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Critical Review

RhoA, a Possible Target for Treatment of Airway Hyperresponsiveness in Bronchial Asthma

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Abstract. Airway hyperresponsiveness to nonspecific stimuli is one of the characteristic features of allergic bronchial asthma. An elevated contractility of bronchial smooth muscle has been considered as one of the causes of the airway hyperresponsiveness. The contraction of smooth muscles including airway smooth muscles is mediated by both Ca^{2+}-dependent and Ca^{2+}-independent pathways. The latter Ca^{2+}-independent pathway, termed Ca^{2+} sensitization, is mainly regulated by a monomeric GTP-binding protein, RhoA, and its downstream target Rho-kinase. In animal models of allergic bronchial asthma, an augmented agonist-induced, RhoA-mediated contraction of bronchial smooth muscle has been suggested. The RhoA/Rho-kinase signaling is now proposed as a novel target for the treatment of airway hyperresponsiveness in asthma. Herein, we will discuss the mechanism of development of bronchial smooth muscle hyperresponsiveness, one of the causes of the airway hyperresponsiveness, based on the recent studies using animal models of allergic bronchial asthma and/or cultured airway smooth muscle cells. The possibility of RhoA as a therapeutic target in asthma, especially airway hyperresponsiveness, will also be described.

Keywords: RhoA, bronchial smooth muscle, airway hyperresponsiveness, allergic bronchial asthma

1. Introduction

The dramatic increase in the number of asthma cases over the last decades is of great concern for public health in the world (1). Increased airway narrowing in response to nonspecific stimuli is a characteristic feature of human obstructive pulmonary diseases, including bronchial asthma. This abnormality is an important sign of the disease, although the pathophysiological variations leading to the hyperresponsiveness remain unclear. It has been suggested that one of the factors that contribute to the exaggerated airway narrowing in asthmatics is an abnormality of the properties of airway smooth muscle (2). Rapid relief from airway limitation in asthmatic patients by β-stimulant inhalation may also suggest an involvement of augmented airway smooth muscle contraction in the airway obstruction. Thus, it may be important for development of asthma therapy to understand changes in the contractile signaling of airway smooth muscle cells associated with the disease.

Asthmatic patients have an increased contractility of airway smooth muscle (3 – 6). Asthmatic animal models also have hyperresponsiveness of airway smooth muscles (7, 8). Similarly, an increased responsiveness of bronchial smooth muscle has been demonstrated in a rat model of airway hyperresponsiveness induced by repeated antigen inhalation (9 – 12). In this animal model of airway hyperresponsiveness, the bronchial smooth muscle contraction induced by receptor agonists such as acetylcholine (ACh), but not by high-K^+ depolarization, is markedly augmented (9 – 11). Similar results were also obtained in a mouse model of allergic bronchial asthma (13, 14). Moreover, it has also been demonstrated that muscarinic receptor density and antagonist affinity of airway smooth muscle are at normal levels (10). Thus, it is possible that the mechanisms responsible for the

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airway hyperresponsiveness exist, at least in part, in the downstream pathway of muscarinic receptor signaling, including agonist-mediated Ca^{2+} sensitization.

Increasing evidence suggest that RhoA, a monomeric GTP-binding protein, is a key protein in the contraction of smooth muscles, including airway smooth muscles. The signaling of RhoA and its downstream Rho-kinases are now considered as a therapeutic target of asthma (15 – 18). In the current brief review, we will discuss the mechanism of development of bronchial smooth muscle hyperresponsiveness, one of the causes of the airway hyperresponsiveness, based on the recent studies using animal models of allergic bronchial asthma and/or cultured airway smooth muscle cells. The possibility of RhoA as a therapeutic target in asthma, especially airway hyperresponsiveness, will also be discussed.

