Repeated allergen inhalation induces phenotypic modulation of smooth muscle in bronchioles of sensitized rats

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Asthma is a disorder that affects the tracheobronchial tree from large to small airways (22). One of the basic characteristics of asthma is airway hyperresponsiveness (AHR) that is demonstrated by increased responses to inhaled bronchoconstrictors such as methacholine and that can be observed following allergen exposure. Induction and perpetuation of AHR may result from repeated inflammatory events involving a complex and coordinated response of multiple inflammatory and structural cells, mediators, connective tissue elements, and cytokines whose actions lead ultimately to persistent changes in airway wall structure (5). This remodeling includes epithelial cell damage and mucus gland hypertrophy, reticular basement membrane thickening, alterations in connective tissue composition, and an increase in the content of smooth muscle in the airway wall as a result of hyperplasia and hypertrophy (5).

Differentiation of smooth muscle in the developing lung is characterized, as in other tissues, by the progressive replacement of nonmuscle cytoskeletal and contractile proteins with smooth muscle-specific isoforms, leading to a range of distinctive smooth muscle cell phenotype subpopulations (25, 27, 37). In intact, healthy mature blood vessels and airways, the majority of smooth muscle cells exist in a quiescent and fully differentiated contractile phenotype. Little attention has so far been paid to modulation of the contractile state in airway disease, although parallels in airway and pulmonary vascular remodeling are increasingly being sought (4, 16, 18, 37). In vitro cell culture systems have demonstrated that exposure of airway smooth muscle (ASM) cells to a mitogenic stimulus results in a reversible modulation in contractile phenotype to a more synthetic state (16, 19) in which there are accelerated proliferation and loss, and subsequent reexpression at confluence, of specific contractile phenotype markers (17, 19, 29). The synthetic phenotype is characterized by ultrastructural changes such as a progressive loss in smooth muscle microfilament content and increased biosynthetic organelle density and by reduced biochemical expression of specific contractile smooth muscle marker proteins such as smooth muscle (sm)-α-actin and sm-myosin heavy chain (MHC) and a reduced ability to contract (6, 27).

Although the function of the contractile smooth muscle phenotype is generally accepted as a regulator of airway caliber, the existence and function of the syn-

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thetic phenotype are less clear. In systemic vascular disease, the synthetic smooth muscle cell is thought to be central to the pathogenesis of the repair-injury response of the atherosclerotic lesion (31) and is responsible for the production of paracrine and autocrine factors and the synthesis and deposition of extracellular matrix components (27). Substantial evidence from in vitro cell culture-based systems (reviewed in Refs. 8 and 21) now exists to support the possibility that ASM is likewise an important source of proinflammatory and bronchoprotective mediators, particularly in asthma. The presence of such a functionally altered phenotype may be of particular relevance in asthma where the content of ASM is increased. However, the unequivocal demonstration of smooth muscle phenotype modulation has not been established in vivo.

Repeated antigen challenge of the actively sensitized Brown Norway (BN) rat is a well-established model that replicates many of the pathological features of the airway-remodeling process in chronic allergic asthma, including AHR, T cell and eosinophilic inflammation, and increased ASM content (30, 32, 33). Thus, the present study was designed to identify and then quantify in small airways the occurrence of smooth muscle phenotype modulation in vivo after repeated exposure to inhaled allergen and to examine whether there were associated changes in ASM cell structure and contractile function ex vivo. With the possible exception of specific sm-MHC isoforms, no single marker is expressed unequivocally in smooth muscle (27). For this reason, and because extreme phenotypic states such as those reported in cell culture (19) might not be present in vivo, we examined expression of a panel of proteins commonly expressed in smooth muscle and previously used to indicate smooth muscle phenotype (17). Additionally, we investigated whether alterations in contractile function could resolve over time and whether concomitant resolution in biochemical and structural changes also occurred.

METHODS

Animal sensitization and allergen exposure. Pathogen-free male BN rats (Harlan, Bicester, UK) weighing 220–250 g were actively sensitized by intraperitoneal injections on 3 consecutive days of a suspension containing 1 mg/kg ovalbumin (OA) in 0.9% saline containing 100 mg of Al(OH)₃ as adjuvant (32). Rats were challenged on day 6 and then every 3 days for a total of six 15-min exposures. Challenges took place in a 0.8-m³ chamber with free-breathing animals (n = 5 in each group) being exposed to either saline or 1% OA aerosol mist, generated by a DeVilbiss PulmoSonic nebulizer (DeVilbiss Healthcare, Feltham, UK). The aerosol mist was pumped into the box at 600 ml/min using a small animal ventilator. At all times, animals were housed in a caging system that received clean filtered air only, with food and water supplied ad libitum. After 24 h, 7 days, or 35 days, animals were killed following intraperitoneal injection of pentobarbital sodium (50 mg/kg) and the trachea and lungs were rapidly removed.

