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The effects of repeated allergen challenge on airway smooth muscle structural and molecular remodeling in a rat model of allergic asthma

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Labonté I, Hassan M, Risse PA, Tsuchiya K, Laviolette M, Lauzon AM, Martin JG. The effects of repeated allergen challenge on airway smooth muscle structural and molecular remodeling in a rat model of allergic asthma. *Am J Physiol Lung Cell Mol Physiol* 297: L698–L705, 2009. First published July 31, 2009; doi:10.1152/ajplung.00142.2009.—The effects of remodeling of airway smooth muscle (SM) by hyperplasia on airway SM contractility in vivo are poorly explored. The aim of this study was to investigate the relationship between allergen-induced airway SM hyperplasia and its contractile phenotype. Brown Norway rats were sensitized with ovalbumin (OVA) or saline on day 0 and then either OVA-challenged once on day 14 and killed 24 h later or OVA-challenged 3 times (on days 14, 19, and 24) and killed 2 or 7 days later. Changes in SM mass, expression of total myosin, SM myosin heavy chain fast isoform (SM-B) and myosin light chain kinase (MLCK), tracheal contractions ex vivo, and airway responsiveness to methacholine (MCh) in vivo were assessed. One day after a single OVA challenge, the number of SM cells positive for PCNA was greater than for control animals, whereas the SM mass, contractile phenotype, and tracheal contractility were unchanged. Two days after three challenges, SM mass and PCNA immunoreactive cells were increased (3- and 10-fold, respectively; $P < 0.05$), but airway responsiveness to MCh was unaffected. Lower expression in total myosin, SM-B, and MLCK was observed at the mRNA level ($P < 0.05$), and total myosin and MLCK expression were lower at the protein level ($P < 0.05$) after normalization for SM mass. Normalized tracheal SM force generation was also significantly lower 2 days after repeated challenges ($P < 0.05$). Seven days after repeated challenges, features of remodeling were restored toward control levels. Allergen-induced hyperplasia of SM cells was associated with a loss of contractile phenotype, which was offset by the increase in mass.

phenotype; animal model; myosin heavy chain isoform

STRUCTURAL AIRWAY REMODELING has been widely reported in both human asthma (4, 13) and animal models of experimental asthma (11, 23, 37). Most tissues of the airway wall are affected by remodeling, and the observed changes include shedding of the epithelium, thickening of the reticular basement membrane, increased mucous gland size and number, goblet cell differentiation, angiogenesis, and augmentation of the airway smooth muscle (SM) mass (4). The changes in airway wall tissues are likely to be of clinical importance since they may control airway caliber, airway elasticity, and responsiveness and may cause symptoms related to mucus overproduction (4). Airway SM remodeling is a reported feature of several animal models of allergic asthma (11, 12, 22), including the Brown Norway (BN) rat (19, 26, 31). The increase in

SM mass, observed in asthma of varying severity (5, 29, 36), has been proposed to be sufficient to account for altered airway responsiveness (16). The augmentation of airway SM mass has been attributed to hyperplasia through evidence of cell proliferation quantified by bromodeoxyuridine incorporation or PCNA expression in vivo (11, 19, 28, 37). Susceptibility to SM remodeling may be a risk factor for asthma; cultured airway SM cells from asthmatic subjects were shown to have a higher proliferation rate than cells from normal subjects (14), similarly to SM cells from hyperresponsive rats (38).

Experimental work on cultured SM cells suggests that the proliferative and contractile phenotypes are inversely related (6, 27). Cell culture in the presence of serum modulates the SM cell contractile apparatus toward nonmuscle contractile protein isoforms. A loss in SM α -actin (α -SMA), SM myosin heavy chain (sm-MHC), calponin, h-caldesmon, desmin, myosin light chain kinase (MLCK), and tropomyosin was observed following 7 days of culture of airway SM cells compared with freshly isolated cells (12). Loss of muscarinic M_3 receptors is also associated with the proliferative phenotype (25). The exact causes of this phenotypic modulation are still unknown, but cell proliferation per se and modification of the substratum influence this loss in contractile proteins (27). The expression of SM-specific contractile proteins may be regained under specific culture conditions (21).

Moir and coworkers (26) have demonstrated that airway SM remodeling is associated with a reduction in the expression of SM contractile proteins in the bronchioles of repeatedly challenged BN rats. However, the tracheal SM did not undergo phenotypic modulation, raising the question of possible differences in tracheal and intrapulmonary bronchial SM susceptibility to undergo remodeling. Differences in the pharmacological properties of tracheal and peripheral airways have long been known (7). The loss of sm-MHC, in association with airway SM hyperplasia (26), occurs at a time when airway hyperresponsiveness (AHR) is observed in the intact animal, raising the question of whether certain sm-MHC isoforms might be selectively preserved leading to AHR. In this regard, we were particularly interested in assessing the expression of SM-B, a sm-MHC isoform with an extra 7-amino acid insert in the loop above the nucleotide binding pocket (35). The expression of this myosin isoform increases the velocity of muscle shortening, which might be an important factor for AHR (17).

The aims of the present study were to evaluate the structural and phenotypic changes of SM from the trachea to peripheral airways and to measure the relationship between SM molecular remodeling and force generation in allergen-challenged BN rats. We also wished to assess the implications of the structural and molecular remodeling of airway SM for airway responsiveness in vivo. The allergen challenges were performed so as

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to ensure adequate exposure of the entire tracheobronchial tree to the allergen.

