

Endogenous Relaxin Regulates Collagen Deposition in an Animal Model of Allergic Airway Disease

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We examined the relationship among relaxin (a peptide hormone that stimulates collagen degradation), airway fibrosis, other changes of airway remodeling, and airway hyperresponsiveness (AHR) in an animal model of allergic airway disease. Eight- to 10-wk-old relaxin gene-knockout (RLX^{-/-}) and wild-type (RLX^{+/+}) mice were sensitized with ovalbumin (OVA) or saline ip at d 0 and 14 and challenged three times per week for 6 wk with nebulized 2.5% OVA or saline. Saline-treated control RLX^{+/+} and RLX^{-/-} mice had equivalent collagen expression and baseline airway responses. OVA-treated RLX^{-/-} mice developed airway inflammation equivalent to that in OVA-treated RLX^{+/+} mice. However, OVA-treated RLX^{-/-} mice had markedly increased lung collagen deposition as compared with OVA-treated RLX^{+/+} and saline-treated mice (all $P < 0.05$). Collagen was predominantly deposited in

the subepithelial basement membrane region and submucosal regions in both OVA-treated RLX^{+/+} and RLX^{-/-} mice. The increased collagen measured in OVA-treated RLX^{-/-} mice was associated with reduced matrix metalloproteinase (MMP)-9 ($P < 0.02$) expression and failure to up-regulate matrix metalloproteinase-2 expression, compared with levels in OVA-treated RLX^{+/+} mice. Goblet cell numbers were equivalent in OVA-treated RLX^{-/-} and RLX^{+/+} mice and increased, compared with saline-treated animals. Both OVA-treated RLX^{+/+} and RLX^{-/-} mice developed similar degrees of AHR after OVA treatment. These findings demonstrate a critical role for relaxin in the inhibition of lung collagen deposition during an allergic inflammatory response. Increased deposition of collagen *per se* did not influence airway epithelial structure or AHR. (*Endocrinology* 147: 754–761, 2006)

STRUCTURAL CHANGES in the airway wall (airway remodeling) are characteristic features of allergic airway disease (AAD) that can occur early in the course of disease (1), contribute to airway hyperresponsiveness (AHR) (2), and lead to irreversible airway obstruction (1, 2). Airway remodeling is characterized by increased deposition of collagen (fibrosis) in the subepithelial basement membrane region and in the submucosal layers (3), smooth muscle hypertrophy and hyperplasia, fibroblast hyperplasia, epithelial metaplasia, and goblet cell proliferation (1). Collagen deposition in the subepithelial basement membrane region is a hallmark of airway remodeling in AAD (4); however, the cellular and molecular events that lead to the remodeling process are poorly understood. In particular, the factors regulating collagen deposition associated with airway remodeling in AAD have not been clearly defined, and the relationship between collagen deposition and other structural changes in the epithelium remain unclear. Furthermore, although corticosteroid antiinflammatory treatment can reduce symptoms, improve lung function, and modify bronchial hyperresponsiveness, these agents are not effective in

reversing established airway collagen deposition or other structural changes of remodeling (5, 6). Consequently, there are currently no treatments that effectively reverse established collagen deposition or other structural changes in AAD.

Relaxin is a small dimeric peptide hormone secreted by the ovary into the blood in highest amounts during pregnancy and generally associated with female reproduction (7). However, the most consistent biological effect of relaxin is its ability to stimulate the breakdown of collagen. Relaxin stimulates collagen breakdown in preparation for successful parturition (7). Relaxin treatment has been demonstrated to inhibit fibrosis in a bleomycin-induced model of lung fibrosis (8), by inhibiting TGF β -induced matrix protein production (8) and increasing the expression of the collagen-degrading enzymes, matrix metalloproteinases (MMPs) (8, 9). Furthermore, relaxin administration has been reported to prevent the development of collagen deposition associated with airway inflammation in ovalbumin (OVA)-sensitized guinea pigs (10) and OVA-sensitized challenged BALB/c mice (11), highlighting the antifibrotic potential of exogenous relaxin therapy. However, the role that endogenous relaxin plays in airway fibrosis and remodeling is yet to be determined.

Recently we generated a relaxin gene knockout (RLX^{-/-}) mouse to further our understanding of the physiological actions of relaxin (12), which lacks the major stored and circulating form of relaxin. RLX^{-/-} mice developed an age-related progression of fibrosis in several tissues (13–15), including the airway and lung that was first detectable at 6–9 months of age (15). Furthermore, treatment of RLX^{-/-} mice

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Abbreviations: AAD, Allergic airway disease; AB-PAS, Alcian blue-periodic acid Schiff; AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; BM, basement membrane; MCh, methacholine; MMP, matrix metalloproteinase; OVA, ovalbumin; RLX^{+/+}, relaxin wild type; RLX^{-/-}, relaxin gene knockout.

