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Rho-kinase and contractile apparatus proteins in murine airway hyperresponsiveness

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Abstract

Airway hyperresponsiveness (AHR) is a hallmark of bronchial asthma. Increased expression of smooth muscle contractile proteins or increased responsiveness of the contractile apparatus due to RhoA/Rho-kinase activation may contribute to AHR.

BALB/c mice developed AHR following systemic sensitization by intraperitoneal injections of 20 µg ovalbumin (OVA) in presence of 2 mg Al(OH)₃ on days 1 and 14, and airway challenge by 1% OVA-inhalation for 20 min each on days 28, 29 and 30. As assessed by Western blot, protein expression of RhoA, MLC (myosin light chain) and smMLCK (smooth muscle myosin light chain kinase) was increased in lungs of OVA/OVA-animals with AHR, as well as in lungs of OVA-sensitized and sham-challenged animals (OVA/PBS) without AHR, compared with lungs of PBS/PBS-animals. Pretreatment with the specific Rho-kinase inhibitor Y-27632 reduced MLC-phosphorylation and AHR. Contribution of Rho-kinase to bronchoconstriction was increased in lungs of OVA/OVA-animals compared with OVA/PBS- and PBS/PBS-animals, respectively. Furthermore, bronchoconstriction following MCh stimulation was significantly reduced after Y-27632 application.

In conclusion, systemic allergen-sensitization increased pulmonary expression of proteins involved in smooth muscle contraction, which may contribute to development of AHR. However, this observation was independent from local allergen challenge, suggesting that additional cofactors may be required for the activation of Rho-kinase and thereby the induction of AHR.

Rho-kinase may play an important role in murine AHR, and the bronchodilating action of Rho-kinase inhibition may offer a new therapeutic perspective in obstructive airway disease.

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Keywords: Asthma; Airway hyperresponsiveness; Isolated perfused mouse lung; Contractile apparatus proteins; Rho-kinase

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Introduction

Airway hyperresponsiveness (AHR) is a hallmark of bronchial asthma (National Heart LaBI and GINA Executive Committee, 2004). However, the mechanisms contributing to this exaggerated narrowing of the airways to a variety of non-specific stimuli are not exactly known (O'Byrne and Inman, 2003). Abnormalities of airway smooth muscle (ASM) have been postulated to play an important role for AHR (Martin et al., 2000; Seow et al., 1998). Hyperplasia or hypertrophy of ASM cells, as well as enhanced activation of the contractile apparatus and augmented expression of contractile proteins may contribute to AHR (Black, 2004; Solway and Fredberg, 1997).

Ca^{2+} /calmodulin activated myosin light chain kinase (MLCK) is responsible for the phosphorylation of the 20-kD regulatory light chains of myosin (MLC) (Kamm and Stull, 2001) leading to muscle contraction. However in AHR, the role of this key regulator of contraction is not unequivocally clear. MLCK gene expression was increased in bronchial biopsies from human subjects with asthma (Ma et al., 2002), as well as in immortalized human ASM cells isolated from asthmatic airways (Zhou et al., 2004). Furthermore, allergen-sensitization and -challenge increased MLCK quantity and activity in canine ASM (Jiang et al., 1992a). In contrast, MLCK expression was unaltered in ASM of human subjects with moderate asthma (Woodruff et al., 2004), and decreased MLCK expression was observed in rat bronchioles subsequent to allergen-sensitization and -challenge (Moir et al., 2003).

In addition to MLCK activation following cytosolic Ca^{2+} rise, supplementary mechanisms contribute to further contractile force at fixed Ca^{2+} concentrations (Somlyo and Somlyo, 1994). This Ca^{2+} -sensitization mechanism mainly depends on myosin regulatory light chain (MLC) phosphorylation (Somlyo and Somlyo, 2003), which is determined by the ratio of activities of MLCK to MLC phosphatase (MLCP) (Somlyo and Somlyo, 2003). MLCP activity is basically regulated by small GTP-binding proteins (Somlyo and Somlyo, 2003), including the small G protein RhoA and its target protein Rho-kinase (ROCK) (Kimura et al., 1996). ROCK furthermore increases MLCK activity (Somlyo and Somlyo, 2003). Accordingly, highly specific ROCK inhibition prevented acute agonist-induced G protein-mediated Ca^{2+} -sensitization in a variety of smooth muscle preparations (Uehata et al., 1997; Yamagata et al., 2000), including airway smooth muscle *in vitro* and *in vivo* (Yamagata et al., 2000; Yoshii et al., 1999). Notably, increased RhoA protein expression was observed in rats (Chiba et al., 1999) and guinea pigs (Schaafsma et al., 2004) with AHR.