Tissue collection. Intact small bronchioles comprising branches of the distal subsegmental bronchi of the right lung were dissected free of parenchyma and connective tissue for assessment of contractile function and airway wall morphology or, along with cartilage-free and epithelium-denuded trachealis, were snap-frozen and stored at −80°C for later determination of contractile protein content by Western immunoblot.

Contractile tension development. Intact small bronchioles (internal diameter 250–400 μm; length 1–2 mm), free of all visible traces of parenchyma, were mounted onto the jaws of a Mulvany-Halpern small vessel myograph (Danish Myo Technology, Aarhus, Denmark) as described previously (7) and bathed with a physiological salt solution at 37°C, containing 118 mM NaCl, 24 mM NaHCO₃, 1 mM MgSO₄, 4 mM KC1, 5.56 mM glucose, 5 mM sodium pyruvate, 0.435 mM NaH₂PO₄, 1.8 mM CaCl₂, pH 7.4, equilibrated with 5% CO₂ in air. After equilibration for 30 min with washing every 5 min, the degree of stretch was normalized for bronchiole size by setting each preparation to 80% of the stretch necessary to obtain the peak of the active length-tension relationship (80% Lmax), which was determined by the response to 75 mM KCl (equivolumar substitution for Na⁺) following each incremental stretch. At 80% Lmax, an equilibration period of 30 min was then allowed before the start of each experiment after which contraction was induced by 75 mM KCl. This was repeated twice more at 10-min intervals and resulted in contractions of similar magnitude. After washing for 15 min, cumulative concentration-response relationships were generated for each agonist under investigation. Developed tension was expressed in milliNewtons per millimeter length of bronchiole (mN/mm) and normalized for smooth muscle content (mN/mm²). At the end of the experiment, bronchiole tissue segments were maximally relaxed with papaverine (100 μM), fixed in 10% formal saline for 60 min, and stored at 4°C in 70% ethanol until being paraffin embedded for quantitative histological assessment.

Quantitative histology. Smooth muscle content in isolated bronchiole preparations was determined by morphometric analysis of 5-μm transverse sections after staining with Masson’s Trichrome according to the manufacturer’s recommended protocol but substituting Celestine Blue and Mayer’s Hematoxylin in place of Weigert’s Iron Hematoxylin (Sigma, Poole, UK). Area occupied by smooth muscle (WAₘ) and basement membrane perimeter (Pₘ) in each bronchiole section were measured by a single observer using commercially available imaging software (KS300, Carl Zeiss Systems, London, UK) by drawing around the muscle bundles and measuring the area after setting the threshold color of the muscle (red in the case of Masson’s Trichrome stain) as described previously (32). Pₘ was measured twice in each bronchiole, and WAₘ was normalized to this value to account for airway size as well as folds and grooves in the reticular basement membrane (20). With the use of this method, smooth muscle content of three to seven semiserial sections from each of five to seven bronchioles was calculated from four animals in each treatment group. In some sections, the collagen-staining step was omitted from the trichrome protocol to facilitate morphological identification and counting of dark elongated smooth muscle cell nuclei in the muscle bundles. Nuclei that were clearly round were excluded from the counts.

Protein extraction, electrophoresis, and Western immunoblotting. For each treatment group (i.e., sensitized control or sensitized OA challenged), snap-frozen bronchioles (5 bronchiole segments/animal) and trachealis muscle (epithelium-denuded) from each of five animals were pulverized using a liquid nitrogen precooled Bessman Tissue Pulverizer (Fisher Scientific, Chicago, IL). Tissue protein extracts were prepared by addition of RIPA buffer (PBS containing 1% Igepal, 0.5% sodium deoxycholate, 1% SDS) containing 10% protease inhibitors (Protease Inhibitor Cocktail Set III, Calbiochem) and then were heated to 90°C for 10 min and clarified by centrifugation (10,000 g for 5 min). Total tissue protein...
content in supernatants was estimated using the bicinchoninic acid assay (36), and proteins (5 to 10 μg/lane) were separated by SDS/PAGE on 10% or 4 to 12% acrylamide precast gels (Invitrogen, Paisley, UK). Standard Western immunoblotting procedures and a panel of monoclonal antibodies (all from Sigma) against sm-α-actin (clone 1A4, 1:10,000), β-actin (clone AC15, 1:10,000), calponin (clone hCP, 1:5,000), and sm-MLCK (clone k36, 1:5,000) were used to determine the abundance of contractile, cytoskeletal, and regulatory proteins in trachealis and bronchial tissue lysates. Additionally, an anti-smoothelin antibody (R4A, 1:5) was supplied by Professor G. van Eys (University of Maastricht, The Netherlands). Immunodetection of monoclonal antibodies was performed using a horseradish peroxidase-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA; 1:5,000–1:20,000). To separate different MHC isoforms, total tissue proteins were electrophoresed on 4 to 12% acrylamide precast gels (Invitrogen). A monoclonal antibody from Santa Cruz Biotechnology was used to detect sm-MHC (clone G4, 1:200). This antibody recognizes both SM1 and SM2 sm-MHC isoforms but does not distinguish between SMA or SMB or recognize nm-MHC isoforms. A rabbit polyclonal IgG antibody (Biomedical Technologies, Stoughton, MA or Biogenesis, Bournemouth, UK) was used to detect nm-MHC (1:1,000) followed by a horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology; 1:5,000). Signals were visualized by enhanced chemiluminescence (Amersham-Pharmacia) and were quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA) on autoradiographs that depicted bands within a linear range of exposure.