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with recombinant human relaxin-2 relaxin successfully reversed established fibrosis in the airways and lung (15). These findings suggested an important role for endogenous relaxin in regulating collagen deposition within the lung and further confirmed the potential role for exogenous relaxin in the treatment of fibrosis associated with airway and lung disorders. Based on these combined findings, we hypothesize that endogenous relaxin protects the airways and lung from fibrosis in response to AAD.

The 9-wk murine model of AAD mimics a majority of the changes observed in human asthma including: the recruitment of eosinophils, goblet cell hyperplasia, and/or metaplasia and a distinctive pattern of subepithelial fibrosis (16–19), although no changes in smooth muscle thickening are detected. Whereas this model reflects several changes seen in human AAD, it is strain dependent (16–20). In the present study, we examined the role of endogenous relaxin in the regulation of collagen deposition and airway remodeling using this model of AAD on $RLX^{-/-}$ mice. The relationship among airway fibrosis, other structural changes of remodeling, and AHR was also examined. The $RLX^{-/-}$ mouse is an ideal model to study the specific contributions of endogenous relaxin to airway fibrosis in AAD.

Materials and Methods

Animals

Relaxin knockout mice were generated as described previously (12). All female relaxin wild-type ($RLX^{+/+}$) and $RLX^{-/-}$ mice used in this study were generated from relaxin heterozygous (C57BLK6J/129SV) parents (12) and were age-matched littermates. Eight- to 10-wk-old $RLX^{+/+}$ and $RLX^{-/-}$ mice with similar baseline collagen levels (15) were used in all studies. The animals were housed in a controlled environment and maintained as detailed before (15). These experiments were approved by the Howard Florey Institute and the Royal Children's Hospital Animal Experimental Ethics Committees, which adhere to the Australian Code of Practice for the care and use of laboratory animals for scientific purposes.

OVA-induced allergic airway disease model

An OVA-induced model of AAD was used as previously described (21). Eight- to 10-wk-old $RLX^{+/+}$ and $RLX^{-/-}$ mice were sensitized with an ip injection of 10 μ g of grade V OVA (Sigma Chemical, St. Louis, MO) and 1 mg of aluminum potassium sulfate (Sigma) in 0.5 ml saline on d 0 and 14. Mice were then challenged 3 d/wk for 6 wk (from d 21 to 63) with an aerosol of 2.5% (wt/vol) OVA in saline solution for 30 min (21). Vehicle-treated mice received 0.5 ml saline alone ip on d 0 and 14 and were challenged with saline aerosols.

Analysis of AHR

Methacholine (MCh)-induced airway reactivity was assessed 24 h after the final aerosol challenge by invasive plethysmography (Buxco Electronics, Troy, NY). Briefly, mice were anesthetized by ip injection of ketamine (200 μ g/g) and xylazine (10 μ g/g), tracheostomized, and the jugular vein 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/min in a mouse plethysmograph chamber. Increasing MCh doses were delivered iv and airway resistance and compliance measured (Biosystem XA version 2.7.9; Buxco Electronics) for 2 min after each dose. Results are expressed as the maximal resistance after each dose of MCh minus baseline (PBS alone) resistance.

Determination of OVA-specific antibody titers by ELISA

Serum was obtained 24 h after the final OVA or saline challenge and stored at -20°C . Levels of OVA-specific IgE were determined by ELISA, as described previously (21).

Bronchoalveolar lavage (BAL)

After measurement of airway reactivity, BAL was carried out, as detailed previously (21). Total viable cell counts were determined in a hemocytometer using 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) after counting 300 cells. Results are expressed as total cell number $\times 10^4$.

Tissue collection

Lung tissues were weighed (total lung weight) and then separated into individual lobes for various analyses including hydroxyproline analysis, histological analysis, and MMP zymography.

Lung histopathology

The right lung lobe and trachea were fixed in 10% neutral buffered formalin for 18–24 h and routinely processed. Serial 5- μ m sections taken every 100 μ m were stained with hematoxylin and eosin for assessment of peribronchial inflammation, with Masson trichrome for assessment of collagen and Alcian blue-periodic acid Schiff (AB-PAS) for assessment of goblet cells.

Morphometric analysis of structural changes

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 μ m luminal diameter were analyzed per mouse for the parameters described below 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). Total 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 (micrometers) of basement membrane to account for variation in bronchial diameters. Goblet cells were counted in AB-PAS stained sections and expressed as number of cells per 100 μ m of basement membrane.

Hydroxyproline analysis of lung collagen

A portion of each lung sample from saline and OVA-treated $RLX^{+/+}$ and $RLX^{-/-}$ mice were treated as described previously to determine their hydroxyproline contents (22). Hydroxyproline values were then converted to collagen content by multiplying by a factor of 6.94 (based on hydroxyproline representing approximately 14.4% of the amino acid composition of collagen, in most mammalian tissues) (23) and further expressed as a proportion of the tissue dry weight (collagen concentration).

Zymography of MMP expression

Total MMPs were extracted from a similar portion of each lung tissue from saline and OVA-treated $RLX^{+/+}$ and $RLX^{-/-}$ mice and analyzed by gelatin zymography as described previously (24). 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). The mean $\pm$ SE density of each MMP was graphed and expressed as the relative ratio of the values in the saline-treated $RLX^{+/+}$ group, which was expressed as 1.

