

Intranasally administered serelaxin abrogates airway remodelling and attenuates airway hyperresponsiveness in allergic airways disease

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Summary

Background The peptide hormone relaxin plays a key role in the systemic hemodynamic and renovascular adaptive changes that occur during pregnancy, which is linked to its anti-remodelling effects. Serelaxin (a recombinant form of human gene-2 relaxin) has been shown to inhibit lung fibrosis in various disease models and reverse airway remodelling and airway hyperresponsiveness (AHR) in allergic airways disease (AAD).

Objective Although continuous systemic delivery of exogenous serelaxin alleviates allergic fibrosis and AHR, more direct routes for administration into the lung have not been investigated. Thus, intranasal administration of serelaxin was evaluated for its ability to reverse airway remodelling and AHR associated with AAD.

Methods Female Balb/c mice were subjected to a 9-week model of chronic AAD. Subgroups of animals ($n = 12/\text{group}$) were then treated intranasally with serelaxin (0.8 mg/mL) or vehicle once daily for 14 days (from weeks 9–11). Saline-sensitized/challenged mice treated with intranasal saline served as additional controls. Differential bronchoalveolar lavage (BAL) cell counts, ovalbumin (OVA)-specific IgE levels, tissue inflammation, parameters of airway remodelling and AHR were then assessed.

Results Chronic AAD was associated with significant increases in differential BAL cell counts, OVA-specific IgE levels, inflammation, epithelial thickening, goblet cell metaplasia, TGF- β 1 expression, epithelial Smad2 phosphorylation (pSmad2), subepithelial collagen thickness, total lung collagen concentration and AHR (all $P < 0.05$ vs. respective measurements from saline-treated mice). Daily intranasal delivery of serelaxin significantly diminished AAD-induced epithelial thickening, epithelial pSmad2, subepithelial and total lung collagen content (fibrosis) and AHR (all $P < 0.05$ vs. vehicle-treated AAD mice).

Conclusions and Clinical Relevance Intranasal delivery of serelaxin can effectively reduce airway remodelling and AHR, when administered once daily. Respirable preparations of serelaxin may have therapeutic potential for the prevention and/or reversal of established airway remodelling and AHR in asthma.

Keywords airway hyperresponsiveness, airway remodelling, allergic airways disease, asthma, intranasal, serelaxin

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Introduction

Asthma is a chronic inflammatory disease of the airways that affects both adults and children [1, 2]. Certain symptoms and airway inflammation can be effectively controlled using inhaled aerosols of beta agonists and/or corticosteroids or other anti-inflammatory

agents. However, in many cases, these therapies are not sufficient to eliminate progression of long-term structural remodelling of the airways [3, 4]. Airway remodelling consists of epithelial thickening and goblet cell metaplasia, subepithelial fibrosis, smooth muscle thickening and angiogenesis; which can occur early in asthma pathogenesis. Finding a therapy that reverses or

even prevents airway remodelling is critical, as remodelling contributes to airway hyperresponsiveness (AHR) and fixed airway obstruction independently of inflammation [5, 6], and is likely associated with disease severity and steroid resistance.

In this regard, we and others have shown that the naturally occurring hormone relaxin has pleiotropic effects that effectively attenuate remodelling and related dysfunction in several organs [7–9]. Mainly produced in reproductive organs of women and men, relaxin expression has also been identified in the mammalian lung, particularly in epithelial cells, fibroblasts and airway smooth muscle cells [10]. In addition to softening the pelvic ligaments and relaxing the uterus in preparation for parturition [11], relaxin augments the hemodynamic and renovascular adaptive changes that occur during pregnancy [12] through its vasorelaxant and matrix remodelling properties [7, 9]. Consistent with this, other preclinical studies have shown the antifibrotic actions of relaxin in several organs including the lung [7–11].

Of further relevance to its actions in the allergic lung, relaxin has been found to inhibit the infiltration of pro-inflammatory cells [13, 14], promote dilation of alveolar blood capillaries, reduce the thickness of the air-blood barrier [14] and inhibit airway fibrosis and AHR [10, 15] in models of allergic airways disease (AAD) that undergo several features of human asthma. Furthermore, serelaxin (a recombinant form of human gene-2 relaxin) markedly attenuated the fibrosis associated with interstitial and parenchymal lung disease [16, 17] and hypoxic pulmonary hypertension [18] in experimental models, suggesting that its antiremodelling actions are independent of aetiology. The proposed mechanisms involved in serelaxin's actions include its ability to inhibit the profibrotic influence of transforming growth factor (TGF)- β 1 [16] and myofibroblast contractility [17], which in turn limits myofibroblast-mediated extracellular matrix (ECM)/collagen deposition. Additionally, serelaxin promotes the activity of various matrix metalloproteinases (MMPs) that facilitate collagen degradation [10, 16].

