

## Mechanisms underlying the pathogenesis of hyper-contractility of bronchial smooth muscle in allergic asthma

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### Abstract

Airway hyperresponsiveness (AHR) and inflammation are key pathophysiological features of asthma. Enhanced contraction of bronchial smooth muscle (BSM) is one of the causes of the AHR. It is thus important for development of asthma therapy to understand the change in the contractile signaling of airway smooth muscle cells associated with the AHR. In addition to the Ca<sup>2+</sup>-mediated phosphorylation of myosin light chain (MLC), contractile agonists also enhance MLC phosphorylation level, Ca<sup>2+</sup>-independently, by inactivating MLC phosphatase (MLCP), called Ca<sup>2+</sup> sensitization of contraction, in smooth muscle cells including airways. To date, involvements of RhoA/ROCKs and PKC/Ppp1r14a (also called as CPI-17) pathways in the Ca<sup>2+</sup> sensitization have been identified. Our previous studies revealed that the agonist-induced Ca<sup>2+</sup> sensitization of contraction is markedly augmented in BSMs of animal models of allergen-induced AHR. In BSMs of these animal models, the expression of RhoA and CPI-17 proteins were significantly increased, indicating that both the Ca<sup>2+</sup> sensitizing pathways are augmented. Interestingly, incubation of BSM cells with asthma-associated cytokines, such as interleukin-13 (IL-13), IL-17, and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), caused up-regulations of RhoA and CPI-17 in BSM cells of naive animals and cultured human BSM cells. In addition to the transcription factors such as STAT6 and NF- $\kappa$ B activated by these inflammatory cytokines, an involvement of down-regulation of miR-133a, a microRNA that negatively regulates RhoA translation, has also been suggested in the IL-13- and IL-17-induced up-regulation of RhoA. Thus, the Ca<sup>2+</sup> sensitizing pathways and the cytokine-mediated signaling including microRNAs in BSMs might be potential targets for treatment of allergic asthma, especially the AHR.

**Key words:** bronchial asthma, airway hyperresponsiveness, Ca<sup>2+</sup> sensitization, RhoA, CPI-17 (Ppp1r14a)

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## Introduction

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Pathophysiology of allergic asthma is characterized by the combination of airway hyperresponsiveness (AHR), inflammation, and remodeling (1-3). The AHR is defined by increased airway narrowing in response to a wide range of stimuli, and is responsible for recurrent episodes of wheezing and breathlessness. Enhanced bronchial smooth muscle (BSM) contraction is one of the causes of AHR. In addition, the AHR correlates with the severity of asthma (4) and with the amount of treatment needed to control symptoms (5). The severity of hyperresponsiveness is associated with severer symptoms and a steeper fall in forced expiratory volume in 1 second (FEV<sub>1</sub>) (6).

Allergic asthma is a Th2 lymphocyte-mediated inflammatory airway disease. Cytokines derived from Th2 lymphocytes play a key role in the pathophysiology of asthma through the induction of eosinophilic airway inflammation. These lead to variable airway obstruction and AHR to nonspecific stimuli (7). The  $\beta$  adrenergic drugs are the most potent dilators of BSMs currently approved for clinical use against asthma. Among the  $\beta$  adrenergic agonists, the individual agents vary in their rapidity of onset and action duration. Inhaled, short-acting, selective  $\beta_2$  adrenergic agonists (SABAs) are the mainstay of acute asthma therapy, whereas inhaled, long-acting, selective  $\beta_2$  adrenergic agonists (LABAs), in combination with inhaled glucocorticoids, play a role in long-term control of moderate to severe asthma. Rapid relief from airway limitation in asthmatic patients by SABA inhalation suggests the involvement of augmented airway smooth muscle contraction in the airway obstruction. Thus, it is important to understand the changes in the contractile signaling of airway smooth muscle cells associated with AHR for the development of asthma therapy. In this review, we will describe the pathophysiological mechanisms of augmented BSM contraction in AHR.