Statistical analysis

The results were analyzed using a one-way ANOVA; with Newman-Keuls tests for multiple comparisons between groups. Lung functional studies were analyzed with a two-way ANOVA, with Bonferroni post-test. In this paper $P < 0.05$ is described as statistically significant. Mor-

phometry was expressed as median with 95% confidence interval and analyzed using the Mann-Whitney test.

Results

RLX^{-/-} mice have normal sensitization and airway inflammation responses to OVA

To confirm that both $RLX^{+/+}$ and $RLX^{-/-}$ mice were adequately sensitized to OVA, serum levels of OVA-specific IgE were measured in mice treated with either OVA or saline (controls). Serum levels of OVA-specific IgE in OVA-treated $RLX^{-/-}$ mice were equivalent to those of OVA-treated $RLX^{+/+}$ mice, and both were significantly ($P < 0.001$) higher than levels in saline-treated controls (Fig. 1). As expected saline-treated mice did not develop any IgE response to OVA.

Both $RLX^{+/+}$ and $RLX^{-/-}$ mice demonstrated increased total and differential BAL cell counts after OVA sensitization-treatment. There were no significant differences in total cell counts between OVA-treated $RLX^{+/+}$ and $RLX^{-/-}$ mice. There was a marked increase in the total number of BAL eosinophils in both the $RLX^{+/+}$ and $RLX^{-/-}$ OVA-treated mice ($P < 0.001$), compared with saline-treated mice (Table 1). The increased numbers of eosinophils confirmed that the chronic model of AAD (17) could be induced in $RLX^{+/+}$ and $RLX^{-/-}$ mice. Increases in BAL neutrophils ($P < 0.001$), monocytes/macrophages ($P < 0.001$), and lymphocytes ($P < 0.05$) were also observed in OVA-treated mice, compared with that measured from saline-treated animals (Table 1). These combined data demonstrated that $RLX^{+/+}$ and $RLX^{-/-}$ mice were able to mount IgE and airway inflammation responses after OVA sensitization.

Histologic analysis of lung tissues from $RLX^{+/+}$ and $RLX^{-/-}$ mice

Lung tissue sections from saline or OVA-treated $RLX^{+/+}$ and $RLX^{-/-}$ mice were stained with Masson trichrome to determine the relative amount and distribution of collagen deposition within the airway/lung of $RLX^{-/-}$ and $RLX^{+/+}$ mice. Saline-treated $RLX^{+/+}$ (Fig. 2A) and $RLX^{-/-}$ (Fig. 2B) mice had minimal collagen staining in the airway and lung.

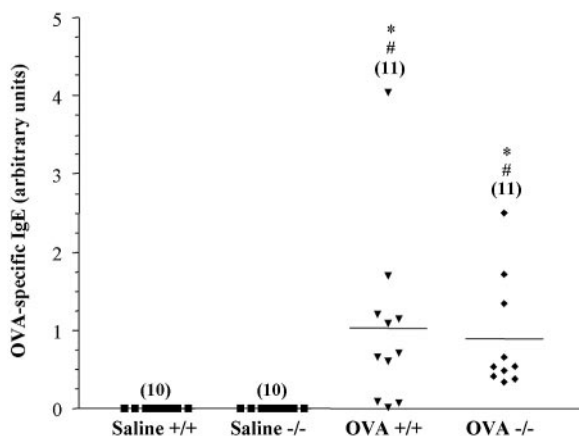


FIG. 1. ELISA analysis of OVA-specific IgE responses. Data are presented as scatter plots with means shown as a bar. *, $P < 0.001$ when compared with saline-treated $RLX^{+/+}$ group and #, $P < 0.001$ when compared with saline-treated $RLX^{-/-}$ group.

OVA-treated $RLX^{+/+}$ mice demonstrated a modest increase in collagen staining in the airways (Fig. 2C), when compared with saline-treated $RLX^{+/+}$ controls. A further marked increase in collagen deposition was observed in OVA-treated $RLX^{-/-}$ mice (Fig. 2D), compared with that observed in OVA-treated $RLX^{+/+}$ mice. The increase in collagen was observed predominantly in the subepithelial basement membrane (BM) regions of the airway wall and in the submucosal regions surrounding airway structures and the larger blood vessels, with minimal deposition in the lung parenchyma (Fig. 2D).

Morphometric analysis of the total area of collagen deposition in lung tissue sections confirmed these changes. There was significantly increased collagen in the airways of OVA-treated $RLX^{+/+}$ mice, compared with that in saline-treated $RLX^{+/+}$ mice ($P < 0.02$; Fig. 2E). A further marked increase in total area of collagen was observed in OVA-treated $RLX^{-/-}$ mice, compared with OVA-treated $RLX^{+/+}$ mice ($P < 0.001$; Fig. 2E).

