumulation of transforming growth factor-β1 and epithelial cytoplasmic immunoreactivity for nuclear factor-κB. Treatment with pentoxifylline inhibited only eosinophil recruitment and epithelial thickening. Roflumilast and dexamethasone slightly decreased AHR, whereas this was significantly reduced by pentoxifylline. Thus, in this model of chronic asthma, both roflumilast and dexamethasone were potent inhibitors of airway inflammation and remodeling. Roflumilast did not diminish accumulation of transforming growth factor-β1, suggesting that it might affect remodeling by mechanisms distinct from glucocorticoids.

Asthma is characterized by chronic inflammation of the airways with superimposed acute exacerbations, together with a variety of structural changes such as subepithelial fibrosis, mucous cell hyperplasia/metaplasia and increased smooth muscle mass, which are collectively referred to as airway wall remodeling (Bousquet et al., 2000; Howarth, 2001; Kumar, 2001). Changes of remodeling correlate with the development of airway hyper-reactivity (AHR) (Chetta et al., 1996; Hoshino et al., 1998a; Howarth, 2001) and with persistent airflow limitation in chronic asthmatics (Niimi et al., 2000). The progressive decline in lung function may be inhibited by maintenance therapy with inhaled glucocorticoids, presumably because of inhibition of the progression of airway wall remodeling (Howarth, 2001). Moreover, long-term titration of inhaled glucocorticoid therapy to reduce AHR, which yields substantially improved clinical outcomes, is associated with significant reduction in subepithelial fibrosis (Sont et al., 1999). These observations lend support to the notion that therapeutic agents able to efficiently inhibit airway wall remodeling may be of particular value in asthma.

Phosphodiesterase (PDE) isoenzymes control the degradation of cyclic AMP and thus play key roles in regulating compartmentalized signalling responses to intracellular gradients of cyclic AMP (Houslay and Adams, 2003). The PDE4 family of enzymes, encoded by four *PDE4* genes which generate at least 16 different isoforms, exclusively hydrolyzes cyclic AMP and accounts for most of this activity within cells (Conti et al., 2003; Houslay and Adams, 2003). Selective inhibitors of PDE4, which are directed against the catalytically active site, inhibit the function of a variety of inflammatory and immunomodulatory cells (Torphy, 1998). For example, these compounds decrease the production of numerous relevant inflammatory mediators, including histamine and leukotrienes (Weston et al., 1997), pro-inflammatory cytokines such as tumor necrosis factor-α and interleukin-1β (Verghese et al., 1995), as well as T-lymphocyte-derived cytokines such as interleukin-4 and interleukin-5 (Souness et al., 1999).

The potential value of selective PDE4 inhibitors in the treatment of inflammatory disease of the airways has been demonstrated in preliminary studies of their use in chronic obstructive pulmonary disease (Compton et al., 2001). PDE4 inhibitors may also be useful as alternatives or adjuncts to glucocorticoids in the treatment of asthma, because they exhibit significant anti-inflammatory activity in a variety of in vivo models of allergic bronchopulmonary inflammation (Schudt et al., 1995; Torphy, 1998). However, assessment of their capacity to modulate airway wall remodeling has hitherto been limited by the lack of a suitable model of chronic asthma that replicates these structural changes.

We have described an experimental model of asthma in mice, in which BALB/c mice are systemically sensitized to ovalbumin and subjected to inhalational challenge with low mass concentrations of aerosolized antigen for at least 6 weeks (Temelkovski et al., 1998). This model replicates most of the features that are typical of the chronic human disease, including accumulation of numerous intraepithelial eosinophils; chronic inflammation in the lamina propria; together with epithelial hypertrophy, mucous cell hyperplasia/metaplasia and subepithelial fibrosis. Unlike other chronic exposure models, there is no evidence of down-regulation of inflammatory or immunologic responses. Furthermore, there is no associated parenchymal inflammation, so that development of AHR can be attributed to abnormalities of the airways rather than to parenchymal lesions. Using gene-targeted animals and long-term administration of antibodies, we have employed this model to investigate the role of various mediators and cell populations in the pathogenesis of chronic asthma (reviewed in Kumar and Foster, 2002).

In the present study we examined the ability of roflumilast, a novel potent PDE4-selective inhibitor (Hatzelmann and Schudt, 2001), to inhibit airway inflammation, remodeling and AHR in this model. Roflumilast has been demonstrated to be an effective anti-inflammatory agent in acute allergen challenge models in animals and is capable of inhibiting antigen-induced bronchospasm (Bundschuh et al., 2001). Its potential usefulness in human asthma has been

demonstrated in clinical studies (Timmer et al., 2002). We compared the therapeutic response to roflumilast with that to pentoxifylline (a non-specific PDE inhibitor) and the glucocorticoid dexamethasone, and investigated the effects of these drugs on underlying mechanisms potentially involved in airway inflammation and remodeling.

