

Relaxin Reverses Airway Remodeling and Airway Dysfunction in Allergic Airways Disease

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Mice deficient in the antifibrotic hormone relaxin develop structural changes in the airway that resemble airway remodeling, and demonstrate exaggerated remodeling changes in models of allergic airways disease (AAD). Relaxin expression in asthma has not been previously studied. We evaluated the efficacy of relaxin in the treatment of established airway remodeling in a mouse model of AAD. Relaxin expression in mouse AAD was also examined by immunohistochemistry and real-time PCR. BALB/c mice with established AAD were treated with relaxin or vehicle control (sc for 14 d), and effects on airway remodeling, airway inflammation, and airway hyperresponsiveness (AHR) were assessed. Relaxin expression was significantly reduced in the airways of mice with AAD compared with controls. Recombinant relaxin treatment in a mouse model of AAD reversed collagen deposition and epithelial thickening, and significantly improved AHR (all $P < 0.05$ vs. vehicle control), but did not influence airway inflammation or goblet cell hyperplasia. Relaxin treatment was associated with increased matrix metalloproteinase-2 levels, suggesting a possible mechanism for its antifibrotic effects. Endogenous relaxin expression is decreased in murine AAD, whereas exogenous relaxin represents a novel treatment capable of reversing established airway remodeling and AHR. (*Endocrinology* 150: 2692–2699, 2009)

Asthma is a chronic inflammatory airway disease that is associated with airway remodeling and airway hyperresponsiveness (AHR). In most subjects, symptoms are effectively managed with inhaled corticosteroids for control of airway inflammation and bronchodilators as needed. However, in a subset of patients, significant ongoing symptoms persist despite optimal treatment with corticosteroid and bronchodilator therapy, likely due to airway remodeling changes that contribute to persistent airway obstruction (1) and increased AHR (2–4). Such patients must accept debilitating symptoms and a markedly reduced quality of life (5).

Airway remodeling changes include subepithelial fibrosis, smooth muscle hyperplasia/hypertrophy, goblet cell hyperplasia, and neovascularization, and collectively these lead to a thickened airway wall that exacerbate AHR and result in fixed airway obstruction (6). The mechanisms controlling the pathogenesis of airway remodeling are poorly understood. Although repeated

episodes of airway inflammation may contribute to airway remodeling, the remodeling process can occur early and progress independently of inflammation (7–9). Corticosteroids have only limited ability to reverse or prevent the progression of aspects of airway remodeling (10).

Airway wall fibrosis in asthma occurs in the subepithelial region (the lamina reticularis) and in the submucosa (11). Airway fibrosis may drive other airway remodeling changes (12), and extracellular matrix (ECM) components may bind pro-inflammatory growth factors that exacerbate inflammation and fibrosis (13). These findings suggest that treatments that prevent or reverse airway fibrosis may be effective in minimizing the extent of airway remodeling, and the resultant AHR and loss of lung function.

The extent of fibrosis that develops in tissues is determined by the balance between ECM production and degradation. In the airway wall, myofibroblasts produce collagen in response to pro-

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Abbreviations: AAD, Allergic airways disease; AHR, airway hyperresponsiveness; AU, arbitrary unit; BAL, bronchoalveolar lavage; ECM, extracellular matrix; H2, human gene-2; MMP, matrix metalloproteinase; OVA, ovalbumin; RXFP, relaxin family peptide receptor; SAL, saline.

fibrotic cytokines such as TGF β 1, whereas collagen degradation is regulated by matrix metalloproteases (MMPs) and tissue inhibitors of MMPs (14). Relaxin is a dimeric peptide hormone with known antifibrotic properties. We have recently shown tissue-specific roles for relaxin in regulating ECM deposition in organs such as the kidney, heart, liver, and lung (15).

Radiolabeling studies demonstrated binding of relaxin targeted to the lung (16). We have demonstrated relaxin and relaxin receptor [relaxin family peptide receptor (RXFP) 1] mRNA expression in the normal wild-type mouse lung (17, 18), and RXFP1 mRNA is present in the human lung (19) suggesting that cells in this organ are capable of secreting as well as binding the hormone. However, the specific cellular localization and tissue distribution of relaxin and RXFP1 have not been characterized within the mouse lung.

We have demonstrated an important role for relaxin in the regulation of airway fibrosis and airway remodeling in mouse allergic airways disease (AAD) (17, 20, 21). Aged relaxin-deficient mice develop spontaneous airway fibrosis and bronchial epithelial thickening (17), and these changes closely resemble those of airway remodeling in mouse models of AAD and human asthma. Young relaxin-deficient mice develop exaggerated airway subepithelial fibrosis and total airway wall thickness in a chronic model of AAD when compared with wild-type controls (21).