Hydroxyproline analysis of total lung collagen, reflected what was observed by histological and morphometric examination (Fig. 2F). The lungs of saline-treated $RLX^{+/+}$ and $RLX^{-/-}$ mice had similar levels of collagen (approximately 850 $\mu\text{g}/\text{lung}$). OVA-treated $RLX^{+/+}$ mice had a 15% increase in lung collagen content, which was not significantly different ($P = 0.16$) from that measured from saline-treated animals. However, OVA-treated $RLX^{-/-}$ mice had a 26% ($P < 0.05$; Fig. 2F) increase in total lung collagen content, compared with lung tissues obtained from the saline-treated groups. Taken together, these findings indicate that OVA sensitization and treatment of mice was associated with increased airway fibrosis that was exaggerated in the absence of relaxin.

MMP expression and activity in saline or OVA-treated $RLX^{+/+}$ and $RLX^{-/-}$ mice

Gelatin zymography of lung tissue from saline and OVA-treated $RLX^{+/+}$ and $RLX^{-/-}$ mice was used to demonstrate changes in the expression and activity of MMP-9 (gelatinase-B) and MMP-2 (gelatinase-A). Densitometric analysis of the zymographs demonstrated no significant differences in either MMP-9 (Fig. 3A) or MMP-2 (Fig. 3B) expression in the lungs of saline-treated $RLX^{+/+}$ mice and saline-treated $RLX^{-/-}$ mice. OVA treatment of $RLX^{+/+}$ mice had no obvious effects on MMP-9 expression (Fig. 3A) but did induce a significant increase (60%, $P < 0.05$) in MMP-2 expression, compared with that measured in the lung of saline-treated mice (Fig. 3B). Conversely, in OVA-treated $RLX^{-/-}$ mice, a significant decrease in MMP-9 expression ($P < 0.02$) was observed, compared with levels measured in OVA-treated $RLX^{+/+}$ mice (Fig. 3A), whereas no changes in MMP-2 expression were observed, compared with that measured in the lungs of saline-treated animals (Fig. 3B). As a result, the levels of both matrix-degrading proteinases, MMP-9 and MMP-2 (both $P < 0.02$), were significantly lower in OVA-treated $RLX^{-/-}$ mice, compared with levels detected in OVA-treated $RLX^{+/+}$ mice.

TABLE 1. BAL differential cell counts (cells per milliliter BAL fluid $\times 10^4$)

	Saline ^{+/+} (mean $\pm$ SE)	Saline ^{-/-} (mean $\pm$ SE)	OVA ^{+/+} (mean $\pm$ SE)	OVA ^{-/-} (mean $\pm$ SE)
Total cells	476.63 $\pm$ 27.14	541.80 $\pm$ 92.76	942.58 $\pm$ 73.55 ^a	728.81 $\pm$ 55.77
Neutrophils	13.72 $\pm$ 4.26	24.49 $\pm$ 14.19	87.77 $\pm$ 48.69 ^b	71.65 $\pm$ 25.96
Eosinophils	2.26 $\pm$ 1.4	2.55 $\pm$ 1.65	91.52 $\pm$ 41.39 ^b	67.03 $\pm$ 48.17 ^c
Lymphocytes	31.72 $\pm$ 10.77	35.22 $\pm$ 13.55	99.45 $\pm$ 32.46 ^a	90.28 $\pm$ 21.65 ^a
Monocytes/macrophages	428.93 $\pm$ 23.58	479.56 $\pm$ 95.84	663.84 $\pm$ 70.66 ^b	499.85 $\pm$ 66.89

BAL was performed, and total viable cell counts and differential counts of neutrophils, eosinophils, lymphocytes, and monocytes/macrophages were determined from individual mice ($n = 6$ per group).

^a $P < 0.05$ and ^b $P < 0.001$ when compared with saline-treated RLX^{+/+}.

^c $P < 0.001$ when compared with saline-treated RLX^{-/-} mice.