The murine model of allergen-sensitization and airway-challenge mimics several important features of

allergic airway inflammation, including airway hyperresponsiveness, eosinophil infiltration of the airways, production of allergen-specific IgE and increased Th2-cytokines (Hamelmann et al., 1997, 1999). However, the regulation of contractile apparatus proteins in allergic mouse lungs and the “therapeutic” potential of ROCK inhibition in bronchoconstriction have not been investigated.

In the current study, we analyzed the role of pulmonary RhoA protein expression and ROCK activity for airway responsiveness in isolated, ventilated and perfused lungs of allergen-sensitized and -challenged mice, and examined the bronchodilating action of ROCK inhibition. Furthermore, the contribution of allergen-sensitization and -challenge to alterations in MLC and MLCK protein expression as well as MLC-phosphorylation was studied.

Methods

Experimental protocol

All experimental procedures were approved by local authorities (LaGeSo, Berlin). Pathogen-free female BALB/c mice (20–23 g) (BgVV, Berlin, Germany) were maintained on OVA-free diet. Systemic sensitization with OVA (20 µg per injection) (Sigma, Deisenhofen, Germany) adsorbed to 2 mg $\text{Al}(\text{OH})_3$ (Pierce, Rockford, IL, USA) by intraperitoneal injections on days 1 and 14, and repeated airway challenges with aerosolized 1% OVA (wt/vol) in PBS (20 min) on days 28, 29 and 30 were performed as described (Gerhold et al., 2003).

Immunoglobulin measurements

Plasma levels of IgE were measured using ELISA kits (Bethyl, Montgomery, TX, USA) according to the manufacturer's instructions.

Whole body plethysmography

In unrestrained animals enhanced pause (Penh) was quantified as an empiric index of *in vivo* airway obstruction by barometric whole body plethysmography (WBP) (Buxco[®], EMKA Technologies, Paris, France) as described (Hamelmann et al., 1997). Briefly, each mouse was placed in a main chamber of the body plethysmograph. Inhalative airway provocation was performed with aerosolized PBS (baseline) and increasing doses of methacholine (MCh, 6.25–50 mg/ml; Fährhaus, Hamburg, Germany) for 3 min each. Pressure differences between the main and a reference chamber caused by volume and resultant pressure changes during

inspiration and expiration of the mouse were measured and averaged for 3 min after each nebulization.

Ex vivo airway responsiveness in isolated perfused mouse lungs (IPML)

Mouse lungs were prepared, ventilated and perfused as described (von Bethmann et al., 1998; Witzenrath et al., 2006). Briefly, anesthetized mice were tracheotomized and ventilated. After sternotomy and cannulation of left atrium and pulmonary artery, lungs were perfused with 37 °C sterile Krebs–Henseleit–hydroxyethylamylpectine buffer (1 ml/min) (Serag, Germany) in a non-recirculating system, and left atrial pressure was adjusted at +2.2 cmH₂O. Pulmonary arterial and left atrial pressure were monitored continuously. Upon isolation, lungs were ventilated by negative pressure (−4.5 to −9.0 cmH₂O, 90 breaths/min) in a closed chamber. Hyperinflation (−24 cmH₂O) was performed at 4-min intervals. A differential pressure transducer continuously measured the chamber pressure, and airflow velocity was monitored by means of a pneumotachograph connected to a second differential pressure transducer. All data were amplified and analyzed with Pulmodyn[®] software, and the data for airway resistance were analyzed as described (von Bethmann et al., 1998). All hardware and software was purchased from HSE Harvard Apparatus (March-Hugstetten, Germany). Aerosol was generated using a Pari LL nebulizer (Pari, Starnberg, Germany) connected to a custom inhalation device.

Expression of contractile proteins in murine lungs

On day 32, lungs were *ex vivo* ventilated and perfused. Following a steady-state period of 30 min, perfusion was changed to buffer containing Y-27632 or solvent, and 10 min later, MCh (100 μM) was administered to the perfusate for 5 min. Lungs were then snap frozen in liquid nitrogen and stored at −80 °C. Lung homogenates were lysed in buffer containing Triton X-100, subjected to SDS–PAGE and blotted on Hybond-ECL membrane (Amersham Biosciences, Freiburg, Germany), as described (Schmeck et al., 2004). Immunodetection was carried out with specific antibodies detecting MLC or phosphorylated MLC (Santa Cruz Biotechnologies, Santa Cruz, CA), smMLCK (Sigma-Aldrich, Munich, Germany) or RhoA (BD Translab, Heidelberg, Germany), respectively. In all experiments, ERK2 kinase (Santa Cruz Biotechnologies, Santa Cruz, CA) was detected simultaneously to confirm equal protein load. Proteins were visualized by incubation with secondary IRDye 800- or Cy5.5-labeled antibodies, respectively (Odyssey infrared imaging system, LI-COR Inc.) (Brell et al., 2005).