The beneficial effects of serelaxin have previously been demonstrated by systemic administration via microinfusion pumps [10, 15–19]. In the setting of systemic sclerosis (scleroderma), systemic delivery was critical for serelaxin effects [20]. However, the use of systemically delivered relaxin for the treatment of asthma in the patient setting poses significant barriers, and a more feasible means for drug delivery to the target organ (such as intranasal delivery) is required [21]. The purpose of this study was to evaluate the efficacy of intranasally administered serelaxin once daily in a murine model of chronic AAD [22, 23] that presents with the three central features of asthma: airway inflammation, airway remodelling and AHR.

Materials and methods

Animals

Six to eight-week-old female Balb/c mice were obtained from the Walter and Eliza Hall Institute (Melbourne, Victoria, Australia), housed in the Murdoch Children's Research Institute (MCRI) Animal Facility under specific pathogen-free conditions and maintained on a 12 h light–12 h dark cycle. All procedures were approved by the MCRI Animal Ethics Committee, which adheres to the Australian Code of Conduct for care and use of laboratory animals for scientific purposes.

Induction of chronic AAD

A chronic model of ovalbumin (OVA)-induced AAD [22] was established in mice ($n = 24$). Mice were sensitized i.p. on day 0 and 14 with 10 μ g Grade V chicken egg ovalbumin (Sigma–Aldrich Corp., St. Louis, MO, USA) and 0.4 mg aluminium potassium sulphate (alum) in 0.5 mL saline, then challenged by whole body inhalation exposure to aerosolized 2.5% OVA (weight/volume of saline) three times a week from days 21–63 (30 min per session) using an ultrasonic nebulizer [23]. Control mice ($n = 12$) were sensitized with 0.4 mg alum in 0.5 mL saline and challenged with nebulised saline.

Intranasal treatment

Ovalbumin (OVA)-sensitized/challenged mice (with established AAD) were treated once daily with vehicle (20 mM sodium acetate buffer, pH 5.0; $n = 12$) or 0.8 mg/mL serelaxin (equivalent to the 0.5 mg/kg/day dose that was previously shown to be effective for the treatment of AAD [10]; kindly provided by Corthera Inc, San Carlos, CA, USA; a subsidiary of Novartis Pharma AG, Basel, Switzerland; $n = 12$) from days 64–77 via intranasal administration. Briefly, mice were lightly anesthetized with isoflurane inhalation, held in a supine position and administered 50 μ L of vehicle or serelaxin peptide solution into both nostrils (25 μ L per nostril) using an automatic pipette. A fourteen-day treatment period was chosen to replicate the time frame used to evaluate the effects of systemic serelaxin treatment [10]. Saline-sensitized/challenged control mice received 50 μ L of saline intranasally, once daily over the 14-day treatment period.

Measurement of AHR

Twenty-four hours after the last vehicle/drug administration, methacholine-induced airway reactivity was assessed by invasive plethysmography as described before [10, 23]. Mice were anaesthetized intraperitoneally

with 200 µg/g ketamine and 10 µg/g xylazine. Tracheotomy was performed using an 18-gauge tracheotomy tube and jugular vein cannulated with a 0.61 × 0.28 mm polyethylene tube (Microtube Extrusions, North Rocks, NSW, Australia). Mice were then placed in a plethysmograph chamber (Buxco Research Systems, Wilmington, NC, USA) where increasing concentrations of acetyl-β-methacholine (from 31.25 µg/kg to 500 µg/kg) were delivered intravenously in five doses. After every dose, airway resistance and compliance were measured (Biosystem XA version 2.7.9; Buxco Electronics Inc, Wilmington, NC, USA). The change in airway resistance calculated by the maximal resistance after each dose minus baseline resistance (PBS alone) was plotted against each dose of methacholine evaluated.

Bronchoalveolar lavage (BAL)

Three 0.5 mL lavages were pooled in ice-cold 20% FCS/PBS before red blood cells were lysed, and cells were washed twice in 5% FCS/PBS. Total viable cell counts were performed manually by trypan blue exclusion. Cytospin smears (2×10^4 cells) were prepared, fixed with methanol and stained with modified Wright's stain (Hema-Tek®; Bayer Diagnostics, Leverkusen, Germany). Differential counts of eosinophils, neutrophils, lymphocytes and macrophages were determined using light microscopy (×40 magnification, 100 cells counted) in a blinded manner.

Determination of OVA-specific IgE and serelaxin levels by ELISA

Serum was obtained by cardiac puncture 24 h after the last vehicle/drug administration and stored at -80°C . Serum OVA-specific IgE titres were determined from the three groups of animals studied by ELISA, as previously described [24]. Additionally, serum and BAL serelaxin levels were determined from serelaxin-treated mice (24 h after the last drug administration; $n = 5$), using the human relaxin-2 Quantikine ELISA kit (R&D Systems, Minneapolis, MN, USA); as per the manufacturer's instructions.