Functional consequences of relaxin deficiency

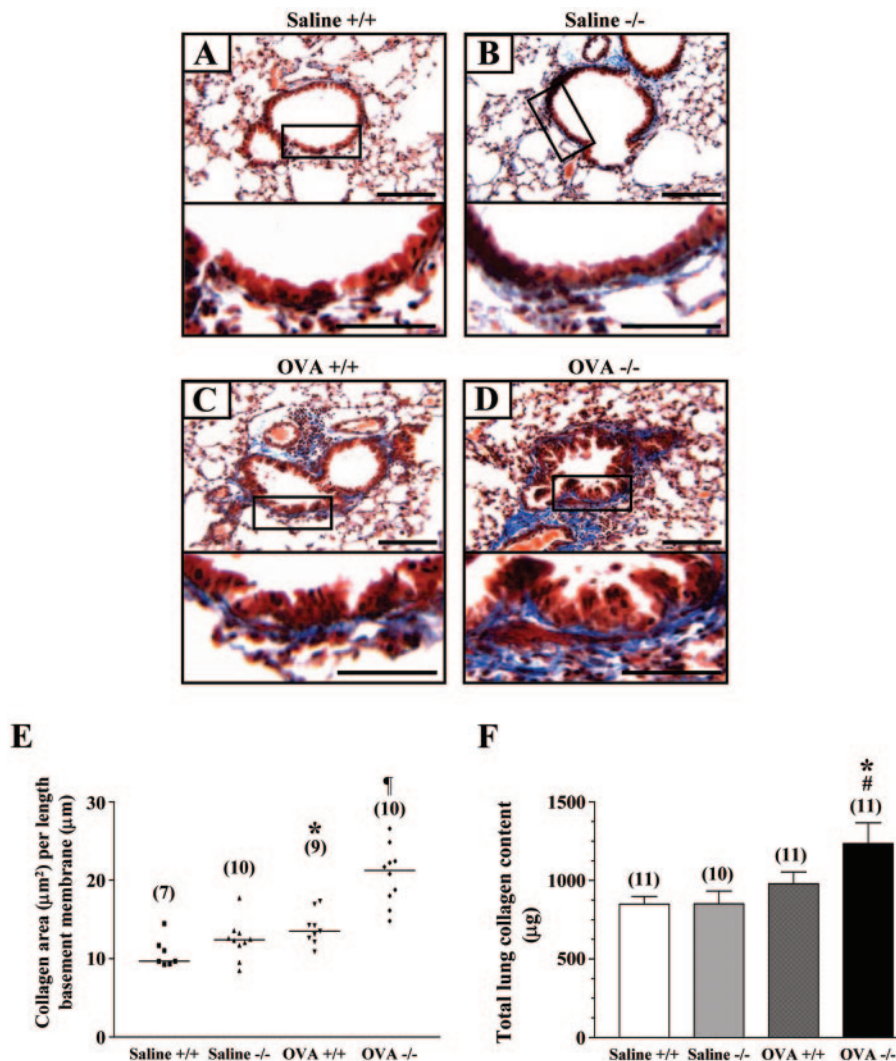
To determine whether the observed differences in airway collagen deposition were associated with changes in lung function, AHR was determined by invasive plethysmography in RLX^{+/+} and RLX^{-/-} mice. Both saline-treated RLX^{+/+} and RLX^{-/-} mice exhibited standard responses to MCh that were not significantly different (Fig. 4). Both groups of OVA-treated mice developed increased reactivity to MCh, with an increased gradient in the dose-response curve as compared with values obtained from respective

saline-treated control mice ($P < 0.001$) (Fig. 4). AHR in OVA-treated RLX^{-/-} mice was equivalent to that of OVA-treated RLX^{+/+} mice. Therefore, OVA treatment was associated with increased airway reactivity in both RLX^{+/+} and RLX^{-/-} mice that was independent of the influence of relaxin.

Other airway remodeling changes: airway epithelial thickness and goblet cell hyperplasia

Lung tissue sections from RLX^{+/+} and RLX^{-/-} mice were also examined for other structural changes associated with

FIG. 2. Lung collagen distribution and content in saline- and OVA-treated mice. Masson trichrome stained lung tissue sections were examined to assess collagen distribution in saline-treated RLX^{+/+} (A) and RLX^{-/-} (B) mice and OVA-treated RLX^{+/+} (C) and RLX^{-/-} (D) mice. Bar, 100 μ m in upper panels and 50 μ m in lower panels. E, Morphometric analysis was performed and results expressed as the area of collagen per basement membrane length ($n = 7$ –10 samples per group). *, $P < 0.02$ when compared with saline-treated RLX^{+/+} mice; †, $P < 0.001$ when compared with OVA-treated RLX^{+/+} mice. F, Hydroxyproline analysis of collagen content was also performed on the four groups studied ($n = 10$ –11 samples per group) and was expressed as the total collagen content per group. *, $P < 0.05$ when compared with saline-treated RLX^{+/+} mice and †, $P < 0.05$ when compared with saline-treated RLX^{-/-} mice.