Methods

Experimental design

The protocols we employed for sensitization and inhalational challenge have previously been described in detail (Temelkovski et al., 1998; Foster et al., 2000; Kumar et al., 2002). Briefly, specific pathogen-free female BALB/c mice (aged approximately 8 weeks at the commencement of experimental studies) were sensitized by intraperitoneal injections of 50 μg of alum-precipitated chicken egg ovalbumin (Sigma Australia, Sydney; unless otherwise specified, all chemicals were obtained from this source) 21 days and 7 days before inhalational exposure. They were maintained in a laminar flow holding unit (Gelman Sciences, Sydney, Australia) and housed in autoclaved cages on autoclaved bedding in an airconditioned room on a 12 hour light/dark cycle. Irradiated food and acidified water were provided *ad libitum* throughout. Mice were exposed to 10-20 mg/m^3 of aerosolized ovalbumin for 30 min/day on 3 days/week for 6 weeks in a whole-body inhalation exposure chamber (Unifab Corporation, Kalamazoo, MI). All experimental procedures complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and additional requirements of the Animal Care and Ethics Committee of the University of New South Wales (ref. no. 01/34.1).

Groups of 8 animals were treated with roflumilast (ALTANA Pharma, Konstanz, Germany) (5 mg/kg/day by gavage, suspended in 2.5% polyethylene glycol-4% methylcellulose solution), dexamethasone (1 mg/kg/day by gavage, cyclodextrin compound in saline) or pentoxifylline (50 mg/kg/day by intraperitoneal injection, in saline) on 5 days/week for the last two weeks of the inhalational exposure. For each of these drugs, this was the lowest dosage that was effective in inhibiting an inflammatory response. The dosage of roflumilast was based on studies of its anti-inflammatory activity in a model of collagen-induced arthritis in mice (Barsig et al., 2001) and on kinetic studies which demonstrated that 5 mg/kg/day produces therapeutically relevant blood levels in these animals (ALTANA Pharma, unpublished data). The dosages of pentoxifylline and dexamethasone, which are

similar to those used in other investigations of their anti-inflammatory effects (Kremsner et al., 1991; Blyth et al., 1998), were selected on the basis of preliminary experiments in the chronic asthma model, in which a range of doses was compared with respect to ability to inhibit accumulation of eosinophils and/or chronic inflammatory cells. These experiments also established that pentoxifylline was relatively ineffective when administered to mice by gavage. Drugs were administered 30 minutes prior to aerosol challenge. Vehicle-treated control animals received polyethylene glycol-methylcellulose by gavage and naïve unexposed controls of the same age were assessed in parallel. We have previously shown that sensitized and chronically exposed control animals treated with intraperitoneal injections of saline develop lesions comparable to those of untreated animals (unpublished data) and that chronic exposure of non-sensitized mice is not associated with development of significant airway inflammation, remodeling or AHR (Foster et al., 2000).

Airway reactivity

As a screening procedure for assessment of changes in airway reactivity, responsiveness to methacholine was assessed in conscious, unrestrained mice by whole-body plethysmography (Buxco, Troy, NY) approximately 24 hours after administration of the last dose of drug. The apparatus yields a measure of changes in respiratory pattern known as enhanced Pause (Penh), which correlates with and can be used to monitor airway resistance (Hamelmann et al., 1997). Responses to methacholine (aerosolized from solutions of 3.125 to 50 mg/ml) were measured as described (Foster et al., 2000). We have demonstrated that in this chronic exposure model, increased Penh correlates with increased specific airway resistance measured using a low frequency forced oscillation technique (Collins RA et al, submitted for publication).

Histomorphometry and immunohistochemistry

The trachea and lungs were collected 18 hours after the last inhalational exposure, fixed in 10% buffered formalin overnight and embedded in paraffin. Although changes of inflammation and remodeling are demonstrable in the trachea, main

bronchi and intrapulmonary airways in this chronic exposure model, morphometric quantification of airway changes was performed in sections of the longitudinally oriented trachea for convenience of sampling and measurement. Numbers of eosinophils within the airway epithelial layer and of nuclear profiles in the lamina propria were counted in hematoxylin & eosin-stained sections of the trachea. The thickness of the subepithelial zone of collagenization and of the epithelial layer were assessed in reticulin-stained sections. Mucus-secreting goblet cells were quantified in intrapulmonary airways in sections stained with Alcian blue-PAS. The validity and reliability of the morphometric techniques we employed have been established in previous reports (Temelkovski et al., 1998; Foster et al., 2000; Kumar et al., 2002).