Data and statistical analysis. Data in the text and figure legends are expressed as means ± SE of observations obtained in tissues from n animals in each treatment group. EC50 values and extrapolated maximum responses were estimated for individual concentration-response curves using nonlinear least-squares regression (SigmaPlot, SPSS, Chicago, IL) where appropriate. EC50 values were converted to negative logarithmic values (pD2) for all statistical analysis, although for ease of comprehension, EC50 values are given in the text. Statistical differences in the morphometry and contraction data were determined using two-way ANOVA followed by Bonferroni’s post hoc t-test to determine statistical differences after multiple comparisons (SigmaStat, SPSS). Densitometric data were expressed as a percentage of contractile protein content in control bronchioles from saline-challenged animals. Contractile protein expression in bronchioles and trachealis was often variable even after normalization. Thus, statistical analysis was limited to comparisons between treatment (i.e., saline vs. OA) for either bronchiole or trachealis tissue at each time point using one-way ANOVA. A probability value of <0.05 was considered significant.

RESULTS

ASM content. Morphometric measurements were obtained in three to seven semiserial histological sections from each of five to seven bronchioles from a total of four animals at each time point (24 h, 7 days, and 35 days) in each experimental group (OA and saline). Repeated OA exposure induced a small (~1.44-fold) but significant (P < 0.05, n = 5) increase in ASM content in bronchioles obtained from the lungs 24 h after the last of six repeated OA challenges, compared with animals challenged with saline (Fig. 1, A-C). Statistical differences in ASM content were not found in bronchioles from saline- or OA-challenged animals when the lungs were removed at 7 days or 35 days despite an apparent reduction (~1.23-fold, P > 0.05, n = 5) in small bronchiole muscle content in the OA-challenged group (Fig. 1, D and E). Numbers of elongated nuclei in smooth muscle bundles were also counted and found not to differ significantly (P > 0.05, n = 5) between the two treatment groups at any of the time points investigated (Table 1).

Contractile responses. To investigate whether the observed increase in ASM content after repeated allergen exposure was associated with changes in ASM responsiveness in vitro, responses were examined to receptor- (carbachol, serotonin) and nonreceptor-mediated (4β-phorbol 12,13-dibutyrate [4β-PDBu], KCl) agonists in isolated rat small bronchiole preparations obtained from sensitized control and sensitized OA-challenged animals at either 24 h, 7 days, or 35 days after the last challenge.

Responses at 24 h after challenge. In five separate experiments, no difference in the contractile sensitivity to carbachol (i.e., the concentration that induced 50% of the maximal developed tension, EC50) was detected in 10 bronchiolar preparations removed from lungs at 24 h after the last exposure to OA compared with saline [EC50: saline (Sal) 2.00 ± 0.3 μM vs. OA 2.22 ± 0.24 μM, P > 0.05, n = 5]. However, the maximum developed contractile force (Tmax) induced by carbachol was significantly greater in OA compared with saline-treated animals (Tmax: Sal 2.91 ± 0.28 mN/mm vs. OA 3.77 ± 0.29 mN/mm, P < 0.05, n = 5; Fig. 2A), and this was paralleled by increases in 4β-PDBu-induced contraction (Tmax: Sal 2.43 ± 0.24 mN/mm vs. OA 3.47 ± 0.37 mN/mm, P < 0.05, n = 5) and contraction induced by direct depolarization with KCl (Tmax: Sal 1.33 ± 0.14 mN/mm vs. OA 1.82 ± 0.17 mN/mm, P < 0.05, n = 5; Fig. 2, C and D). Conversely, there was no significant increase in Tmax for serotonin-induced contraction (Fig. 2B). In each case where an increase in Tmax was found, it was ~1.3- to 1.4-fold and was similar in magnitude to the observed 1.44-fold increase in ASM content found in bronchioles removed from OA-challenged animals compared with their saline-challenged counterparts (Fig. 1A). Thus, when the preparations were corrected for differences in ASM content, increases in maximum developed contractile force to carbachol, 4β-PDBu, and KCl in the isolated bronchioles from OA-challenged animals were abrogated (Fig. 2, A-C insets, and D). EC50 and Tmax values for each of the agonists tested on isolated bronchioles from rats killed 24 h after the last inhaled challenge with saline or OA appear in Tables 2 and 3, respectively.