Lung histopathology

The right lung was fixed in 10% neutral buffered formalin for 24–48 h before it was transversely cut into three pieces, processed and embedded routinely in paraffin wax. Representative sections of tissue, 3 µm each, were taken and stained with haematoxylin and eosin (for assessment of lung inflammation), alcian blue-periodic acid-Schiff (for assessment of goblet cell metaplasia) and Masson trichrome (for assessment of epithelial thickness and subepithelial collagen deposition). All

analyses of histological sections were performed in a randomized and blinded fashion.

Histological evaluation of inflammation

Sections were stained with Mayer's haematoxylin and eosin Y (Grate Scientific, Melbourne, Vic., Australia) and observed under low-power light microscopy (×50 magnification). Histological grading of inflammation severity from 0 to 4 was assigned to every slide (0 = no detectable inflammation; 1 = occasional inflammatory cell aggregates, pooled size $< 0.1 \text{ mm}^2$; 2 = some inflammatory cell aggregates, pooled size $\sim 0.2 \text{ mm}^2$; 3 = widespread inflammatory cell aggregates, pooled size $\sim 0.3 \text{ mm}^2$; 4 = widespread and massive inflammatory cell aggregates, pooled size $\sim 0.6 \text{ mm}^2$); and was performed blinded by the same investigator. The area of inflammatory cell aggregates was measured using Image Pro Discovery software (Media Cybernetics, Silver Spring, MD, USA) in μm^2 and converted to mm^2 .

Assessment of goblet cell metaplasia

Sections were stained with alcian blue-periodic acid-Schiff (ABPAS) to enumerate goblet cells. Mucin-positive cells observed at high power (×400 magnification) were manually counted from four to five different airways per section and expressed as the mean number of goblet cells per 100 µm of basement membrane (Image Pro Discovery).

Masson trichrome staining

Masson trichrome staining was performed to detect subepithelial collagen deposition in the airways. Briefly, dewaxed sections (passed through xylene, absolute ethanol, 70% ethanol and tap water) were post-fixed in Bouin's fixative (Grate Scientific) for 1 h; stained with Weigert's haematoxylin for 10 min; incubated with Masson's Red for 45 min; followed by 7 min treatment with phosphotungstic acid and 15 min incubation with aniline blue. Slides were then rinsed briefly, dehydrated, cleared and mounted.

Immunohistochemistry

Paraffin-embedded lung sections were immunohistochemically stained for TGF-β1 [23, 25] and phosphorylation of Smad2 (pSmad2; an intracellular protein that promotes TGF-β1 signal transduction/activity) [25], using primary antibodies to TGF-β1 (sc146; 1 : 200 dilution; Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) and pSmad2 (#3108; 1 : 250 dilution; Cell Signaling Technology, Danvers, MA, USA) as described before. Detection of antibody staining was completed

using the DAKO EnVision anti-rabbit kit (DAKO Corp., Carpinteria, CA, USA) and 3,3'-diaminobenzidine (DAKO Corp.); where sections were counterstained with haematoxylin.

Morphometric analysis of structural changes

Changes in airway TGF- β 1 expression and epithelial pSmad2 levels were assessed by image analysis of corresponding immunohistochemical-stained sections, while changes in epithelial thickness and subepithelial collagen (fibrosis) around the airway lumen were assessed from Masson trichrome-stained sections; which were all captured (at $\times 100$ magnification) using a SPOT digital camera (Q Imaging, Burnaby, BC, Canada) and analysed with Image Pro Discovery (Media Cybernetics). Four to five airways (150–350 μ m in diameter) per mouse were assessed. Epithelial thickness and subepithelial collagen regions were traced with a digital pen and the thickness of each region calculated by the imaging software. Results were expressed as mean thickness (μ m) of the 4–5 airways sampled. Airway TGF- β 1 staining was calculated as a percentage of the total area within each field analysed, while the number of pSmad2 positively stained nuclei per epithelial layer was determined and expressed per 100 μ m basement membrane length.

Hydroxyproline analysis

The second largest lung lobe from each mouse was treated as described previously [10, 19] for the determination of hydroxyproline content. Hydroxyproline values were estimated based on a standard curve constructed with serial dilutions of a 0.1 mg/mL stock of trans-4-hydroxyproline-L-proline (Sigma-Aldrich) and expressed per 2nd largest lung lobe (μ g).

Statistical analysis

All data were expressed as the mean $\pm$ SEM and analysed using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). A Mann–Whitney *U*-test was used to compare differences between non-parametric groups in all experiments performed except for the analysis of the lung function (AHR) data, which was assessed by a two-way ANOVA with Bonferroni's *post hoc* test. $P < 0.05$ was considered to be statistically significant.