Relaxin has prevented the development of airway fibrosis in a bleomycin-induced model of lung injury (22), and in guinea pig (23) and mouse (24) models of AAD, however, relaxin's effects as a treatment to reverse established airway remodeling changes in AAD have not been examined. This is relevant because airway remodeling changes occur early in the course of asthma, may be well established when asthma diagnosis is confirmed, and are prominent in patients with steroid-resistant asthma who might benefit most from a new treatment. Therefore, a clinically useful antiremodeling treatment must be effective in reversing established airway remodeling in asthma. In aged relaxin-deficient mice, treatment with recombinant relaxin reversed fibrosis and normalized collagen levels, demonstrating the potential for relaxin to be applied as a treatment for established fibrosis *in vivo* (17).

In the current study, we aimed to determine the expression and tissue distribution of relaxin in the airway/lung in mouse AAD, and to evaluate the effect of systemic relaxin treatment on established changes of airway remodeling in a murine model of AAD.

Materials and Methods

Animals

Six-week-old female Balb/c mice were housed under specific pathogen free conditions, and maintained on a fixed 12-h light, 12-h dark lighting schedule, with free access to food and water at all times. Female mice were used in these studies because they demonstrate better responses in ovalbumin (OVA) AAD models (25–27). All experimental procedures were approved by the Murdoch Children's Research Institute Animal Ethics Committee and followed the Australian Guidelines for the Care and Use of Laboratory Animals for Scientific Purposes.

Mouse model of chronic AAD

An established model of OVA-induced chronic AAD was used as previously described (21, 28). The model was chosen to investigate the effect of endogenous relaxin treatment on airway remodeling changes because it includes many of the pathological features of human asthma. This model displays AHR, increased allergic responses indicated by increased IgE against OVA (OVA-specific IgE), and remodeling changes, including epithelial remodeling and peribronchial fibrosis. However, it does not display smooth muscle thickening (21, 28). Briefly, mice were sensitized with 10 μ g grade V OVA (Sigma Chemical, St. Louis, MO) and 1 mg aluminum potassium sulfate adjuvant in 500 μ l saline (SAL) ip on d 0 and 14, and then challenged with nebulized 2.5% (wt/vol) OVA in SAL (3 d/wk) for 6 wk from d 21. OVA-exposed mice were then treated with either relaxin (OVA relaxin), vehicle control (OVA vehicle), or left untreated (OVA). Mice exposed to SAL instead of OVA served as additional controls (SAL). For treatment experiments, 20 mice were included in each of the four experimental groups: OVA relaxin, OVA vehicle, OVA, and SAL. For relaxin expression experiments, 15 mice from the OVA and SAL groups were examined.

Systemic relaxin treatment

On d 64, 24 h after the final nebulization, OVA mice were treated with recombinant human gene-2 (H2) relaxin (0.5 mg/kg $\cdot$ d; kindly provided by Corthera Inc., San Mateo, CA) or vehicle (sodium acetate buffer; 20 μ mol/ml) by continuous infusion for 14 d. Both agents were loaded into osmotic mini-pumps (model 2002; Durect Corp., Cupertino, CA.) and implanted sc. The pumps remained in the mice until the end of the experiment.

Methacholine-induced AHR

On d 78, 24 h after relaxin or vehicle treatment, AHR was measured by invasive plethysmography using a mouse plethysmograph (Buxco Electronics, Troy, NY), as described previously (21). Briefly, mice were anesthetized by ip injection of ketamine (200 μ g/g) and xylazine (10 μ g/g), tracheostomized, and the jugular vein was cannulated. Mice were ventilated with a small animal respirator (Harvard Apparatus, Holliston, MA) delivering 0.01 ml/g body weight at a rate of 120 strokes per minute in a mouse plethysmograph chamber. Increasing methacholine doses were delivered iv, and airway resistance was measured (Biosystem XA version 2.7.9; Buxco Electronics) for 2 min after each dose. Results were then expressed as the maximal resistance after each dose of methacholine minus baseline (PBS alone) resistance.

Bronchoalveolar lavage (BAL)

After measurement of airway reactivity, BAL was performed as described previously, and differential cell counts of inflammatory cells were determined (21, 29). Total viable cell counts were determined using a hemocytometer with Trypan blue exclusion. Differential counts of eosinophils, neutrophils, lymphocytes, and monocytes/macrophages were determined on cytospin smears of BAL samples (4×10^5 cells) from individual mice stained with DiffQuick (Life Technologies, Auckland, New Zealand) and identified by standard morphological criteria after counting 300 cells.