MATERIALS AND METHODS

Experimental design. Male BN rats (Harlan Industries, Indianapolis, IN) weighing between 150 and 180 g were sensitized on day 0 with a subcutaneous injection of either sterile PBS (control animals) or 1-mg ovalbumin (OVA; Sigma-Aldrich, St. Louis, MO) diluted in sterile PBS and containing 100 mg of Al(OH)₃ (EM Industries, Hawthorne, NY) as adjuvant and with an intraperitoneal injection of 2×10^9 of heat-killed *Bordetella pertussis* bacilli (supplied by Dr. Thomas Issekutz, Dalhousie University, Halifax, Nova Scotia, Canada). On days 14, 19, and 24, control and OVA BN rats were anaesthetized with xylazine hydrochloride (14 mg/kg ip; Novopharm, Toronto, Ontario, Canada) and pentobarbital (30 mg/kg ip; CEVA Sante Animale, Lenexa, KS), intubated, and challenged with nebulized 5% OVA for 5 min (Hudson RCI, Arlington Heights, IL). Three groups of animals were studied: rats challenged once and killed 24 h after challenge and rats that received three challenges and were killed 2 or 7 days after the final challenge with an overdose of intraperitoneal pentobarbital. Lung and tracheal tissues were harvested for morphometry, real-time quantitative PCR (qPCR), Western blots, and force measurements. Bladder tissues were used as reference organs for Western blots. All experimental protocols involving animals were approved by the McGill University Animal Care Committee and complied with the guidelines of the Canadian Council on Animal Care.

Morphometry. Lungs were fixed in 10% buffered formalin at a hydrostatic pressure of 25 cmH₂O for 24 h. Tissues were then paraffin-embedded. Sampling of tissues for morphometric analysis was done using a systematic biased sampling strategy as previously reported (11). Five-micrometer-thick midsagittal and parahilar sections were taken from the lungs to sample small (<1-mm basement membrane perimeter (Pbm)), and medium-sized (1- to 2-mm Pbm), and large airways (>2-mm Pbm), respectively. We analyzed 5–8 airways per animal, sectioned transversely, as defined by a long-to-short axis ratio of 1.5 or less. Sections were also prepared from the lower third of the trachea. Sections were double-immunostained for α -SMA and PCNA. α -SMA (Sigma-Aldrich) staining was revealed with Vector Red (Vector Laboratories, Burlington, Ontario, Canada) and was considered as an indicator of the SM mass. Airway SM surface area was measured with Image-Pro Plus (Media Cybernetics, Bethesda, MD), and the results were normalized to the square of the Pbm² to correct for airway size. The Pbm was measured by tracing a line along the base of the epithelial cells. PCNA immunoreactivity (Calbiochem, San Diego, CA) was developed with BCIP/NTB (Vector Laboratories), and the violet-stained nuclei within the airway SM bundles were counted as a measurement of proliferation. Results were also normalized to Pbm². All measurements were performed by a single trained observer (M. Hassan) blinded as to group status.

Airway responsiveness to MCh in vivo. To study OVA-induced effects on airway responsiveness, we measured respiratory parameters induced by the administration of aerosolized methacholine (MCh). Animals were anesthetized with xylazine hydrochloride (14 mg/kg ip)

and pentobarbital (30 mg/kg ip). A tracheostomy tube connected the rat to a computer-controlled small animal ventilator (flexiVent; SCIREQ, Montreal, Quebec, Canada) with the following ventilatory parameters: tidal volume, 8 ml/kg; frequency, 90 breaths/min; positive end-expiratory pressure (PEEP), 2.0 cmH₂O. Muscle paralysis was induced with pancuronium bromide (1.0 mg/kg ip). Baseline measurements were recorded after the aerosol administration of saline, followed by the administration of MCh (Sigma-Aldrich) diluted with saline in doubling doses from 4 to 128 mg/ml. MCh was delivered for 10 s at each concentration using the Aeroneb ultrasonic nebulizer (SCIREQ) on the flexiVent system. Total respiratory resistance (*R*) and total respiratory elastance (*E*) were assessed using the single compartment model and measured every 15 s, eight times at each MCh dose. The peak response is shown at each concentration of MCh.

RNA extraction and qPCR experiments. Lung parenchyma was removed from the intrapulmonary airways by gently scraping the tissues with a safety razor blade held at 90° while holding the trachea with a forceps to avoid as much vascular SM contamination as possible. RNA was extracted from the intrapulmonary airways, using a commercial kit (RNeasy Mini Kit; Qiagen, Mississauga, Ontario, Canada). RNA quantity and quality were assessed by spectrophotometer (Biomate 3; Thermo Spectronic, Rochester, NY). Reverse-transcription was done with 0.5 mg of RNA for each sample. qPCR was performed with 25 ng/ μ l cDNA per reaction with 10 μ l of 2 $\times$ QuantiTect SYBR Green PCR (Qiagen), 1 μ l of forward and reverse primers (5 μ M), and 7 μ l of RNase-free water for a total volume of 20 μ l per capillary (see Table 1 for primer sequences and PCR product length). The samples were amplified in StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA). A negative control (RNase-free water) was added to each qPCR run. Individual data were normalized for their corresponding housekeeping gene (ribosomal S9). PCR product length was verified on an agarose gel.

Protein extraction and Western blotting. A total protein extract was obtained from the snap-frozen trachea and the reference organ bladder. Tissues were immersed in liquid N₂, ground with a mortar and pestle, homogenized, and extracted with a pyrophosphate extraction buffer. A Bradford assay was performed to determine protein content. Electrophoresis of 20 μ g of total protein for each sample and the reference organ bladder, 5 μ g of chicken gizzard as a positive control, and a kaleidoscope ladder (Bio-Rad, Mississauga, Ontario, Canada) was done on a 7% acrylamide gel using a Laemmli buffer system. Proteins were electroblotted to polyvinylidene difluoride membranes (Bio-Rad). The membranes were cut just below the 82-kDa level as estimated with the ladder. The upper membranes were first probed for SM-B (SMB₆₊₇, 1:1,000, provided by Dr. A. Rovner, University of Vermont; Ref. 20) for 2 h at 37°C and then incubated with the matching secondary antibody, an anti-rabbit-IgG coupled to horseradish peroxidase (HRP; GE Healthcare Biosciences, Baie d'Urfé, Quebec, Canada). Signal detection was done with ECL Plus system (GE Healthcare Biosciences). The upper membranes were subsequently stripped in 60 mM Tris·HCl, 70 mM SDS, pH 6.7, with 70.5 μ l/10 ml 2-mercaptoethanol for 30 min at 55°C, carefully rinsed, and probed overnight at 4°C for total myosin (BT-562; Biomedical Technologies, Stoughton, MA) followed by the anti-rabbit-IgG-HRP. The upper