Results

Validation of the mouse model of chronic AAD

Mice subjected to the chronic model of AAD were confirmed to be adequately sensitized to OVA, by demon-

strating increased BAL cell counts and OVA-specific IgE levels compared to saline-treated controls (both $P < 0.05$ vs. saline group; Table 1). Of the BAL cell counts determined, eosinophil cell numbers ($P < 0.01$ vs. saline group) and monocytes ($P < 0.05$ vs. saline group) in particular, were significantly higher in lung washouts of OVA-vehicle mice compared with that measured in saline-treated animals (Table 1), indicative of OVA-induced allergic disease.

As expected, vehicle-treated OVA mice (OVA-vehicle) were also associated with markedly increased inflammation (Fig. 1), epithelial thickening (Fig. 2), goblet cell metaplasia (Fig. 3b,d), subepithelial collagen deposition (Fig. 4b,d), lung hydroxyproline content (Fig. 4e), TGF- β 1 expression (Fig. 5b,g), epithelial pSmad2 levels (Fig. 5e,h) and AHR (Fig. 6) compared with respective measurements obtained from saline-treated controls (all $P < 0.05$ vs. saline group), validating the murine model of chronic AAD [10, 22, 23].

Effects of intranasal serelaxin treatment on inflammation, airway remodelling and AHR in chronic AAD Airway inflammation

Confirmation that serelaxin (0.8 mg/mL) treatment (OVA-serelaxin) was delivered to mice was established by the ELISA measurements of serelaxin in the BAL (39.81 ± 5.89 ng/mL) and serum (9.02 ± 0.93 ng/mL) of OVA-serelaxin animals. BAL differential cell counts (Table 1) and inflammation score of H&E-stained lung sections (Fig. 1) from OVA-serelaxin mice were not significantly different to corresponding measurements obtained from OVA-vehicle animals.

Epithelium morphology

Epithelial thickness in Masson trichrome-stained sections from OVA-serelaxin mice was significantly reduced compared with that measured in OVA-vehicle

Table 1. Evaluation of allergic responses by bronchoalveolar lavage (BAL) differential cell counts ($\times 10^4$ cell/mL) and OVA-specific IgE by ELISA

Groups	Saline	OVA-vehicle	OVA-serelaxin
Total cells*	12.33 ± 1.89	$25.50 \pm 4.10^\dagger$	$25.38 \pm 2.85^\dagger$
Eosinophils*	0.08 ± 0.31	$1.05 \pm 0.64^\dagger$	$0.85 \pm 0.27^\dagger$
Neutrophils*	0.28 ± 0.56	0.56 ± 0.13	0.69 ± 0.27
Lymphocytes*	2.38 ± 2.04	5.58 ± 1.66	5.73 ± 1.44
Monocytes*	9.56 ± 2.61	$18.31 \pm 1.40^\dagger$	$18.09 \pm 1.33^\dagger$
OVA-specific IgE (AU)*	0 ± 0.01	$0.37 \pm 0.08^\dagger$	$0.4 \pm 0.04^\dagger$

*Values are the mean $\pm$ SEM cells or OVA-specific IgE analysed from 9 to 12 mice/group.

$^\dagger P < 0.05$ and $^\ddagger P < 0.01$ vs. corresponding value from saline group.

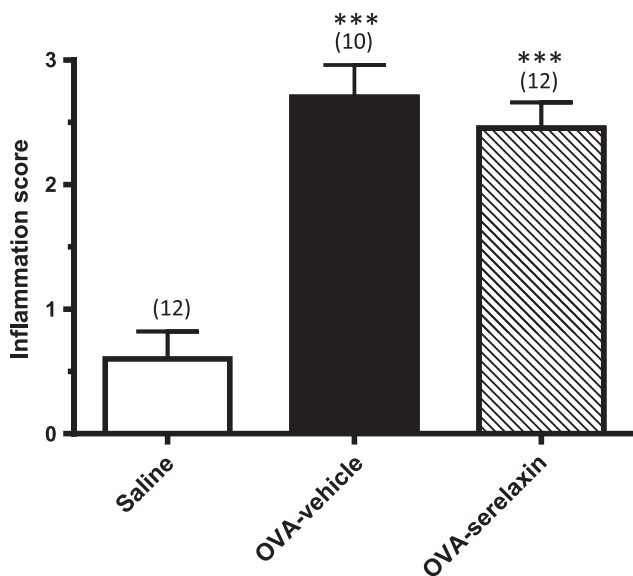


Fig. 1. Effect of intranasal serelaxin treatment on overall lung inflammation in chronic allergic airways disease. Shown in the mean $\pm$ SEM inflammation score in H&E-stained lung tissue sections obtained from saline, OVA-vehicle and OVA-serelaxin mice. Sections were scored for the number and distribution of inflammatory aggregates on a scale of 0 (no apparent inflammation) to 4 (severe inflammation). Numbers in parentheses represent number of mice analysed per group. *** P < 0.001 vs. saline group.