Quantitation of serum OVA-specific IgE levels

On d 78, serum was obtained by lethal cardiac puncture of anesthetized mice and stored at -70°C for measurement of OVA-specific IgE by ELISA (2, 21, 30). OVA-specific IgE levels were expressed as arbitrary units (AUs), where 1 AU = OD of 1:50 dilution of positive control serum.

Tissue collection

Lung tissues were weighed (total lung weight) and then separated into individual lobes for hydroxyproline analysis, histological analysis, and MMP zymography (2, 17, 21).

Lung histopathology

The right lung lobe and trachea were fixed in formalin, embedded in paraffin, and routinely processed (2, 17, 21). Sections were stained with Masson trichrome for assessment of epithelial and subepithelial collagen thickness, and Alcian blue-periodic acid Schiff for assessment of goblet cells.

Morphometric analysis of structural changes

Morphometric evaluation of lung tissue sections was determined as described previously (2, 20, 21). Images of lung tissue sections were captured using a Digital camera (Q Imaging, Burnaby, British Columbia, Canada). A minimum of five bronchi measuring 150–350 μm luminal diameter was analyzed per mouse for the parameters described below (epithelial thickness and subepithelial collagen thickness) using Image Pro-Discovery software (Media Cybernetics, Silver Spring, MD), which was calibrated with a reference micrometer slide. The thickness of the bronchial epithelial layer was measured by tracing around the basement membrane and the luminal surface of epithelial cells and calculating the area between these lines, using a digitizer (Aiptek, Irvine, CA). Subepithelial collagen thickness was similarly measured by tracing around the outer extent of the total collagen layer in the submucosal region and around the basement membrane and the area between these lines calculated. Total areas were calculated by subtracting the inner area from the outer area. These areas were expressed per length (μm) of basement membrane to account for variation in bronchial diameters. Goblet cells were counted in Alcian blue-periodic acid-Schiff stained sections and expressed as the number of cells per 100 μm basement membrane.

Hydroxyproline analysis of lung collagen

A portion of each lung sample was treated as described previously to determine hydroxyproline content (31). Total hydroxyproline content (μg) was converted from a linear standard curve of 4-hydroxyproline (Sigma-Aldrich, Sydney, New South Wales, Australia).

Zymography of MMP expression

Total MMPs were extracted from a similar portion of each lung tissue and analyzed by gelatin zymography as described previously (32). The resulting bands on the zymograph were analyzed by densitometry using a GS 710 densitometer (Bio-Rad Laboratories, Richmond, CA) and Quantity-One software (Bio-Rad Laboratories). The mean $\pm$ SE density of each MMP was graphed and expressed as the relative ratio of the values in the SAL-treated group, which was expressed as one.

Immunohistochemistry

Immunohistochemistry for relaxin was performed using a monoclonal mouse antibody (clone 2F1 fred; Immunodiagnostik, Bensheim, Germany) to H2 relaxin. Detection of staining in mouse tissues was with an animal research kit (Dakocytomation, Carpinteria, CA) with 3-3'-diaminobenzidine. Ovary tissue was used as a positive control for relaxin expression. Negative controls consisted of omission of primary antibody. To confirm specificity, primary antibodies against relaxin were preabsorbed with an excess concentration of recombinant H2 protein. The 1:10 solution of antibody to peptide was mixed and incubated at 4 C overnight before incubation on tissues. For comparison of SAL controls and chronic AAD relaxin staining, sections from 10 mice were used per group. Intensity and extent of immunohistochemical staining were scored zero for negative and +++++ (1–3) for positive staining by two independent blinded observers, and the means were calculated.

Real-time PCR

Mouse lung tissue and pregnant mouse ovary tissue were dissected and stored in RNA later (Ambion, Inc., Austin, TX). The amount of mouse relaxin mRNA was determined by real-time PCR (ABI Prism 7000; Applied Biosystems, Foster City, CA), as described previously (17). The RNA was extracted using RNeasy (Ambion) extraction buffer in accordance with the manufacturer's instructions. RNA purity was determined and cDNA synthesis conducted using a TaqMan reverse-

transcriptase kit (Applied Biosystems). The amount of mouse relaxin mRNA was determined by real-time PCR (ABI Prism 7000), whereas 18S was used in separate PCRs to control for quality and equivalent loading of cDNA. Analyses of standards, lung and ovary tissue samples were performed in triplicate; 18S samples were performed in duplicate. The amount of relaxin mRNA expression is represented as a ratio of mouse relaxin to 18S.