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## Involvement of augmented Ca<sup>2+</sup> sensitization in BSM hyper-contraction in allergic asthma

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To elucidate the pathogenesis of allergic bronchial asthma, various animal models have been used by investigators including us. In an allergic asthma model using rats (8), that were actively sensitized with 2,4-dinitrophenylated *Ascaris suum* extract antigen and repeatedly challenged with aerosolized antigen, a marked augmentation of airway responsiveness to inhaled acetylcholine (ACh), i.e., the AHR, was observed. In this animal model of asthma, the ACh responsiveness of the isolated BSMs was also enhanced significantly. Similarly, in a mouse model of allergic asthma in which ovalbumin was used as an antigen, both the *in vivo* AHR and the *in vitro* BSM hyperresponsiveness have also been shown (9, 10). These observations remind us of an idea that the hyper-contractility of BSM *per se* is a cause of the AHR. Indeed, the hyperresponsiveness of airway smooth muscles was also suggested in asthmatics (11). At least, the BSM hyperresponsiveness to ACh observed in the antigen-induced AHR animals is not explained simply by changes in its receptor number: no significant difference in the muscarinic receptor density was observed between the AHR and control animals (12). Furthermore, the ACh-mediated increase in cytosolic Ca<sup>2+</sup> concentration measured using the Fura-2-loaded BSMs was within normal level whereas the contraction induced by ACh was much enhanced in the AHR animals (13).

We have tried to uncover the mechanism of the BSM hyperresponsiveness in allergic asthma. We shows evidence that the agonist-induced Ca<sup>2+</sup> sensitization of contraction is augmented in BSMs of the AHR animals (14). In BSMs that were pre-incubated with ACh (10<sup>-3</sup> M) under Ca<sup>2+</sup>-free condition (in the presence of 10<sup>-6</sup> M nifedipine and 0.05 mM EGTA), addition of Ca<sup>2+</sup> induced a concentration-dependent contraction. The

contractile response to  $\text{Ca}^{2+}$  of the ACh-stimulated BSMs isolated from the OA-challenged mice was markedly augmented as compared to that from the sensitized control animals. By contrast, no significant difference in the response to  $\text{Ca}^{2+}$  of BSMs depolarized with 60 mM  $\text{K}^+$  (in the absence of nifedipine and presence of 0.05 mM EGTA and  $10^{-6}$  M atropine) was observed between the groups. These findings suggest that, although the contraction mediated by  $\text{Ca}^{2+}$  itself is not changed, the ACh-mediated contractile signaling independent of cytosolic  $\text{Ca}^{2+}$  concentration, i.e.,  $\text{Ca}^{2+}$  sensitization of contraction, is augmented in BSMs of the AHR animals. To confirm it in more detail, the BSM contractility was also determined by using  $\alpha$ -toxin-permeabilized BSM preparations in mice (9). When the  $\text{Ca}^{2+}$  concentration was clamped at pCa 6.0, application of ACh ( $10^{-5}$ – $10^{-3}$  M) in the presence of GTP ( $10^{-4}$  M) caused a further contraction, i.e., ACh-induced  $\text{Ca}^{2+}$  sensitization, in an ACh concentration-dependent manner. The ACh-induced  $\text{Ca}^{2+}$  sensitization was significantly greater in BSMs of the AHR mice as compared to those of control animals. Similar results were also obtained when a rat model of antigen-induced AHR was used (15). It is thus strongly suggested that the  $\text{Ca}^{2+}$  sensitization of contraction is augmented in BSMs of the AHR animals.