Impact of Y-27632 on airway responsiveness and bronchoconstriction in IPML

Lungs of allergen-sensitized mice were *ex vivo* ventilated and perfused. Following a steady-state period of 30 min, MCh was administered to the perfusate for 30 s at 12-min intervals. Nine minutes before MCh administration, Y-27632 (5 μM) or solvent was admixed to the buffer fluid. Airway resistance values were determined 30 s before the administration of MCh, as well as at the maximum level of resistance increase. In another set of experiments, MCh was nebulized for 30 min. Six minutes after onset of nebulization, Y-27632 was administered to the perfusate and airway resistance values were monitored continuously. The change in airway resistance was expressed as fold airway resistance.

Data analysis

Data are expressed as mean ± S.E.M. Differences were analyzed by one-way analysis of variance (ANOVA), followed by posthoc Student–Newman–Keuls test. *P*-values of less than 0.05 were considered to represent a significant difference. All calculations were performed with GraphPad 4 Software (San Diego, CA, USA).

Results

Allergen-induced sensitization and airway inflammation

IgE serum levels were increased following systemic allergen-sensitization with OVA compared to sham-sensitization with PBS, regardless of subsequent airway challenges (Fig. 1A). Allergen airway challenges of sensitized mice induced eosinophilic airway inflammation in BAL fluid and lung tissues (data not shown). Airway hyperresponsiveness as estimated by increased Penh values after MCh provocation was observed in OVA/OVA mice compared to OVA/PBS and PBS/PBS placebo treated mice (Fig. 1B).

Expression of contractile proteins in murine lungs

Lungs of the different groups of mice were analyzed following pretreatment with Y-27632 (or solvent) and subsequent MCh perfusion. Protein expression of RhoA, MLC and MLCK was increased in lungs of OVA/OVA, as well as in lungs of OVA/PBS mice, compared to non-sensitized controls (PBS/PBS). Y-27632 distinctly reduced MLC-phosphorylation, while protein expression of RhoA, MLC or MLCK was unaltered (Fig. 2).

