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Abrogation of Airway Hyperresponsiveness but not Inflammation by Rho kinase Insufficiency

David I. Kasahara¹, Fernanda M.C. Ninin¹, Allison P. Wurmbrand¹, James K. Liao², and Stephanie A. Shore¹

¹Department of Environmental Health, Harvard School of Public Health, Boston, MA, USA

²Department of Medicine, University of Chicago, Chicago, IL, USA

Abstract

Background—Major features of allergic asthma include airway hyperresponsiveness (AHR), eosinophilic inflammation, and goblet cell metaplasia. Rho kinase (ROCK) is a serine/threonine protein kinase that regulates the actin cytoskeleton. By doing so, it can modulate airway smooth muscle cell contraction and leukocyte migration and proliferation. This study was designed to determine the contributions of the two ROCK isoforms, ROCK1 and ROCK2, to AHR, inflammation and goblet cell metaplasia in a mast-cell dependent model of allergic airways disease.

Methods and Results—Repeated intranasal challenges with OVA caused AHR, eosinophilic inflammation, and goblet cell hyperplasia in wildtype (WT) mice. OVA-induced AHR was partially or completely abrogated in mice haploinsufficient for ROCK2 (ROCK2^{+/-}) or ROCK1 (ROCK1^{+/-}), respectively. In contrast, there was no effect of ROCK insufficiency on allergic airways inflammation, although both ROCK1 and ROCK2 insufficiency attenuated mast cell degranulation. Goblet cell hyperplasia, as indicated by PAS staining, was not different in ROCK1^{+/-} versus WT mice. However, in ROCK2^{+/-} mice, goblet cell hyperplasia was reduced in medium but not large airways. Maximal acetylcholine-induced force generation was reduced in tracheal rings from ROCK1^{+/-} and ROCK2^{+/-} versus WT mice. The ROCK inhibitor, fasudil, also reduced airway responsiveness in OVA-challenged mice, without affecting inflammatory responses.

Conclusion—In a mast cell model of allergic airways disease, ROCK1 and ROCK2 both contribute to AHR, likely through direct effects on smooth muscle cell and effects on mast-cell degranulation. In addition, ROCK2 but not ROCK1 plays a role in allergen-induced goblet cell hyperplasia.

Keywords

asthma; IL-13; mucus; ROCK; eosinophil

INTRODUCTION

The pathological features of allergic asthma include bronchoconstriction, airway hyperresponsiveness (AHR), airway infiltration with immune cells, especially T lymphocytes and eosinophils, and airway remodeling including goblet cell hyperplasia. Data from animal models indicate that Rho-associated coiled-coil forming kinases (ROCKs), serine/threonine protein kinases activated by GTP-bound RhoA, may be involved in the pathogenesis of asthma (1-4). ROCKs have pleiotropic effects that can contribute to asthma, including the ability to augment smooth muscle contraction (5, 6), as well as effects on immune cell proliferation and migration (2, 7). Furthermore, in allergen sensitized mice, ROCK activation occurs after airway challenge with allergen (4), consistent with the ability of many of the inflammatory mediators induced by allergen challenge to cause ROCK activation (8, 9). Importantly, in allergen sensitized and challenged animals, ROCK inhibitors have been shown to reduce AHR and to suppress airway inflammation (2).

There are two ROCK isoforms: ROCK1 and ROCK2 (10, 11). Their kinase domains are highly (92%) homologous, though their Rho binding domains are less (55%) homologous (10). There are tissue specific differences in ROCK1 and ROCK2 expression, as well as differences in the subcellular localization of ROCK1 and ROCK2 (10, 12, 13). In addition, accumulating evidence indicates differences in the regulation of ROCK1 and ROCK2 (14). Collectively, these data suggest that ROCK1 and ROCK2 may serve different functions depending on the cell type. Understanding the cellular targets and individual contributions of each ROCK isoform to the pathophysiological manifestations of asthma might allow for the development of asthma therapeutics that target this pathway.

We have previously reported that both ROCK1 and ROCK2 contribute to the pathological features of allergic airways disease in mice (4). ROCK1 and ROCK2 deficient mice typically die either *in utero* or soon after birth (15, 16), but heterozygous mice (ROCK1^{+/-} and ROCK2^{+/-} mice) are viable (17, 18). Pulmonary expression of ROCK1 or ROCK2 is about 50% of wildtype (WT) levels in ROCK1^{+/-} and ROCK2^{+/-} mice respectively, with no changes in the other isoform (4). We have shown that ovalbumin (OVA) aerosol challenge results in ROCK activation in the airways of OVA sensitized and challenged mice (4) and both basal and OVA-induced ROCK activation are reduced by approximately 50% in ROCK1^{+/-} or ROCK2^{+/-} versus WT mice, indicating that both isoforms are activated after allergen challenge. Importantly, OVA-induced AHR was virtually abolished in both ROCK1^{+/-} and ROCK2^{+/-} versus WT mice (4). Pulmonary inflammation and goblet cell hyperplasia were also reduced in ROCK1^{+/-} and ROCK2^{+/-} versus WT mice, though there were differences in the role of each ROCK isoform, with ROCK1 insufficiency resulting in greater reductions in Th2 cytokines and lymphocyte recruitment to the airways and ROCK2 insufficiency causing greater reductions in goblet cell hyperplasia (4).

