Inhaled Calcilytics: Effects on Airway Inflammation and Remodeling

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SUMMARY

Chronic obstructive pulmonary disease is a major cause of mortality and there is a lack of available drugs that reduce the decline in lung function seen with disease progression. Therefore, there is clearly an unmet need for new therapeutics. Previously, we have shown that calcium-sensing receptor (CaSR) activation elicits airways hyper-responsiveness and inflammation in pre-clinical *in vivo* murine models of asthma. However, the role of CaSR in the progression of COPD is currently unknown. In this presentation, a role for CaSR and topical calcilytic therapies will be proposed and discussed to reduce COPD pathogenesis and disease progression. The proposal is supported by new data on the anti-inflammatory effects of the inhaled negative allosteric CaSR modulator or calcilytic, NPS89636. The effects of NPS89636 were studied in an *in vivo* model of COPD induced in guinea pigs by inhalation of lipopolysaccharide (LPS); as in human subjects with COPD, pulmonary inflammation, airways obstruction and remodeling in the guinea pig lungs were previously shown to be insensitive to inhaled corticosteroids. Here, we show that treatment with NPS89636 reduced inflammation, specifically leukocyte and neutrophil infiltration, in the airways of LPS-treated animals. In addition, calcilytic treatment reduced lung interstitial wall thickening. These effects were unlikely attributable to off-target calcilytic actions on the parathyroid glands, as free ionised blood calcium levels were not altered for up to 24 hours after calcilytic inhalation. Together, these observations suggested that topically delivered calcilytics may represent a novel treatment strategy for COPD.

INTRODUCTION

The World Health Organization reports that chronic obstructive pulmonary disease (COPD) is currently the 5th leading cause of death worldwide. The disease is characterised by progressive and irreversible decline in lung function caused by airway remodeling associated with chronic inflammation of the small airways and lung parenchyma [1]. The disease is triggered by an abnormal response to inhaled particles and gases, most commonly cigarette smoke [1]. Unlike asthma, the airways inflammation in COPD appears to be driven by neutrophils, which are not susceptible to inhibition by high doses of corticosteroids [1, 2], so that current therapeutic strategies are largely palliative. Clearly, there is an unmet clinical need to identify novel therapeutic agents that may alter the natural history of the disease [1].

New therapeutic strategies for COPD targeting airways hyper-responsiveness, remodeling or inflammation, are under investigation. These include anticholinergic bronchodilators, drugs suppressing mitogen activated protein kinase (MAPK), phosphoinositide-3 kinase (PI-3K), Rho kinase (RhoK) and phosphodiesterase-4, leukotriene and chemokine receptor blockers, neutrophil elastase inhibitors and antibodies against TNF-α [1, 3]. In addition, there is evidence that activation of arginase participates in the airways remodeling and inflammatory response in patients with COPD and animal surrogates [4, 5], suggesting that arginase could be targeted for COPD treatment.

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Recently, we have identified a possible link between the activation of arginase and the development of airways inflammation and hyperresponsiveness via L-arginine-derived polyamines and activation of the airways calcium-sensing receptor (CaSR) [6]. CaSR is a G protein-coupled receptor that signals by augmenting intracellular calcium [6-8], activating RhoK [8, 9], phosphorylating MAPK/ERK, activating PI-3K/Akt [6-8] and reducing intracellular cAMP concentrations [6-8]. While Ca^{2+} is the physiological ligand of the CaSR, many other polyvalent cations and polycations can activate it, including divalent cations (Cd^{2+} and Ni^{2+}), trivalent cations (Gd^{3+} and La^{3+}) and arginase-derived polyamines (spermine, spermidine, putrescine) [6, 7]. Other polycationic compounds implicated in the pathogenesis of obstructive airways disease, including eosinophil-derived major basic protein [10] and other cationic proteins [11, 12], are also agonists of the CaSR [6]. The CaSR is pivotal in extracellular Ca^{2+} homeostasis and its expression is highest in the tissues involved in the control of mineral ion metabolism, namely the parathyroid glands, kidneys and bone [7, 13]. Indeed, pharmacological CaSR activators have been developed for the treatment of genetic and acquired disorders of mineral ion metabolism caused by defective CaSR expression or function [13]. For example, calcimimetics such as cinacalcet (Sensipar, Mimpara) have been on the market since 2004 for the treatment of tertiary hyperparathyroidism. Conversely, calcilytics were developed by NPS Pharmaceuticals (now Shire), GSK, Novartis and Japan Tobacco initially as possible novel therapeutic agents for the prophylaxis of osteoporosis, but their development has since been discontinued owing to lack of efficacy [14, 15]. NPS/Shire is currently repurposing its calcilytics for a rare disease (autosomal dominant hypocalcaemia with hypercalciuria) caused by activating CaSR mutations

