

Up-regulation of Rac1 in the bronchial smooth muscle of murine experimental asthma

Yuki Kai¹ | Momoko Motegi¹ | Yuta Suzuki¹ | Hiroto Takeuchi¹ | Yui Harada¹ |
Fumiaki Sato² | Yoshihiko Chiba³ | Junzo Kamei¹ | Hiroyasu Sakai¹

¹Department of Biomolecular Pharmacology, School of Pharmacy, Hoshi University, Tokyo, Japan

²Department of Analytical Pathophysiology, School of Pharmacy, Hoshi University, Tokyo, Japan

³Department of Physiology and Molecular Sciences, School of Pharmacy, Hoshi University, Tokyo, Japan

Correspondence

Hiroyasu Sakai, Department of Biomolecular Pharmacology, School of Pharmacy, Hoshi University, Tokyo, Japan.
Email: sakai@hoshi.ac.jp

Funding information

MEXT-Supported Program for the Strategic Research Foundation at Private Universities, Grant/Award Number: S1411019

Abstract

There has been considerable research on the involvement of RhoA/Rho kinase signalling in smooth muscle contractions. However, only a few reports have addressed the specific role of Rac1, which is a member of the Rho GTPase superfamily. Therefore, this study investigated the role of Rac1-related pathways in bronchial smooth muscle (BSM) contractions. Bronchial rings isolated from mice were suspended in an organ bath, and the isometric contractions of circular smooth muscles were monitored. The phosphorylation of myosin light chains (MLCs) was analysed by immunoblotting. The Rac1 inhibitor EHT1864 inhibited carbachol (CCh)-induced BSM contractions, although high K⁺ depolarization-induced BSM contractions were not significantly attenuated by EHT1864. Moreover, high K⁺- and phorbol 12,13-dibutyrate (PDBu; PKC activator)-induced contractions were not attenuated by Rac1 inhibition, whereas sodium fluoride (NaF)-induced force development was inhibited by EHT1864. The gene and protein expression of Rac1 was increased in the BSM of a murine model with antigen-induced airway hyper-responsiveness (AHR). In addition, an increased force of the BSM contractions in AHR was suppressed by EHT1864 treatment, suggesting that the up-regulation of Rac1 is involved in AHR. These findings suggest that an increase in Rac1-mediated signalling is involved in the augmented contractions of BSMs in antigen-induced AHR mice.

1 | INTRODUCTION

The contractile response of bronchial smooth muscles (BSMs) is primarily mediated by the phosphorylation of myosin light chains (MLCs). Recent studies have demonstrated that smooth muscle contractions are regulated by both Ca²⁺-dependent and Ca²⁺-independent pathways. The Ca²⁺-dependent pathway is triggered by a Ca²⁺ influx into the cytoplasm. The binding of up to four Ca²⁺ ions with one calmodulin (CaM) molecule activates CaM/myosin light chain kinase (MLCK), which results in the phosphorylation of MLCs. Conversely, the Ca²⁺-independent pathway can elicit contractions in the absence of changes in intracellular Ca²⁺ levels.

Both RhoA/Rho kinase and protein kinase C (PKC)/17-kDa PKC-potentiated inhibitory protein of protein phosphatase I (CPI-17) pathways induce smooth muscle contractions mediated by Ca²⁺-independent pathways.¹ The RhoA/Rho kinase pathway induces the phosphorylation of MLCs by inhibiting myosin light chain phosphatase (MLCP). Because GTPγS, a non-hydrolysable GTP analogue that activates G proteins, inhibits the activity of MLCP, G proteins are implicated in sensitization. RhoA is activated by Rho guanine nucleotide exchange factors (RhoGEFs) when the GDP-bound form of RhoA is converted to its GTP-bound form.²

Bronchial asthma is a chronic respiratory disorder affecting the airways and is characterized by variable and recurring

symptoms. It is usually associated with airflow obstruction, inflammation and bronchial hyper-responsiveness.³ The role of airway inflammation and hyper-responsiveness in causing bronchial asthma was originally suggested by the correlation between the extent of airway inflammation and the severity of the illness.⁴ Therefore, a deep understanding of the fundamental mechanisms of airway inflammation and bronchial hyper-responsiveness is essential in the development of novel medical treatments for asthma. We have previously demonstrated that repeated antigenic challenge induces a distinct airway hyper-responsiveness (AHR) in the BSM of actively sensitized rats and mice.⁵