2. Smooth muscle contraction in normal state

Traditionally, smooth muscle contraction is mainly mediated by an increase in cytosolic Ca^{2+} via the activation of plasma membrane Ca^{2+} channels and/or Ca^{2+} release from sarcoplasmic reticulum (SR). When plasma membrane receptors coupled with heterotrimeric GTP-binding proteins (G proteins), such as G_q and $G_{12/13}$, are activated, agonist-receptor – G protein coupling occurs and the exchange of GDP for GTP in the α subunit of G protein is promoted. The binding of GTP to the α subunit induces a dissociation of the $\alpha\beta\gamma$ holomer to free, activated GTP-bound α subunit. Subsequently, the GTP-bound α subunit activates phospholipase C (PLC) to generate inositol 1,4,5-trisphosphate (IP_3), which binds to its receptors on SR, leading to Ca^{2+} release from the SR. The increased cytosolic Ca^{2+} forms the $4Ca^{2+}$-calmodulin – myosin light chain kinase (MLCK) complex and activates MLCK. The activated MLCK phosphorylates the 20-kDa myosin light chain (MLC), leading to smooth muscle contraction (Fig. 1).

In addition to the Ca^{2+}-dependent phosphorylation of MLC, MLC phosphorylation is also regulated by MLC phosphatase, Ca^{2+}-independently, and thus further contraction occurs, which is termed Ca^{2+} sensitization of smooth muscle contraction (19, 20). The agonist-induced Ca^{2+} sensitization of contraction has been demonstrated in various types of smooth muscles including airway ones (13, 21 – 26). The RhoA/Rho-kinase plays an important role in the regulation of MLC phosphatase activity. The MLC phosphatase removes phosphate from the phosphorylated MLC to induce smooth muscle relaxation. The MLC phosphatase is a holoenzyme and consists of three subunits: a 37-kDa catalytic subunit, a 20-kDa variable subunit, and a 110- to 130-kDa myosin-binding subunit. The myosin-binding subunit, when phosphorylated, inhibits the enzymatic activity of MLC phosphatase, allowing the light chain of myosin to remain phosphorylated, thereby promoting contraction. The Rho-kinase, a serine/threonine kinase, phosphorylates the myosin-binding subunit of MLC phosphatase, resulting in an