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[16]. With our recent report of the efficacy of calcilytics in the airway [6], there is now increasing interest in the repurposing of CaSR modulators for lung disease.

In addition to its prominent role in the regulation of free ionized Ca^{2+}, the CaSR is expressed in tissues not known to be involved in divalent cation homeostasis such as airways epithelial and smooth muscle cells, as well as in monocytes and eosinophils [6, 17, 18]. Based on these findings, we have demonstrated that calcilytics delivered directly to the airways exhibit the potential to abrogate hyperresponsiveness and inflammation in allergic asthma [6]. Furthermore, the CaSR has been implicated in regulating epithelial cell adhesion [9], particularly in human oesophageal epithelial cells where CaSR stimulation decreases the expression of intercellular adhesion molecules [19]. Polycations can also enhance epithelial permeability in guinea pig trachea [20], indicating a possible role for the CaSR signalling in mediating the loss of epithelial cell integrity, as observed in obstructive airways diseases including COPD. Finally, mucus overproduction is one of the cardinal clinical features of obstructive airways diseases, particularly COPD. It was demonstrated recently that CaSR activation underlies hypoxia-induced mucus hypersecretion in cultured human epithelial cells [21], providing yet another precedent for therapeutic benefit calcilytics in COPD.

Despite these accumulating data there is presently no direct evidence that the CaSR is involved in the pathogenesis of COPD and that CaSR inhibition can abrogate COPD-associated airways inflammation and remodeling. Consequently, we set out in this study to establish such evidence in a pre-clinical surrogate of COPD *in vivo*, incorporating delivery of the CaSR inhibitor by inhalation, which is putatively most appropriate to the management of airways disease and is likely to minimise systemic unwanted effects.

METHODS

Animal experiments

All animal procedures were approved by local ethical review and conformed to the regulations of the UK Home Office according to the Animals (Scientific Procedures) Act 1986 and ARRIVE guidelines [22]. Male, Dunkin-Hartley guinea pigs obtained from Harlan were housed in a room with a 12 hour dark/light cycle, and access to food and water *ad libitum*. Induction of COPD-like chronic neutrophilic inflammation was adapted from Toward *et al*, 2001 [23, 24]. Briefly, animals were exposed to nebulized lipopolysaccharide (LPS, 30 µg/ml in saline) delivered by a Pulmostar nebuliser (DeVilbiss, Sunrise Medical) at 0.3 mL/min driven by a constant pressure of 20 lb p.s.i. to a Perspex exposure chamber for one hour, on alternate days over eighteen days. Animals received drugs/vehicles for nine days commencing after the fifth LPS exposure using the same aerosol delivery technique: NPS89636 3 µM (vehicle: 0.03% DMSO in saline) or budesonide 0.6−1.2 mg/ml (vehicle: 30% DMSO, 30% ethanol in saline), 30 min before LPS exposure (Figure 1 A).