Statistical analysis

The data were analyzed using a one-way ANOVA, with Newman-Keuls tests for multiple comparisons between groups. Lung function studies were analyzed with a two-way ANOVA, with Bonferroni post-test. Morphometry was expressed as median with 95% confidence interval and analyzed using the Mann-Whitney *U* test.

Results

Expression of relaxin in mouse AAD

Examination of lung tissue from Balb/c mice with chronic AAD and control mice revealed that relaxin expression was localized to bronchial epithelial cells, fibroblasts, and airway smooth muscle in control mice (SAL) (Fig. 1, A and B). Relaxin protein expression was markedly reduced in mice with AAD (OVA) (Fig. 1, C, D, and F) compared with SAL mice ($P = 0.0089$). No staining was observed in any antibody negative controls or preabsorption controls (Fig. 1E).

Relaxin mRNA expression in mouse AAD

The amount of relaxin mRNA was determined by real-time RT-PCR in mice with AAD (OVA) and control mice (SAL) ($n = 7$ per group) (Fig. 1G). Mouse relaxin mRNA in lung tissues from mice with AAD was reduced by 64% ($P = 0.03$) compared with that in control mice (relaxin to 18S ratio $\pm$ SE $1.6 \times 10^{-7} \pm 3.6 \times 10^{-8}$ OVA *vs.* $4.4 \times 10^{-7} \pm 1.3 \times 10^{-7}$ SAL).

Relaxin treatment of established airway fibrosis in a mouse model of AAD

Airway inflammation

The chronic AAD model used in the current study showed remodeling changes consistent with those reported previously (2, 28), although small numbers of BAL eosinophils present at 2 wk after final allergen challenge were not reported in one similar study at 3 wk (33). At d 78, 24 h after relaxin or vehicle treatment, serum levels of OVA-specific IgE measured by ELISA were significantly increased in all OVA mice groups compared with SAL control mice ($P < 0.001$), confirming adequate sensitization to OVA (Table 1).

All groups of OVA mice also showed significantly higher total cell counts in BAL compared with SAL controls ($P < 0.01$). The number of eosinophils, neutrophils, lymphocytes, and monocytes was significantly higher in all OVA groups compared with that in the SAL control group but did not differ among the OVA groups studied (Table 1).

Airway/lung collagen deposition

Untreated OVA mice (Figs. 2B and 3A) and vehicle-treated OVA mice (Figs. 2C and 3A) had an increased area of subepi-