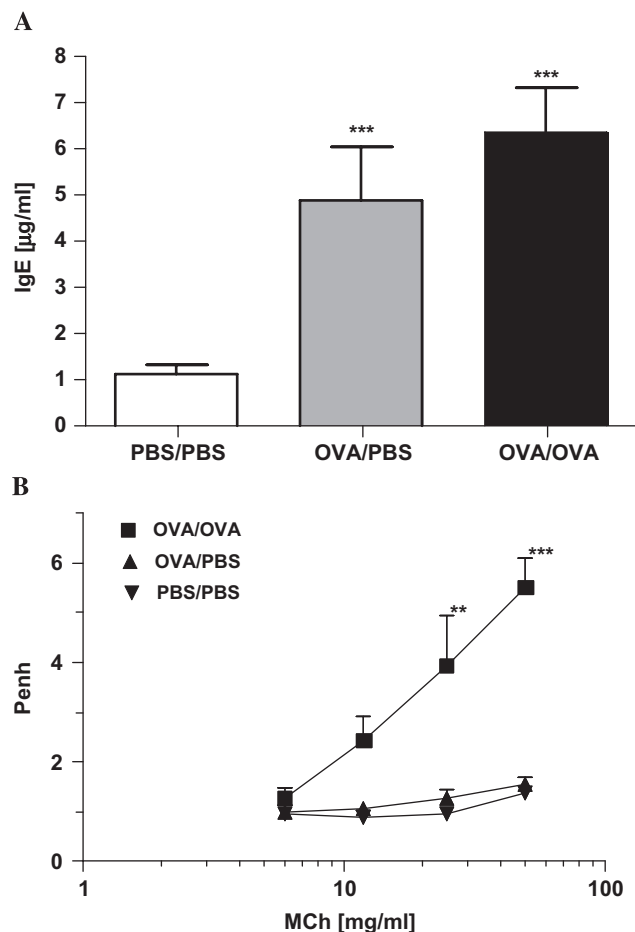


Fig. 1. Detection of allergic inflammation in intact mice. Animals were sensitized and challenged as described. (A) On day 31, airway responsiveness to inhalative methacholine (MCh) was examined *in vivo*. MCh was nebulized for 3 min and Penh was determined in the following 3 min. (B) On day 32, plasma IgE levels were measured by ELISA. Values are given as mean $\pm$ S.E.M. ($n = 6$ each). Error bars are missing when falling into symbols. ** $P < 0.01$; *** $P < 0.001$ vs. all other groups (A); *** $P < 0.001$ vs. PBS/PBS (B).

Y-27632 diminished AR in IPML

In order to examine the impact of the Rho-kinase pathway on the MCh-induced airway response, lungs were *ex vivo* perfused with the Rho-kinase inhibitor Y-27632 (5 μ M). In the absence of Y-27632, isolated lungs of OVA/OVA mice showed distinctly enhanced AR in response to intravascular MCh provocation compared to OVA/PBS and PBS/PBS lungs (Fig. 3A). Rho-kinase inhibition reduced AR dramatically to similar levels in all groups (Fig. 3A). The relative attenuation by Y-27632 of the MCh-provoked airway response was significantly more pronounced in OVA/OVA lungs, compared to OVA/PBS and PBS/PBS lungs (Fig. 3B).

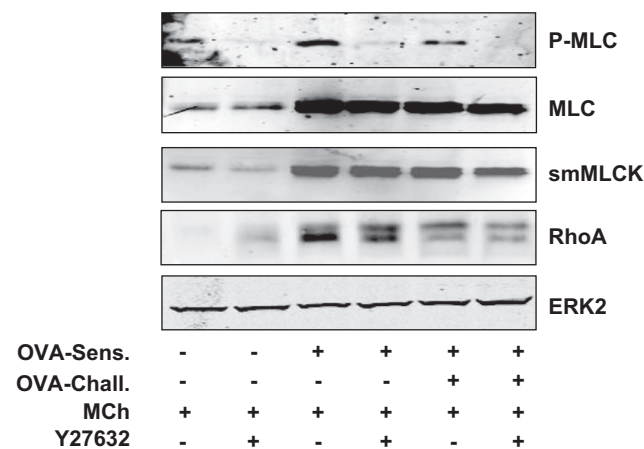


Fig. 2. Increased expression of MLC, smMLCK and RhoA following systemic allergen-sensitization, and decrease of MLC-phosphorylation by ROCK inhibition in mice lungs. Lungs of OVA/OVA, OVA/PBS or PBS/PBS mice were perfused with methacholine (MCh) following pretreatment with the ROCK inhibitor Y-27632 or solvent, respectively. Protein expression was analyzed by Western blot. Representatives of four independent experiments are shown.

Bronchodilation by the Rho-kinase inhibitor Y-27632 in IPML

In order to directly prove the importance of Rho-kinase activation for the development of increased airway responsiveness, we utilized a specific inhibitor of the Rho-kinase, Y-27632. MCh (12.5 mg/ml) was continuously aerosolized into isolated lungs of allergen-sensitized and -challenged mice, causing a sustained increase of airway resistance. Six minutes after onset of MCh inhalation, Y-27632 (5 μ M) or solvent was added to the perfusion buffer. This means of Rho-kinase inhibition induced a significant and persistent decrease of airway resistance compared to lungs without addition of the inhibitor (Fig. 4).

Discussion

The main findings of the current study were (i) expression of important contractile apparatus proteins was increased in mouse lungs following allergen-sensitization and -challenge, along with the development of airway hyperresponsiveness; (ii) systemic sensitization per se was sufficient to increase expression of contractile apparatus proteins including RhoA, but did not evoke airway hyperresponsiveness; (iii) the particular importance of ROCK for AHR development was demonstrated in lungs of mice undergoing both allergen-sensitization and