BALF analysis

Animals were euthanized 24 hrs after the ninth exposure with intraperitoneal sodium pentobarbital (Euthatal, 400 mg/kg). Bronchoalveolar lavage was performed to determine total and differential cell counts in the bronchoalveolar lavage fluid (BALF) as described previously [25]. Briefly, phosphate buffered saline (PBS) (1 mL per 100g weight of animal) was instilled into the airways through a polypropylene cannula inserted into trachea, and then gently aspirated, and the procedure was repeated twice. Total BALF cells were counted immediately using a Neubauer haemocytometer. For differential cell counts, cells in the BALF were immobilised on

slides using the Shandon Cytospin 3 and then submerged in 1.5% Leishman's stain in methanol, and cells were counted using microscope with a 100x oil immersion objective. For immunofluorescence, the BALF cells were allowed to adhere to a glass slide for 1 hour at room temperature, and then fixed with 2% formalin for 15 mins. Cells were blocked and permeabilized with 0.1% Tween-20 and 1 mg/ml bovine serum albumin in PBS for 2 hours, and samples incubated with rabbit anti-CaSR antibody (1:100, Abcam) for 2 hours. After washing, the secondary antibody (goat anti-rabbit Alexa 594, 1:200 Invitrogen, Thermo Fisher Scientific, Waltham, MA USA) was applied for 1 hour and nuclei were counterstained with Hoechst (1:10000) for 20 minutes. Negative control staining was performed by omitting the primary antibody. Images were acquired using an Olympus BX61 upright epifluorescence microscope and a 100x oil objective.

Blood calcium analysis

Whole blood was collected from the carotid artery, allowed to clot overnight at 4 °C and then centrifuged (3,000g for 10 min at 4 °C). Free Ca^{2+} determinations were performed on supernatant serum by the Clinical Biochemistry service (Heath hospital, Cardiff, UK) using a calcium electrode (Radiometer ABL800 Flex).

Histology

Half of the lung was clamped and infused with neutral buffered formalin solution, then placed in a tube containing the solution for a further 24 hours. Fixed lungs were then embedded in paraffin wax. Five μm sections were cut and stained with haematoxylin and eosin (H&E) using a Leica Autostainer XL (Leica Microsystems, Milton Keynes, United Kingdom) according to the manufacturer's protocol, and

histopathology scoring was performed by a pathologist who was ignorant of the treatments. Grades of 4 to 5 were given for samples that had increased fibrosis with definite damage to lung architecture with formation of fibrous bands or small fibrous mass.

Drugs and materials

NPS89636 was a gift from NPS Pharmaceuticals, Inc. All other chemicals purchased from Sigma-Aldrich (Dorset, UK) unless otherwise stated.

Data analysis

Data were analysed using Microsoft Office Excel (Microsoft Corporation) and Prism 6 (Graph Pad Inc.). Student's two-sided, unpaired t-test was used to compare groups of two data sets, and one-way ANOVA with the Bonferroni *post hoc* test used to compare three or more data sets. All results are shown as mean ± standard error of the mean (SEM), with n being the number of animals.

RESULTS AND DISCUSSION

It is well established that corticosteroids have little effect on COPD progression. Specifically, inhaled corticosteroids do not reduce neutrophilic inflammation in patients with COPD [1]. Neutrophil infiltration into the airways of guinea pigs following acute exposure to LPS, an agent present in tobacco smoke, was shown to be insensitive to treatment with inhaled budesonide [26], and was only partially sensitive to systemic dexamethasone [23]. Here we show that inhaled budesonide does not significantly alter total, macrophage, neutrophil, eosinophil and lymphocyte infiltration into the BALF of guinea pigs chronically treated with LPS (Figure 1),