### RhoA/ROCK pathway as a therapeutic target of asthma

Increased bronchial tone plays an important role in the pathophysiology of airway diseases including asthma. Bronchial tone is mainly regulated by the contraction of BSM cells (BSMCs). Smooth muscle contraction is mediated by the phosphorylation of the regulatory myosin light chain (MLC). The MLC phosphorylation level increases when MLC kinase (MLCK) is activated, whereas the level decreases when MLC phosphatase (MLCP) is activated (16). MLCP dephosphorylates MLC, leading to the smooth muscle relaxation (17). The MLCP activity is highly regulated both by contraction and relaxation signaling pathways. A monomeric GTPase RhoA plays a key role in the  $\text{Ca}^{2+}$  sensitization of contraction in smooth muscles. Contractile agonists, such as G protein-coupled receptor (GPCR) agonists, have an ability to activate RhoA. The precise nature of the activation of RhoA by GPCR is not yet uncovered but involves guanine nucleotide exchange factors RhoGEFs, such as p115RhoGEF, PDZ-RhoGEF and LARG (18). The RhoGEFs activate RhoA by exchanging GDP- to GTP-bound form of RhoA (19). The activated GTP-bound form of RhoA activates its downstream Rho associated coiled-coil containing protein kinases (ROCKs: also called as Rho-kinases) (20–22), which in turn phosphorylates myosin phosphatase targeting protein (MYPT), leading to an inhibition of MLCP activity (23). When the MLC phosphatase is inhibited, the phosphorylated MLC cannot be dephosphorylated, resulting in a promotion of contractile state, that is  $\text{Ca}^{2+}$  sensitization of smooth muscle contraction.

As described above, the ACh-induced  $\text{Ca}^{2+}$  sensitization was significantly augmented in BSMs of mice with allergic asthma. The augmented  $\text{Ca}^{2+}$  sensitization was sensitive to *Clostridium botulinum* C3 exoenzyme, an inhibitor of RhoA, and Y-27632, an inhibitor of ROCK (15, 24), indicating that the RhoA/ROCK pathway is involved in the  $\text{Ca}^{2+}$  sensitizing signaling. Interestingly, protein expression of RhoA in the BSMs was markedly increased in rat models of allergic asthma (9, 15). In addition, when BSMs were stimulated with contractile GPCR agonists such as ACh and endothelin-1, a higher expression of active form of RhoA was observed in the AHR animals (25). An augmented RhoA-mediated  $\text{Ca}^{2+}$  sensitization in smooth muscle contraction has been reported in experimental animal models of diseases such as hypertension (11, 15, 26), and coronary (9, 27, 28) and cerebral (29–31) vasospasms. Thus, the signaling of RhoA and its downstream ROCKs are now considered as a therapeutic target of asthma (28–31), although the exact mechanism of up-regulation of RhoA is still unclear.

## Inflammatory cytokines upregulate RhoA expression in BSMs

Pro-inflammatory cytokines such as interleukin-1 $\beta$  (IL-1 $\beta$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) could mediate the inflammatory response in asthma and are linked to the disease severity. IL-1 $\beta$  and TNF- $\alpha$  have been shown to play a prominent role in developing airway responsiveness and airway inflammation in bronchial asthma. Increased amounts of these cytokines have been detected in bronchoalveolar lavage (BAL) fluid (32, 33), and in the culture supernatants of alveolar macrophages from asthmatic patients (34, 35). Furthermore, it has been reported that inhaled TNF- $\alpha$  enhanced airway responsiveness to methacholine in humans (36). Therefore, we examined the effect of TNF- $\alpha$  on the BSM contractility. Treatment of rat BSMs with TNF- $\alpha$  resulted in a significant upward shift of the concentration-response curve to ACh, but not to high K<sup>+</sup>, compared with control tissues. The TNF- $\alpha$ -induced hyperresponsiveness to ACh was completely blocked by a p42/44 MAPK inhibitor, U0126, and a protein synthesis inhibitor, cycloheximide, but not by a p38 MAPK inhibitor, SB203580. TNF- $\alpha$  also caused a phosphorylation of p42/44 MAPK and an upregulation of RhoA in the BSMs: the TNF- $\alpha$ -induced upregulation of RhoA was abolished by U0126 pretreatment (37).