Immunohistochemical staining was performed as previously described (Kumar et al., 2002), with affinity-purified rabbit polyclonal primary antibodies to transforming growth factor-β1 (TGF-β1) (sc-146) or the RelA subunit of nuclear factor-κB (NF-κB) (sc-372) (Santa Cruz Biotechnology, Santa Cruz, California) and a peroxidase-antiperoxidase detection system. For NF-κB staining, antigen retrieval was performed by boiling deparaffinized sections in 0.01M citrate buffer (pH 6.0) for 10 min in a microwave.

Because of the non-linear relationship between amount of antigen and accumulation of immunoperoxidase reaction product, intensity of immunoreactivity was assessed semi-quantitatively rather than by image analysis. Grading was performed by a single observer blinded to the identity of the samples and slides were presented in random order for examination. The scale used was 0 = no staining, 1 = weak staining, 2 = moderate staining and 3 = strong staining.

Statistical analysis

In general, one-way analysis of variance followed by Newman-Keuls multiple comparison test was used to examine differences between groups. Where grading was used for assessment, a non-parametric Kruskal-Wallis test followed by Dunn's test was employed. GraphPad Prism 3.03 (GraphPad Software, San Diego, California) was used for all data analysis.

Results

No side effects of drug treatment were observed in any of the experimental groups studied.

Airway inflammation

We have previously shown that whereas eosinophils are rarely identifiable within the tracheal epithelium of normal BALB/c mice, sensitized mice exhibited recruitment of numerous intraepithelial eosinophils after long-term inhalation exposure to aerosolized ovalbumin (Temelkovski et al., 1998). This finding was reproduced in sensitized mice treated with vehicle alone ($p < 0.001$ compared to unexposed controls, Fig 1A). Treatment with each of the three drugs tested led to a reduction in the accumulation of eosinophils ($p < 0.001$ compared to vehicle-treated controls for roflumilast, $p < 0.01$ for the other two drugs, Fig 1A).

Sensitized chronically exposed BALB/c mice treated with vehicle alone developed widespread multifocal accumulation of lymphocytes, plasma cells and other chronic inflammatory cells in the lamina propria of the trachea (Fig 2A) and the increase in cell numbers was similar to that previously described in untreated animals ($p < 0.001$ compared to unexposed controls, Fig 1B). Accumulation of lamina propria cells was significantly diminished in mice treated with roflumilast (Fig 2B) or dexamethasone ($p < 0.01$ for both compared to vehicle-treated controls, Fig 1B), but was unaffected in mice treated with pentoxifylline.

Airway wall remodeling

As has previously been demonstrated in untreated animals (Temelkovski et al., 1998), sensitized mice exposed to antigen and treated with vehicle alone exhibited accumulation of subepithelial collagen (Fig 3A), leading to significant thickening of the reticulin-stained zone ($p < 0.001$ compared to unexposed controls, Fig 4A). In mice treated with roflumilast, the thickness of this zone was similar to that in unexposed controls (Fig 3B) and the decrease in mean thickness was statistically significant ($p < 0.001$ compared to vehicle-treated controls, Fig 4A). Dexamethasone also decreased the subepithelial accumulation of collagen but the effect was less

marked ($p < 0.05$). There was no significant diminution of subepithelial fibrosis in mice treated with pentoxifylline (Fig 4A).

Treatment with each of the three drugs prevented the development of thickening of the airway epithelium that was observed in mice treated with vehicle alone ($p < 0.001$ compared to vehicle-treated controls, Fig 4B).

Mucus-secreting goblet cells, which are virtually absent in the intrapulmonary airways of naïve mice, were strikingly increased in sensitized exposed mice treated with vehicle only, with a median grade of 4. Treatment with roflumilast or pentoxifylline had little effect on the number of mucous cells, but there was a modest reduction in the proportion of goblet cells in the airways of dexamethasone-treated mice, which had a median grade of 3 ($p < 0.05$).

Airway responsiveness

As reported previously for sensitized chronically exposed BALB/c mice (Foster et al., 2000), animals treated with vehicle exhibited a left-shifted Penh dose-response curve and increased maximal reactivity, characteristic of airway hyper-reactivity (Fig 5). Animals treated with roflumilast or with dexamethasone exhibited a modest decrease in airway responsiveness, but this was not statistically significant. Animals treated with pentoxifylline demonstrated statistically significant reductions in Penh at methacholine concentrations of 6.25, 12.5 and 50 mg/ml (Fig 5).

Immunohistochemistry

We have recently shown (Kumar et al, submitted for publication) that in sensitized BALB/c mice chronically exposed to inhaled antigen, there is a progressive increase in subepithelial immunoreactivity for TGF-β1, which precedes but is spatially associated with collagen accumulation. To investigate possible mechanisms of inhibition of remodeling in drug-treated animals, we semi-quantitatively assessed immunoreactivity for TGF-β1. In vehicle-treated animals, subepithelial immunostaining was comparable to that previously observed in untreated mice and was significantly increased compared to naïve animals ($p < 0.001$, Fig 6A). The median grade of immunoreactivity was significantly diminished in dexamethasone-

treated mice ($p < 0.05$ compared to vehicle-treated controls, Fig 6A) but not in animals treated with the other drugs.