echoing observations in patients with COPD [1]. In contrast, inhalation of the calcilytic, NPS98636 (3 µM) prior to LPS exposure significantly reduced total leukocyte counts, as well as neutrophil, eosinophil and lymphocyte infiltration into airways of LPS-treated animals (Figure 2 A, C ,D ,E). Moreover, calcilytic inhalation significantly reduced LPS-induced interstitial wall thickening (Figure 3).
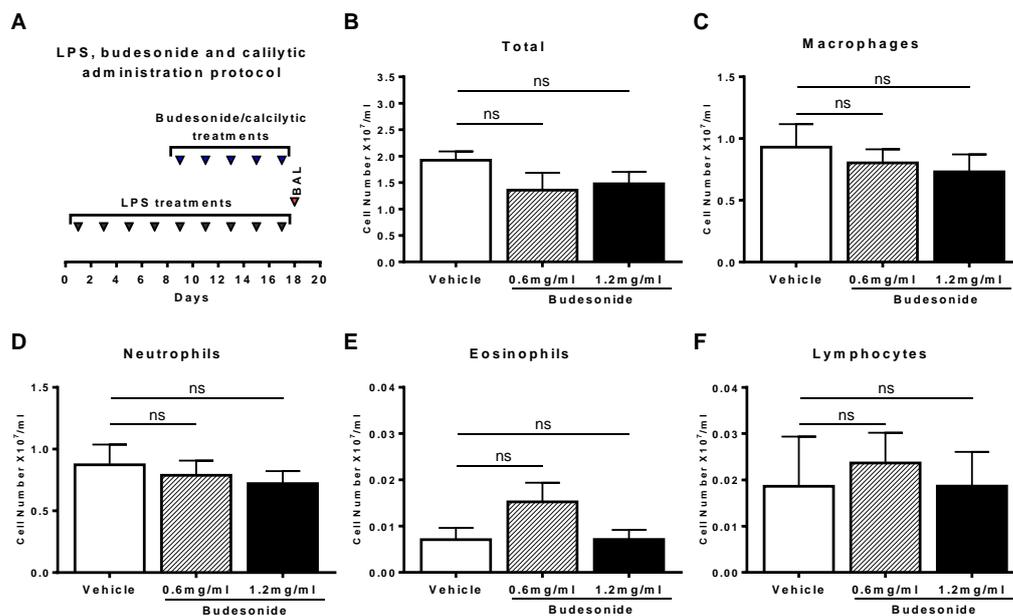