Table 1. Primer sequences and PCR product length used for qPCR

Target Gene	Forward Primer	Reverse Primer	PCR Product Length, bp
Total myosin	5'-CCAATCCTGGCTTTTCG-3'	5'-CTCTCGTCCCTAGCATGTC-3'	170
SM-B	5'-CAAGGCCCATCTTTTGCCTAC-3'	5'-CTCTCGTCCCTAGCATGTC-3'	224
MLCK	5'-ACATCCGTCAGGAGATCAGC-3'	5'-CACTCCCCTCTGTTAGCTC-3'	148
M ₃ receptor	5'-GGAACCTAGCCTGCCACCTC-3'	5'-GCCAGACCAATCATCACACC-3'	166
S9	5'-AGGATTTCTTGGAGAGAAGGCTGC-3'	5'-CTTCTGAGAGTCCAGGCGAACAAT-3'	157

qPCR, quantitative PCR; SM-B, smooth muscle myosin heavy chain fast isoform; MLCK, myosin light chain kinase; M₃ receptor, muscarinic receptor; S9, housekeeping gene.

membranes were finally incubated with an anti-MLCK (Sigma-Aldrich) for 90 min at room temperature followed by an anti-mouse-IgG-HRP. The lower membranes were probed for the housekeeping protein GAPDH (Affinity Bioreagents, Golden, CO) for 1 h at room temperature and then incubated with anti-mouse-IgG-HRP (GE Healthcare Biosciences). Signal quantification was performed with the FluorChem 8500 imaging system (Alpha Innotech, San Leandro, CA). The result for each target protein was normalized for the reference organ value on each membrane and for their respective housekeeping protein GAPDH.

Isometric force measurements. The lower part of the trachea (3–5 mm) was harvested, cleaned of connective tissue in Krebs-Henseleit solution (118 mM NaCl, 4.7 mM KCl, 2.4 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.17 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.17 mM KH_2PO_4 , 11.1 mM glucose, and 25 mM NaHCO_3 , pH 7.4), and measured for length and width. Each tracheal ring preparation was suspended on hooks connected to a dual-mode transducer (force displacement; Grass Instrument, Longueuil, Quebec, Canada) in 10-ml Krebs-Henseleit solution gassed with 95% O_2 -5% CO_2 and maintained at 37°C. Isometric tension was recorded on a polygraph (BBC Goerz Metrawatt), and the preload was set at 1.5 g. During a 60-min period, the tissues were allowed to stabilize and were frequently washed with fresh Krebs-Henseleit solution. Then, cumulative concentration-response curves to MCh (10^{-7} to 10^{-3} M) were measured.

Data analysis. Normalization for airway SM volume (approximated from airway SM area^{3/2}) was done to correct the mRNA and protein expression of SM-specific contractile proteins for the increased mass of SM that followed repeated OVA challenges. Since the area fraction of the trachea represented by SM was only $5.56 \pm 0.61\%$ ($n = 3$) and in the intrapulmonary airways $13.8 \pm 1.06\%$ ($n = 4$) after three OVA challenges correction of expression for housekeeping genes was not adequate to account for changes in SM mass, and an additional correction was applied to better assess expression levels per unit volume of SM.

Data were expressed as means $\pm$ SE. SM morphometry, proliferation, qPCR, Western blot, and all respiratory parameters were analyzed using a repeated-measures two-way ANOVA. Data from force and stress were analyzed using a repeated-measures mixed model. The values were log-transformed to normalize variances, and the reported P values are based on these transformations. Statistical significance was defined as $P \leq 0.05$. All analyses were conducted using the statistical package SAS version 9.1.3 (SAS Institute, Cary, NC).

RESULTS

Airway SM mass and hyperplasia. A single OVA challenge had no influence on SM mass for both trachea and intrapulmonary airways measured 24 h later (Fig. 1). An approximately threefold increase in airway SM mass was observed in both trachea and intrapulmonary airways for the OVA-sensitized/OVA-challenged rats compared with the controls, 2 days after repeated challenges ($n = 4$, $P \leq 0.0001$, and $n = 6$, $P \leq 0.0001$, respectively). By 7 days after the repeated challenges, the tracheal SM mass was reduced to control levels compared with the 2-day time point ($n = 6$; $P < 0.01$). The intrapulmonary SM mass was also significantly reduced at 7 days compared with the 2-day time point ($n = 6$; $P < 0.001$) but was still greater compared with the control rats ($n = 6$; $P < 0.05$). Hyperplasia was assessed by PCNA immunoreactivity in SM cells (Fig. 2). A single OVA challenge doubled the tracheal SM PCNA immunoreactivity after 24 h ($n = 4$; $P < 0.01$) but did not reach statistical significance for the intrapulmonary airways ($n = 4$; $P = 0.06$). Repeated allergen challenges induced a 10-fold increase in PCNA-positive tracheal SM cells after 2 days ($n = 4$; $P \leq 0.0001$), which was back to control levels