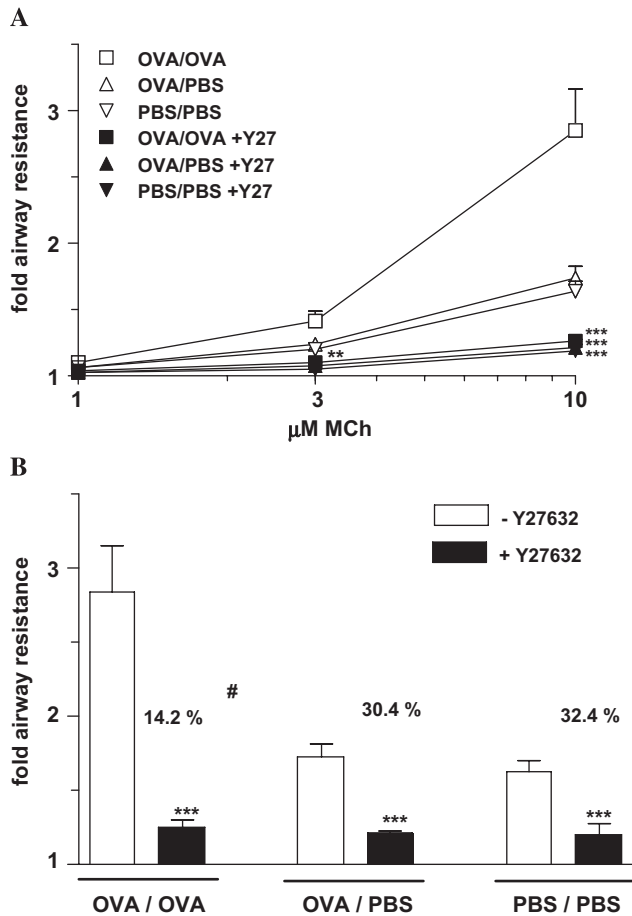


Fig. 3. Airway responsiveness (AR) is reduced by preceding Y-27632 perfusion. Mice were sensitized and challenged as described. Perfusion of the isolated lungs was repetitively changed to buffer containing MCh (30 s each dose), and AR was determined considering baseline values 30 s before MCh application, and at the maximum airway resistance (A). Attenuation of airway increase in response to MCh (10 μM) by Y-27632 pretreatment, relating to untreated lungs (B). Values are given as mean ± S.E.M. ($n = 5$ each). Error bars are missing when falling into symbols. ** $P < 0.01$ vs. OVA/OVA; *** $P < 0.001$ vs. corresponding control group; # $P < 0.05$ vs. OVA/PBS and PBS/PBS.

-challenge, providing evidence for a dissociation of RhoA expression and ROCK activation; and (iv) ROCK inhibition following MCh-provocation evoked bronchodilation, therefore possibly providing a therapeutic perspective in obstructive airway disease.

In the currently employed, well characterized murine model of allergic airway inflammation, production of allergen-specific IgE and AHR were induced as expected, and eosinophilic infiltration of the airways as well as increased Th2-cytokines have been observed previously (Hamelmann et al., 1997, 1999). To verify allergen-induced changes in Penh *in vivo* prior to isolation of lungs, whole body plethysmography was

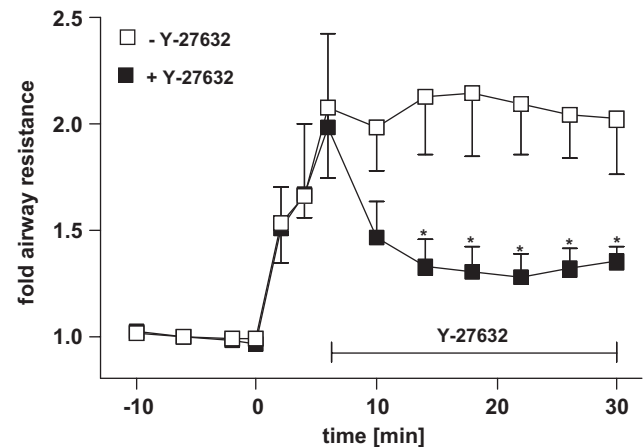


Fig. 4. Y-27632 reduced increased airway resistance. Mice were treated as described. Lungs were *ex vivo* perfused and ventilated. Following a steady-state period of 30 min, MCh was nebulized continuously (12.5 mg/ml) to induce a sustained airway resistance increase. After 6 min, Y-27632 or solvent was added to the perfusate.

performed. As it has been questioned whether Penh is a robust measure of airway responsiveness (Adler et al., 2004; Schwarze et al., 2005), AHR was also quantified in isolated lungs (Witzenrath et al., 2006).