The acute allergen sensitization and challenge protocol used the study discussed above (4) requires T cells but not mast cells for the induction of the asthma-like phenotype (19, 20). However, mast cells can play a role in some patients with allergic asthma (21). Accordingly, the purpose of this study was to examine the requirement for ROCK1 and ROCK2 in an allergic airways disease model in which mast cells *are* required. To that end, we sensitized

and challenged WT, ROCK1^{+/-}, and ROCK2^{+/-} mice using a mast cell dependent model (20, 22) involving intraperitoneal OVA sensitization *without* alum, followed by weekly intranasal instillations of OVA. Our results indicate a role for both ROCK1 and ROCK2 in allergen induced AHR but not inflammation in this model. Our data also indicate a role for ROCK2 but not ROCK1 in mucous cell hyperplasia.

MATERIALS AND METHODS

Animals

The Harvard Medical Area Standing Committee on Animals approved this study. The generation of ROCK1^{+/-} and ROCK2^{+/-} mice was previously described (18, 23). ROCK1^{+/-} and WT (C57BL/6) mice were bred to yield ROCK1^{+/-} mice and littermate WT controls. Similarly, ROCK2^{+/-} and WT mice were bred to yield ROCK2^{+/-} mice and littermate WT controls. WT mice from the two types of litters were combined into one WT group. Mice in all 3 groups (ROCK1^{+/-}, ROCK2^{+/-} and WT) were studied at the same time.

Allergen Sensitization and Challenge

Six-week old female ROCK1^{+/-}, ROCK2^{+/-} and WT mice were sensitized on days 1, 4 and 7 by i.p. injection of 50 µg OVA in 0.1 ml of PBS *without* alum. Starting on day 12, mice were challenged weekly for 4 weeks by i.n. instillation of either sterile PBS or OVA (20 µg in 30 µl PBS) as previously described (24). Mice were studied 24 h after the last OVA or PBS challenge.

In vivo ROCK inhibition with fasudil

Fasudil (HA-1077) is a potent inhibitor of Rho-kinase which prevents ROCK from phosphorylating the myosin bind subunit of myosin light chain phosphatase (MLCP) (25). It has been used *in vivo* to promote vasodilation (26, 27), axon regeneration in model of spinal cord injury (28), and to attenuate fibrosis (29). Using the same OVA sensitization and challenge protocol described immediately above, WT mice were treated with PBS or with fasudil (10mg/kg, i.p.; LC Labs, USA) dissolved in PBS 30 minutes before each i.n. OVA challenge. Fasudil was also administered 30 minutes before measurements of pulmonary mechanics and airway responsiveness to ensure ROCK inhibition during methacholine challenge since many agonists of G-protein coupled receptors can induce ROCK activation. Fasudil was used in the same dose range and with a similar treatment regimen (i.e. just prior to each challenge) as reported by others to inhibit allergic AHR in a non mast cell dependent model of allergic airways disease in mice (2).

Measurement of pulmonary mechanics and airway responsiveness

Twenty-four hours after the last OVA challenge, mice were anesthetized with pentobarbital and xylazine and instrumented for the measurement of pulmonary mechanics using the forced oscillation technique as previously described (30, 31). Prior to measurements, we twice inflated the lungs to 30 cmH₂O airway opening pressure (total lung capacity (TLC)) to establish a standardized volume history. Static elastance (Estat) was calculated from pressure-volume loops and baseline pulmonary mechanics was assessed using the forced oscillation technique, as previously described (32). One minute after an inflation to TLC, we

measured total lung impedance (Z_L) with a 3s optimized pseudorandom volume signal containing frequencies from 0.25 to 19.63 Hz. We then used a parameter estimation model (33) to partition Z_L into components representing Newtonian resistance (R_n), which largely reflects the conducting airways, the coefficients of lung tissue damping (G) and lung tissue elastance (H), which reflect the small airways and pulmonary parenchyma respectively. This sequence was repeated 3 times and the baseline values averaged. We then measured airway responsiveness as follows. We used an ultrasonic nebulizer (Aeroneb, SCIREQ) to deliver aerosols of PBS or increasing concentrations of methacholine dissolved in PBS (in half log increments from 0.3 to 100 mg/ml) to the lungs. Each aerosol delivery occurred one minute after an inflation to TLC. After each dose, Raw, G and H were measured every 15 seconds for 3 minutes. At each dose, the 3 highest values of Raw, G and H were averaged and used to construct dose response curves.