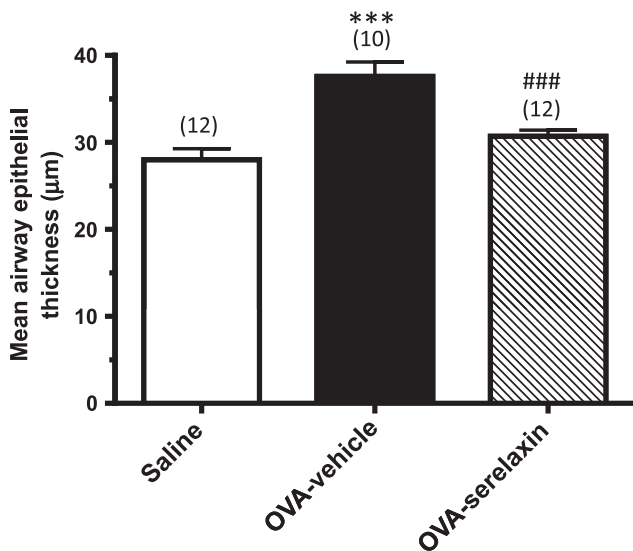


Fig. 2. Effect of intranasal serelaxin treatment on airway epithelial thickness in chronic allergic airways disease. Shown is the mean $\pm$ SEM airway epithelial thickness (μ m) in Masson trichrome-stained lung sections from saline, OVA-vehicle and OVA-serelaxin mice; as measured by morphometric analysis. Numbers in parentheses represent number of mice analysed per group. *** P < 0.001 vs. saline group, ### P < 0.001 vs. OVA-vehicle group.

animals (P < 0.001 vs. OVA-vehicle group; and approached that of saline-sensitized/challenged mice (no significant difference to saline group; Fig. 2).

However, no differences in goblet cell metaplasia (ABPAS-positive cells) were observed between OVA-serelaxin (Fig. 3c,d) and OVA-vehicle (Fig. 3b,d) mice (both P < 0.001 vs. saline group; Fig. 3a,d).

Airway remodelling and fibrosis

Subepithelial collagen deposition and lung hydroxyproline levels (Fig. 4) were used as measures of airway remodelling and fibrosis in the chronic model of AAD. Subepithelial collagen deposition was significantly lower in OVA-serelaxin mice (Fig. 4c,d) as compared to OVA-vehicle mice (Fig. 4b,d; P < 0.001 vs. OVA-vehicle group), but remained significantly greater than that measured in saline-treated (Fig. 4a,d) animals (P < 0.05 vs. saline group). Remarkably, lung hydroxyproline content in OVA-serelaxin mice was normalized back to levels measured in saline-treated controls (P < 0.01 vs. OVA-vehicle group; no significant difference to saline group; Fig. 4e).

TGF- β 1 and pSmad2 levels were additionally assessed to determine the mechanisms by which collagen deposition (fibrosis) was significantly diminished in OVA-serelaxin mice. While airway TGF- β 1 staining was unchanged between OVA-vehicle (Fig. 5b,g) and OVA-serelaxin (Fig. 5c,g) mice, epithelial pSmad2 nuclear staining was significantly lower in OVA-serelaxin mice (Fig. 5f,h) compared with that measured in OVA-vehicle mice (Fig. 5e,h), but remained significantly greater than that measured in saline-treated mice (Fig. 5d,h; P < 0.01 vs. OVA-vehicle group; P < 0.01 vs. saline group).

AHR

Airway reactivity was significantly lower in OVA-serelaxin mice compared with OVA-vehicle animals (three highest doses of methacholine were all P < 0.05 vs. OVA-vehicle group; Fig. 6). However, airway reactivity in OVA-serelaxin mice remained significantly higher than that measured in saline-treated control animals (4 highest doses of methacholine were all P < 0.01 vs. saline group; Fig. 6).

Discussion

The OVA-induced model of chronic AAD used in the present study resembles clinical asthma and is highly suitable for studying the pathophysiology of airway remodelling and its contribution to AHR [10, 23]. We observed significantly increased inflammation, epithelial thickness, goblet cell metaplasia, subepithelial collagen deposition, lung hydroxyproline content, TGF- β 1 and pSmad2 staining as well as AHR in mice sensitized and challenged with OVA, as previously characterized