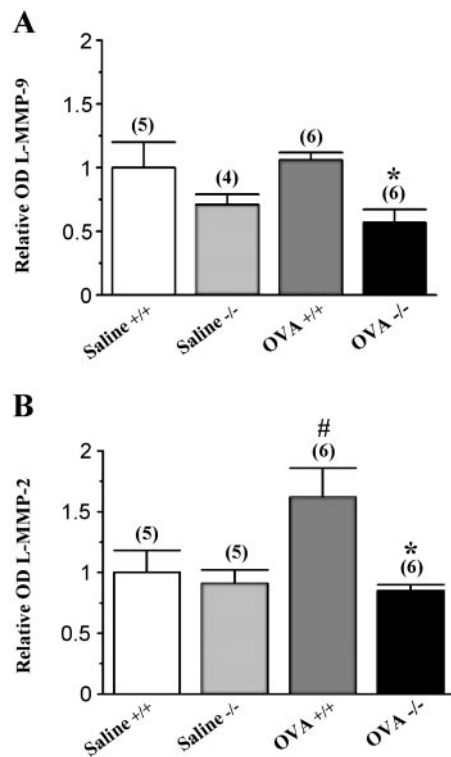


FIG. 3. MMP expression in lung tissues from saline- and OVA-treated $RLX^{+/+}$ and $RLX^{-/-}$ mice. Gelatin zymography of lung tissues from saline- and OVA-treated $RLX^{+/+}$ and $RLX^{-/-}$ mice ($n = 4-6$) was performed to detect changes in MMP-9 (A) or MMP-2 (B) expression. The levels of latent MMP in each group was determined by densitometry and expressed as a relative ratio to the mean value for the saline-treated $RLX^{+/+}$ group, which was expressed as 1. #, $P < 0.02$ when compared with saline-treated $RLX^{+/+}$ mice and *, $P < 0.02$ when compared with OVA-treated $RLX^{+/+}$ mice.

airway remodeling. Morphometric examination of lung tissue sections from saline- and OVA-treated $RLX^{+/+}$ and $RLX^{-/-}$ mice revealed that there were no significant alterations in epithelial thickness in any of the groups studied (Fig. 5A). There was, however, a significant increase in goblet cell numbers in both the OVA-treated $RLX^{+/+}$ and OVA-treated $RLX^{-/-}$ mice ($P < 0.001$), compared with the saline-treated control animals (Fig. 5B). There was no significant difference in goblet cell numbers between OVA-treated $RLX^{+/+}$ and $RLX^{-/-}$ mice. These findings indicated that relaxin deficiency was associated with increased airway fibrosis but did not influence these other structural changes of airway remodeling in this animal model of AAD.

Discussion

In this study, we have demonstrated a number of key findings. First, long-term OVA treatment of mice was associated with increased collagen deposition and airway fibrosis, which was exaggerated in the absence of relaxin, *i.e.* OVA-treated $RLX^{-/-}$ mice demonstrated significantly higher airway collagen deposition, compared with OVA-treated $RLX^{+/+}$ mice. These findings confirm an important role for endogenous relaxin in the regulation of lung collagen turnover in response to AAD. The distribution of collagen in OVA-treated $RLX^{-/-}$ mice was predominantly in the epi-

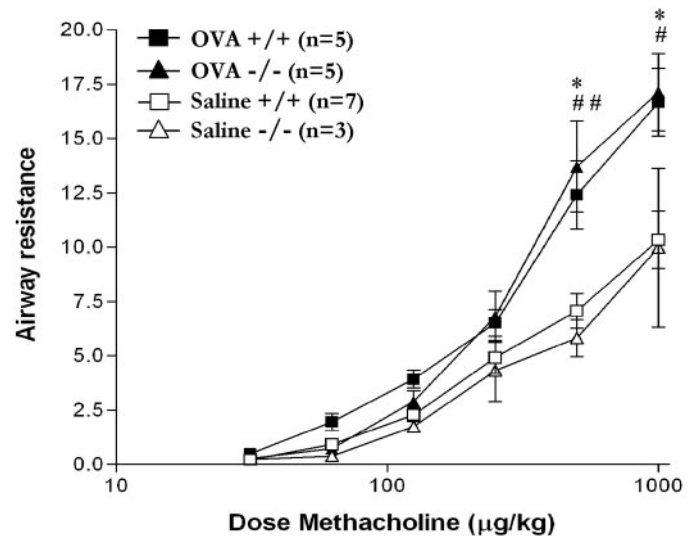


FIG. 4. MCh-induced airway responses. AHR in saline- and OVA-treated $RLX^{+/+}$ and $RLX^{-/-}$ mice. Mice were tracheostomized and the jugular vein cannulated. MCh doses were delivered iv and airway resistance was measured. OVA-treated $RLX^{+/+}$ (A) and OVA-treated $RLX^{-/-}$ (B) mice demonstrated increased MCh-induced AHR when compared with the respective saline-treated controls. No differences in AHR were observed when OVA-treated $RLX^{+/+}$ and $RLX^{-/-}$ mice were compared. *, $P < 0.001$ when compared with saline-treated $RLX^{+/+}$ mice. #, $P < 0.01$ and ##, $P < 0.001$ when compared with saline-treated $RLX^{-/-}$ mice; $n = 3-7$ samples were analyzed per group.

thelial BM regions of the airway wall and in the submucosal regions surrounding the airways consistent with the consequences of airway remodeling in asthma (3). Second, the increased collagen detected in OVA-treated $RLX^{-/-}$ mice (compared with levels detected in OVA-treated $RLX^{+/+}$ mice) was mediated in part by a failure for up-regulation of MMP-2 and a significant reduction in MMP-9 expression. Third, the increased collagen detected in OVA-treated mice did not affect AHR or other structural changes within the airway. These combined findings confirm that relaxin down-regulates collagen expression in adverse AAD.