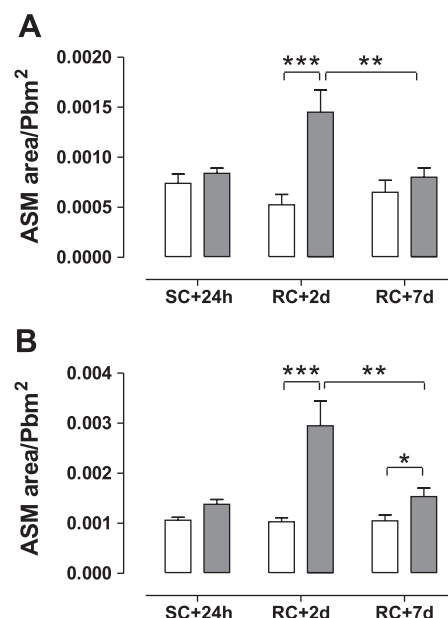


Fig. 1. Airway smooth muscle (ASM) mass. No difference in ASM mass was observed at 24 h after a single challenge in trachea (A) and intrapulmonary airways (B). Two days after repeated ovalbumin (OVA) challenges, SM mass was increased in both trachea and intrapulmonary airways, which was back to control level at 7 days. Data are reported as means of ASM area normalized for the square of the basement membrane perimeter (Pbm²). White bars, control animals; gray bars, treated animals (OVA-sensitized/OVA-challenged) for each time point; SC, single challenge; RC, repeated challenges; d, days. * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.0001$.

after 7 days ($n = 6$; $P \leq 0.0001$). For the intrapulmonary airways, a 3-fold increase in proliferating SM cells was observed for the repeatedly challenged animals killed 2 days later ($n = 4$; $P \leq 0.001$), which was also restored to control levels at 7 days after the final challenge ($n = 6$; $P \leq 0.01$).

Airway responsiveness to MCh. To study the association between airway SM mass increase and airway responsiveness *in vivo*, we measured respiratory parameters induced by aerosol MCh at 2 and 7 days after repeated challenges. Although both airway SM mass and PCNA immunoreactivity were significantly increased in OVA-treated rats 2 days after challenges, there were no significant differences between control and OVA-treated rats in respiratory resistance and elastance at either 2 or 7 days after repeated OVA challenges ($n = 6-8$; $P > 0.05$; Fig. 3).

mRNA levels encoding contractile proteins. Three genes coding for the contractile proteins in the intrapulmonary airways were studied with qPCR: total myosin, SM-B, and MLCK. Results without SM volume normalization showed that OVA challenges did not influence the mRNA expression from the intrapulmonary airways of each gene studied at each time point (Fig. 4, A, C, and E). With normalization for SM volume (Fig. 4, B, D, and F), 2 days after repeated challenges, a substantial reduction in total myosin, SM-B, and MLCK expression ($n = 6$, $P \leq 0.01$, $P \leq 0.01$, $P \leq 0.001$, respectively) was observed compared with the control animals. However, this reduction in mRNA expression coding for contractile proteins was restored 7 days after repeated challenges for all tested genes ($n = 6$).

Contractile protein expression. The analysis of tracheal contractile protein expression by Western blotting (see Fig. 5G

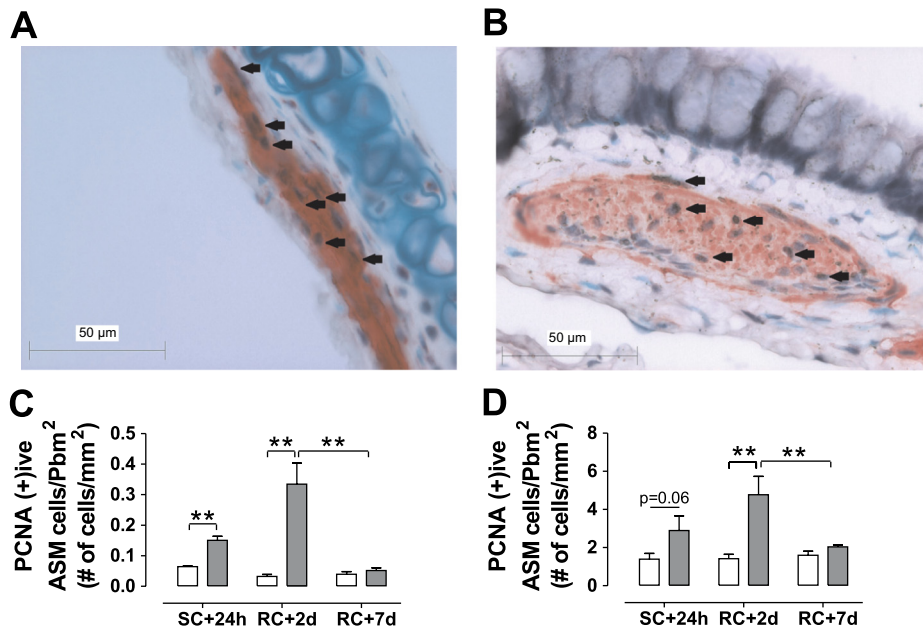


Fig. 2. ASM hyperplasia. Morphometric analysis of PCNA-positive [(+)ive] SM cells of trachea (A) and intrapulmonary airways (B). The red staining corresponds to the SM α -actin (α -SMA), indicating the SM bundles, and the violet staining corresponds to the PCNA-positive cells (as indicated by the arrowheads). A single OVA challenge doubled SM cell proliferation in trachea (C) and in intrapulmonary airways (D). After repeated OVA challenges, a 10-fold increase in PCNA-positive SM cells in trachea and a 3-fold increase in intrapulmonary airways were observed 2 days after the final challenge, and both were reduced to control levels after 7 days. Data are reported as means of PCNA-positive ASM cells normalized for Pbm². White bars, control animals; gray bars, treated animals (OVA-sensitized/OVA-challenged) for each time point. ** $P \leq 0.01$.

for sample blots) was also performed with and without normalization for SM volume. Without the SM volume normalization, no significant alteration in any of the proteins was observed at 24 h after a single challenge, and no difference in total myosin or MLCK was observed at 2 and 7 days after repeated challenges (Fig. 5, A and E). Only SM-B showed a reduced expression 7 days after multiple OVA challenges ($n = 7$; $P < 0.05$; Fig. 5C). When normalized for SM volume, again no statistical difference in contractile protein expression was observed 24 h after a single challenge. However, total myosin and MLCK protein expression was reduced 2 days after the repeated challenges (respectively, $n = 4$, $P < 0.05$, and $n = 4$, $P < 0.001$) but recovered to baseline levels after 7 days

(respectively, $n = 5$, $P > 0.05$, and $n = 5$, $P > 0.05$) (Fig. 5, B and F). The correction for SM volume abrogated the variations in SM-B expression 7 days after repeated challenges (Fig. 5D).