To investigate possible mechanisms of inhibition of inflammation in drug-treated animals, we semi-quantitatively assessed expression of the RelA (p65) protein of the NF-κB pathway. This revealed constitutive immunoreactivity in the cytoplasm of tracheal epithelial cells of naïve mice (confirmed using another polyclonal antibody to p65, data not shown) and significant upregulation of the intensity of immunoreactivity in sensitized BALB/c mice chronically exposed to inhaled antigen and treated with vehicle only ($p < 0.05$, Fig 6B), but no evidence of nuclear immunoreactivity. Cytoplasmic immunostaining was markedly diminished in dexamethasone-treated mice, with a median grade lower than that in unexposed controls ($p < 0.001$ compared to vehicle-treated controls, Fig 6B). Treatment with pentoxifylline reduced staining for p65 to the levels in unexposed control animals ($p < 0.05$ compared to vehicle-treated controls, Fig 6B). Staining was also reduced in mice treated with roflumilast, but this effect did not achieve statistical significance.

Discussion

In this study we employed a mouse model of chronic asthma, which replicates many of the features of the human disease with a high degree of fidelity (Kumar and Foster, 2002), to investigate inhibition of the airway lesions by PDE inhibitors as compared to glucocorticoids. We have previously shown that in this model, changes of inflammation and airway wall remodeling are established by 4 weeks and progress with continuing exposure to antigen (Temelkovski et al., 1998). Therefore, we were able to assess whether these drugs could inhibit progression of or reverse these changes by treatment during weeks 5 and 6 of exposure.

Both roflumilast and dexamethasone were potent inhibitors of airway inflammation and remodeling. At the doses tested, the drugs suppressed the intraepithelial accumulation of eosinophils and the accumulation of chronic inflammatory cells in the lamina propria of the airways, as well as reversing subepithelial collagenization and epithelial hypertrophy. Inhibition of remodeling by inhaled glucocorticoids is well-documented in human asthma (Hoshino et al., 1998b; Sont et al., 1999) and administration of glucocorticoids has also been shown to inhibit changes of airway fibrosis in other animal models (Blyth et al., 2000; Vanacker et al., 2001). However, our study demonstrates for the first time that comparable suppression of subepithelial fibrosis and epithelial hypertrophy can be achieved following treatment with a PDE4-selective inhibitor.

Although both roflumilast nor dexamethasone caused modest inhibition of airway responsiveness to methacholine in our model of chronic asthma, these effects were not statistically significant at the doses tested. The same dose of roflumilast is effective in inhibiting both antigen-induced bronchoconstriction and AHR in an acute allergen challenge model of asthmatic inflammation in rats (Bundschuh et al., 2001; Wollin et al., 2002) and a comparable dose of dexamethasone similarly decreases AHR to methacholine in acute models (Trifilieff et al., 2000). However, our studies in the chronic model of asthma using gene-targeted mice have clearly demonstrated that different mechanisms are involved in chronic AHR (Kumar and Foster, 2002). Given that the doses administered were

chosen on the basis of anti-inflammatory activity, it is entirely possible that higher doses of these drugs would be able to significantly suppress AHR. This might be analogous to the need for higher doses of inhaled glucocorticoids to achieve effective control of AHR in human asthma (Sont et al., 1999).

In contrast to the inhibition of mucous cell hyperplasia/metaplasia by dexamethasone, which replicates previous observations in a murine model (Blyth et al., 1998), treatment with roflumilast did not suppress goblet cell change in this model. This result was in contrast to observations in an acute exposure model of asthmatic inflammation, in which the PDE4-selective inhibitor rolipram was demonstrated to inhibit the goblet cell response (Kanehiro et al., 2001) and further highlights that phenotypically similar lesions may be differentially modified in acute and chronic disease.

The non-selective PDE inhibitor pentoxifylline had lesser effects on chronic airway inflammation and remodeling than either roflumilast or dexamethasone, being able to inhibit only eosinophil accumulation and epithelial thickening at the dose tested. Despite this, pentoxifylline significantly decreased AHR in this model of chronic asthma. We speculate that this could be related to the ability of this agent to suppress production of Th1 cytokines (Rott et al., 1993), among which interferon-γ has been identified as possibly contributing to AHR (Fleming et al., 2001). Alternatively, pentoxifylline might act via a direct effect on airway smooth muscle as a consequence of inhibition of other PDE isoenzymes (Schmidt et al., 2000).