Acetylcholine (ACh)-induced contractions in the permeabilized BSM preparations of antigen-induced airway hyper-responsive rats were inhibited by the RhoA inhibitor C3 toxin. In addition, RhoA is reportedly activated by ACh stimulation in BSM preparations. Furthermore, CPI-17 phosphorylation induced by ACh was increased in the BSM of AHR rats. In these studies, we have demonstrated that an increase in the force of the contractions induced by the stimulation of GPCRs in the BSM of AHR rats is primarily mediated by both Ca^{2+} -independent and Ca^{2+} -dependent pathways; however, the Ca^{2+} -dependent pathway is not changed.⁵

The RhoGTPase superfamily can be categorized primarily into three subfamilies: Rho, Rac and Cdc42. These subfamilies mediate a range of cytoskeletal, cell motility and other cellular responses.⁶ The augmentation of RhoA-mediated Ca^{2+} sensitization in smooth muscle contractions has been observed in the animal models of diseases such as hypertension,⁷ coronary artery spasm⁸ and cerebral vasospasms.⁹ There are few reports focusing on the role of Rho superfamily members other than RhoA in smooth muscle contractions; however, Rac1 signalling appears to be involved in aortic smooth muscles contractions in rats.¹⁰ In rat BSMs, Rac1 is involved in the Ca^{2+} -sensitive MLCP pathway,¹¹ which mediates muscle contractions. Moreover, the inhibition of Rac1 prevents bronchoconstriction and AHR.¹² In the current study, we investigated the role of Rac1 in the BSM contractions of AHR mice.

2 | MATERIALS AND METHODS

2.1 | Animals

Male BALB/c mice (7–8 weeks old, Tokyo Laboratory Animals Science Co., Ltd., Tokyo, Japan) were used in this study. All procedures using animals were carried out according to protocols approved by the Animal Care Committee of the Hoshi University (Tokyo, Japan; accreditation No. 30-091).

2.2 | Functional studies

The isolation of left BSM and suspension in organ baths were performed as described previously.¹³ Fifteen minutes

after the last wash, an ascending concentration series of carbachol (CCh) was applied; each consecutive concentration was administered once a plateau response to the previous concentration was attained. To evaluate the effect of Rac1 inhibitor on CCh-induced BSM contractions, tissues were treated with EHT1864 (10 $\mu\text{mol/L}$; Cayman Chemical, MI, USA) for 20 minutes. before the successive applications of 30 nmol/L (3×10^{-8} mol/L)–100 $\mu\text{mol/L}$ (10^{-4} mol/L) CCh. To evaluate the effects of Rac1 inhibitor on high K^{+} depolarization-induced BSM contractions, tissues were treated with EHT1864 (10 $\mu\text{mol/L}$) for 20 minutes. before the successive applications of 10, 30 and 60 mmol/L KCl. To measure the contractile responses to phorbol 12,13-dibutyrate (PDBu) with 60 mmol/L K^{+} -treatment, the responsiveness of BSM to high K^{+} was also measured by applying an isotonic preparation of 60 mmol/L K^{+} . After the contraction reached a plateau, 1 $\mu\text{mol/L}$ of the direct PKC activator PDBu was administered. Thereafter, 3 $\mu\text{mol/L}$ of the PKC inhibitor GF109203X was administered. In the presence of atropine (1 $\mu\text{mol/L}$), force development was observed in response to all constriction-inducing agents administered, with the exception of CCh. The Rac1 inhibitor EHT1864 reportedly inhibits Rac1 activation selectively in vitro,¹⁴ and 10 $\mu\text{mol/L}$ EHT1864 is used by many studies.^{10–12,14–16} Therefore, 10 $\mu\text{mol/L}$ EHT1864 was used in this study.

2.3 | Western blotting

Western blotting was performed as described previously.¹¹ Briefly, the samples were homogenized with T-PERTM Tissue Protein Extraction Reagent (Pierce). After the samples (20 μg) were subjected to 15% SDS-polyacrylamide gel electrophoresis, Western blot was performed. The membranes (polyvinylidene difluoride membranes) were incubated with the following primary antibodies: rabbit anti-p-MLC (Ser19, 1:1000 dilution; Cell Signalling Technology, MA, USA), rabbit anti-MLC (1:1000; Cell Signalling Technology, MA, USA), rabbit anti-MYPT1 (1:1000, Cell Signalling Technology, MA, USA), rabbit phospho-MYPT1 (Thr696) antibody (1:1000, Cell Signalling Technology, MA, USA), rabbit phospho-MYPT1 (Thr853, 1:1000, Cell Signalling Technology, MA, USA) or anti-Rac1 (1:1000, Merck Millipore, MA, USA). Membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin (Ig) G (1:5000 dilution; GE healthcare) and detected with an ECL system. The ratio of corresponding p-MLC/MLC or p-MYPT/MYPT was calculated as an index of p-MLC or p-MYPT.