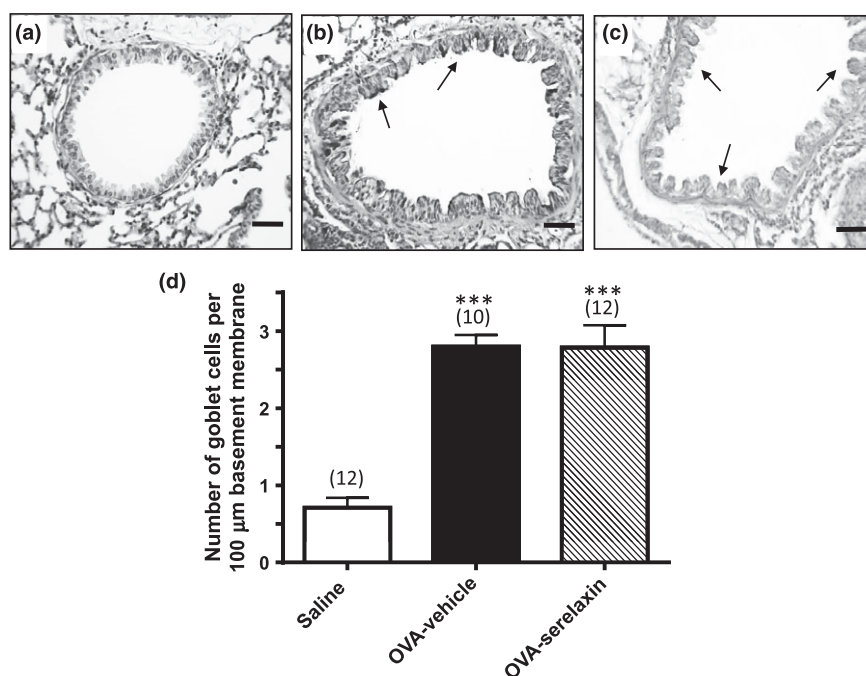


Fig. 3. Effect of intranasal serelaxin treatment on goblet cell metaplasia in chronic allergic airways disease. Representative photomicrographs of ABPAS-stained airways taken from (a) saline, (b) OVA-vehicle and (c) OVA-serelaxin mice. Scale bar = 100 μm. Also shown is mean $\pm$ SEM goblet cell numbers/100 μm basement membrane length (d) from each of the groups studied. Numbers in parentheses represent number of mice analysed per group. *** P < 0.001 vs. saline group.

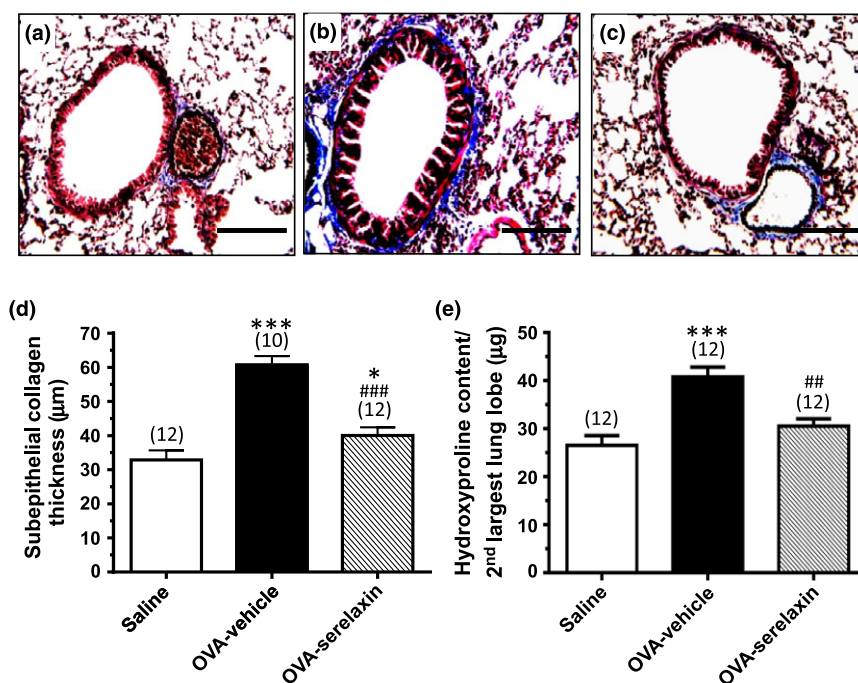


Fig. 4. Effect of intranasal serelaxin treatment on measures of fibrosis in chronic allergic airways disease. Representative photomicrographs of Masson trichrome-stained lung airway sections of (a) saline, (b) OVA-vehicle and (c) OVA-serelaxin mice. Scale bar = 300 μm. Also shown is the mean $\pm$ SEM subepithelial collagen thickness (μm) in the lamina reticularis (d) and lung hydroxyproline content (e) from each of the groups studied. Numbers in parentheses represent number of mice analysed per group. * P < 0.05, *** P < 0.001 vs. saline group, ** P < 0.01, *** P < 0.001 vs. OVA-vehicle group.