The mechanism of inhibition of remodeling by roflumilast and dexamethasone is unclear, although inhibition of growth factors for fibroblasts seems plausible. In human studies, treatment with inhaled glucocorticoids has been demonstrated to decrease expression of insulin-like growth factor-1 (Hoshino et al., 1998b). Epithelial cell-derived growth factors of the TGF-β family are considered to be particularly important in the remodeling of the airways in asthma (Davies et al., 2003). We have recently shown (Kumar et al, submitted for publication) that in sensitized BALB/c mice chronically exposed to inhaled antigen, there is a progressive increase in subepithelial immunoreactivity for TGF-β1, which precedes the development of

subepithelial fibrosis. Furthermore, interleukin-13-deficient mice and mice depleted of $CD4^+$ T cells by chronic administration of a monoclonal antibody, in both of which remodeling was abrogated, failed to exhibit accumulation of TGF-β1. We therefore investigated the relationship between inhibition of remodeling and expression of TGF-β1 by immunohistochemistry. Interestingly, this revealed that whereas treatment with dexamethasone significantly decreased subepithelial immunoreactivity for TGF-β1, roflumilast had no such effect. This result suggests that it might affect remodeling by mechanisms distinct from glucocorticoids, for example by a direct effect on fibroblast accumulation and function (Kohyama et al., 2002).

The NF-κB pathway, which is a major signaling mechanism involved in regulation of the inflammatory response, is inhibited by glucocorticoids and PDE inhibitors (Almawi and Melemedjian, 2002; Haddad et al., 2002). Airway epithelial cell expression of the RelA subunit of NF-κB is upregulated following antigen challenge of sensitized animals (Poynter et al., 2002). We therefore investigated whether the activity of roflumilast or dexamethasone was related to inhibition of expression of this protein. Mice chronically exposed to inhaled antigen and treated with vehicle control exhibited enhanced epithelial cytoplasmic expression of RelA protein. Whereas treatment with dexamethasone strikingly diminished the intensity of immunoreactivity for RelA, the effect of pentoxifylline was less marked, while the effect of roflumilast did not achieve statistical significance. The difference between pentoxifylline and roflumilast might be attributed to the more effective inhibition of NF-κB signaling by nonspecific PDE inhibitors than by PDE4-selective agents (Haddad et al., 2002).

In conclusion, we have demonstrated that in experimental chronic asthma, treatment with the novel PDE4-selective inhibitor roflumilast effectively inhibits both acute and chronic airway inflammation, as well as changes of remodeling. The mechanisms of the effects of roflumilast appear to differ from those of glucocorticoids. Our findings lend support to the notion that PDE4-selective inhibitors could be of value in the treatment of asthma, either as alternatives or as adjuncts to conventional glucocorticoid therapy.

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Footnotes:

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Legends for Figures

Fig. 1 (A) Intraepithelial eosinophils and (B) inflammatory cells in the lamina propria of the trachea of sensitized, chronically exposed mice treated with vehicle alone compared to unexposed animals or to animals treated with 5 mg/kg/day roflumilast, 1 mg/kg/day dexamethasone or 50 mg/kg/day pentoxifylline. Significant differences compared to unexposed controls are shown as ** = $p < 0.01$, *** = $p < 0.001$, significant differences compared to vehicle-treated controls shown as ## = $p < 0.01$, ### = $p < 0.001$.

Fig. 2 Photomicrographs demonstrating that accumulation of chronic inflammatory cells in the lamina propria in vehicle-treated mice (A) is diminished in roflumilast-treated mice (B) (hematoxylin & eosin stain).

Fig. 3 (A) Subepithelial collagenization and (B) hypertrophy of the tracheal epithelium in sensitized, chronically exposed mice treated with vehicle alone compared to unexposed animals or animals treated with roflumilast, dexamethasone or pentoxifylline. Significant differences compared to unexposed controls are shown as * = $p < 0.05$, ** = $p < 0.01$, *** = $P < 0.001$, significant differences compared to vehicle-treated controls shown as # = $p < 0.05$, ### = $p < 0.001$.

Fig. 4 Photomicrographs demonstrating that thickening of the subepithelial zone of collagen (arrow) in vehicle-treated mice (A) is diminished in roflumilast-treated mice (B) (reticulin stain).

Fig. 5 Airway reactivity of unexposed and sensitized chronically exposed mice treated with vehicle alone or with roflumilast, dexamethasone or pentoxifylline, assessed by change in Penh in response to increasing concentrations of aerosolized β-methacholine. Significant differences compared to vehicle-treated controls are shown as * = $p < 0.05$, *** = $p < 0.001$.

Fig. 6 (A) Subepithelial immunoreactivity for TGF-β1 and (B) cytoplasmic immunoreactivity for NF-κB in airway epithelial cells of unexposed and sensitized chronically exposed mice treated with vehicle alone or with roflumilast, dexamethasone or pentoxifylline. Bars indicate median grades and significant differences are as shown.