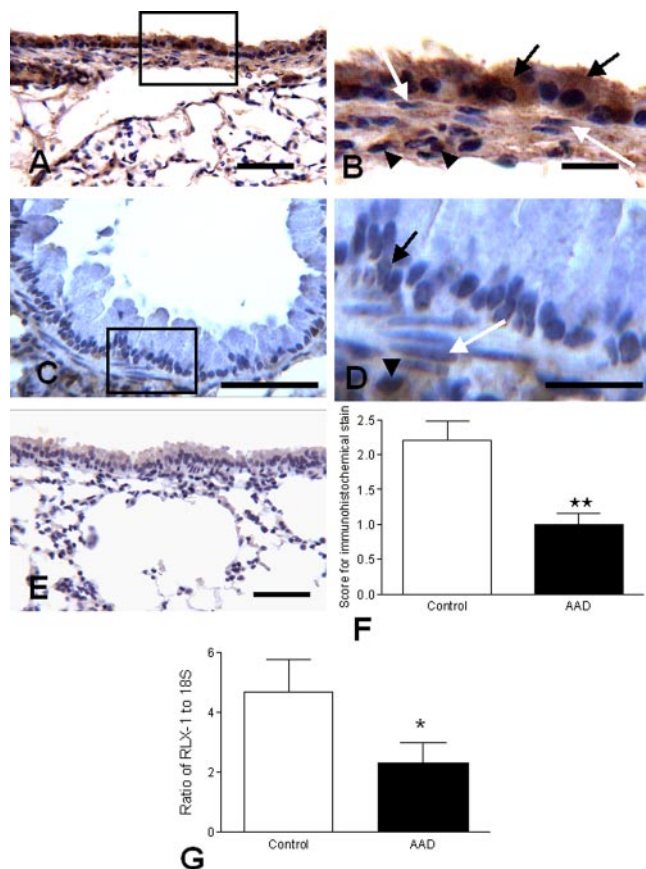


FIG. 1. Relaxin expression in lungs from control and AAD mice. A, There is strong relaxin staining in the control mouse airway (bar, 100 μ m). Inset in A shows that staining is strongest in bronchial epithelial cells (black arrows), and is moderate in fibroblasts (black arrowheads) and airway smooth muscle cells (white arrows) (bar, 25 μ m) (B). C, Relaxin staining is reduced in AAD (bar, 100 μ m). Inset in C shows that staining is weak in bronchial epithelial cells (black arrow), and in fibroblasts (black arrowhead) and airway smooth muscle cells (white arrow) (bar, 25 μ m) (D). E, Staining is absent in mouse control tissue incubated with preabsorbed with an excess of recombinant relaxin peptide (bar, 100 μ m). F, The immunohistochemical staining score is lower in AAD vs. controls (n = 10 per group). **, $P = 0.0089$. G, Relaxin mRNA was also significantly lower in AAD vs. controls (*, $P = 0.03$).

thelial collagen deposition in the bronchial wall (both $P < 0.001$) compared with SAL mice (Figs. 2A and 3A). In contrast, the area of subepithelial collagen deposition in relaxin-treated OVA mice (Figs. 2D and 3A) was not different from SAL mice, and significantly reduced compared with untreated OVA ($P < 0.001$) and vehicle-treated OVA groups ($P < 0.001$).

Lung hydroxyproline content in the four groups studied (Fig. 3B) demonstrated findings consistent with those described for morphometric analysis of subepithelial collagen deposition. Lung hydroxyproline content in the untreated OVA ($P < 0.05$) and vehicle-treated OVA ($P < 0.01$) groups was markedly elevated compared with SAL mice, however, hydroxyproline levels in relaxin-treated OVA mice were similar to SAL mice, and significantly lower than untreated OVA ($P < 0.001$) and vehicle-treated OVA ($P < 0.001$) mice (Fig. 3B).

Other airway remodeling changes

Epithelial thickness was significantly reduced in relaxin-treated OVA mice compared with untreated OVA ($P < 0.01$) and

TABLE 1. Number of BAL eosinophils, neutrophils, lymphocytes, and monocytes ($\times 10^{-2}$), and serum levels of OVA-specific IgE (AU)

	Eosinophils (%)	Neutrophils (%)	Lymphocytes (%)	Monocytes (%)	OVA-specific IgE (AU)
Saline	1.26 $\pm$ 0.30 (0.86 $\pm$ 0.28)	3.40 $\pm$ 0.58 (2.04 $\pm$ 0.28)	2.15 $\pm$ 0.40 (1.50 $\pm$ 0.28)	142.86 $\pm$ 17.79 (89.16 $\pm$ 2.45)	0.012 $\pm$ 0.002
OVA	20.17 $\pm$ 5.65 (6.89 $\pm$ 1.65) ^a	18.60 $\pm$ 4.22 (6.43 $\pm$ 1.11) ^a	18.96 $\pm$ 4.41 (6.48 $\pm$ 1.14) ^a	245.50 $\pm$ 25.25 (89.17 $\pm$ 2.5) ^a	0.582 $\pm$ 0.068 ^b
OVA vehicle	15.53 $\pm$ 3.94 (5.44 $\pm$ 0.76) ^a	14.20 $\pm$ 2.86 (5.17 $\pm$ 0.59) ^a	14.27 $\pm$ 2.62 (5.42 $\pm$ 0.88) ^b	243.20 $\pm$ 35.05 (90.51 $\pm$ 1.8) ^c	0.554 $\pm$ 0.052 ^b
OVA relaxin	14.53 $\pm$ 1.56 (5.82 $\pm$ 0.71) ^b	13.18 $\pm$ 0.84 (5.39 $\pm$ 0.57) ^b	13.92 $\pm$ 0.73 (5.62 $\pm$ 0.46) ^b	226.00 $\pm$ 14.30 (88.37 $\pm$ 2.83) ^a	0.527 $\pm$ 0.086 ^b

Values are expressed as mean $\pm$ SEM (range).

^a $P < 0.01$ vs. saline; ^b $P < 0.001$ vs. saline; ^c $P < 0.05$ vs. saline.