Bronchoalveolar Lavage (BAL)

After measurements of pulmonary mechanics were complete, mice were euthanized with an overdose of sodium pentobarbital, blood was drawn by cardiac puncture, and serum stored at -20°C for subsequent measurement of OVA-specific IgE and IgG1 by ELISA (Cayman Chemical, Ann Arbor, MI). BAL was performed as previously described (34). Total BAL cells were counted with a hemacytometer. An aliquot of BAL cells was spun onto glass slides, stained, and differential cell counts were performed on at least 300 cells. BAL supernatant was stored at -80°C and subsequently analyzed by ELISA for IL-5, IL-13, and IP-10 (R&D Systems Inc., Minneapolis, MN). IL-5, IL-13, and IP-10 were examined because a screening multiplex assay (Eve Technologies, Calgary, Canada (31)) identified each of these cytokines to be elevated in BAL fluid of OVA versus PBS challenged WT mice sensitized and challenged in the manner described above. We also measured mouse mast-cell protease 1 (mMCP-1) in the BAL by ELISA (eBiosciences, San Diego, CA).

RNA Extraction and Real-Time PCR

After BAL, the left lung was excised and placed in RNA later, and subsequently homogenized for RNA extraction with RNeasy (Qiagen). Total RNA (1 μg) was reverse transcribed using random hexamer primers and Superscript III (Invitrogen). We used real-time PCR and SYBR Green (Applied Biosystems) to assess mRNA expression for each of the following genes: *Retnla* (resistin like 1 alpha), *Itln1* (intelectin 1), *Muc5ac*, and *Clca3* (Gob5). Primers have been previously described (24). Gene expression values were normalized to 18s and expressed relative to WT PBS values, using the $\Delta\Delta\text{Ct}$ method (35). For each set of primers, melt curve analysis yielded a single peak consistent with one PCR product.

Histology and Morphometry

In a separate cohort, mice were euthanized 24 h after the last OVA or PBS challenge. The lungs were inflated in situ with 4% paraformaldehyde at 20 cm of water, the trachea was tied off and the lungs removed. The left lung was sliced, first sagittally and then transversely. Slices were embedded in paraffin, sectioned, and stained with Periodic acid-Schiff (PAS) by the Rodent Histology Core (Harvard Medical School, Boston, MA). Sections were also used

to assess the inflammation index, a product of the severity and prevalence of inflammation (36). The number of inflammatory cell infiltrate layers surrounding the airways was used to assign a severity score as follows: 0, no cells; 1, 3 cell layers; 2, 4-9 cell layers; 3, 10 cell layers. For each airway, we also assigned a mucous hyperplasia index as follows: 0, no PAS staining cells; 1, 25%; 2, 25-50%; 3, >50% of the airway circumference consisted of PAS staining cells. All airways were analyzed that had a lumen area that covered more than 40% of the visual field when viewed at 250x magnification in a brightfield microscope. We classified the size of airways as “medium” for airways with lumens that covered more than 40 but less than 80% of the visual field when viewed at 250x. “Large” airways were those with lumens that covered more than 80% of the visual field at 250x. The total area of the visual field calculated at 250x was 0.255mm². For both inflammation and goblet cell hyperplasia, a mean value for each mouse was computed and used for statistical analysis.

Tracheal contractility assay

Naïve WT, ROCK1^{+/-} and ROCK2^{+/-} mice were euthanized and their tracheas were harvested. Tracheal rings approximately 3-5 mm in length were excised and placed in iced Krebs buffer (pH 7.4) under aeration with 5% CO₂/95% O₂. Tracheal rings were attached with to a metal wire connected at one end to the bottom of the glassware. Another metal wire connected the ring to a force transducer (FT03, Grass, Warwick, RI). Once connected, rings were maintained at 37°C. Tracheal rings were stretched to a baseline tension of 0.5g, and left to stabilize for 45-60 minutes. Force was measured before and after addition of increasing concentrations of acetylcholine (10⁻⁹ to 10⁻⁴ M). Force was recorded after transformation of analog to a digital signals using data acquisition software (DATAQ, Akron, OH). At the end of the experiment, tissues were dried to obtain a dry weight to normalize force generation across tracheal rings. The concentration of acetylcholine required to cause an increase of 50% of the maximal tension (EC50) was calculated for each ring.