In addition, cytokines derived from Th2 lymphocytes, including IL-4, IL-5, IL-9, IL-13 and IL-25, play a key role in the pathophysiology of asthma. In the AHR rat model, IL-4, IL-6 and IL-13 in the BAL fluid were markedly and significantly increased compared with the control rats (38). An increased expression of IL-4 has been demonstrated in the BAL fluid after segmental allergen challenge to asthmatic patients (39). IL-4 promotes eosinophilic airway inflammation by increasing eotaxin expression and inhibiting eosinophil apoptosis (40). IL-4 induces mucus hypersecretion (41), which also contributes to the airway obstruction. Interestingly, IL-4 also acts on airway smooth muscles directly, and can cause hyperresponsiveness of airway smooth muscles (42). We thus examined the effect of IL-4 on the expression level of RhoA in cultured human BSMCs (hBSMCs). Incubation of hBSMCs with IL-4 induced a distinct phosphorylation of signal transducer and activator of transcription 6 (STAT6), a major signal transducer activated by IL-4, indicating that IL-4 is capable of activating signal transduction in hBSMCs directly. IL-4 also induced a significant increase in the expression level of RhoA (43).

There is increasing evidence that IL-13 is also a central mediator of AHR induction (44–47). The human *IL-13* gene is located on chromosome 5q in a region that has been linked to asthma (48, 49). An increased expression of IL-13 has been demonstrated in BAL cells obtained from patients with symptomatic asthma (50, 51). In addition, overexpression of IL-13 in mouse airway epithelial cells using the Clara cell 10-kD protein gene promoter induced AHR to aerosolized methacholine (52). Intratracheal instillation of recombinant IL-13 to naive mice also evoked AHR to inhaled methacholine (53) and intravenously administered ACh (44). To elucidate the role of IL-13 in the induction of BSM hyperresponsiveness, the effects of IL-13 on contractility and RhoA expression in BSMs were investigated. *In vivo* treatment of airways with IL-13 by intranasal instillation induced a BSM hyperresponsiveness with RhoA upregulation in BSMs of naive mice. Moreover, IL-13 induced RhoA upregulation. The IL-13-induced upregulation of RhoA was inhibited by leflunomide, a STAT6 inhibitor, in cultured hBSMCs (54).

## Involvement of microRNAs (miRNA) in RhoA expression in smooth muscle cells

MicroRNAs (miRNAs), a class of small non-coding RNA, are associated with a variety of basic biological processes (55–58). Mature miRNAs regulate the expression of protein-coding genes by targeting their mRNA, leading to translational inhibition or RNA degradation (59). Interestingly, recent studies also revealed a pos-