2.4 | Rac1 pull-down assay

The pull-down assay was performed as described previously.¹¹ Briefly, bronchial tissues were isolated as described above and equilibrated in oxygenated Krebs-Henseleit

solution at 37°C for 1 hour. After the equilibration period, tissues were stimulated with 1 µmol/L CCh for 15 minutes, quickly frozen with liquid nitrogen and lysed in lysis buffer with the following composition: 25 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 1% IGEPAL CA-630, 10.0 mmol/L MgCl₂, 1.0 mmol/L EDTA and 2% glycerol. Active Rac1 in tissue lysates (200 µg protein) was precipitated with 22 µg of GST-tagged Rac1-binding domain [amino acid residues 67-150 of human p21-activated protein kinase-1 (PAK-1); Millipore, Billerica, MA, USA], which was expressed in *Escherichia coli* and bound to glutathione-agarose beads. Precipitates were washed three times in lysis buffer. Samples were mixed with SDS loading buffer and boiled for 4 minutes. Bound proteins were resolved on 5%-20% polyacrylamide gels, transferred to PVDF membranes and immunoblotted with an anti-Rac1 antibody (Millipore).

2.5 | RT-PCR analysis

RT-PCR was performed as described previously.¹⁷ Briefly, total RNA was extracted from each sample with a one-step guanidinium thiocyanate-phenol-chloroform extraction procedure using TRI Reagent™ (Sigma-Aldrich, Saint Louis, MO, USA). cDNA was prepared from total RNA (1.0 µg) with a QuantiTect® Reverse Transcriptase kit (Qiagen, Hilden, Germany) after incubation with gDNA wipeout buffer at 42°C for 2 minutes to remove contaminating genomic DNA. Reaction mixtures (3 µL) were subjected to PCR (50 nmol/L forward and reverse primers; Fast SYBR Green Mastermix; Applied Biosystems, Foster City, CA, USA) in a final volume of 10 µL. The PCR primer sets used are shown in Table 1. The thermal cycle profile used was (a) denaturing for 30 seconds at 95°C and (b) annealing for 30 seconds at 60°C. PCR amplification was done for 40 cycles. Data are expressed as expression relative to that of mRNA for the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using the $2^{-\Delta\Delta CT}$ method.

2.6 | Sensitization and antigenic challenge

Sensitization and antigenic challenges for models of mice with allergic bronchial hyper-responsiveness were performed as described previously.¹⁸ In brief, BALB/c mice (8 weeks

of age) were actively sensitized by intraperitoneal injections of 8 µg ovalbumin (OA; Seikagaku Co., Tokyo, Japan) with 2 mg Imject Alum (Pierce Biotechnology, Inc, Rockford, IL, USA) on Day 0 and Day 5. The sensitized mice were challenged with aerosolized OA-saline solution (5 mg/mL) for 30 minutes on Days 12, 16 and 20. A control group (sensitized control; SC) of mice received the same immunization procedure but inhaled saline aerosol instead of OA challenge. The aerosol was generated with a compressor nebulizer (MiniElite™: Philips Respironics, NV, USA) and introduced to a Plexiglas chamber box (130 × 200 mm, 100 mm height) in which the mice were placed. Twenty four hours after the last OA challenge, the mice were killed by exsanguination from abdominal aorta under anaesthesia.

2.7 | Statistical analysis

Results are expressed as mean ± SEM. After a one-way ANOVA was performed to evaluate overall between-group differences, the Bonferroni post hoc test was performed to test for differences between individual groups using GraphPad Prism 5 for Max OS X (GraphPad Software, Inc, CA, USA). The indicators of statistical significance and error bars on the response curves were generated using GraphPad Prism 5 for Max OS X. P values <0.05 were considered to be statistically significant.