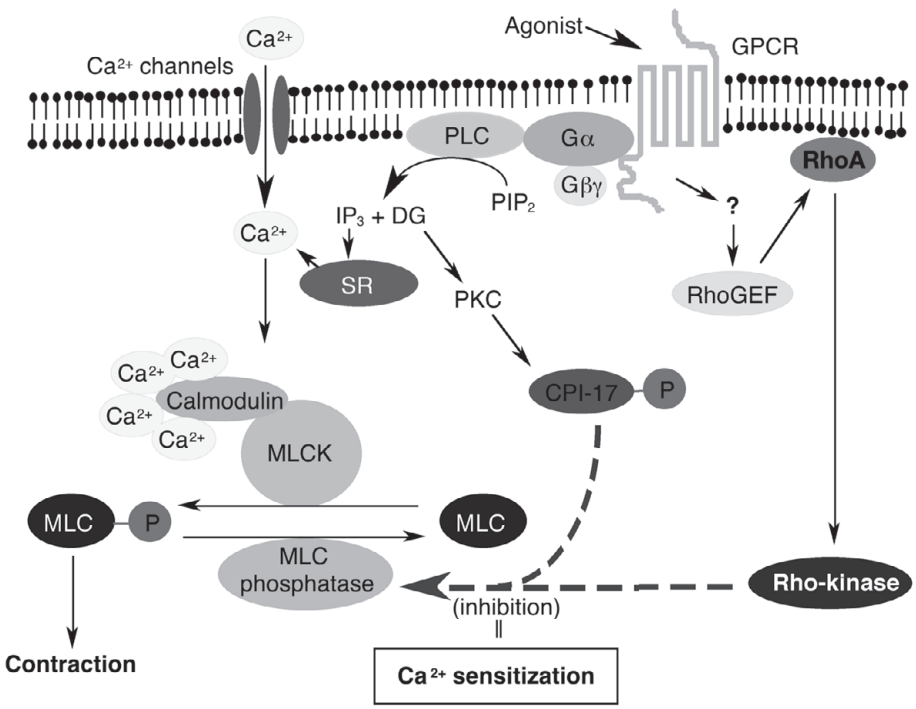

Fig. 1. Schematic diagram of the regulation of smooth muscle contraction. GPCR: G protein–coupled receptor, PLC: phospholipase C, PIP_2: phosphatidylinositol 4,5-bisphosphate, IP_3: inositol 1,4,5-trisphosphate, DG: diacylglycerol, SR: sarcoplasmic reticulum, PKC: protein kinase C, CPI-17: 17-kDa PKC–potentiated protein phosphatase 1 inhibitor protein, RhoGEF: RhoA guanine nucleotide exchange factor, MLC: myosin light chain, and MLCK: MLC kinase.

inhibition of its activity and thus promoting the phosphorylated state of the MLC (Fig. 1). Pharmacological inhibitors of Rho-kinase, such as Y-27632 and fasudil, block its activity by competing with the ATP-binding site on the enzyme and prevent RhoA-mediated MLC phosphatase inhibition, resulting in smooth muscle relaxation (27 – 29). In addition to the RhoA/Rho-kinase system, an involvement of protein kinase C (PKC) and its downstream, a 17-kDa PKC-potentiated protein phosphatase 1 inhibItor protein (CPI-17), in agonist-induced Ca^{2+} sensitization has also been suggested (20) (Fig. 1).

3. RhoA-mediated Ca^{2+} sensitization in diseased smooth muscles

In experimental animal models of several human diseases, an augmented RhoA/Rho-kinase-mediated Ca^{2+} sensitization in smooth muscle contraction has been reported. Uehata and colleagues (27) originally demonstrated an involvement of Rho-kinase signaling in the pathogenesis of hypertension. They showed that inhibition of Rho-kinase by Y-27632 reduced the elevated blood pressure in spontaneously hypertensive rats (SHR) and renal and deoxycorticosterone acetate-salt–induced hypertensive rats but not the normal blood pressure in normotensive control animals (27). Mukai and colleagues (30) reported an increase in Rho-kinase mRNA and activity in vascular smooth muscle of the SHR model. Furthermore, Seko and colleagues (31) demonstrated an augmented activation of RhoA, that is, an increase in GTP-RhoA level, in vascular smooth muscle of various hypertension models, including the SHR. In coronary arterial smooth muscle, Satoh and colleagues (23) firstly demonstrated an augmented agonist-induced, G protein–mediated Ca^{2+} sensitization in coronary vasospasm of the SHR model, although the involvement of RhoA/Rho-kinase signaling had not yet been identified. Then Shimokawa and colleagues (32) showed that hypercontraction and enhanced MLC phosphorylation induced by serotonin in a swine model of coronary artery spasm were inhibited by a Rho-kinase inhibitor, hydroxyfasudil, suggesting that the RhoA/Rho-kinase pathway plays a central role in the pathogenesis of coronary artery spasm. An upregulation of Rho-kinase by inflammatory stimuli, such as interleukin-1β and angiotensin II, was also demonstrated in the coronary artery smooth muscle (33, 34). The RhoA/Rho-kinase signaling is also remarkable in cerebral vasospasm. Experimental cerebral vasospasm induced by subarachnoid hemorrhage was accompanied by elevated Rho-kinase activity and phosphorylation of myosin phosphatase at its myosin-binding subunit (35). In the SHR model, cerebral vasodilation induced by Y-27632 was significantly greater than that in the normotensive control (36). It is thus possible that the RhoA/Rho-kinase–mediated signaling is the key for understanding the abnormal contraction of diseased vascular smooth muscles.