Responses at 7 days after challenge. Agonist-induced contractile responses were also examined in isolated small bronchioles from animals killed 7 days after the last inhaled challenge with either OA or saline. No differences were found (P > 0.05, n = 5) between the two treatment groups for either the sensitivity (EC50) or Tmax obtained for each of the agonists (Tables 2 and 3). Because the increase in ASM in bronchioles from
OA-challenged animals appeared to be resolved at 7 days (Fig. 1B), correction for differences in smooth muscle content did not change this situation (Table 3).

**Responses at 35 days after challenge.** Finally, we examined agonist-induced contractile responses in isolated small bronchioles from animals killed 35 days after the last inhaled challenge with either OA or saline. In tissues not corrected for smooth muscle content, differences were not detected in the values for either EC50 or Tmax for any of the agonists examined between the two treatment groups (P > 0.05, n = 5; Tables 2 and 3), although the concentration-response curves obtained from OA-treated animals were consistently to the left of those treated with saline (Fig. 3, A-C). However, after correction for differences in the muscle content at 35 days between the treatment groups, significant increases (~1.32- to 1.42-fold, P < 0.05–0.01, n = 5) in Tmax values of the preparations from OA-treated animals were observed with each of the agonists examined, compared with saline-treated animals (Fig. 3, A-C insets, and 3D, and Table 3).

**Contractile protein expression.** To investigate the biochemical contractile phenotype of the ASM in vivo after repeated OA challenge, expression by semiquantitative Western immunoblots of a panel (see Ref. 17) of smooth muscle-specific marker proteins [sm-α-actin, sm-MHC, calponin, smoothelin, sm-myosin light chain kinase (MLCK)] as well as their nonmuscle isoforms (nm-MHC, β-actin) was examined in trachealis and in small bronchiole preparations from sensitized control and sensitized OA-challenged animals at either 24 h, 7 days, or 35 days after the last challenge.

### Table 1. Temporal changes in bronchiolar smooth muscle cell nuclei counts in sensitized Brown Norway rats following repeated antigen challenge

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Airway Smooth Muscle Cell Number, nuclei/mm basement membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 h</td>
</tr>
<tr>
<td>Sal</td>
<td>45.53 ± 1.91</td>
</tr>
<tr>
<td>OA</td>
<td>41.61 ± 5.46</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5 for all groups. Sal, saline; OA, ovalbumin. Only elongated smooth muscle nuclei were included in counts. Two-way ANOVA did not detect significant temporal differences or changes due to treatment.

Fig. 1. Representative photomicrographs depicting airway smooth muscle (ASM) bundles (arrows) in bronchioles removed from sensitized Brown Norway rats 24 h after the last challenge with saline (A) or aerosolized 1% ovalbumin (OA; B). Calibration bars represent 100 μm. Bar charts show temporal increases in ASM content in isolated bronchioles removed 24 h (C), 7 days (d, D), and 35 d (E) after the last challenge with saline (open bars) or OA (filled bars). Bars represent means ± SE of measurements obtained in 3–7 semiserial histological sections from each of 5–7 bronchioles from a total of 4 animals at each time point (24 h, 7 d, and 35 d) in each experimental group. NSP > 0.05 (not significant), *P < 0.05 compared with saline by Bonferroni’s t-test.

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lated to the BN strain. Other contractile markers such as the 204-kDa band of SM1 sm-MHC, calponin (single band at 34 kDa), smoothelin-A (59 kDa), and the 138-kDa band of sm-MLCK were unchanged by repeated OA exposure (Fig. 4, B-D). The smoothelin-B isoform (~115 kDa) was also detected but its expression in trachealis was too weak for reliable quantification (not shown).

In contrast to trachealis, in bronchiole preparations the content of sm-α-actin, SM1 sm-MHC, calponin, and smoothelin-A was significantly reduced (*P < 0.05 compared with saline) by repeated OA challenge (Fig. 2, insets). Statistical comparisons for other agonists appear in Table 2.