Isometric force measurements. No difference in either maximal force development or response expressed as a percentage of maximal force at each dose of MCh was observed in single- and multiple-challenged animals (2 and 7 days). The EC_{50} for OVA-sensitized/OVA-challenged animals was not statistically different from controls [respectively: 4.75×10^{-7} M vs. 6×10^{-7} M ($n = 5$) for 24 h after a single challenge; 1×10^{-6} M vs. 7.5×10^{-7} M ($n = 5$) for 2 days after repeated challenges; and 5.7×10^{-7} M vs. 6.3×10^{-7} M ($n = 6$) for 7 days after

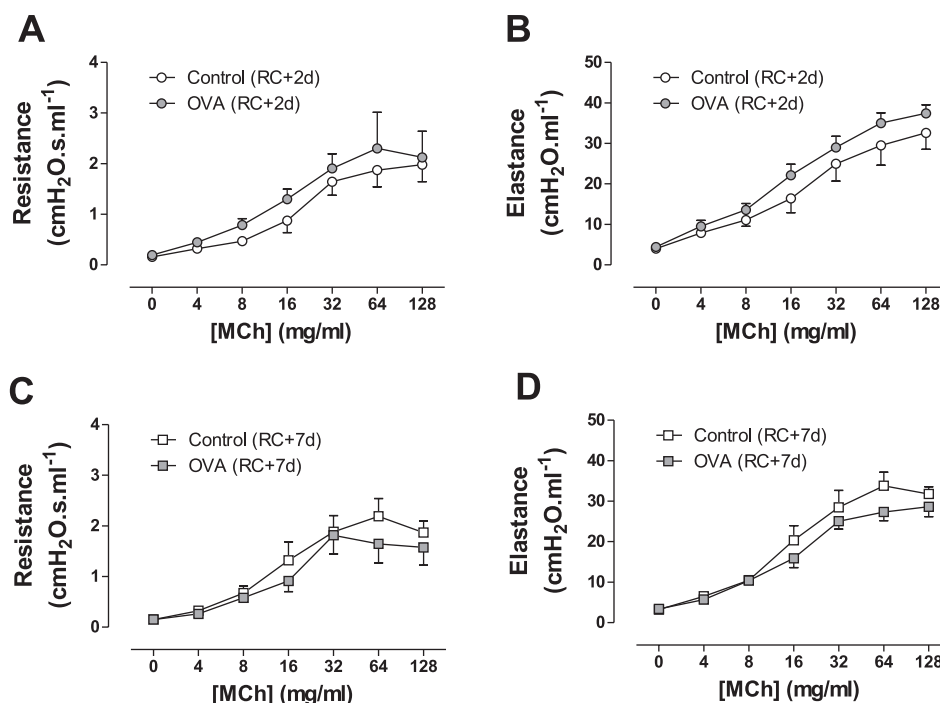


Fig. 3. Airway hyperresponsiveness (AHR) measurements. Total respiratory resistance in control and OVA-treated rats 2 days (A) and 7 days (C) after repeated challenges. Total respiratory elastance in control and OVA-treated rats 2 days (B) and 7 days (D) after repeated challenges. Data are expressed as means $\pm$ SE ($n = 6-8$ per group). MCh, methacholine.

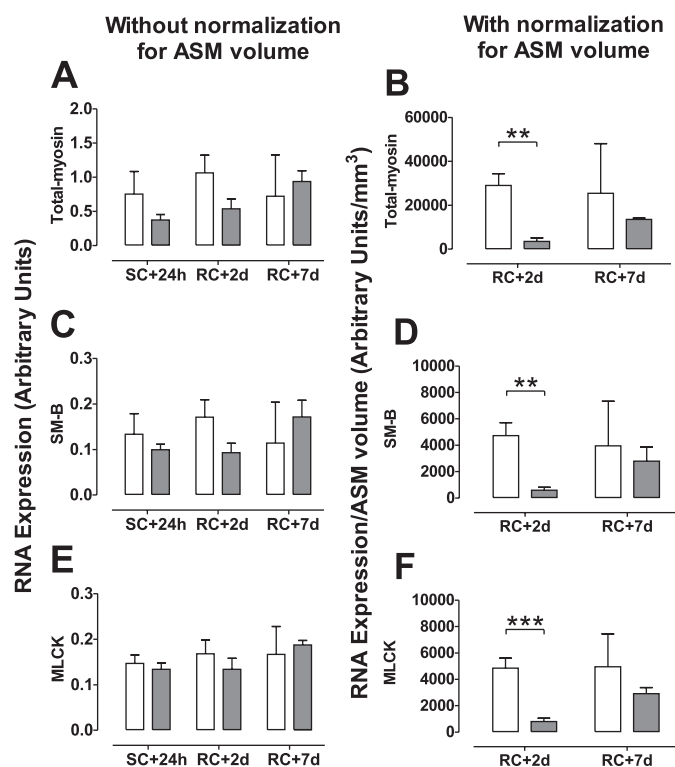


Fig. 4. mRNA expression coding for contractile proteins. There was no significant variation in mRNA coding for the measured contractile proteins in the intrapulmonary airways without normalization for ASM volume [A: total myosin; C: SM myosin heavy chain fast isoform (SM-B); E: myosin light chain kinase (MLCK)], but a substantial reduction was observed at 2 days postfinal challenge when normalization for ASM volume was applied (B: total myosin; D: SM-B; F: MLCK). These reductions were restored at 7 days postfinal challenge. A single challenge did not influence the mRNA expression for each studied gene (SC+24h). The data were corrected for the housekeeping gene S9 and expressed as means $\pm$ SE ($n = 6$). White bars, control animals; gray bars, treated animals (OVA-sensitized/OVA-challenged) for each time point. ** $P \leq 0.01$ and *** $P \leq 0.001$.