Statistical Analysis

Data were analyzed by factorial ANOVA (BAL and serum moieties, histological analyses, and lung mRNA expression) using STATISTICA software (StatSoft®; Tulsa, OK) with genotype (WT, ROCK1^{+/-}, ROCK2^{+/-}) and treatment (OVA or PBS) as main effects. BAL cells were log transformed in order to conform to a normal distribution. Fisher's LSD test was used for post hoc comparisons. For comparison between two groups we used a 2-tailed t-test. A p value < 0.05 was considered statistically significant.

RESULTS

ROCK insufficiency attenuates allergen-induced AHR but not inflammation

Compared to PBS, repeated OVA challenge increased airway responsiveness to methacholine (Figs 1A and 1B, WT OVA versus PBS, p<0.05). This AHR was most pronounced for changes in G and H (parameters reflecting changes in the lung periphery), whereas methacholine-induced changes in Rn (Newtonian resistance, reflecting mostly large airways) were minimally affected by OVA (Supl Fig 1). In PBS challenged mice, there was no significant difference in airway responsiveness among WT, ROCK1^{+/-} and ROCK2^{+/-} mice. ROCK1 insufficiency completely abrogated OVA-induced AHR regardless of

whether G or H was used as the index of response (Figs 1A and 1B center). In *ROCK2*^{+/-} mice, OVA challenge still resulted in an increase in AHR when G was used as the index of response, although the maximal response (at 100 mg/ml) was significantly lower than that observed in WT mice challenged with OVA (Fig. 1A, left panel). When H, an indicator of small airway closure, was used as the index of response, OVA challenge no longer resulted in significant AHR in *ROCK2*^{+/-} mice (Fig. 1B, left panel). These differences in lung mechanics were not caused by lack of sensitization to allergen since WT, *ROCK1*^{+/-} and *ROCK2*^{+/-} challenged with OVA had similarly increased levels of OVA specific IgE and IgG compared to PBS controls (Figs 2A and 2B respectively).

Compared to PBS challenged mice, intranasal OVA challenge induced a significant increase in BAL eosinophils, neutrophils, and lymphocytes (Figs 3A, B, and C). BAL macrophages were not affected by OVA challenge (Fig 3D). Compared to WT mice, *ROCK1*^{+/-} or *ROCK2*^{+/-} mice had similar OVA-induced increases in BAL eosinophils and neutrophils. However, BAL lymphocytes were significantly lower in OVA treated *ROCK2*^{+/-} versus WT mice, suggesting that ROCK2 may be required for the recruitment of these cells. Histopathological evaluation confirmed an increase in inflammatory cells around the airways by repeated OVA challenges compared to PBS control (Fig. 3E), but the inflammation score was similar in WT, *ROCK1*^{+/-} and *ROCK2*^{+/-} OVA challenged mice (Fig 3E). We also observed a significant increase in perivascular inflammation by OVA versus PBS challenge that was equal magnitude in all three genotypes (Fig 3F).

Attenuation of AHR is not due to changes in Th2 cytokines

The Th2 cytokine, IL-13, is required for OVA-induced AHR (37, 38) and ROCKs have been reported to play a role in lymphocyte activation (39-41). Consequently, we measured BAL IL-13 (Fig. 4A). Compared to PBS, OVA caused a significant increase in BAL IL-13, but there was no effect of ROCK1 or ROCK2 insufficiency (Fig 4A). To determine whether differences in signaling events downstream of IL-13 might contribute to the loss of OVA-induced AHR in ROCK1 mice, we measured pulmonary mRNA expression of *Retnla* and *Itln1*. IL-13 is required for allergen-induced increases in expression of both these genes in the lung (42). In WT mice, OVA caused a marked increase in both *Itln1* and *Retnla* mRNA abundance (Figs 4B and C). There was no significant difference in *Itln1* and *Retnla* mRNA in WT versus *ROCK1*^{+/-} mice. However, in *ROCK2*^{+/-} mice, OVA exposure failed to increase *Itln1*, although *Retnla* expression was induced to the same level as in WT mice (Fig 4B and C). OVA-induced increases in IL-5, an eosinophil survival factor, and IP-10, a neutrophil chemotactic factor, were also not different in *ROCK1*^{+/-} or *ROCK2*^{+/-} versus WT mice (Figs. 4D and 4E respectively).

Mast cell activity is dependent on ROCK

The OVA sensitization and challenge model used in this study produces allergic airways responses that are dependent on mast cells (19, 20). To assess degranulation of mast cells we measured BAL concentrations of mast-cell protease/tryptase 1 (mMCP-1) (Fig. 4F). In WT mice, BAL mMCP-1 was greater in mice that had been challenged with OVA versus PBS (Fig 4F), consistent with mast cell degranulation. BAL mMCP-1 was significantly lower

OVA challenged ROCK1^{+/-} or ROCK2^{+/-} mice than in WT mice (Fig.4F), consistent with the observations of others that that ROCK has a role in mast-cell activation (43).