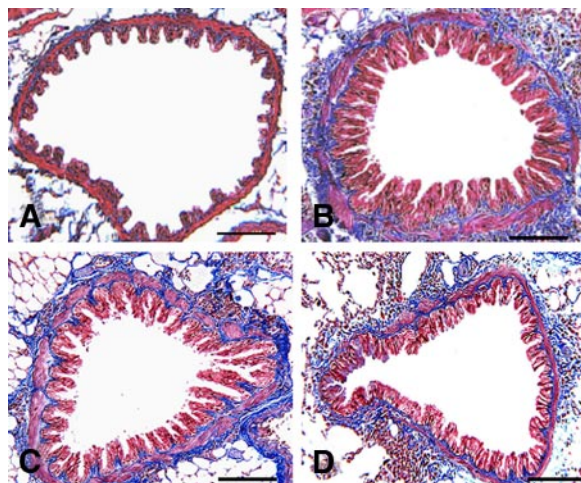


FIG. 2. Representative Masson trichrome-stained sections from SAL (A), untreated OVA (B), vehicle-treated OVA (C), and relaxin-treated OVA (D) mice. Epithelial thickness and subepithelial collagen deposition were increased in untreated and vehicle-treated OVA mice compared with SAL mice. Relaxin treatment decreased epithelial thickness and subepithelial collagen deposition compared with untreated and vehicle-treated OVA mice. Bar, 100 μ m.

vehicle-treated OVA mice ($P < 0.01$) but was not significantly different from the SAL mice (Fig. 4).

Relaxin treatment had no significant effect on goblet cell number. The mean number of goblet cells per 100 μ m bronchial basement membrane was significantly higher in untreated OVA (4.67 ± 0.58 ; $P < 0.001$), vehicle-treated OVA (4.43 ± 0.43 ; $P < 0.001$), and relaxin-treated OVA (3.99 ± 0.29 ; $P < 0.001$) mice compared with SAL mice (0.072 ± 0.02 ; mean $\pm$ SEM). However, there were no differences in goblet cell number between vehicle-treated, relaxin-treated, and untreated OVA groups.

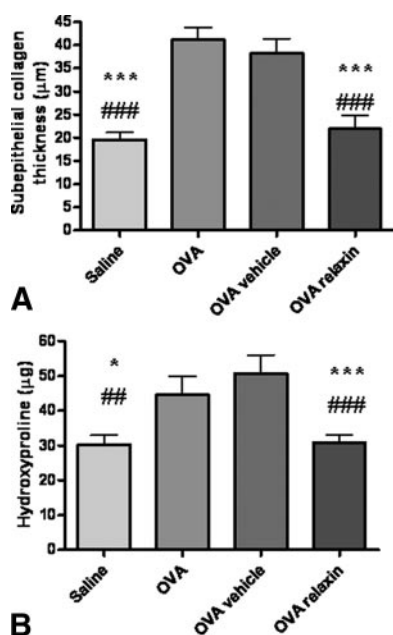


FIG. 3. Morphometric analysis of subepithelial collagen deposition (A) and lung hydroxyproline content (B) in SAL, untreated OVA, and vehicle and relaxin-treated OVA mice. Results are expressed as the mean $\pm$ SE thickness of subepithelial collagen deposition (A) and hydroxyproline (μ g) (B). *, $P < 0.05$ and ***, $P < 0.001$ compared with untreated OVA; ##, $P < 0.01$ and ###, $P < 0.001$ compared with vehicle-treated OVA groups ($n = 20$ per group).

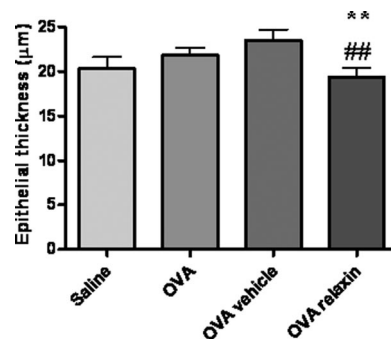


FIG. 4. Morphometric analysis of bronchial epithelial thickness in lung tissue sections from SAL, untreated OVA, vehicle-treated OVA, and relaxin-treated OVA mice. Results are expressed as mean $\pm$ SE thickness for individual airways. **, $P < 0.01$ compared with untreated OVA; ##, $P < 0.01$ compared with vehicle-treated OVA groups ($n = 20$ per group).

AHR

Untreated OVA and vehicle-treated OVA mice showed significantly increased methacholine-induced AHR compared with SAL mice at the three highest doses of methacholine (Fig. 5). Airway resistance in relaxin-treated mice was significantly lower than untreated OVA and vehicle-treated OVA mice at the three highest doses of methacholine.