Table 2. Temporal changes in sensitivity (EC_{50}) of small bronchiole preparations from sensitized Brown Norway rats following repeated antigen challenge

<table>
<thead>
<tr>
<th>Treatment</th>
<th>EC_{50}, μM</th>
<th>24 h</th>
<th>OA</th>
<th>35 days</th>
<th>Sal</th>
<th>OA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbachol</td>
<td>2.00 ± 0.33</td>
<td>2.22 ± 0.24</td>
<td>2.20 ± 0.25</td>
<td>1.83 ± 0.17</td>
<td>2.56 ± 0.30</td>
<td>2.25 ± 0.30</td>
</tr>
<tr>
<td>Serotonin</td>
<td>2.02 ± 0.61</td>
<td>3.65 ± 0.80</td>
<td>2.59 ± 0.44</td>
<td>2.66 ± 0.59</td>
<td>2.52 ± 0.40</td>
<td>3.90 ± 0.13</td>
</tr>
<tr>
<td>4β-PDBu</td>
<td>0.58 ± 0.17</td>
<td>1.01 ± 0.40</td>
<td>0.40 ± 0.06</td>
<td>0.48 ± 0.06</td>
<td>0.38 ± 0.04</td>
<td>0.43 ± 0.05</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5 for all groups. Two-way ANOVA did not detect significant temporal differences or changes due to treatment; 4β-PDBu, 4β-phorbol 12,13-dibutyrate.

Statistical comparisons for other agonists appear in Table 2.
lysates (kDa) were not detected in either trachealis or bronchiole proteins such as smoothelin-A, H9252 At 7 days after OA or saline challenge, levels human airways (1). detected in either trachealis or in bronchiole lysates, ever, nm-MLCK isoforms (210 to 220 kDa) were not and nm-isoforms of MLCK (discussed in Ref. 1). How-
were blotted. The MLCK antibody used detects both sm-
cance (P = 0.001, n = 5) after repeated OA exposure compared with saline (Fig. 4). An apparent reduction in sm-MLCK con-
tent was also detected but this did not achieve signifi-
cance (P = 0.132, n = 5). Changes in nm isoforms of these proteins such as β-actin (42.5 kDa) and nm-MHC (198 kDa) were not detected in either trachealis or bronchiole lysates (P > 0.05, n = 5), confirming that reductions in smooth muscle contractile protein content were not the result of gel-loading artifacts or in the preparation of lysates and that equal amounts of total lysate protein were blotted. The MLCK antibody used detects both sm-
and nm-isoforms of MLCK (discussed in Ref. 1). How-
ever, nm-MLCK isoforms (210 to 220 kDa) were not detected in either trachealis or in bronchiole lysates, consistent with similar findings in smooth muscle from human airways (1).

Smooth muscle protein expression at 7 days after challenge. At 7 days after OA or saline challenge, levels of expression of sm-α-actin, sm-MHC, calponin, smoothelin-A, β-actin, and nm-MHC in trachealis or in bronchioles were not different between the two groups (P > 0.05, n = 5; Fig. 5). The only exception to this was in the expression of sm-MLCK, which was reduced (P < 0.05, n = 5) in lysates from bronchioles, but not from trachealis of OA-challenged animals (Fig. 5D).

Smooth muscle protein expression at 35 days after challenge. At 35 days, the content of sm-MHC, calpo-
nin, smoothelin-A, and sm-MLCK was reduced in bron-
chioles from OA-challenged animals (P < 0.05–P < 0.01, n = 5). sm-α-Actin, β-actin, and nm-MHC levels were not different between the two groups (P > 0.05, n = 5). Likewise, in trachealis, none of the contractile protein markers differed in their expression between the two groups (P > 0.05, n = 5; Fig. 6).

**DISCUSSION**

In this study, using morphometric techniques coupled with myography, we demonstrate that ASM con-
tent and maximal isometric tension development to several agonists (carbachol, KCl, and 4β-PDBu) are increased in isolated bronchioles removed 24 h after repeated antigen challenge of actively sensitized BN rats. In addition to producing an increase in bronchio-
lar smooth muscle content and increased maximal iso-
metric tension development ex vivo, repeated exposure to aerosolized OA reduced the abundance of several smooth muscle contractile, cytoskeletal, and regulatory proteins (sm-α-actin, calponin, smoothelin-A, and SM-1 MHC) in bronchioles but had little effect on nonmuscle protein content (β-actin and nm-MHC). Analogous changes in smooth muscle protein content were not detected in trachealis. The increase in bronchiolar muscle content and force generation and reduction in smooth muscle contractile marker protein content recovered 7 days after the last allergen exposure. However, at 35 days after the last OA challenge, increased maximal isometric force in the bron-
chioles occurred with each of the agonists examined after correction for negligible differences in muscle content. Again, these increases in force-generating capacity were accompanied by reduced smooth muscle marker but not nonmuscle protein content and were reminiscent of the unexpected changes seen earlier at 24 h.