Abnormalities of the RhoA/Rho-kinase signaling have also been suggested in preterm labor and erectile dysfunction. During pregnancy, the uterus undergoes major functional and structural remodeling. The myometrium normally remains relatively quiescent but is able to generate powerful contractions at the time of parturition. Niiro and colleagues (37) reported an upregulation of RhoA/Rho-kinase associated with the augmented smooth muscle contractility in rat myometrium during pregnancy. Similar results have also been obtained in pregnant rabbit myometrium (38). Penile erection was induced by increased corpus cavernosum pressure resulting from increased blood flow into the penis, which is mediated by relaxation of the smooth muscle cells in the cavernosal arterioles and sinuses. Chitaley and colleagues (39) reported that Y-27632 increased corpus cavernosum pressure in an in vivo rat model, suggesting that RhoA/Rho-kinase–mediated Ca^{2+} sensitization of corpus cavernosum smooth muscle maintains the flaccid (contracted) state of penis. This is further supported by the other investigations (40 – 43). Interestingly, topical application of Y-27632 to the surface of the tunica albuginea or to the glans penis and surrounding skin was effective to cause penile erection in rats (44). Thus, the RhoA/Rho-kinase signaling pathway might also represent potential targets for the development of new treatments for preterm labor and erectile dysfunction.

4. Upregulation of RhoA in bronchial smooth muscle of experimental asthma

The Ca^{2+} sensitization of airway smooth muscle has been reported in canine (25), porcine (45), and rabbit tracheae (46) and human bronchus (46, 47). Likewise, we have also demonstrated that the Ca^{2+} sensitization is inherent in BSMs of rats (26) and mice (13), as determined by permeabilized muscle strips. Since the Ca^{2+} sensitization induced by ACh is sensitive to C3 exoenzyme (13, 26) and Y-27632 (29), the RhoA/Rho-kinase pathway is involved in the signaling. RhoA and Rho-kinases are also expressed in BSMs of these animals (13, 26, 29). Activation of RhoA by ACh stimulation has also been demonstrated in the bronchial smooth muscles of rats (48) and mice (13).

In animal models of allergic bronchial asthma (7, 8, 10, 26) and patients with asthma (3), an increase in airway smooth muscle responsiveness to muscarinic agonists has been demonstrated, whereas no change in the levels of plasma membrane receptors was observed (7, 8,

10). In addition, the agonist-induced increase in cytosolic Ca^{2+} level has been reported to be at the normal level even in hyperresponsive bronchial smooth muscles (49, 50). The findings remind us that the Ca^{2+} sensitization induced by agonist stimulation in bronchial smooth muscle might be elevated in airway hyperresponsiveness. We have previously demonstrated an augmented ACh-induced, RhoA-mediated Ca^{2+} sensitization of bronchial smooth muscle contraction in a rat model of allergic bronchial asthma (26). In this animal model, the ACh-induced Ca^{2+}-sensitizing effect, measured in permeabilized muscle strips under a constant Ca^{2+} concentration, was augmented. The augmented Ca^{2+} sensitization induced by ACh stimulation was almost completely blocked by *Clostridium botulinum* C3 exoenzyme, which ADP-ribosylates and inhibits RhoA protein activation. It is noteworthy that the expression level of RhoA protein was markedly increased in bronchial smooth muscles of the airway hyperresponsive rats (26). An upregulation of RhoA mRNA was also found in this animal model of allergic bronchial asthma (51). Similar findings have also been observed in a murine model of antigen-induced airway hyperresponsiveness (13, 52 – 54). It is thus possible that the change in the transcriptional regulation of the RhoA gene is one of the causes for the development of bronchial smooth muscle hyperresponsiveness in allergic asthma.

5. Transcriptional regulation of the RhoA gene

The RhoA gene encodes 193–amino acid proteins, and the sequence of these amino acids has been highly conserved during evolution (Fig. 2). The sequence of rat RhoA protein shows 99.5% and 100% sequence similarity with the human and mouse RhoA proteins, respectively. The rat RhoA protein shows 95% sequence similarity even with the RhoA protein of lower organisms such as *Xenopus* and zebrafish. This high homology indicates that RhoA is involved in a key biological pathway that is evolutionarily conserved. As compared to other regions, the RhoA C-terminus (amino acids 187 – 193) shows a low level of similarity between mammals and nonmammals (Fig. 2). The C-terminus of all Rho families is post-translationally processed by prenylation for membrane binding (55, 56). In addition, serine residue 188 (absent in nonmammalian RhoA) of RhoA is phosphorylated by several kinases (57 – 59). This phosphorylation leads to the suppression of Rho-mediated stress fiber formation or inhibition of the membrane translocation of RhoA (5). Thus, post-translational regulation is likely to be different between mammals and non-mammals.