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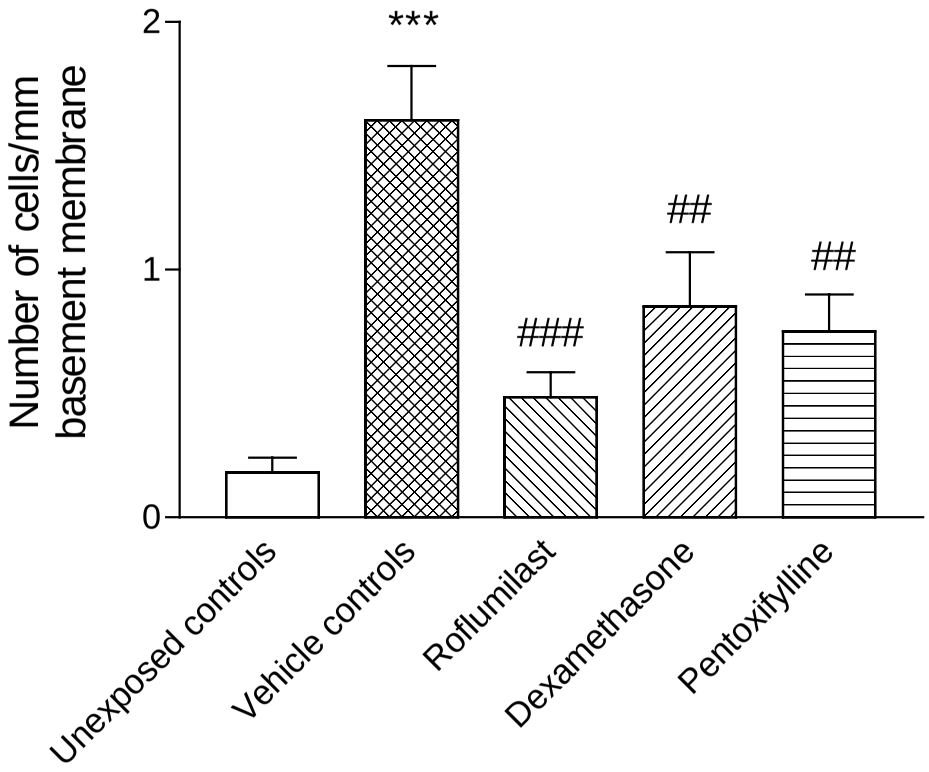
Figure 1

A.



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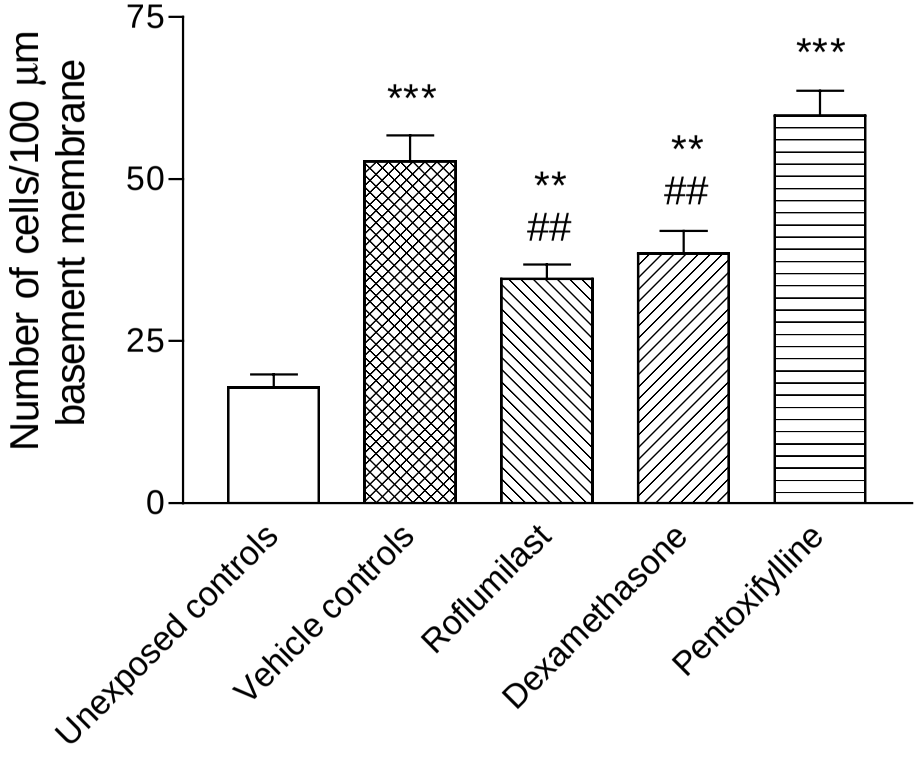