With the use of the actively sensitized BN rat model, several groups demonstrated increased ASM content and increased DNA synthesis in both large- and me-
dium-sized airways after repeated allergen exposure (30, 32, 33). In the present study, changes in ASM content were assessed only in bronchioles that comprised branches of the distal subsegmental bronchus of the right lobe of the rat lung whose internal diameter rarely exceeded 400 μm. A significant increase in smooth muscle content was found 24 h after the last allergen exposure, which contrasts with other studies where no change was reported in small airways (28, 30), despite reported increases in DNA synthesis (30). A possible explanation may reside in the protocol we used to sensitize and challenge the animals, which is more extreme with more challenges in a shorter time period compared with other studies (30) [and levels of IgE remain high at the time of the final OA exposure (32)]. The inflammatory response this produces may be more extreme with the potential for many mediators to be involved in the changes seen. The resolution in the increased bronchiolar smooth muscle content may reflect reduced survival of myocytes (12) following the initial growth changes at 24 h, perhaps as a result of resolution of the acute inflammation. We did not detect increases in the total number of elongated cell nuclei in the muscle bundles, suggesting the absence of hyperplas-

cal smooth muscle content and increased maximal iso-
metric tension development ex vivo, repeated exposure to

<table>
<thead>
<tr>
<th>Treatment</th>
<th>24 h</th>
<th>7 days</th>
<th>35 days</th>
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<tbody>
<tr>
<td>Carbachol</td>
<td>2.91 ± 0.29</td>
<td>3.77 ± 0.30‡</td>
<td>3.66 ± 0.39</td>
</tr>
<tr>
<td>Serotonin</td>
<td>803 ± 79</td>
<td>720 ± 57*</td>
<td>694 ± 74</td>
</tr>
<tr>
<td>4β-PDBu</td>
<td>1.91 ± 0.24</td>
<td>3.34 ± 0.19*</td>
<td>2.08 ± 0.25</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5 for all groups. Data in italics are corrected for differences in smooth muscle content (mN/mm²). Equivalent data for KCl appear in Figs. 2D and 4D. *P > 0.05 (not significant), †P < 0.05, ‡P < 0.01 vs. Sal at each time point by 2-way ANOVA; Tₘₙ, temporal changes in maximum tension development.
phic growth, thought to contribute to increased ASM content of peripheral airways in a subset of asthma deaths (11), may account for the increase in muscle content in bronchioles after repeated OA exposure. However, these data should be interpreted cautiously because only elongated nuclei were counted in the muscle bundles, and we did not examine other indexes of proliferation such as labeling with bromodeoxyuridine.

The mechanisms underlying AHR may include enhanced ASM content and contractility and changes in airway epithelial function and/or integrity (2, 5, 10). Repeated OA challenge in sensitized BN rats increases epithelial cell DNA synthesis (30), supporting activation of injury and repair mechanisms in our model (32). Mechanical disruption of the airway epithelium increases the sensitivity of tracheal preparations to cholinesterase-sensitive cholinomimetics (acetylcholine and methacholine) and to serotonin and histamine, but not to KCl, phorbol esters, or to cholinesterase-resistant cholinomimetics (carbachol, betanechol) (3, 35). This contrasts with the profile of increased agonist responsiveness we observed at 24 h with carbachol, KCl, and the phorbol ester 4β-PDBu, suggesting that altered epithelial function is unlikely to be a major factor in determining increased force generation after OA challenge. Indeed, AHR to inhaled muscarinic agonists after repeated OA challenge (28, 32) may be due to increased maximum force generation of the ASM itself in small airways, since we observed an increase in maximal isometric tension with the cholinesterase-resistant cholinomimetic, carbachol in isolated bronchioles at 24 h after the last challenge. With the exception of serotonin, whose actions

![Fig. 3. Isometric tension development induced by carbachol (A), serotonin (B), 4β-PDBu (C), and KCl (75 mM; D) in isolated small bronchiole preparations removed 35 d after repeated challenge of sensitized Brown Norway rats with saline (○ and open bars) or OA (● and filled bars). Insets: similar data after correction for differences in ASM (see Fig. 1). NSP > 0.05, *P < 0.05 compared with saline by Bonferroni's t-test for KCl response. Statistical comparisons for other agonists appear in Table 2. See Fig. 2 legend for additional information.](http://ajplung.physiology.org/content/images/154-AAJPLUNG-2003-00314/Fig3)
involve prejunctional modulation of cholinergic and peptidergic neurotransmitter release in addition to activation of multiple serotonergic receptor subtypes on ASM (15), increased force generation of bronchiolar preparations ex vivo was found at 24 h with all other agonists. This likely reflects a generalized intrinsic change in the ability of the bronchiole preparations to generate force as these agonists induce contraction by discrete receptor and nonreceptor constrictor mechanisms. Thus, increased isometric force was also observed in relation to KCl, which initiates smooth muscle contraction largely independently of receptor activation by direct depolarization of the plasma membrane, allowing opening of voltage-dependent channels, influx of extracellular Ca\(^{2+}\), and activation of the contractile motor, as well as to 4β-PBDu, which activates PKC isoforms to initiate contraction by sensitization of the contractile machinery to Ca\(^{2+}\) (reviewed in Ref. 39). These differing contractile