AHR can be defined as an exaggerated response of the airways to non-specific stimuli, resulting in airway obstruction (O'Byrne and Inman, 2003). In hyperresponsive airways, the amount of smooth muscle may be increased (James and Carroll, 2000), but it is not unequivocally clear whether hyperplasia, hypertrophy, hypercontraction, or a combination of these factors is the leading cause of augmented airway responsiveness (Black, 2004). However, it is generally agreed that altered function in ASM contraction is not evoked by increased force generation of the single ASM cell (Solway, 2000), but an increased ASM shortening velocity (Fernandes et al., 2003). The velocity of muscle contraction is partly determined by MLC-phosphorylation (Dillon et al., 1981), which is a pre-requisite for the actin and myosin cross-bridge cycling that shortens the myocyte (Fernandes et al., 2003). Increased shortening velocity was demonstrated in canine allergic lung inflammation (Jiang et al., 1992a,b), as well as in ASM cells isolated from human asthmatics, where it was accompanied by enhanced mRNA quantity of MLCK (Ma et al., 2002). Besides MLCK-induced phosphorylation, RhoA-dependent activation of ROCK, resulting in inhibition of MLCP, increases phospho-MLC levels by decreasing MLC dephosphorylation.

The development of AHR following allergic-sensitization and -challenge was accompanied by increased pulmonary RhoA protein expression in mice. Since enhanced RhoA protein expression was also observed in

rats (Chiba et al., 1999) and guinea pigs (Schaafsma et al., 2004) with allergen-induced AHR, increased RhoA protein expression may be a common and important phenomenon in this disorder. Furthermore, pulmonary smMLCK and MLC protein expression was increased in allergic lung inflammation. Interestingly, increased smMLCK, MLC and RhoA protein levels were also observed in lungs of systemically sensitized, but not locally challenged animals, which did not display AHR. Hence, these findings suggest that over-expression of contractile apparatus proteins, though often associated with AHR may not necessarily be accompanied by a hypercontractile phenotype of ASM.

To further investigate the contribution of Ca^{2+} -sensitization to murine AHR, lungs of allergen-sensitized and -challenged animals were *ex vivo* perfused and ventilated, and the specific ROCK inhibitor Y-27632 (Ishizaki et al., 2000) was employed. The isolated, blood-free perfused and ventilated mouse lung (IPML) provides an intact organ with functional integrity, which is independent of metabolic and humoral impact of other organs (von Bethmann et al., 1998; Witzernath et al., 2006). Lung function parameters are accessible to accurate measurement (Witzernath et al., 2006). ROCK inhibition reduced MLC-phosphorylation following MCh perfusion, as confirmed by Western blot analysis. Moreover, following ROCK inhibition, the MCh-induced airway responses were decreased to a significantly higher degree in lungs of OVA/OVA as compared to lungs of OVA/PBS or PBS/PBS mice, suggesting increased ROCK activity subsequent to allergen-sensitization and -challenge. This observation was in line with previous studies reporting enhanced contribution of ROCK to histamine-induced bronchoconstriction in allergen-sensitized guinea pigs (Schaafsma et al., 2004). Remarkably, RhoA protein expression was also increased in lungs of OVA/PBS mice, but not ROCK activity. This novel finding suggests the dissociation of RhoA expression and ROCK activity. Several mediators have been associated with increased ROCK activity, including TNF α (Hunter et al., 2003), epidermal growth factor (Schaafsma et al., 2005) and platelet-derived growth factor (Schaafsma et al., 2005), which may be increased following local allergen challenge. However, the exact mechanism underlying ROCK activation independent of RhoA expression has still to be determined.

Considering the augmentation of ROCK-dependent Ca^{2+} -sensitization in allergen-induced AHR, ROCK inhibition may possibly provide a therapeutic perspective in obstructive lung disease. Some evidence for this assumption is given by the fact that suppression of bronchoconstriction by oral and inhalative Y-27632 treatment prior to airway provocation was observed in mice (Hashimoto et al., 2002) and guinea pigs (Schaafsma et al., 2006), respectively. The current study supports

and expands this view by the observation that MCh-constricted airways of allergen-sensitized and -challenged lungs dilated following intravascular treatment with Y-27632. Thus, in addition to a preventive employment of Y-27632, ROCK inhibition may provide a therapeutic option in asthma attacks.

In conclusion, these data provide evidence that allergic lung inflammation and AHR is associated with increased pulmonary expression of several key modulatory contractile apparatus proteins in mice. However, over-expression of the investigated proteins alone was not sufficient to induce the hypercontractile phenotype of ASM. Further, enhanced contribution of ROCK-dependent Ca^{2+} -sensitization was exclusively observed in AHR. ROCK inhibition following MCh-provocation evoked bronchodilation and may thus provide a promising therapeutic perspective in obstructive airway disease.

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