Figure 1. *The inhaled corticosteroid, budesonide, did not affect leukocyte infiltration into the BALF of LPS-treated guinea pigs sampled at Day 18*. LPS and budesonide or calcilytic treatment protocol used in the present study (**A**). Effect of vehicle, 0.6 mg/ml, or 1.2 mg/ml budesonide inhalation on total (**B**) and differential (macrophages (**C**), neutrophils (**D**), eosinophils (**E**), and lymphocytes (**F**)) cell counts in BALF from chronically LPS-treated guinea pigs. Each data point represents mean ± SEM, n=4−10. Performed using a one-way ANOVA with Bonferroni *post hoc* test; ns = not significant ($p > 0.3$).
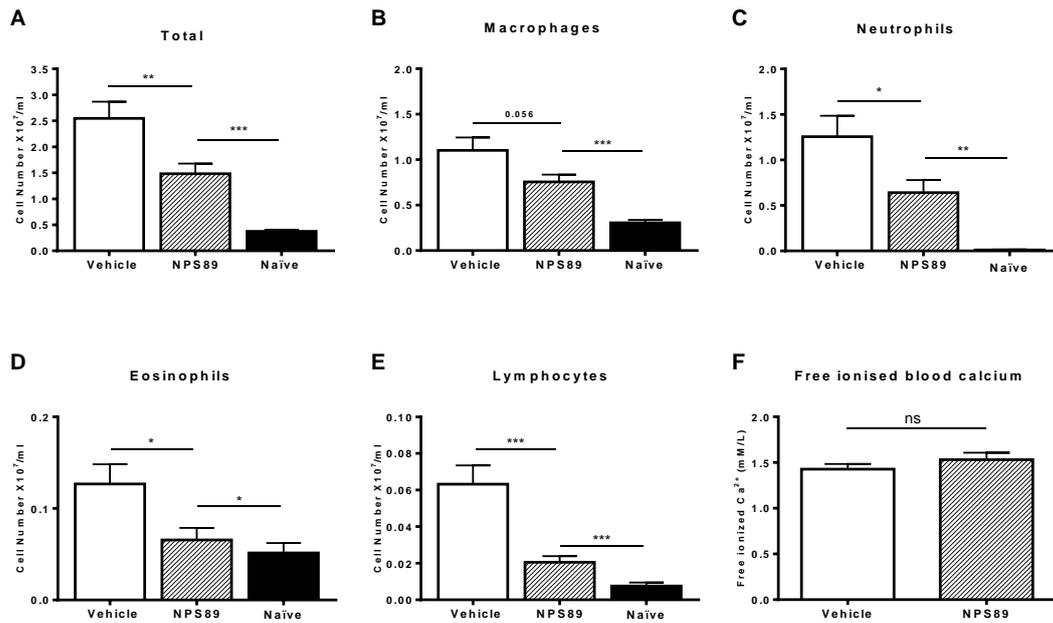
Figure 2. *An inhaled calcilytic, NPS89636, suppressed leukocyte infiltration into the BALF of LPS-treated guinea pigs on Day 18 of the protocol shown in Fig 1.* Effect of NPS89636 (NPS89, 3 µM) on total (**A**), and differential macrophage (**B**), neutrophil (**C**), eosinophil (**D**) and lymphocyte (**E**) cell counts in the BALF, and free ionised calcium concentration in blood (**F**) in chronic LPS-treated and untreated (naïve) guinea pigs. Each data point represents the mean ± SEM, n=5−12. Statistical comparison done by one-way ANOVA with Bonferroni *post hoc* test; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.
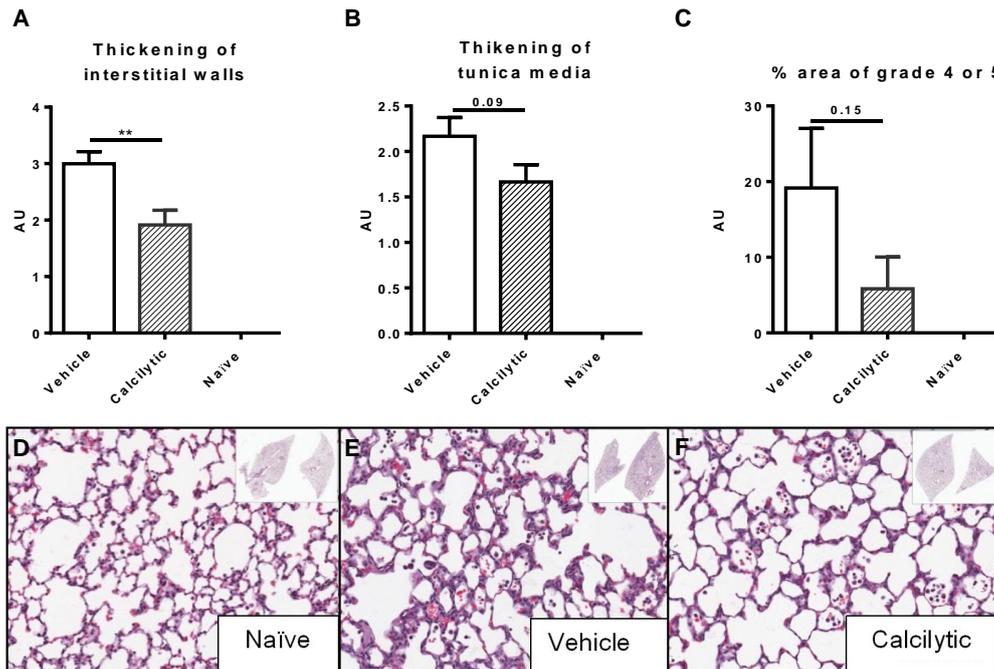
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Figure 3. *Histology of lungs from LPS-treated guinea pigs from Figure 2.*