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**Fig. 4.** Representative Western immunoblots showing smooth muscle (sm)-α-actin and β-actin (A), sm-mysin heavy chain (MHC) and nonmuscle (nm)-MHC (B), calponin and smoothelin (C), and sm-mysin light chain kinase (MLCK; D) protein content in trachealis (Trach; lanes 1 and 2) and bronchioles (Bronch; lanes 3 and 4) removed from sensitized Brown Norway rats 24 h after the last challenge with either saline (Sal; lanes 1 and 3, open bars) or 1% OA (lanes 2 and 4, filled bars). In the bar charts, the first bar of each pair shows means ± SE of data representative of the top autoradiograph, and the second bar shows mean data for the bottom autoradiograph. Mean data are derived from densitometric quantification of autoradiographs using ImageQuant software and are set relative to protein content in control bronchioles from Sal-treated rats (lane 3). B: where multiple anti-myosin-reactive bands occurred, only the SM1 MHC (204 kDa in top autoradiograph) and nm-MHC (198 kDa in bottom autoradiograph) bands were quantified. Autoradiographs and data in graphs are representative of independent experiments using tissues from 5 animals. NS: P > 0.05, *P < 0.05, ***P < 0.001 compared with Sal by Bonferroni’s t-test.
agonists converge on a final pathway in smooth muscle with the interaction of sm-α-actin and myosin and the onset of cross-bridge cycling (39).

The agonist-induced increases in maximal isometric force generation in the bronchioles at 24 h were abrogated when differences in ASM content were accounted for. With the assumption that increases in force development reflect a similar increase in the force-generating capacity of the smooth muscle embedded within the tissue, these data imply that at 24 h the increased maximal force development is unrelated to changes in the intrinsic contractility of individual ASM cells and instead reflects the increased total muscle content in bronchioles from the OA-exposed animals. Such a relationship between increased maximal force generation and muscle content is also supported by our data.

Fig. 5. Representative Western immunoblots showing sm-α-actin and β-actin (A), sm-MHC and nm-MHC (B), calponin and smoothelin (C), and sm-MLCK (D) protein content in Trach (lanes 1 and 2) and Bronch (lanes 3 and 4) removed from sensitized Brown Norway rats 7 days after the last challenge with either Sal (lanes 1 and 2, open bars) or 1% OA (lanes 3 and 4, filled bars). NSP > 0.05, *P < 0.05 compared with Sal by Bonferroni’s t-test. See Fig. 4 legend for additional information.
obtained at 7 days where no difference in force generation or in muscle content occurred between the saline and OA groups. The onset of additional mechanisms may also be important at 35 days when increased force generation of the bronchiole preparations was observed with each of the agonists (including serotonin) in the OA-challenged group following normalization for the small differences in smooth muscle content (Fig. 3, insets). One possibility is that changes in the intrinsic contractile properties of the smooth muscle may be more important than changes in its overall content beyond 7 days. In turn, this may reflect changes in the overall efficiency of force generation by the bronchiolar smooth muscle after repeated OA challenge, perhaps at the level of tissue-smooth muscle cell connections or in the arrangement of the cytoskeletal and contractile
force-generating units within smooth muscle cells (see later discussion and Refs. 14, 34).