Effect of ROCK insufficiency on goblet cell hyperplasia

Goblet cell hyperplasia is a common feature of allergic asthma ((44) review at (45)) and may contribute to asthma symptoms (46). In both medium and large airways of WT mice, OVA challenge caused an increase in the percentage of the airway wall encompassing by PAS+ cells (Figs 5A, B, E and H). There were no genotype-related differences in OVA-induced goblet cell hyperplasia in large airways (Figs 5B, C, D and H). However, in medium sized airways there was a significant decrease in the mucous hyperplasia index in ROCK2^{+/-} mice compared to WT mice (Fig 5E, G and H), whereas ROCK1^{+/-} and WT mice were not different (Figs 5E, F, and H). Compared to PBS, OVA caused a marked increase in the mRNA abundance of both *Muc5ac* and *Cacl3* (Figs 5I and 5J) in WT mice. *Muc5ac* and *Cacl3* mRNA abundance were not difference in ROCK1^{+/-} and WT mice. There was a trend towards reduced *Muc5ac* and *Cacl3* mRNA in ROCK2^{+/-} mice, but the effect was not statistically significant (Figs 5I and J).

Maximal tracheal contractility is attenuated by ROCK insufficiency

Tracheas from WT, ROCK1^{+/-} and ROCK2^{+/-} were studied *in vitro* under isometric conditions. Compared to WT mice, we observed a significant attenuation of the maximal contractile response to acetylcholine in tracheas of mice with ROCK1 or ROCK2 insufficiency (Fig 6). In contrast, the EC50 was not affected (data not shown). These data are consistent with those of others using combined ROCK1/ROCK2 inhibitors which indicate a role for ROCK in smooth muscle contraction (47-49).

OVA-induced AHR but not inflammation was attenuated by the ROCK inhibitor, fasudil

ROCK1^{+/-} or ROCK2^{+/-} mice have only 50% reductions in ROCK1 or ROCK2 respectively with no change in the other ROCK isoform (4). To examine the impact of *combined total* ROCK1 and ROCK2 inhibition, we treated mice with fasudil (10mg/kg), a potent pharmacological inhibitor of both ROCK1 and ROCK2 (50) using a treatment regimen similar to that shown by others to inhibit both inflammation and AHR in a non mast cell dependent protocol (2). Fasudil was administered just before each OVA challenge and also immediately before measurements of lung function. Compared to mice treated with PBS, OVA challenged mice treated with fasudil had a robust reduction in AHR (Figs 7A, B, and C). In contrast, fasudil had no effect on OVA-induced increases in BAL neutrophils, eosinophils, or lymphocytes (Fig.7D-F). Furthermore, analysis of histopathological sections of lungs of OVA challenged mice treated with fasudil versus PBS showed no change in PAS score (Fig.8A), airway inflammation (Fig .8B), or perivascular inflammation (Fig 8C).

DISCUSSION

Our data indicate that ROCK plays an important role in the development of AHR in a mouse model of allergic airways disease induced by OVA sensitization and repeated i.n. challenge. The role of ROCK in OVA-induced AHR was unrelated to changes in IgE, eosinophils, or IL-13. However, airway smooth muscle contractility and OVA-induced mast cell

degranulation and were reduced in ROCK1 and ROCK2 insufficient mice, suggesting that ROCK dependent events occurring in these cells contribute to allergic AHR. Our data also indicate a role for ROCK2 but not ROCK1 in the goblet cell hyperplasia observed in this model.

In WT mice, OVA challenge caused a robust increase in AHR (Fig.1), consistent with the results of other investigators who have utilized this model (20, 51, 52). Importantly, OVA-induced AHR was abolished in ROCK1^{+/-} mice (Fig.1, middle panels). This effect of ROCK1 insufficiency was very robust even though the mice still express 50% of normal ROCK1 protein levels in the lungs (4), suggesting a very strong requirement for ROCK1 for OVA-induced AHR in this model. Compared to WT mice, OVA-induced AHR was also attenuated in ROCK2^{+/-} mice (Fig.1, right panels), but the effect was not as profound as it was in ROCK1^{+/-} mice. Inhibiting both ROCK1 and ROCK2 with fasudil also markedly attenuated AHR in OVA-challenged mice (Fig.6).