repeated challenges]. Active stress was calculated from force and tracheal SM cross-sectional area. A single challenge did not influence the force/mm² of muscle, but 2 days after multiple challenges, a substantial reduction in stress was observed for OVA-sensitized/OVA-challenged animals compared with controls (1,361.7 g/mm², $n = 4$ vs. 432.9 g/mm², $n = 5$; $P \leq 0.01$; at 3.0×10^{-7} M MCh). This reduction in active stress was no longer present 7 days after repeated challenges (Fig. 6).

Expression of the M₃ muscarinic receptor. To ensure that the reduced contractility observed 2 days after repeated challenges was not due to a lower expression of the M₃ muscarinic receptor, we tested the mRNA expression in the tracheal tissues using qPCR. There was no significant difference in the mRNA expression for M₃ muscarinic receptor between control and OVA-treated rats 2 days after challenges (respectively, $n = 3$ and 4; $P > 0.05$; Fig. 7A). However, a substantial reduction was observed at 2 days after challenges when normalization for airway SM volume was applied ($P \leq 0.05$; Fig. 7B).

DISCUSSION

The present study demonstrated an increase in airway SM mass and proliferation shortly after repeated allergen chal-

lenges, which were largely back to control levels after 1 wk. No changes in airway responsiveness to MCh occurred in vivo after three OVA challenges, suggesting changes in airway SM properties following remodeling. Consistent with this interpretation, changes in contractile phenotype occurred in both tracheal and intrapulmonary SM as evidenced by a reduction in contractile protein expression. However, a significant reduction of contractile protein was present only after correction for the mass of SM was performed. This reduction in components of the contractile apparatus correlated with a reduction in active stress generation capacity of the tracheal SM, again normalized for the change in cross-sectional area, indicating that the increase in airway SM mass through hyperplasia offset the loss of the contractile phenotype.

Increased SM mass has been reported as a component of the complex structural airway remodeling in asthma (3, 5, 29), and it has been successfully modeled in animals (11, 12, 31). The phenomenon has been shown to affect the whole intrapulmonary airway tree (3, 12). However, there are, to our knowledge, no reports of tracheal SM remodeling. We observed comparable degrees of remodeling in both trachea and intrapulmonary airways: there was a threefold increase in SM mass, shortly after repeated allergen challenges, in both sites. Our results also demonstrated that SM proliferation, quantified by PCNA immunoreactivity, is an early event following allergen challenge, since it was observed 24 h after a single allergen challenge. This proliferative state, which is the likely primary mechanism for the observed increase in SM mass after repeated challenges, was maintained 2 days after multiple challenges in both trachea and intrapulmonary airways. However, these two features were transient as both SM mass and PCNA immunoreactivity were virtually back to control levels 1 wk after the final allergen challenge. The mechanism of the regression of SM mass seems likely to involve apoptosis as the increase in mass was attributable to hyperplasia of SM rather than hypertrophy. We (30) have previously demonstrated alterations in rates of apoptosis following allergen challenge of the BN rat, albeit in an adoptive transfer model, suggesting a possible regulation of SM mass by this process. Presumably, the withdrawal of the stimuli associated with allergen challenge results in SM cell death.

We anticipated a significant increase in airway responsiveness to inhaled aerosolized MCh in vivo as has been previously reported for the BN rat undergoing repeated allergen challenge (2, 19). However, we have also found, in the past, a substantial variability in the responsiveness of BN rats to allergen sensitization and challenge that was dependent on substrain and source (34). A comparison of United Kingdom and United States Ssn substrain BN rats demonstrated that despite comparable levels of serum IgE following identical protocols of OVA sensitization, including adjuvants such as alum and *B. pertussis*, United Kingdom animals had larger late responses and higher numbers of bronchoalveolar lavage neutrophils at 24 h following a single OVA challenge (34). We did not, however, in our previous study explore the effects of multiple challenges on airway responsiveness and did not anticipate the current result. The complete absence of OVA-induced airway responsiveness strengthens the conclusion that under some circumstances remodeling of the airway SM may occur without AHR.

A possible explanation for the lack of association between increase in airway SM mass and airway responsiveness is an