Conceivably, such changes in smooth muscle properties could occur as a result of growth and phenotype modulation toward a less contractile or less differentiated state as suggested in pulmonary vascular remodeling and atherosclerosis where there are accelerated growth of smooth muscle and changes in tissue architecture and reactivity (13, 27, 31). We postulated that if repeated antigen challenge could induce a growth response of ASM and alter its ability to generate force, then in vivo phenotypic modulation of airway wall smooth muscle might contribute, in part, to the response. This is supported by the finding of ~50–60% reduction in expression of several smooth muscle contractile, cytoskeletal, and regulatory protein differentiation markers (17), including sm-α-actin, SM1 sm-MHC, sm-MLCK, and the newly discovered cytoskeletal protein and late marker of smooth muscle differentiation, smoothelin-A (38), at 24 h in bronchioles from sensitized rats challenged with OA. Expression of sm-MHC is restricted to smooth muscle (9) and is considered the most rigorous marker of differentiated smooth muscle (27). We used an anti-myosin antibody that detects both SM1 and SM2 and does not distinguish between the SMA and SMB MHC isoforms; the latter has a unique 7-amino acid insert in the ATP-binding region of the myosin head and is expressed in phasic smooth muscle such as in the gut. Consistent with previous findings in adult rat trachealis (23, 24), we observed bands that corresponded to both the 204-kDa SM1 and 200-kDa SM2 sm-MHC isoforms, with SM1 content being much higher than SM2 (25). However, as with the other contractile marker proteins in trachealis, SM1 and SM2 expression was not changed on repeated OA exposure. It is unclear why the reduction in contractile marker protein content after OA exposure was restricted to bronchioles in contrast to trachealis. A possible explanation is that preferential deposition of OA occurred in the small airways. Nasal breathing could scrub larger particles in the aerosol and permit a greater proportion of small particles to reach more distal airways. However, previous studies reported by us (32) and by others (28, 30, 33) demonstrated in this model that OA aerosol challenge of nasal breathing animals induces inflammatory cell recruitment and smooth muscle bromodeoxyuridine incorporation in both proximal and distal airways. This implies that sufficient OA aerosol particles are deposited to provoke a response in proximal (and distal) airways and so reductions in sm marker proteins in bronchiolar but not in tracheal tissues cannot be explained solely on the basis of preferential OA deposition in small airways. Additional factors might include heterogeneity in the capacity of ASM between large and small airways to respond to OA or release of different proinflammatory mediators at varying levels of the tracheobronchial tree (11). Another possibility to be considered when interpreting these data is that fibrotic changes induced by OA challenge might reduce the proportion of smooth muscle-derived proteins present in total tissue lysates leading to an apparent reduction in their abundance. Although we do not completely exclude this possibility, several features of the response obtained are inconsistent with this notion. For example, the reduction in smooth muscle marker proteins found 24 h after the last OA challenge occurred only with contractile, cytoskeletal, and regulatory protein differentiation markers. Repeated OA challenge did not alter the expression of other proteins expressed by smooth muscle such as β-actin (42 kDa) and nm-MHC (198 kDa). Moreover, the reduction occurred despite an increase in the overall smooth muscle content (Fig. 1) and was found in bronchioles but not in trachealis. In contrast, OA-induced fibrosis in this model, as determined by collagen and fibronectin deposition, is reported to occur in both large and small airways (28).

We propose, therefore, that the selective downregulation of smooth muscle contractile, cytoskeletal, and regulatory proteins by repeated OA exposure indicates the occurrence in vivo of modulation of ASM toward a less differentiated phenotype. This is further supported by the 24-h morphometric data, which showed that despite a reduction in smooth muscle marker protein expression, the actual content of smooth muscle tissue identified by the trichrome stain was increased ~1.44-fold in bronchioles, presumably reflecting increased cellular growth. The finding of increased maximal force generation in the face of reduced smooth muscle contractile protein content such as sm-α-actin and sm-MHC was unexpected and is not easily explained but may indicate a level of redundancy in the contractile motor. Under such conditions, other nonmuscle proteins may play an important role by replacing the function of lost smooth muscle contractile proteins, as was recently proposed for KCl-induced sustained contractions of the bladder from sm-MHC gene knockout mice (26). The antibodies we used detect the total content of smooth muscle contractile marker proteins such as sm-α-actin and sm-MHC but not the proportion of these proteins in the contractile filaments. We can speculate a degree of redundancy exists for total sm-α-actin and sm-MHC and in the levels required to support maximal tension development. Further studies are required to examine whether repeated OA challenge increases the efficiency of tension development, in the face of reduced total content of contractile proteins, by investigating possible differences in filamentous to globular actin ratios or altered recruitment into filaments of monomeric myosins after repeated OA exposure. These putative mechanisms may ultimately regulate the efficiency of smooth muscle optimal force generation during shortening and contraction maintenance (14, 34).

In summary, repeated OA exposure of adult BN rats resulted in increased maximal tension development of bronchiolar smooth muscle preparations ex vivo, increased bronchiole sm content, and decreased the abundance of smooth muscle contractile, cytoskeletal, and regulatory proteins (sm-α-actin, calponin, smoothelin-A, and SM-1 MHC) at 24 h but not 7 days after the last OA challenge. Our results in bronchioles are consistent with the first in vivo demonstration of modulation of ASM phenotype. Whether such changes signify true alter-
ations in the phenotype of individual cells, selective ex-

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