Changes in MMP2 and MMP9 expression in a mouse model of AAD and after relaxin treatment

Zymographic measurement of gelatinase expression and activity in the mouse lung revealed elevated levels of latent and active MMP2 (Fig. 6A) and MMP9 (Fig. 6B) in untreated OVA, vehicle-treated OVA, and relaxin-treated OVA groups compared with SAL controls (all $P < 0.05$). H2 relaxin-treated OVA mice had a further significant increase in both latent and active MMP2 (Fig. 6A) compared with that in untreated ($P < 0.01$) and vehicle-treated OVA groups ($P < 0.01$).

Discussion

In this study we demonstrated that relaxin expression was significantly decreased in mice with AAD. Given the known anti-fibrotic effects of relaxin, these findings suggest that a relative deficiency of relaxin expression in the AAD-affected airway/lung

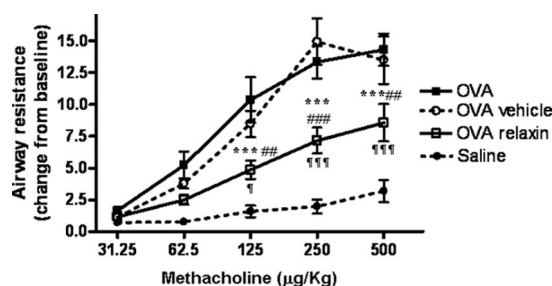


FIG. 5. The mean $\pm$ SE. AHR in SAL, untreated OVA (OVA), vehicle-treated OVA (OVA vehicle), and relaxin-treated OVA (OVA relaxin) mice. AHR was significantly higher in OVA and OVA vehicle compared with SAL mice. Relaxin treatment significantly decreased AHR compared with OVA and OVA vehicle mice. ***, $P < 0.001$ compared with OVA; ##, $P < 0.01$ and ###, $P < 0.001$ compared with OVA vehicle; ¶, $P < 0.05$ and ¶¶, $P < 0.001$ compared with SAL ($n = 20$ per group).

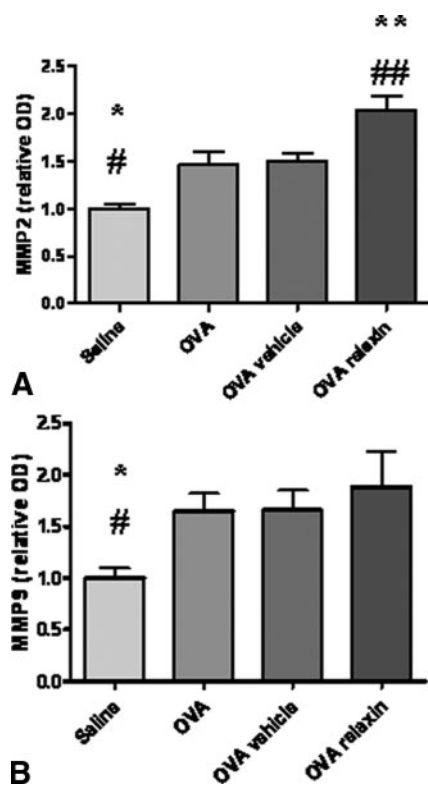


FIG. 6. Gelatin zymography detection of latent and active MMP2 (A) and MMP9 (B) levels in lung tissues ($n = 6$ per group). Active and latent MMP2 and MMP9 in OVA and OVA vehicle mice were significantly higher than SAL. Relaxin treatment increased MMP2 but not MMP9 levels. *, $P < 0.05$ and **, $P < 0.01$ compared with OVA; #, $P < 0.05$ and ##, $P < 0.01$ compared with OVA vehicle groups ($n = 15$ per group).

may contribute to airway fibrosis and remodeling in asthma. Consistent with this, exogenous relaxin treatment was shown to reverse established airway fibrosis (total lung collagen and area of subepithelial collagen deposition), and remarkably, to also reverse AHR in mouse AAD, highlighting the potential for relaxin as an antiremodeling therapy in asthma.

Relaxin is a peptide hormone most highly expressed during parturition but also expressed at low levels in the normal female and in males (34). This study was conducted using female mice only because female mice have had greater responses and pathology than males in OVA AAD models, and the model is optimized in this sex (28). Evidence from previous studies in relaxin-deficient mice suggests that relaxin may play a role in regulating airway remodeling in both sexes (17).