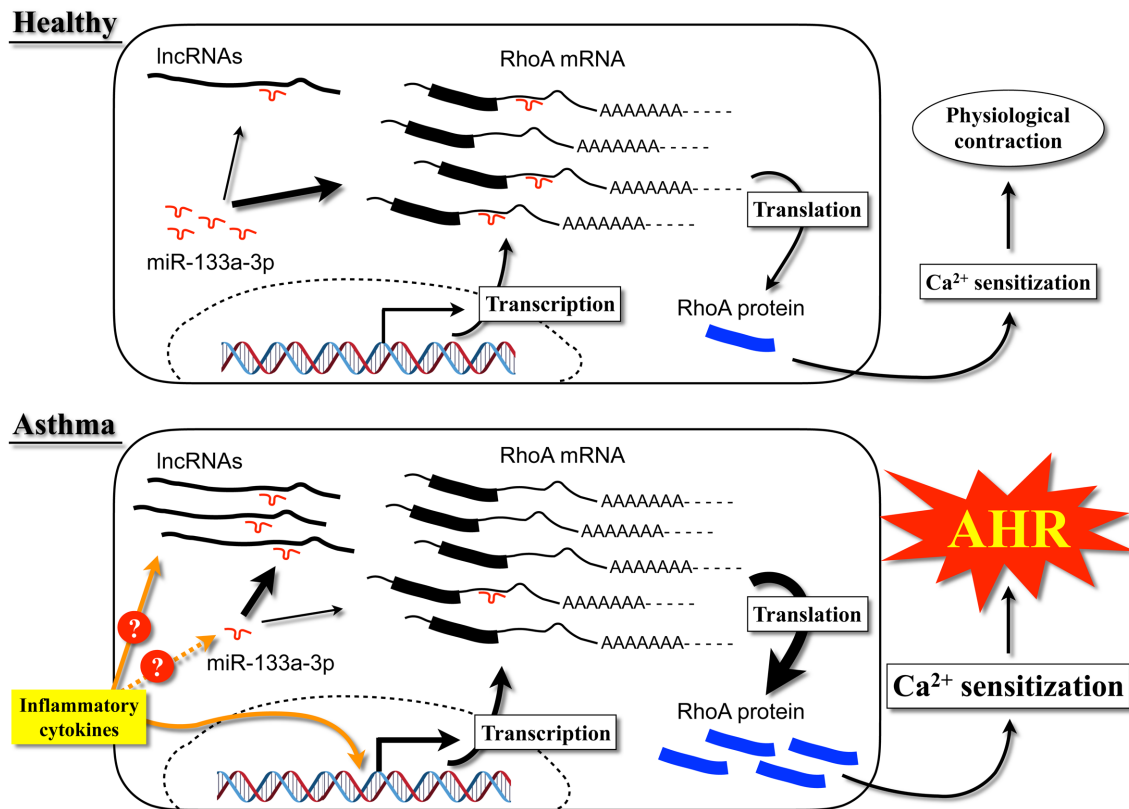


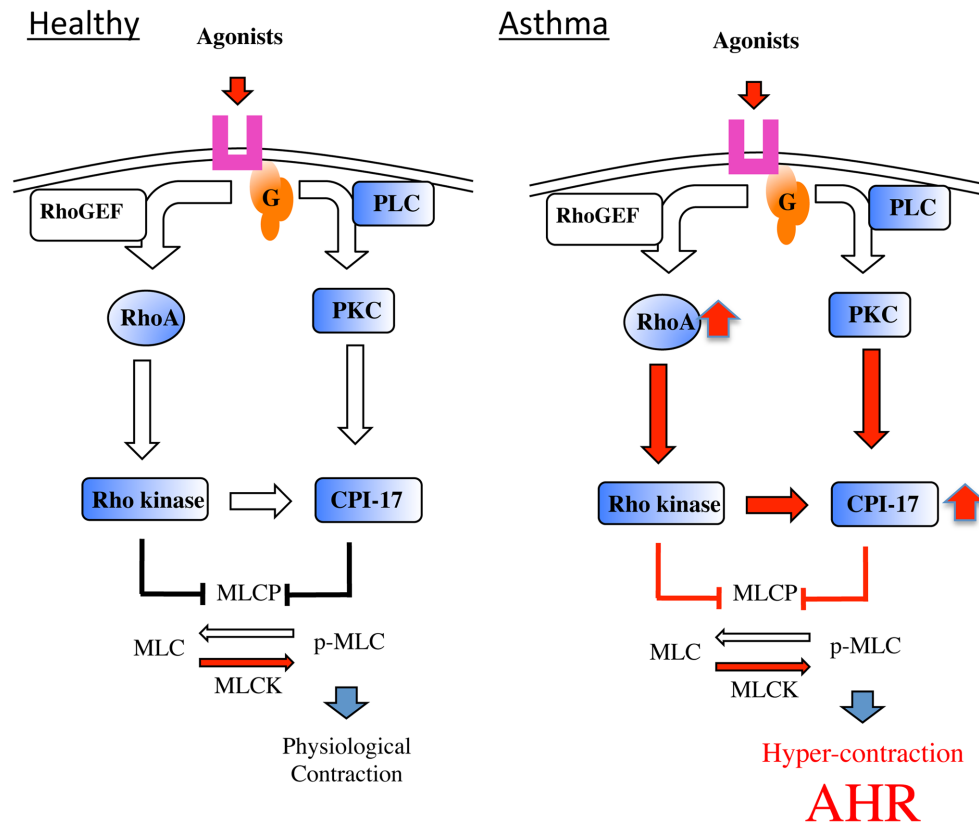
Fig. 1. Schematic diagram of the mechanism of upregulation of RhoA protein in BSM cells of asthmatics.

sible involvement of miRNAs in the contraction of vascular smooth muscle cells (60) and myometrial cells (61) by modulating gene expression.