The data from both the saline and OVA-treated $RLX^{+/+}$ and $RLX^{-/-}$ mice indicate that the long-term model of allergic airway disease was established, as described previously (16–19). OVA-specific IgE was increased in OVA-treated mice but not in saline controls. Furthermore, inflammatory cells were moderately increased in OVA-treated but not saline-treated mice with a significant increase in numbers of eosinophils. This influx of eosinophils during AAD is critical because activated eosinophils are a source of TGF β , which is a potent profibrotic agent. TGF β induces fibroblasts to proliferate and differentiate into myofibroblasts, which are the major source of collagen in active fibrotic sites (26). Furthermore, many of the changes that represent airway remodeling in asthma were observed. The most striking of these was the area of collagen deposition in the subepithelial basement membrane regions of the airway wall in the submucosa surrounding airway structures but not in the lung parenchyma. Goblet cell proliferation was also noted but smooth muscle thickness was unaltered in this model as previously described by Kumar *et al.* (16). There-

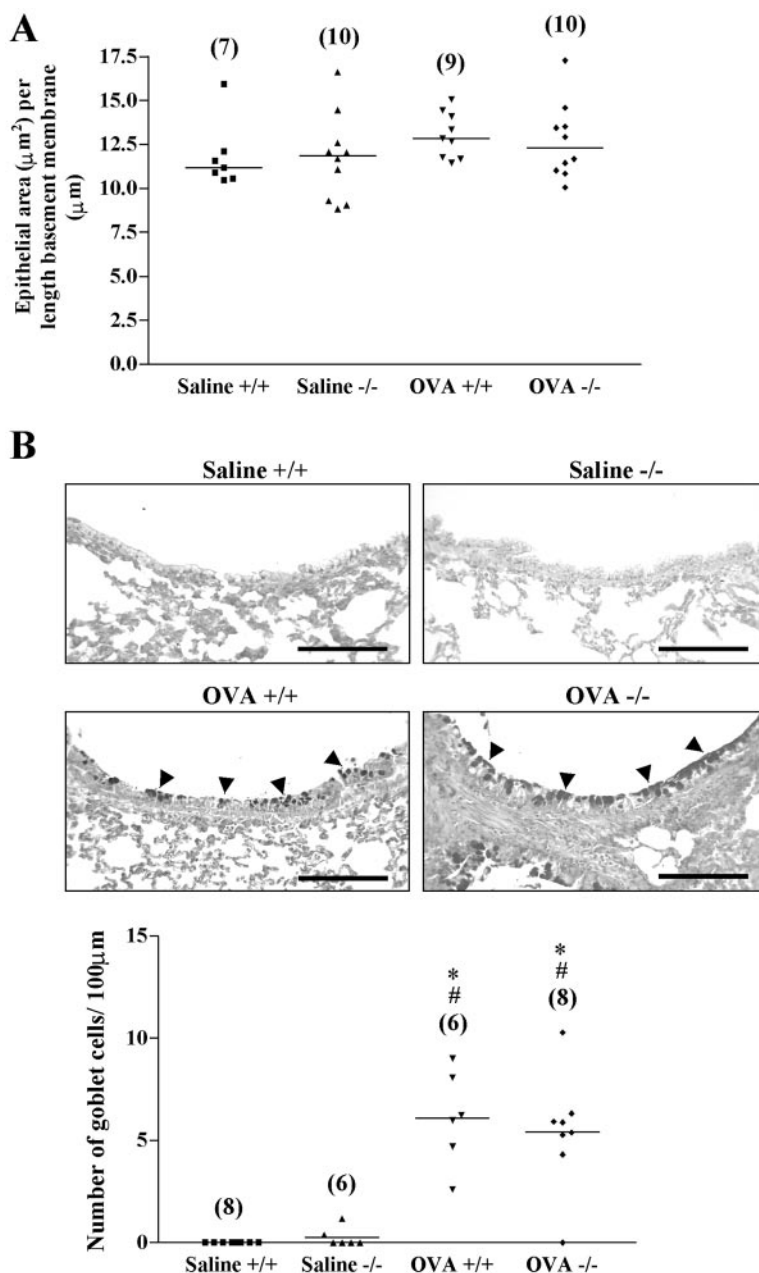


FIG. 5. Morphometric analyses of other structural changes of airway remodeling. Epithelial thickness (A) and goblet cell number (B) were assessed in the saline- and OVA-treated $RLX^{+/+}$ and $RLX^{-/-}$ mice. No differences in epithelial thickness were observed among the four groups. AB-PAS-stained sections were examined to assess goblet cell hyperplasia. Representative sections from saline-treated $RLX^{+/+}$ and $RLX^{-/-}$ mice (demonstrating the absence of goblet cells from the surface epithelium of the bronchus) or OVA-treated $RLX^{+/+}$ and $RLX^{-/-}$ mice (demonstrating goblet cell hyperplasia; indicated by the arrows) are shown. Bar, 100 μ m. Both OVA-treated $RLX^{+/+}$ and $RLX^{-/-}$ mice demonstrated significantly increased numbers of goblet cells, compared with their respective saline-treated controls. *, $P < 0.01$ vs. saline-treated $RLX^{+/+}$ and #, $P < 0.01$ vs. saline-treated $RLX^{-/-}$ mice; $n = 6$ –8 samples were analyzed per group.

fore, this model can be considered to be reflective of human asthma.