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Figure 2

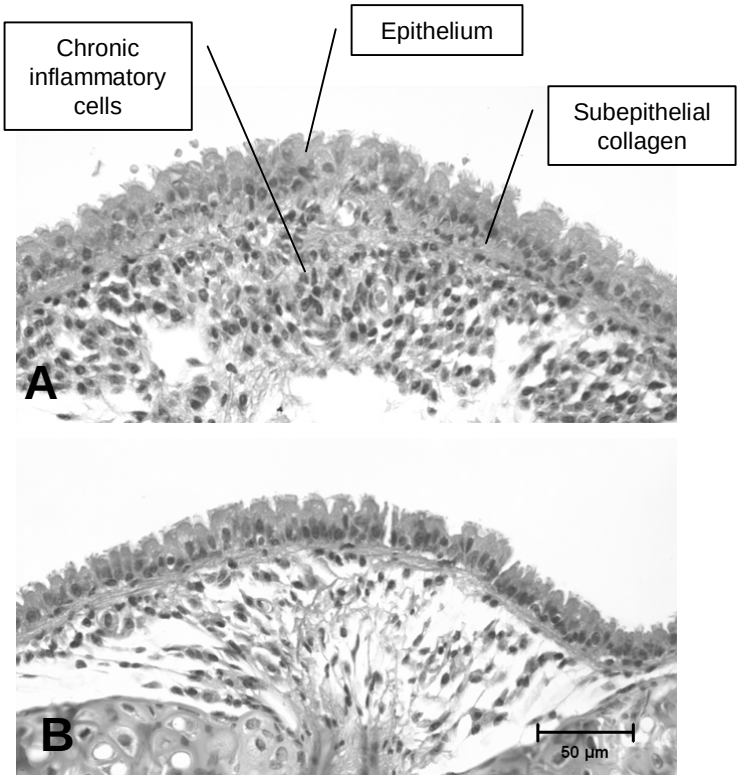

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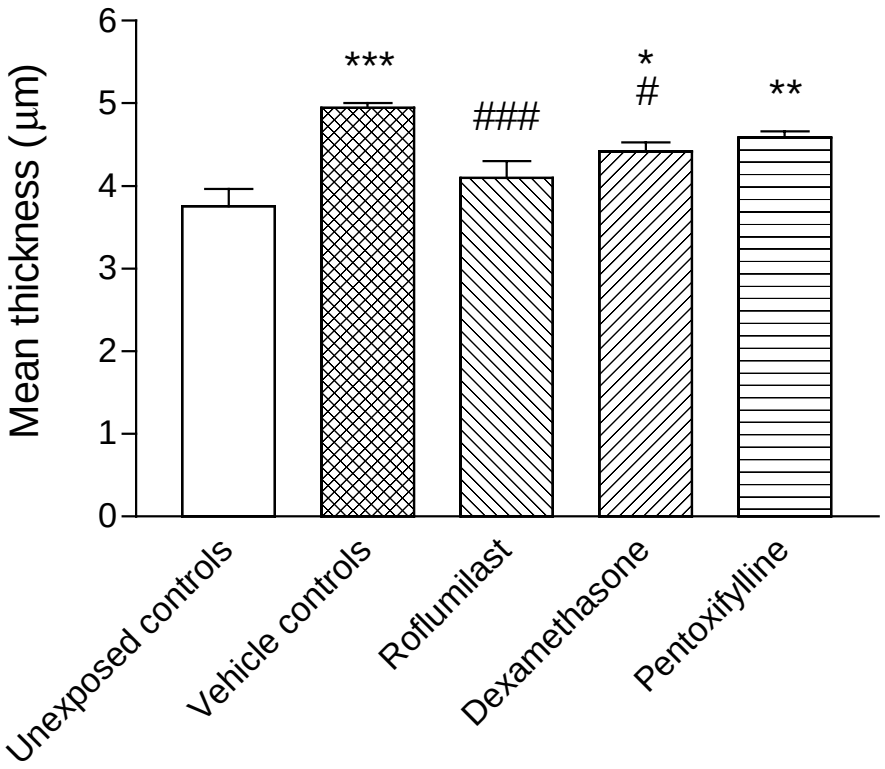
Figure 3

A.