The transcriptional/translational mechanism of RhoA is not well understood. However, it has been suggested that miR-133 negatively regulates RhoA expression in cardiomyocytes (62). RNA-hybrid analysis (<http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/>) (63) of human and mouse *RhoA* mRNA revealed putative binding sites of miR-133a in the 3'-untranslated region (64). Based on this information, we tested the hypothesis that downregulation of miR-133 could induce RhoA upregulation in BSMs. As a result, transfection of BSM cells with a miR-133a antagomir upregulated RhoA protein expression, whereas transfection with pre-miR133a downregulated it (64), suggesting that RhoA protein expression is negatively regulated by miR-133a in BSMs. Interestingly, our data also demonstrated that IL-13 directly acts on BSM cells to cause downregulation of miR-133a and upregulation of RhoA protein (54, 65). It is thus possible that the increased IL-13 level in the airways of asthmatics causes a downregulation of miR-133a, resulting in an upregulation of RhoA protein to induce the augmented BSM contractility, one of the causes of the AHR in allergic bronchial asthma. These findings provide new insight into the role of miR-133a in the BSM contractility and suggest that the miR-133a/RhoA pathway is a putative therapeutic target for asthma (Fig. 1).

### — Involvement of CPI-17 in smooth muscle contraction —

PKC-potentiated inhibitory protein for heterotrimeric MLCP of 17 kDa (CPI-17), which is activated by PKC and acts on an MLCP-specific target, was isolated from pig aorta smooth muscle extracts (66). CPI-17 expression is highly limited to smooth muscle tissues (67). When a contractile agonist binds to GPCR, PKC is



**Fig. 2.** Schematic diagram of the mechanism of BSM hyper-contraction, one of the causes of the airway hyperresponsiveness (AHR) in asthmatics.

activated via an increase in diacylglycerol (DG) and in turn phosphorylates CPI-17. Activated CPI-17 induces MLCP inhibition. CPI-17 is therefore important for the PKC-mediated  $\text{Ca}^{2+}$  sensitization in rabbit arterial smooth muscles (68, 69), human bladder smooth muscles (70), intestinal smooth muscles of rat (71) and mouse (72), human myometrium (73) and rat BSMs (74–76).

In BSM of AHR animals, the mRNA and protein levels of CPI-17 were significantly increased compared with the controls (77). Upon contractile agonist stimulation of hyperresponsive BSMs PKC/CPI-17 signaling activity increased by CPI-17 upregulation. Indeed, the phorbol 12,13-dibutyrate-mediated contraction was markedly augmented in BSMs of AHR animals (78). ACh-induced phosphorylation of CPI-17 at Thr38 was significantly increased in BSMs of AHR animals. Interestingly, pretreatment of AHR animals with Y-27632 or calphostin C, a PKC inhibitor, inhibited the ACh-induced phosphorylation of CPI-17 in bronchial tissues. Moreover, this pretreatment inhibited the ACh-induced phosphorylation of MLC (74). The inhibitory effects of Y-27632 and calphostin C on agonist-induced phosphorylation of CPI-17 have also been examined in rabbit femoral arterial smooth muscles (79). Treatment with glucocorticoids (prednisolone or beclomethasone) significantly inhibited the AHR, and markedly reduced both the protein and mRNA levels of CPI-17 in BSMs. The ACh-induced activation of CPI-17 was also significantly inhibited by glucocorticoids. Glucocorticoids prevented the augmented ACh-induced MLC phosphorylation observed in rat AHR (80). Therefore, glucocorticoids may inhibit AHR through inhibition of CPI-17 overexpression and activation (80). Although the transcription factors involved in CPI-17 expression in BSMs are unclear, an increase in the PKC/CPI-17-mediated signaling might be involved in the augmented BSM. Thus, in addition to the RhoA-mediated signaling described above, the CPI-mediated signaling is also a putative therapeutic target for the AHR in asthmatics (Fig. 2).

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## Conclusions

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We propose that both the upregulations of RhoA and CPI-17 are associated with increased BSM contraction in asthma. Therefore, MLCP inhibitory signaling pathways via RhoA/ROCK, PKC/CPI-17 and their combination may be potential targets for new treatment of AHR in asthma.

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## Conflict of interest

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The authors declare that they have no conflict of interest.

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