The human and mouse RhoA genes are localized to chromosomes 3p21 and 9, respectively. RhoA mRNA is constitutively expressed in a wide variety of tissues and cells. Increased human RhoA mRNA and protein expression levels have been detected in tumor tissue from patients with hepatocellular carcinoma, and these levels were found to be significantly higher than those in the corresponding noncancerous liver tissue (60 – 62). Furthermore, overexpression of human RhoA in rat hepatoma MM1 cells promoted invasion of tumor cells (63). These results indicate that the physiological functions of RhoA are regulated not only by post-translational modification, but also by the mRNA expression level. Therefore, elucidation of the transcriptional regulation of the RhoA gene provides important insights regarding its physiological functions.

As described above, we have shown the upregulations of RhoA protein (26) and mRNA (51) in bronchial smooth muscle of rat experimental asthma. In addition,

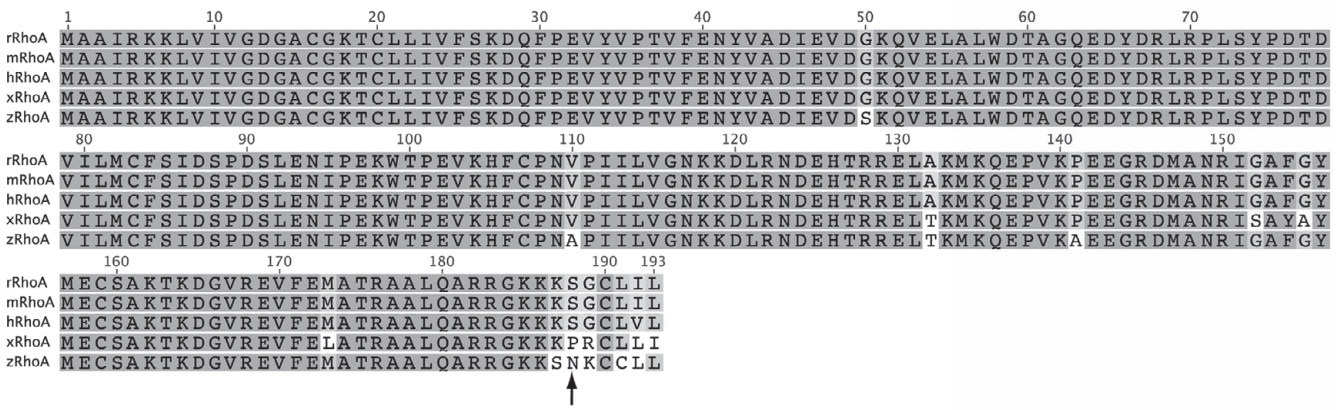

Fig. 2. Sequence alignment of RhoA proteins from several species. Abbreviations for species: r, rat; m, mouse; h, human; z, zebrafish; and x, *Xenopus tropicalis*. The arrow indicates the phosphorylated serine residue in mammalian RhoA. Accession numbers: rRhoA, NP476473; mRhoA, AAC23710.1; hRhoA, BAD96276; zRhoA, AAO65961; and xRhoA, AAD40671.1

our recent studies revealed that both IL-13 and TNF-α have abilities to cause the upregulations of RhoA protein and mRNA, resulting in the induction of bronchial smooth muscle hyperresponsiveness (52, 53, 64, 65). To elucidate the transcriptional mechanism involved in the induction of RhoA by IL-13 and TNF-α, we identified the promoter region of rat RhoA (66) (Fig. 3). The transcription start site (TSS) at the most 5′ end was −243 bp upstream of ATG and −66 bp upstream of the 5′ terminal in the published sequence of rat RhoA cDNA (GenBank accession number: BC061732.1). A typical TATA box was not found in the promoter region, but a GC box–like GC-rich region was observed within the region from −90 to +1 bp.