Effects of ROCK acting within mast cells may contribute to the observed effects of ROCK1 and ROCK2 insufficiency on OVA-induced AHR (Fig.1). Interaction of allergen with IgE bound to its high affinity receptor on mast cells results in mast cell activation and degranulation, and releases substances that have the capacity to induce AHR (21). Data from mast cell deficient mice indicate that mast cells are required for the AHR induced by this type of OVA sensitization and challenge (20, 22). Our data do not indicate ROCK dependent effects on OVA-specific IgE (Fig.2A,B), but our data do indicate reduced BAL mMCP-1 in OVA challenged ROCK1^{+/-} and ROCK2^{+/-} compared to WT mice, consistent with reduced mast cell activation in these ROCK insufficient mice (Fig. 4F). The observation that ROCK inhibitors reduce chemotaxis of rat basophilic leukemia-2H3 cells after engagement of the FcεRI receptor (43) suggests that mast cell recruitment may also be affected by ROCK activation. Clinical studies also support a role for mast cell activation in human asthma: reduced asthma symptoms and improved asthma control are observed in some allergic asthmatics after treatment with omalizumab, which prevents the IgE crosslinking that activates mast cells (53, 54). However, not all patients with allergic asthma respond to such treatment (21), indicating that other mechanisms are also important.

The observation that tracheal contractility was also attenuated in ROCK insufficient mice (Fig.6) suggests that in addition to effects of ROCK within mast cells, the role of ROCK in this model is also, at least partly, at the level of its effects in airway smooth muscle cells, though we cannot rule out effects on other cell types. ROCK promotes smooth muscle contraction by phosphorylating the myosin binding subunit (MBS, also known as MYPT-1) of MLCP (55), thereby inhibiting it. MLCP dephosphorylates the myosin light chain (56), causing the muscle to relax. By inhibiting MLCP, ROCK promotes smooth muscle contraction. Thus, ROCK inhibitors abrogate contractile responses in tracheal rings stimulated with acetylcholine (57). IL-13 receptors are expressed on airway smooth muscle (58) and IL-13 has been shown to promote AHR via direct effects on airway smooth muscle (59, 60), effects that require ROCK (61). Thus, even though IL-13 itself was unaffected by ROCK insufficiency (Fig.4A), it is possible that ROCKs are part of the signaling cascade initiated by IL-13 acting on its receptors on smooth muscle that promotes OVA-induced AHR.

The predominant OVA-induced changes in responses to methacholine occurred in the small airways and lung tissue, since G and H were both substantially impacted (Fig.1, left panels), whereas Rn was only minimally affected (Suppl. Fig. 1 bottom). Such changes are consistent with airway closure (62). While effects on contraction of smooth muscle in small peripheral airways might be expected to promote airway closure, changes in G and H without corresponding effects on Rn can also occur in response to inflammatory thickening of airways or in response to changes in lung stiffness that oppose airway closure (62). Although we did not measure airway thickening per se, the absence of effects of ROCK insufficiency on airway inflammation provide little support for the hypothesis that ROCK-related effects on AHR (Fig.1) were the consequence of differences in inflammatory thickening of airways. Compared to WT mice, there was a small (approximately 15%) increase in lung stiffness (Estat) assessed from quasi static pressure volume curves conducted prior to methacholine challenge in ROCK1^{+/-} but not ROCK2^{+/-} mice treated with PBS (Suppl Fig 1B). Compared to PBS, OVA also caused a small (approximately 15%) but significant increase in Estat in WT mice. Such increases in Estat might be expected to oppose airway closure. However, there were no significant differences in Estat in OVA-challenged ROCK1^{+/-} or ROCK2^{+/-} versus WT mice.

In contrast to the effects of ROCK insufficiency on OVA-induced AHR (Fig. 1), we observed no ROCK-dependent differences in OVA-induced cellular inflammation (Fig.3), except for a reduction in BAL lymphocytes in ROCK2^{+/-} versus WT mice (Fig.3C). This reduction in BAL lymphocytes did not translate into changes in BAL IL-13 or IL-5 (Fig.4), perhaps because ILC2s, which do not typically migrate into the airspaces, are the source of the IL-13 and IL-5 (63-65) rather than Th2 cells. Thus, while ROCK does have the *capacity* to impact eosinophil and lymphocyte activation and migration (8, 39, 40, 66, 67), our results suggest that such events do not contribute to the effects of ROCK1 or ROCK2 insufficiency on OVA-induced AHR (Fig.1) in this model. Dissociations between AHR and airway inflammation have also been observed in asthmatics(68-70).