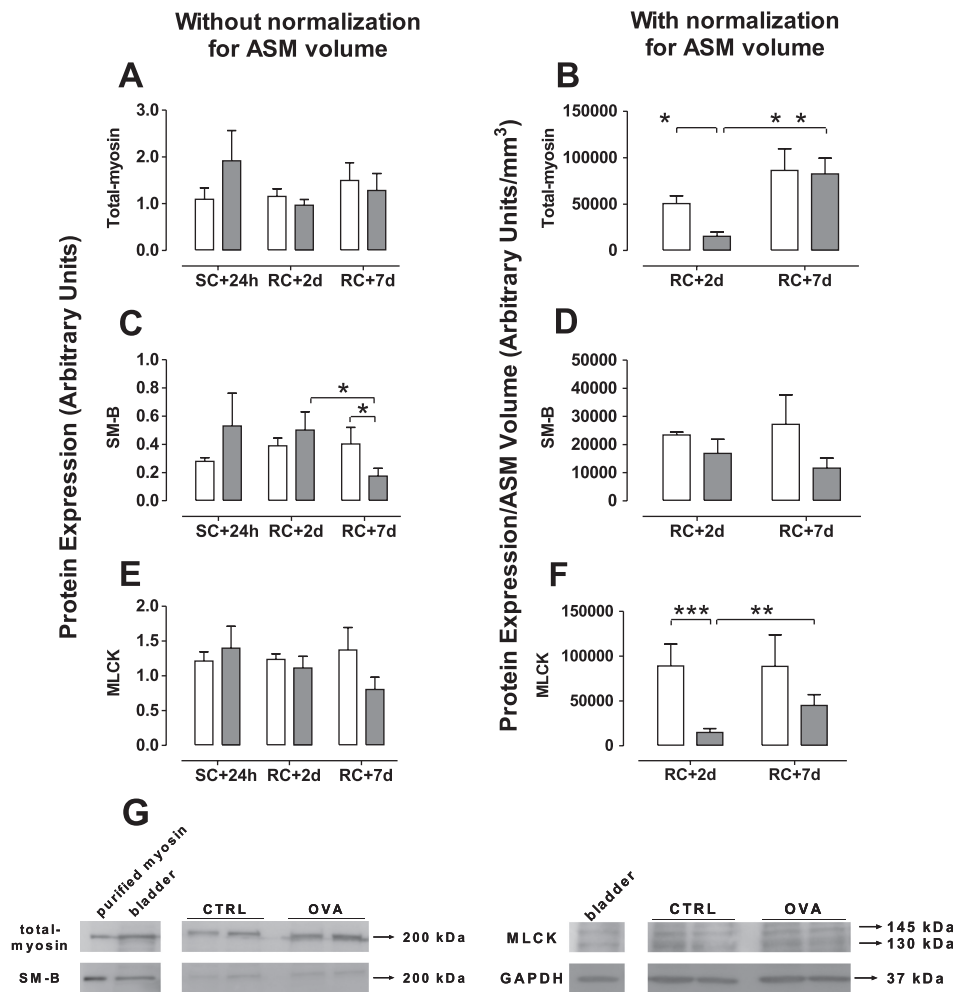


Fig. 5. Contractile protein expression. Protein expression without normalization for SM volume was unchanged for total myosin (A) and MLCK (E) 24 h after a single challenge and 2 and 7 days after repeated challenges. Only SM-B expression (C) was reduced 7 days after repeated challenges in treated rats compared with controls ($n = 9$). When normalization for SM volume was applied, a substantial reduction in total myosin (B) and MLCK expression (F) was observed 2 days after the repeated challenges for treated animals compared with controls ($n = 4$). The contractile protein expression was back to control levels at 7 days ($n = 5$). D: the reduction in SM-B expression was abrogated after SM volume normalization. Data were initially corrected for the reference organ bladder, for the housekeeping protein GAPDH, and expressed as means $\pm$ SE. White bars, control animals; gray bars, treated animals (OVA-sensitized/OVA-challenged) for each time point. CTRL, control. * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.0001$.

alteration in contractile phenotype of SM. We addressed phenotypic remodeling from the standpoint of both the airway as a whole and the individual muscle cells. For the former, information from the trachea or intrapulmonary airways was reported without normalizing for airway SM volume. Overall, we observed no striking changes in the contractile phenotype at each time point despite the evidence of hyperplasia of airway SM. Only SM-B expression was significantly reduced at the protein level, 7 days after repeated challenges in OVA-sensi-

tized/OVA-challenged rats, suggesting the possibility of a selective downregulation of this protein. The 7-amino acid insertion at the NH₂-terminal sequence of the SM-B isoform accounts for an increase in contractile velocity at the molecular level compared with other myosin isoforms in an *in vitro* motility assay (15), and a SM-B knockout mouse has demonstrated differences in the kinetics of bronchoconstriction (33). Also, as demonstrated with bladder myocytes, the expression of SM-B is largely reduced during the proliferation phase of in

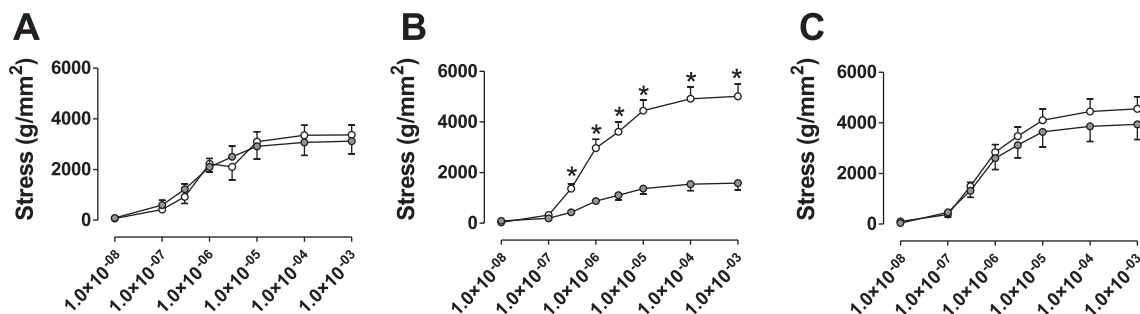
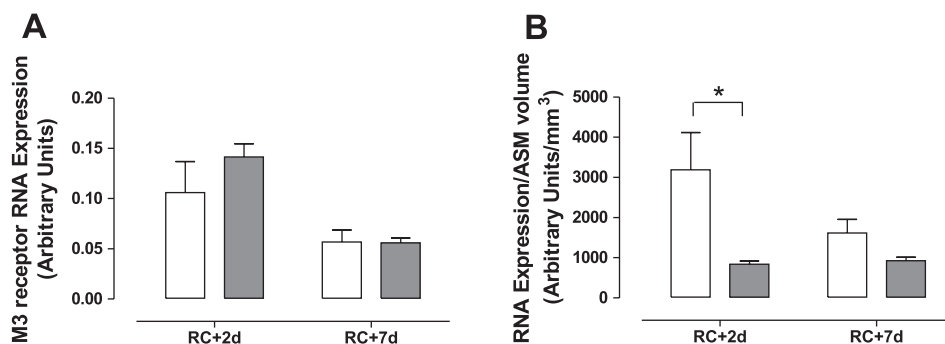