The rat RhoA promoter sequence showed high sequence similarity with the mouse and human RhoA promoter sequences (Fig. 3). Database analysis of the rat RhoA promoter region identified several putative transcription factor binding sites: Sp1, CCAAT/enhancer-binding protein (C/EBP), signal transducers and activator of transcription (STAT), CdxA, and nuclear factor-kappa B (NF-κB). Serially deleted human RhoA luciferase reporter assays revealed that the STAT-binding element, which corresponds to the region extending from −192 to −184 bp in the rat RhoA sequence, is responsible for the induction of promoter activity by IL-13 (64). The putative NF-κB–binding element on the human RhoA promoter, which corresponds to the region extending from −198 to −190 bp in the rat RhoA sequence, partially overlapped with the STAT-binding element (Fig. 3). The NF-κB–binding element was also responsible for induction of luciferase activity by TNF-α (64). It is noteworthy that the functional STAT- and NF-κB–binding sites on the human RhoA promoter were completely conserved in the rat promoter (Fig. 3). The conservation of both *cis*-elements between rats and humans appears to indicate common regulation of STAT and NF-κB by IL-13 and TNF-α.

Our previous studies revealed that IL-13, one of the major cytokines upregulated in the airways of asthmatics (67, 68), is capable of inducing RhoA upregulation via activations of STAT6 (52, 53, 64) and, interestingly, NF-κB (64, 69) in cultured human bronchial smooth muscle cells, and of induction of airway hyperresponsiveness in naive mice (52). Similarly, TNF-α is also capable of inducing RhoA upregulation and bronchial smooth muscle hyperresponsiveness (64, 70 – 73). It is thus possible that IL-13 and TNF-α, generated in the inflamed airways of asthmatics, directly act on the bronchial smooth muscle cells and activate their STAT6 and/or NF-κB, and then upregulate RhoA in the cells, resulting in an augmentation of the agonist-induced, RhoA-mediated contraction of bronchial smooth muscle (Fig. 4).