To investigate the mechanisms by which relaxin may regulate collagen deposition in the airway/lung, MMP-2 and MMP-9 expression was examined in the saline and OVA-treated $RLX^{+/+}$ and $RLX^{-/-}$ mice. Collagen deposition is controlled at several levels, first at the level of collagen gene expression, and in particular, by the balance between the MMPs that degrade fibrillar matrix proteins and the tissue inhibitors of MMPs that inhibit the activity of MMPs. The gelatinases MMP-9 and MMP-2 primarily degrade basement membrane components such as type IV collagen, fibronectin, and elastin but have also been shown to degrade type I collagen (27).

In this study a decrease in MMP-9 was demonstrated in the

lungs of OVA-treated $RLX^{-/-}$ mice (compared with values in OVA-treated $RLX^{+/+}$ mice), and a failure of up-regulation of MMP-2 expression was also identified. This suggests that a relative deficiency of these MMPs in OVA-treated $RLX^{-/-}$ mice was one means by which excessive collagen accumulation (fibrosis) was accelerated during AAD, in the absence of relaxin. These findings are consistent with previous studies, demonstrating that exogenous relaxin administration up-regulates either or both MMP-2 and MMP-9 in other reproductive (28) and nonreproductive (29) organs, with the differential regulation of these enzymes most likely the result of species specificity. Also consistent with this, decreased MMP-9 has been reported in chronic asthma (30) and correlated with increased deposition of collagen and fibronectin. Paradoxically, however, increased MMP-9 and an elevated

MMP-9 to tissue inhibitor of MMP-1 ratio has been demonstrated in acute asthma (31–33). Therefore, MMP-9 may play a dual role in the course of asthma, reflecting its differential regulation in the early *vs.* later stages of disease. Because MMP-9 can degrade matrix proteins of the BM, increased MMP-9 in acute stages of asthma may contribute to the reduced integrity of airway epithelium and the aberrant epithelial repair process, whereas reduced MMP-9 in the later stages of asthma may lead to increased deposition of collagen and fibronectin resulting in BM thickening and airway fibrosis. Furthermore, MMPs are required to allow migration and regression of inflammatory cells (34); MMP-2 is found to be preferentially secreted by fibroblasts and lung epithelial cells, whereas MMP-9 is preferentially secreted by inflammatory cells including macrophages, lymphocytes, neutrophils, and eosinophils (26, 35).

Mucous cell metaplasia and hyperplasia are key features of both acute and chronic asthma with the consequent over-secretion of mucus contributing to the pathology of the disease. There is growing evidence that epithelial and connective tissue changes in asthma are interrelated via the epithelial mesenchymal trophic unit (36). Relaxin is known to influence both epithelial and connective tissues. For example, the vaginal and prostatic epithelium of relaxin-1 null mice is altered (13, 37), and relaxin stimulates bronchial epithelial cell protein kinase activation, migration, and ciliary beating *in vitro* (38) and induces the proliferation of human amniotic epithelium (25). In our study, the increased fibrosis associated with the absence of relaxin did not alter goblet cell numbers or epithelial thickness. There are two possible explanations for this; one possibility is that relaxin and fibrosis *per se* do not drive other structural changes of remodeling. Alternatively, longer periods (>6 wk) of aberrant matrix proteins are required to induce further changes in the epithelium.

AHR is a central feature of asthma and correlates closely with ongoing symptoms. Persistent AHR is a consistent finding in chronic symptomatic asthma; however, the specific contributions by individual components of airway remodeling to AHR are not known. Whereas it has been suggested that airway fibrosis may protect against AHR, in our study, it was found that AHR was not influenced by airway fibrosis *per se*.

We have previously shown that recombinant human relaxin treatment of 9- and 12-month-old RLX^{-/-} mice resulted in reversal of collagen accumulation, which in turn restored lung structure and function, particularly when treatment was applied in the early stages of fibrosis (15). Relaxin also decreased the development of collagen deposition associated with airway inflammation in previously reported OVA sensitized models of AAD (10, 11). In those previous studies (11), collagen accumulation was elevated after 2 wk but was only significantly increased after 4–8 wk of OVA aerosols, which is consistent with what was seen in this model of AAD. Our present findings demonstrate for the first time an important role of endogenous relaxin in the regulation of airway fibrosis and remodeling in AAD and highlight the potential use of relaxin to reverse and/or prevent airway fibrosis in asthma. Our findings indicate that relaxin plays a critical protective role in the inhibition of collagen

deposition associated with a long-term allergic inflammatory response in the airway and suggest that AAD may be associated with a relative deficiency of relaxin expression during the allergic inflammatory response. As such, relaxin may represent an effective therapeutic agent for the prevention or reversal of airway fibrosis in AAD. Relaxin may regulate the extracellular matrix either directly or indirectly via its effects on matrix proteins such as collagen and fibronectin (8–9, 13–15). Additionally, relaxin did not appear to affect other aspects of AAD including AHR and epithelial architecture.

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