Fig. 6. In vitro force generation. Stress development of tracheal rings under cumulative MCh doses. A: a single challenge had no influence on stress development at each MCh dose. B: 2 days after repeated challenges, a reduction in stress generation for OVA-sensitized/OVA-challenged rats was observed starting at a MCh concentration of 3.0×10^{-7} to 1.0×10^{-3} M compared with controls. C: stress generation was returned to control level at 7 days after repeated challenges. Data are expressed as means (g/mm²) $\pm$ SE. White dots, control animals; gray dots, treated animals (OVA-sensitized/OVA-challenged) for each time point. * $P \leq 0.05$.

Fig. 7. M_3 muscarinic receptor mRNA expression. There was no statistical difference for the M_3 receptor mRNA expression at 2 and 7 days after repeated OVA challenges without normalization for ASM volume (A), but a substantial reduction was observed at 2 days after challenges when normalization for ASM volume was applied (B). The data were corrected for the housekeeping gene S9 and expressed as means $\pm$ SE ($n = 3-5$). White bars, control animals; gray bars, treated animals (OVA-sensitized/OVA-challenged) for each time point. $*P \leq 0.05$.



vitro cultured cells (1). There was no corresponding in vivo phenotype to suggest a functional role for the change in SM-B in responsiveness. Correction of the genes of interest for housekeeping genes fails to correct adequately for the increases in SM that occurred. Our reasoning was that after OVA challenges, the fraction of the tissues occupied by SM was very small, justifying a further correction for SM tissue volume. Following this normalization, there were substantial and significant reductions in all three proteins at the mRNA level and in both myosin and MLCK at the protein level. Expression levels were restored by 7 days. Comparable phenotypic modulations associated with cellular proliferation have been described in cultured SM cells from different organs including airways, blood vessels, and bladder (1, 6, 10).

In a similar rat model, Moir and colleagues (26) have previously reported phenotypic modulation toward the expression of nonmuscle isoforms of contractile proteins, although remodeling from the trachea was not observed after repeated challenges. In the present study, we observed phenotypic remodeling throughout the whole airway tree, from trachea to intrapulmonary airways. The tracheal SM was able to undergo both structural and phenotypic remodeling. The 10-fold increase in proliferative response in tracheal muscle, compared with the 3-fold increase observed in intrapulmonary airways, may reflect the central deposition of allergen or a greater sensitivity of tracheal muscle to the challenge. In the study of Moir et al. (26), animals breathed spontaneously in a chamber into which OVA was nebulized. The allergen may have been filtered in the upper airways compared with the technique we employed where the animals were intubated to ensure exposure of the whole tracheobronchial tree to the allergen. To date, there has been no clear phenotypic modulation of the kind we appear to have found. The analysis of mRNA isolated from laser-microdissected SM from mild to moderate asthmatic subjects showed little change in myosin heavy chain or MLCK (33), whereas in another study of mild asthmatics there was both a significant increase in myosin heavy chain, in particular the SM-B isoform, and a very modest increase in MLCK (18). The loss of the contractile proteins as a result of hyperplasia of SM is less likely to be detected in mild asthma as it is likely that few SM cells are affected by hyperplasia at any given time.

Both single and repeated challenges were insufficient to induce changes in isometric force generation by tracheal SM from OVA-sensitized/OVA-challenged rats compared with controls. However, following correction for cross-sectional area, there was a substantial reduction in force 2 days after

repeated challenges, consistent with the loss of contractile protein per unit of muscle. This loss recovered by 1 wk. Different research groups have studied the airway SM force generation in vitro, comparing asthmatic and normal tissues. Force generation was reported to be either unchanged or increased in asthmatic patients compared with normal subjects (32). However, as reviewed by Seow and colleagues (32), the lack of measurements of airway SM dimension and the difficulty of setting the muscle to its optimal length may account for these variations in the results. Our data do not provide an explanation for AHR in association with multiple allergen challenges, although an increase in airway SM mass could certainly contribute but only if sufficient restoration of the contractile proteins occurs to allow the muscle to function as a normal contractile cell. Perhaps a coordinate expression of cytokines such as transforming growth factor- β may be required to restore the contractile phenotype (8). Using the same model but more frequent and a greater number of challenges, McVicker et al. (24) found both an increase in airway responsiveness and a reduction in contractile protein and, to resolve the apparent paradox, proposed that an increase in the ratio of filamentous to globular actin may provide a mechanism to account for the concomitant reduction in contractile protein but increase in airway responsiveness in vivo. Although there was a reduction in mRNA for the M_3 muscarinic receptor 2 days after three OVA challenges, we believe this is a further marker of dedifferentiation of airway SM rather than a cause of the lack of increase in responsiveness to MCh, as there is a very substantial receptor reserve (9).

In conclusion, we report here structural and phenotypic remodeling associated with airway SM hyperplasia in tracheal and intrapulmonary airways, following repeated allergen challenges in a rat model of asthma. Airway responsiveness to MCh challenge in vivo was unaffected despite the substantial airway SM remodeling that occurred. A reduction in the contractile apparatus protein expression may have contributed to a reduced generation of active stress by SM but was completely offset by the increase in SM mass. The increase in airway SM was surprisingly evanescent, and further studies will be needed to elucidate the basis for the reversal of the increase in airway SM mass.

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