Interstitial wall thickening (**A**), thickening of the tunica media (**B**), and % area of grade 4 or 5 (fibrotic histology) (**C**) in absorbance units (AU) were increased in the LPS-treated Guinea pigs (vehicle) compared with naïve animals. These effects were significantly reduced by the calcilytic NPS89636 (NPS89, 3 µM). Each data point represents mean ± SEM, n=2 (naïve) and n=12 (vehicle and NPS98). Representative images of the sections from naïve (**D**), vechicle (**E**) or calcilytic (**F**) pre-treated airways stained with H&E. Unpaired t-test, **$p < 0.01$.

Systemic calcilytics were initially developed to target the parathyroid gland CaSR to evoke oscillating plasma parathyroid hormone (PTH) levels, a known anabolic stimulus [13]. To test whether the beneficial effects of inhaled calcilytics could be ascribed to their systemic actions via the parathyroid CaSR, we measured ionised calcium concentrations in the blood of challenged animals, and found that the mean

concentration was not significantly altered following calcilytic inhalation, indicating that changes in PTH are unlikely to be responsible for the reduction in inflammation produced by inhaled calcilytics (Figure 2F).

Finally, a role for the CaSR receptor signalling in inflammatory responses is becoming more apparent. Being an integrating component of the inflammasome, the CaSR appears to be the key to monocyte and macrophage activation [17, 27, 28] and migration to sites of inflammation [29]. It is also known that CaSR expression itself is upregulated by pro-inflammatory cytokines [6, 30, 31], creating grounds for a vicious cycle that further amplifies the immune response. Here we show that the CaSR is expressed in BALF cells from naïve guinea pigs (Figure 4 A), confirming that it has the propensity to participate in pathological processes in the airways including COPD (Figure 5). Identification of the precise mechanisms whereby calcilytics inhibit these processes in COPD requires further investigation.
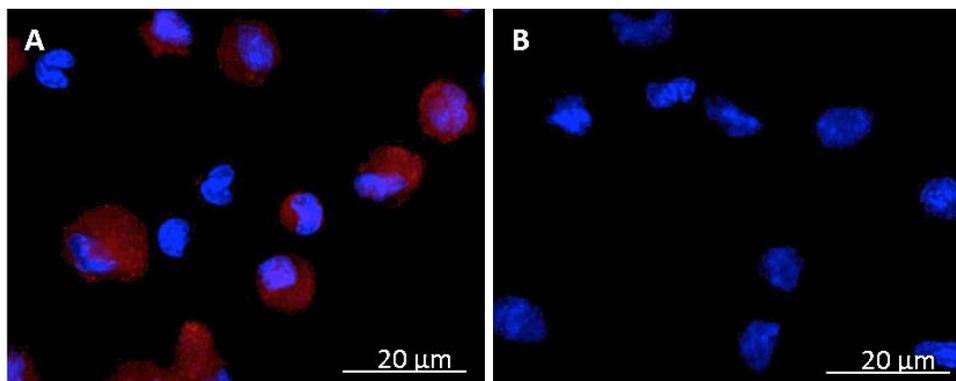