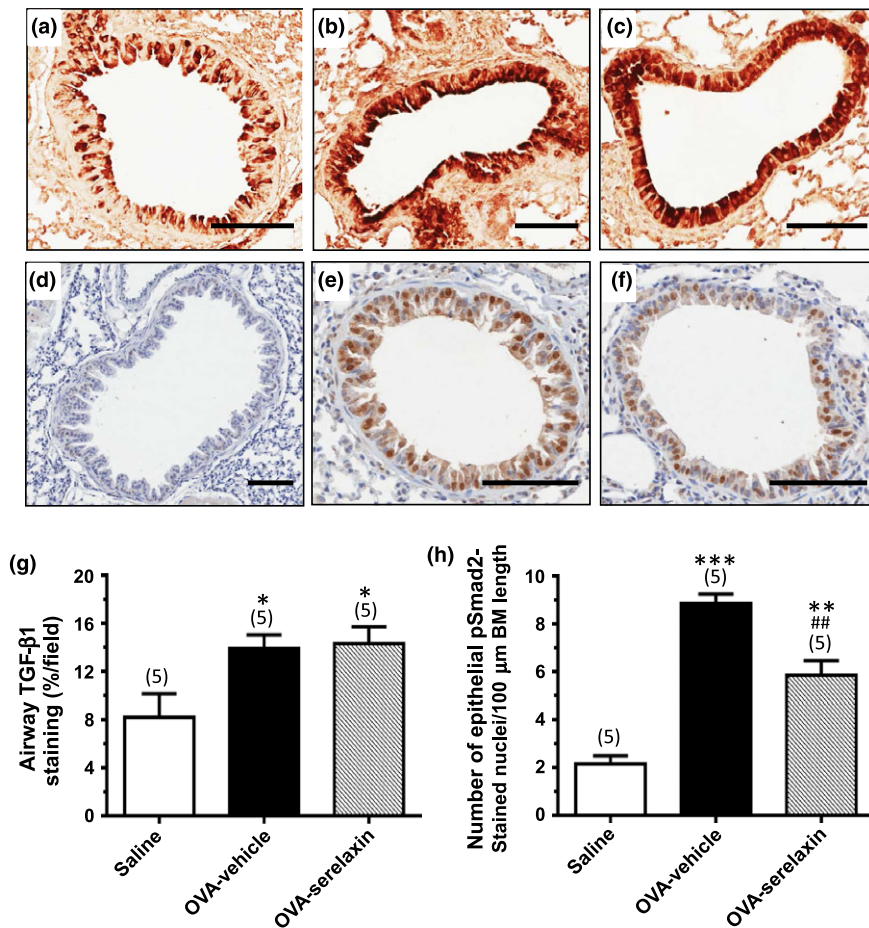


Fig. 5. Effect of intranasal serelaxin treatment on airway TGF-β1 and pSmad2 staining: promoters of collagen deposition in chronic allergic airways disease. Representative photomicrographs of TGF-β1 and pSmad2-stained lung airway sections of (a and d, respectively) saline, (b and e, respectively) OVA-vehicle and (c and f, respectively) OVA-serelaxin mice. Scale bar = 100 μm. Also shown are the mean ± SEM TGF-β1 staining levels expressed as a percentage per field analysed (g) and number of epithelial pSmad2-stained nuclei per 100 μm BM length (h) from each of the groups studied. Numbers in parentheses represent number of mice analysed per group. * $P < 0.05$, *** $P < 0.001$ vs. saline group, ## $P < 0.01$ vs. OVA-vehicle group.

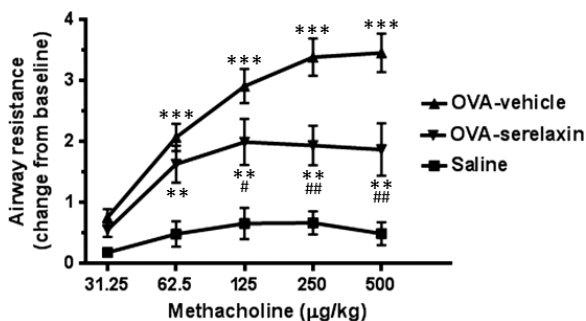


Fig. 6. Effect of intranasal serelaxin treatment on airway hyperresponsiveness (AHR). Shown is the mean ± SEM airway resistance (AHR; change from baseline) in saline, OVA-vehicle and OVA-serelaxin mice ($n = 11$ – 12 mice/group), in response to increasing concentrations of the bronchoconstrictor, methacholine, by invasive plethysmography. ** $P < 0.01$, *** $P < 0.001$ vs. saline group, # $P < 0.05$, ## $P < 0.01$ vs. OVA-vehicle group.

[23]. The key finding of the current study was that intranasal delivery of serelaxin produced detectable levels of the drug in the BAL fluid (~40 ng/mL) that were comparable to circulating levels resulting from its systemic administration [19]; and this intranasal treatment could reverse several structural components of established airway remodelling and improve related lung dysfunction in a mouse model of chronic AAD, when administered once daily over a 14-day treatment period. Serelaxin significantly diminished AAD-induced epithelial thickening, epithelial pSmad2, subepithelial collagen, lung hydroxyproline content (fibrosis) and AHR in the absence of any direct effects on airway inflammation, highlighting a novel and feasible approach to the treatment of airway remodelling in human asthma. Of further note, the ability of intranasal serelaxin delivery to completely reverse AAD-induced lung hydroxyproline content (to that measured in saline-treated control

mice) and reduce AAD-induced AHR by $\sim 50\%$ was comparable to that achieved by its systemic administration [10].