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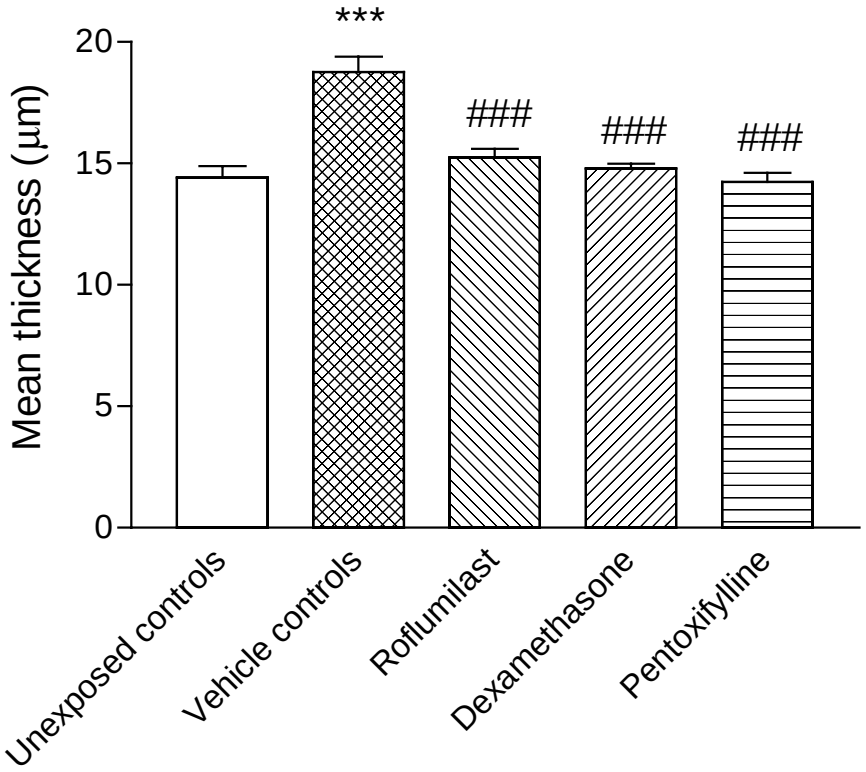

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Figure 4

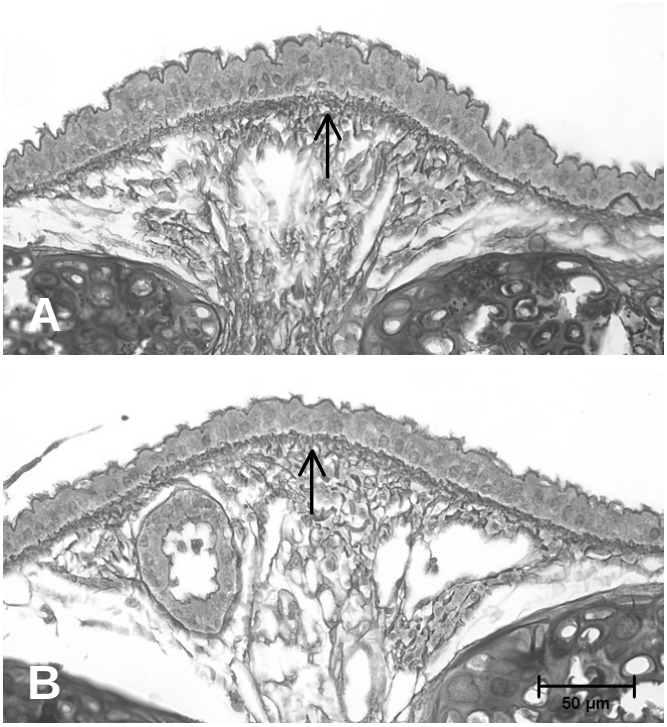

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Figure 5

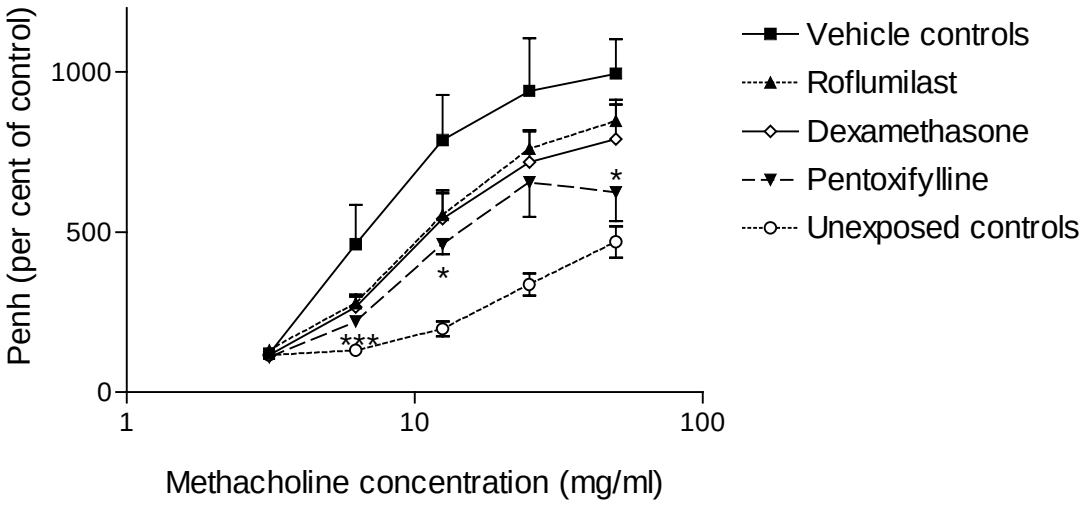

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Figure 6