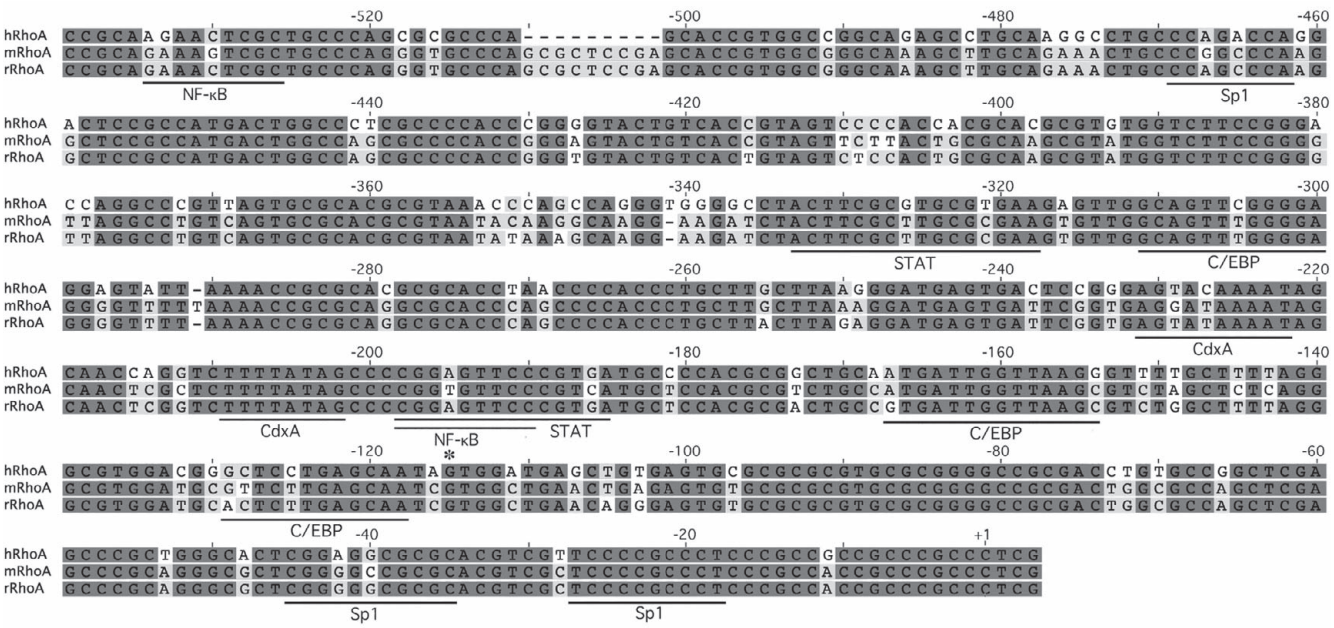

Fig. 3. Sequence alignment of the RhoA promoter regions of rats, mice, and humans. The previously reported transcriptional start site (TSS) of rat RhoA was numbered +1 (Ref. 66). The sequence numbering in the figure was estimated on the basis of the rat TSS (+1). The guanidine base that is marked with an asterisk in the human RhoA sequence was previously predicted to be the TSS (Ref. 64). Putative transcription factor binding sites have been detected by TFSEARCH (http://mbs.cbrc.jp/research/db/TFSEARCHJ.html) and underlined in the rat sequence.

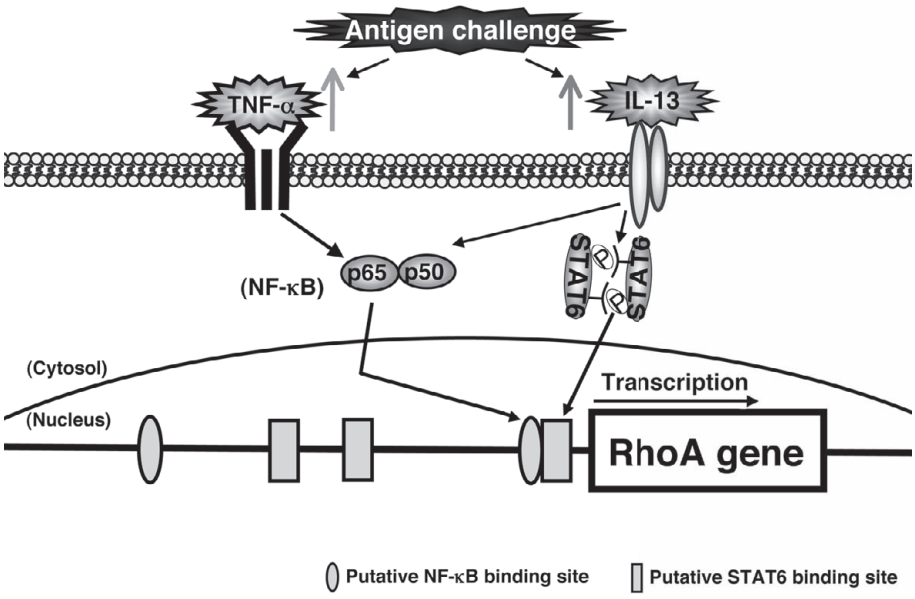

Fig. 4. Schematic model of the upregulation of RhoA in bronchial smooth muscle cells of allergic bronchial asthma. Antigen challenge causes the increases in inflammatory cytokines, such as interleukin-13 (IL-13) and tumor necrosis factor-α (TNF-α), in the airways. The IL-13 and TNF-α directly act on the bronchial smooth muscle cells and activate their transcription factors, signal transducer, and activator of transcription 6 (STAT6) and nuclear factor-kappa B (NF-κB). These transcription factors promote the transcription of RhoA gene and upregulate RhoA protein in the cells. Thus, the agonist-induced, RhoA-mediated contraction is augmented in bronchial smooth muscles of asthmatics.