The intranasal route of delivery is particularly attractive as it can deliver a drug directly to the airways, allowing efficient onset of action. Given that epithelial dysfunction is a fundamental pathology leading to asthma [26], administration of a drug directly to this site would be highly advantageous. Furthermore, an intranasally administered drug offers ease of use and promotes patient adherence to treatment, thereby offering greater feasibility than systemic delivery.

In addition to the current widespread use of inhaled β -agonists and corticosteroids [27] as treatments for asthma, there has been intensive development of inhaled protein therapeutics for other disease conditions. The most prominent example of this technology is inhaled insulin for the treatment of diabetes, which was an important milestone in the development of inhaled therapeutics [28]. Inhaled DNase to breakdown mucus has become the mainstay of therapy for cystic fibrosis, while inhaled interferon- γ is being developed for idiopathic pulmonary fibrosis and there is interest in inhaled $\alpha 1$ -antitrypsin for emphysema [28]. Serelaxin has a two-chain structure [7, 8, 11] and activates a distinct leucine-rich repeat-containing G-protein coupled receptor, Relaxin Family Peptide Receptor 1 (RXFP1). The findings of our current study, along with the identification of strong RXFP1 expression in the airway epithelium [10] confirms that serelaxin can have local effects in the lung, adding to its potential viability as an inhaled treatment.

In this study, intranasal administration of serelaxin abrogated the aberrant airway remodelling changes associated with AAD, which in turn significantly attenuated AHR. Several preclinical studies have shown that serelaxin has potent antifibrotic effects which are mediated by its ability to disrupt the pro-fibrotic actions of TGF- $\beta 1$ [16, 18], while augmenting the activity of various collagen-degrading MMPs [10, 16]. Although its effects on MMP activity were not investigated in the current study, it is likely that serelaxin, regardless of whether it is administered intranasally or systemically [10, 15] inhibits both TGF- $\beta 1$ -induced collagen deposition and promotes MMP-induced collagen degradation to reverse AAD-induced airway fibrosis and related AHR. Consistent with its reported actions in other organs [29, 30], our present findings in the lung demonstrated that serelaxin disrupted the pro-fibrotic actions of TGF- $\beta 1$ on ECM/collagen production by inhibiting the phosphorylation of the intracellular protein, Smad2 (in the absence of any effects on airway TGF- $\beta 1$ expression itself). Serelaxin can also inhibit lung myofibroblast contractility [17]. It is plausible that these antifibrotic actions of serelaxin, in addition to its

ability to normalise epithelial damage (which is emerging as a novel aetiology in asthma pathogenesis [26]) and inhibit the vasoconstricting effects of endothelin-1 [31] to promote dilation [14] all contribute to its ability to significantly attenuate AHR.

The finding that serelaxin administration could not fully reverse AHR may be explained by our current data demonstrating that it did not significantly influence AAD-induced inflammation (as determined by BAL differential counts and inflammation score) and hence, the AHR that is separately caused by airway inflammation. This is consistent with our previous data in this model [10], and its inability to regulate inflammation derived from other aetiologies [32], but differs from separate studies demonstrating that serelaxin significantly attenuated the levels of cardiac and circulating mast cells and histamine release in rat [13], guinea pig [13] and swine [33] models of acute myocardial infarction. This may suggest that the anti-inflammatory properties of serelaxin are species-dependent or dependent on the cell type involved in the pathogenesis of inflammation, as it does not appear to have any effects on eosinophil [10] or macrophage [32] infiltration.

In this regard, it is possible that combining the antiremodelling properties of serelaxin with the anti-inflammatory actions of corticosteroids may yield improved efficacy in reversing AHR in the setting of AAD/asthma. In our recent studies attempting to evaluate such combination therapy [34], we found that serelaxin could modulate a broader range of airway remodelling changes compared with methylprednisolone, but that the combined effects of both treatments offered enhanced control of airway fibrosis and to an extent, AHR. These findings suggest that serelaxin has the potential to supplement corticosteroid treatments, which have powerful anti-inflammatory effects but only modest airway remodelling effects. Thus, intranasal delivery of serelaxin in combination with inhaled corticosteroids would have great potential to help treat the three major components of asthma. Further dose titration and duration studies are warranted though to optimise the therapeutic efficacy of intranasal serelaxin administration.

In conclusion, the data provided in this study demonstrated that serelaxin can be effective in reversing established airway remodelling and AHR when administered intranasally once daily, for as little as a 2-week treatment period. This data further supports the therapeutic potential of serelaxin as a future treatment for asthma and suggests that a once daily intranasal mode of delivery is efficacious enough to target the airway remodelling that contributes to AHR. Future studies combining intranasal serelaxin and corticosteroids may yield further protection from asthma pathogenesis.

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Conflict of interest

The authors declare no conflict of interest.

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