The finding that relaxin treatment could reverse AHR suggests that fibrosis may promote AHR, or alternatively that relaxin may directly modulate airway function (35). Genetic linkage studies in mice have shown that polymorphisms in relaxin are linked to AHR (36, 37). However, it remains uncertain whether AHR is directly regulated by relaxin or whether AHR results from other downstream events regulated by airway fibrosis *per se*. In support of the latter possibility, deposition of ECM proteins in the reticular basement membrane and deeper in the submucosa has correlated with reduced lung function and clinical disease severity (38–41). Furthermore, mathematical models suggest that thickening of any component of the airway wall on the luminal side of the airway smooth muscle layer can result in

increased narrowing of the airway upon smooth muscle contraction that could contribute to AHR (42).

It has been postulated that the development of subepithelial fibrosis in human asthma is able to drive remodeling processes, including epithelial thickening (43). In human asthma, mediators that regulate fibrosis are disrupted, resulting in disequilibrium of ECM production and degradation. During normal homeostasis the turnover of bronchial epithelial cells is relatively low unless injured (44). In asthma, epithelial hyperplasia is believed to develop as a result of increased proliferative capacity, inhibition of apoptosis, or both. Asthma is also associated with reduced epithelial integrity and shedding, and the epithelium of asthma sufferers may have increased susceptibility to damage compared with that of normal individuals (45). *In vitro* work involving primary epithelial cell cultures from bronchial brushings from children with mild asthma has found markedly increased rates of epithelial cell proliferation and doubling times in comparison with primary epithelial cell cultures from control children (46). Studies using human bronchial epithelial cell lines have shown that ECM components are able to promote cell survival (47). Furthermore, fibroblast secretions (such as IGF-I, basic fibroblast growth factor, TGF β 1) can promote differentiation of human epithelial cell lines and epithelial restitution (48, 49). The mechanisms by which relaxin mediates its antifibrotic effects have not been fully elucidated. We have previously shown that relaxin inhibits the effects of the pro-fibrotic cytokine TGF- β 1 on proliferation and differentiation of ECM-producing myofibroblasts, and promotes MMP-induced collagen degradation as part of its collagen remodeling effects (15). Other *in vivo* and *in vitro* studies have shown that relaxin can up-regulate expression of MMPs, including MMP2 and MMP9, and alters the MMP:tissue inhibitor of MMP balance away from airway fibrosis (22, 50, 51). In the current study, levels of two MMPs previously demonstrated to play a role in asthma, MMP9 and MMP2, were examined. Relaxin treatment was associated with higher latent and active MMP2, and a trend toward higher latent and active MMP9 than vehicle treatment. In humans, MMP9 and MMP2 expression is increased in acute asthma but declines in chronic asthma, concurrent with progression of airway remodeling changes (52). Relaxin delivered at this later stage of chronic asthma may be useful in reversing fibrosis, airway remodeling, and AHR. This may be particularly important for the subset of patients with steroid-resistant severe asthma who experience persistent symptoms and AHR despite optimal anti-inflammatory treatment.

The goals of asthma therapy are to obtain long-term control of asthma symptoms, prevent exacerbations, and obtain the best possible lung function for the patient (35). Although there is some evidence that long-term treatment with inhaled corticosteroids is associated with a reduced rate of lung function decline, corticosteroids have limited efficacy in prevention and reversal of airway remodeling (53). Airway remodeling is an important component of asthma leading to AHR, irreversible airway obstruction, and lung function decline (35). The current study provides evidence of a novel antifibrotic therapy that can inhibit AHR and reverse established airway remodeling in AAD.

In summary, our study demonstrates localization of relaxin in the normal mouse lung, and that expression of relaxin protein was significantly decreased in lung tissue from mice subjected to a model of AAD compared with controls. Importantly, we have shown for the first time that recombinant human relaxin is able to reverse airway remodeling changes of aberrant collagen deposition and thickening in an experimental model of AAD, returning these structural changes to levels seen in control animals. Furthermore, AHR (a measure of airway dysfunction in asthma that correlates closely with symptom severity) was markedly reduced in mice treated with relaxin. These results provide strong evidence for the importance of relaxin in the pathogenesis of airway remodeling with expression reduced in a mouse model of chronic AAD, and support the therapeutic application of relaxin as a treatment that targets established airway fibrosis, epithelial remodeling, and AHR. If administered early in pathogenesis, a therapy targeting the relaxin/relaxin receptor system could also potentially slow disease progression, particularly if used as an adjunct therapy in conjunction with inhaled corticosteroids. Further preclinical studies evaluating dosage, delivery, and potential benefits of relaxin therapy are warranted.

Acknowledgments

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