6. Post-transcriptional regulation of the RhoA gene

There is increasing evidence that the microRNAs (miRNAs), a recently discovered class of small, noncoding, single-stranded RNAs, negatively modulate the expression of various genes (74, 75). The miRNAs bind complementarily to the 3′ untranslated region (UTR) of target mRNAs, resulting in mRNA cleavage and/or translation repression (74 – 76). In addition to the transcriptional regulation of the RhoA gene described above, a negative regulation of RhoA protein expression by miR-133a has recently been suggested in cardiomyocytes (77). The RNAhybrid analyses (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/) (78) of human and mouse mRNAs for RhoA revealed putative binding sites of miR-133a in their 3′-UTRs (54). Based on these information, we have recently tested the hypothesis that downregulation of miR-133 may be a cause of the upregulation of RhoA in bronchial smooth muscle.

As a result (54), an upregulation of RhoA protein was observed when the function of endogenous miR-133a was inhibited by its inhibitor, called antagomir-133a, in cultured human bronchial smooth muscle cells (hBSMCs). Conversely, the hBSMCs transfected with precursor miR-133a (pre-miR-133a) showed a downregulation of RhoA protein. On the other hand, neither antagomir-133b nor pre-miR-133b had significant effect on RhoA protein expression. These findings indicate that miR-133a, but not miR-133b, is an endogenous modulator of RhoA protein expression, and that RhoA protein expression is negatively regulated by endogenous miR-133a in hBSMCs.

Our previous studies revealed that IL-13, one of the major cytokines upregulated in the airways of asthmatics (67, 68), is capable of induction of RhoA protein upregulation in hBSMCs (52, 53) and of induction of airway hyperresponisveness in naive mice (52). IL-13 also caused an upregulation of RhoA mRNA and a downregulation of miR-133a in hBSMCs (54). The IL-13–induced upregulation of RhoA mRNA was abolished by coincubation with a STAT6 inhibitor, whereas, interestingly, this treatment had no effect on the downregulation of miR-133a induced by IL-13 (54). These observations suggest that IL-13 is capable of induction of RhoA protein upregulation both by increasing RhoA mRNA via a STAT6-dependent mechanism and by increasing RhoA translation via the STAT6-independent downregulation of miR-133a in hBSMCs (79).

Previous evidence that IL-13 is a key factor for induction of bronchial smooth muscle hyperresponsiveness and upregulation of RhoA protein in a mouse model of allergic bronchial asthma (52) reminds us that miR-133a might also be downregulated in bronchial smooth muscles of the diseased animals. To test this idea, we also determined the expression level of miR-133a in bronchial smooth muscle tissues of the repeatedly antigen-challenged mice and found that there is a downregulation of miR-133a in bronchial smooth muscle tissues of the antigen-challenged mice (54). Taken together, miR-133a might also negatively modulate the expression of RhoA gene in the mouse bronchial smooth muscle, and the downregulation of miR-133a might be a cause of upregulation of RhoA protein in the mouse model of allergic bronchial asthma. It is thus possible that introduction of synthetic miR-133a and/or its mimics specifically into the bronchial smooth muscles of asthmatics might cause

a downregulation of RhoA protein and an amelioration of bronchial smooth muscle hyperresponsiveness, resulting in a suppression of the airway hyperresponsiveness.

In addition to the miR-133a, recent studies also suggest that RhoA protein expression is negatively regulated by miR-155 (80) and miR-31 (81, 82) in cancer cells. Although their expression and function in smooth muscle cells have not been determined to date, whether these miRNAs also downregulate RhoA protein in the bronchial smooth muscle cells is of great interest.

7. Conclusion

In conclusion, it has been suggested that both an increase in the transcription and a decrease in the negative regulation of translation are the causes of the upregulation of RhoA protein in bronchial smooth muscle of allergic bronchial asthma. Since an importance of RhoA and its downstream Rho-kinases has also been demonstrated in the contraction of human bronchial smooth muscles (46), the upregulated RhoA protein would result in an augmentation of the bronchial smooth muscle contraction, one of the causes of the airway hyperresponsiveness. Thus, agents that decrease RhoA transcription, such as STAT6 inhibitors, and/or increase the negative translational regulation, such as synthetic miR-133a mimics, might be useful for the treatment of airway hyperresponsiveness. The issue remaining is targeting these agents specific to bronchial smooth muscle cells, since RhoA is ubiquitously expressed and has key roles in various types of cells.

Acknowledgments

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