Figure 4. *Immunocytochemistry of BALF leucocytes from naïve guinea pigs.*
A) BALF cells stained with CaSR antibody (red) and nuclear stain (Hoechst, blue) show immunoreactivity for CaSR. **B**) Negative control.
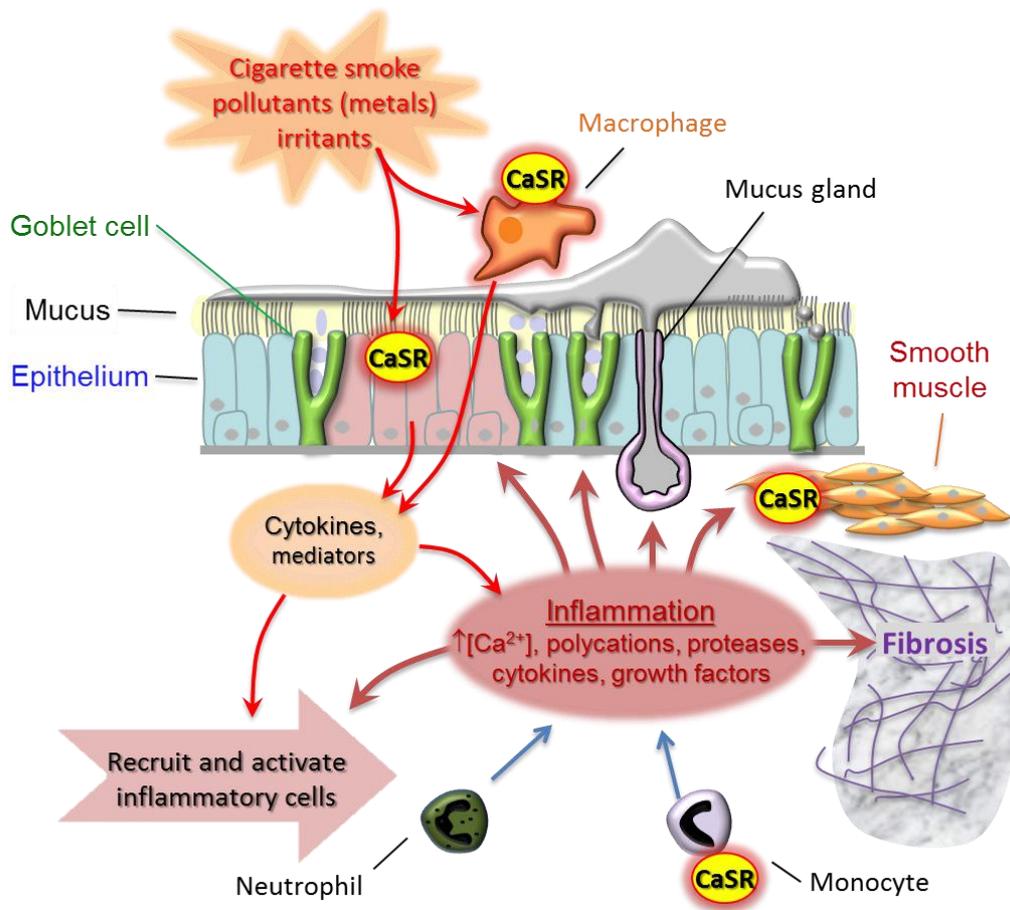
Figure 5. *Proposed CaSR signalling mechanisms contributing to COPD.*

Irritants present in tobacco smoke or pollutants (such as Cd^{2+}, Ni^{2+}, Gd^{3+} and La^{3+}) may stimulate CaSR expressed in macrophages and epithelial cells, activating them and promoting inflammatory mediator secretion. Attracted inflammatory cells release proteases, growth factors and cytokines that can lead to fibrosis, goblet cell hyperplasia, mucus overproduction and smooth muscle cellular proliferation. Polycations released by inflammation, attract monocytes and neutrophils, and further activate smooth muscle and epithelial cells setting the scene for a positive feedback loop sustaining the inflammatory process.

CONCLUSIONS

Our data are consistent with the hypothesis that calcilytics targeting the CaSR represent a novel therapeutic approach in the management of COPD. We demonstrate here that pre-treatment with an inhaled calcilytic can decrease steroid-resistant neutrophilic inflammation and attenuate airways remodeling in an animal COPD surrogate even when administered late in the course of LPS challenge. Calcilytic drugs were demonstrated to have an acceptable safety profile in clinical trials in humans, albeit lacking efficacy for the prophylaxis and treatment of osteoporosis. The re-purposing of calcilytics as topically deliverable drugs targeting chronic obstructive pro-inflammatory airway disorders offers a tantalising alternative to current methods of disease management.

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