We considered the possibility that the only 50% reductions in ROCK1 or ROCK2 observed in these heterozygous mice coupled with the fact that each mouse had only one ROCK isoform affected might have limited our ability to observe an effect on inflammation (Fig.3). However, inhibition of both ROCK1 and ROCK2 by treatment with fasudil prior to each OVA challenge was also without effect on airway inflammation in this model even though it did affect AHR (Fig.6). It is possible that the scheduling of fasudil treatments, in conjunction with the relatively short half-life of this ROCK inhibitor (71) was such that insufficient fasudil remained in the periods between treatments to prevent ROCK activation and subsequent inflammation. However, ROCK activation is most robust immediately after OVA challenge (when fasudil was present) and declines rapidly thereafter (4). Furthermore, others have shown that a similar fasudil treatment regimen *is* effective in reducing allergic airway inflammation in mast cell *independent* models (1, 2).

Our data also indicate an important role for ROCK2, but not ROCK1, in allergen-induced mucous cell hyperplasia in this model (Fig.5). Histological sections showed reduced mucous cell hyperplasia in medium but not large airways in ROCK2^{+/-} versus WT mice exposed to OVA (Fig.5). Similarly, there was a trend towards lower mRNA abundance of *Muc5ac* and

Clca3 (72-74) (Fig. 5I,J), in OVA-challenged ROCK2^{+/-} but not ROCK1^{+/-} mice, although the effect did not reach statistical significance due to high variability in responses among mice. Since only medium and not large airways were affected by ROCK2 insufficiency (Fig. 5), it is possible that the trend towards lower *Muc5ac* and *Cacl3* mRNA expression in ROCK2^{+/-} OVA challenged mice was due to use of RNA extract from total lung tissue without discriminating the size of airways. Interestingly, we did not observe similar decreased PAS+ cells in medium airways of fasudil treated mice (Fig. 8A). Fasudil inhibits both ROCK1 and ROCK2 and it is conceivable that ROCK1 and ROCK2 have opposing effects on goblet cell differentiation. Consistent with this hypothesis, others have shown opposing effects of ROCK1 and ROCK2 knockdown on human keratinocyte differentiation (75). However, it is also possible that the scheduling of fasudil treatments contributed to its lack of efficacy, as discussed above.

We previously reported the effects of ROCK1^{+/-} and ROCK2^{+/-} insufficiency using another model of allergic airways disease (4) that is mast-cell *independent* (19, 76, 77). Reductions in OVA-induced AHR were observed in both models (Fig. 1 and (4)). Since airway narrowing is the ultimate cause of most asthma symptoms, these results bode well for the likely efficacy of ROCK inhibitors in the treatment of human asthma. Indeed, ROCK inhibition with fasudil was effective in reducing AHR in both models (Fig. 7 and (2)). In contrast, the impacts of ROCK1 and ROCK2 insufficiency on OVA-induced inflammation and mucous cell hyperplasia were different in the mast cell dependent and independent models. Here we observed no effect of either ROCK1 or ROCK2 insufficiency or of ROCK inhibition with fasudil on airway eosinophilia (Fig. 3, 7) and effects of ROCK2 but not ROCK1 insufficiency on mucous cell hyperplasia (Fig. 5). In contrast, in a mast cell *independent* model, there were profound reductions in BAL eosinophils with both ROCK1 or ROCK2 insufficiency (4) and with fasudil treatment (2) and reductions in mucous cell hyperplasia in both ROCK1 and ROCK2 insufficient mice. Differences in the roles of ROCK in the mast cell dependent and independent models may be related to the use of alum in the mast cell independent but not dependent models. Use of this adjuvant is likely to create much greater activation of the adaptive immune system which might lead to greater activation of ROCK within lymphocytes. Indeed, a preliminary report from our lab (78) indicates that ROCK activation within CD4 T cells is required for OVA-induced increases in airway eosinophilia, BAL IL-13, and mucous cell hyperplasia in the mast cell independent model. Also consistent with this hypothesis, effects of ROCK inhibition with fasudil or ROCK insufficiency on BAL IL-13 and IL-5 (indicative of lymphocyte activation) were observed in the mast cell independent ((2) and (4)) but not the mast cell dependent (Fig. 3, 7) model.

In conclusion, our data indicate that ROCK plays a key role in allergen-induced AHR and could be a suitable target for preventing bronchoconstriction. In addition, ROCK2 contributes to mucous cell hyperplasia. The ROCK inhibitor, fasudil, is in clinical trials in the US for the treatment of amyotrophic lateral sclerosis, Raynaud's syndrome, and carotid atherosclerosis (see [ClinicalTrials.gov](https://clinicaltrials.gov) NCT01935518, NCT00498615, and NCT00670202), and may prove to also be effective in reducing asthmatic symptoms. Selective ROCK1 and ROCK2 inhibitors are also in development (79). Greater understanding of the specific roles

of ROCK1 and ROCK2 for allergic and other types of asthma may permit their ultimate use for treatment of the airways without unwanted side effects due to cardiovascular and other systemic effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Effect of ROCK insufficiency on airway hyperresponsiveness

Changes in pulmonary mechanics induced by aerosolized metacholine were determined 24 hours after last OVA or PBS challenge. The mechanical parameters determined were: (A) the coefficients of lung tissue damping, G, and (B) the coefficient of lung tissue elastance, H. Results are mean $\pm$ SEM of 6-9 mice per group for PBS challenge and 8 mice per group for OVA challenge. * p <0.05 vs. PBS, # p <0.05 vs. WT, + p <0.05 versus ROCK1.

Figure 2. ROCK insufficiency does not affect allergen sensitization

OVA specific (A) IgE and (B) IgG1 were measured by ELISA in serum from OVA and PBS challenged mice. White bar: WT; grey bar: ROCK1^{+/-}; and black bar: ROCK2^{+/-}. Data are mean±SEM. *p<0.05 versus PBS. n=7-10 mice per group for PBS challenge and 8-9 mice per group for OVA.

Figure 3. Effect of ROCK insufficiency on allergic inflammation

Panels show bronchoalveolar lavage (BAL) (A) Eosinophils, (B) Neutrophils, (C) Lymphocytes, and (D) Macrophages. Also, airway (E) and perivascular (F) inflammation were scored on histological sections. White bar: WT; grey bar: ROCK1^{+/-}; and black bar: ROCK2^{+/-}. * p<0.05 versus PBS challenged, # p<0.05 versus WT. n=7-10 for PBS challenge and 8-9 per group for OVA.

Figure 4. OVA induced pro-inflammatory mediators and degranulation of mast-cells
 (A) BAL IL-13 (PBS is a combination of WT, ROCK1^{+/-} and ROCK2^{+/-} mice in this panel); and (B,C) pulmonary mRNA abundance of two IL-13 dependent genes, Intelectin (*Itih1*), and Resistin like 1 alpha (*Retnla*); (D) BAL IL-5 and (E) BAL IP-10. (F) Mast-cell degranulation protein mMCP-1 was measured by ELISA (F). White bar: WT; grey bar: ROCK1^{+/-}; and black bar: ROCK2^{+/-}. Results are mean±SEM. For ELISA, n=5-10 mice per group. For qPCR, n=6-9 mice per group for qPCR. *p<0.05 vs. PBS, # p<0.05 vs. WT

Figure 5. Goblet cell hyperplasia and mucus related gene expression

Representative micrographs of a large airway of (A) PBS challenged WT mice, and (B-D) OVA challenged (B) WT mouse, (C) ROCK1^{+/-} mouse, and (D) ROCK2^{+/-} mice. (E-G) Micrographs of medium airways from OVA challenged (E) WT mouse, (F) ROCK1^{+/-} mouse and (G) ROCK2^{+/-} mouse. (H) Lung sections of OVA and PBS challenged mice were stained for PAS and scored in medium and large airways (see Methods for definition of medium and large). In a different cohort of mice, genes indicative of mucous production were quantified by qPCR in mRNA extracted from whole lung: (I) *Muc5ac* and (J) *Ccl3*. White bar: WT; grey bar: ROCK1^{+/-}; and black bar: ROCK2^{+/-}. n=4-5 per group for histology and 6-9 per group for qPCR. Results are mean±SEM. *p<0.05 vs. PBS, # p<0.05 versus WT or ROCK1^{+/-}.

Figure 6.

ROCK insufficiency decreases maximal tracheal contractility. Tracheas from WT, ROCK1^{+/-}, and ROCK2^{+/-} mice were studied in tissue baths. Maximal tension produced by tracheal contraction in response to acetylcholine was measured and normalized to a dry weight of each tracheal ring. Results are mean $\pm$ SEM. $n = 5-7/\text{group}$. * $p < 0.05$, single tail T-test.

Figure 7. Effect of the ROCK inhibitor, fasudil, on allergic airways responses

Fasudil decreased airway responsiveness in OVA challenged mice as measured by (A) Newtonian resistance [Rn], (B) coefficient of lung tissue dampening [G], and (C) coefficient of lung tissue elastance [H]. (D) BAL eosinophils, (E) BAL neutrophils, and (F) BAL lymphocytes were not affected by fasudil treatment. Results are mean±SEM. n=7 per group. *p<0.05 versus PBS.

Figure 8. The ROCK inhibitor, fasudil, does not alter goblet cell hyperplasia nor lung inflammation

Histological sections were analyzed in PAS stained slides. (A) Scoring of goblet cells hyperplasia; (B) peribronchial inflammatory cells; and (C) perivascular inflammation. White bar: PBS; and black bar: Fasudil. N=7 per group.