This study was conducted in accordance with the Basic & Clinical Pharmacology & Toxicology policy for experimental and clinical studies.¹⁹

3 | RESULTS

CCh-induced contractions are attenuated by EHT1864, a Rac1 inhibitor in murine BSM (Figure 1A). To test whether Rac1 modulates CCh-induced contractions by activating the Ca²⁺-dependent and/or Ca²⁺-independent pathways, we examined the effect of Rac1 inhibition on high K⁺-induced contractions. In the contractions induced by high K⁺, the K⁺ equilibrium potential is changed, resulting in the activation of L-type Ca²⁺ channels; contractions are then regulated solely by increases in the cytosolic Ca²⁺ concentration.²⁰ The contractions induced by high K⁺ via the Ca²⁺/calmodulin(CaM)/MLCK pathway

TABLE 1 PCR primers used in the present study

Accession number		Primer		Product size (base pairs)
		Deoxyribonucleotide sequences		
GAPDH	NM_008084.2	Forward	CCTCGTCCCGTAGACAAAATG	100
		Reverse	TCTCCACTTTGCCACTGCAA	
Rac1	NM_009007.2	Forward	AGTTGCGTTGTGCTGAGAACA	80
		Reverse	CTCCAGAAGCTGCGGTCTTC	

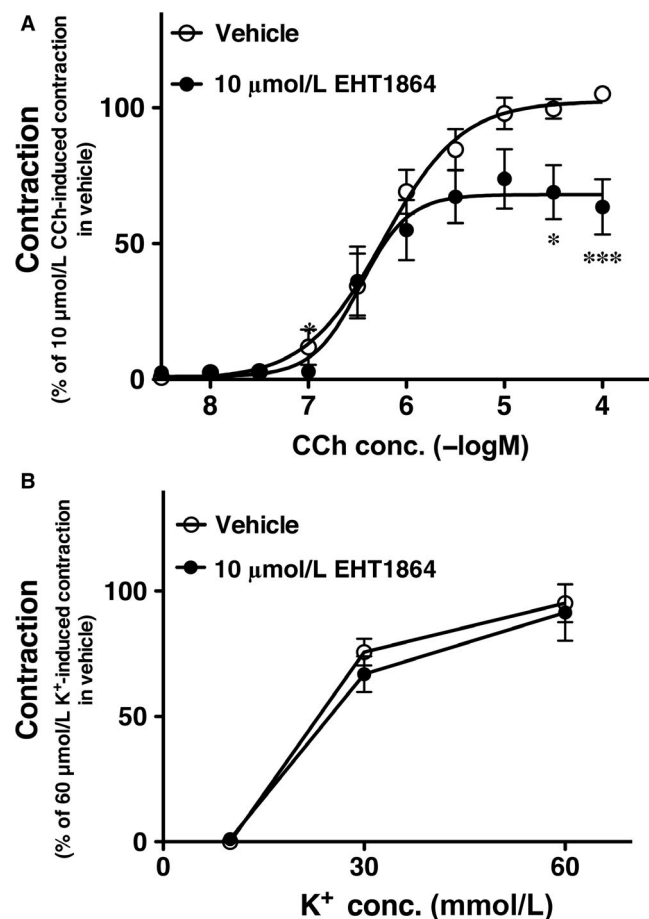


FIGURE 1 Effect of EHT1864, a Rac1 inhibitor, on carbachol (CCh) and high K⁺ depolarization-induced contractions in bronchial smooth muscles (BSMs) of the mouse. The CCh concentrations are expressed as $-\log M$. CCh (A) and high K⁺ (B) responsiveness in the absence or presence of EHT1864. The strength of the contractions is expressed as percentages in response to 10 $\mu\text{mol/L}$ CCh. All values are depicted as mean $\pm$ SEM of 6 independent experiments, respectively. * $P < 0.05$ and *** $P < 0.001$ vs vehicle

were not affected by 10 $\mu\text{mol/L}$ EHT1864 (Figure 1B). These data suggest that the inhibition of Rac1 does not alter the Ca²⁺-dependent pathway of BSM contractions. This finding supports our previous observations regarding the BSM of rats.¹¹

As a result of pull-down assay to examine whether activation of Rac1 is induced by CCh, stimulation by CCh induces activation of Rac1 (GTP-Rac1) and this CCh-induced Rac1 activity was inhibited by the application of EHT 1864 (Figure 2A). Next, we examined whether MLC phosphorylation was modulated by Rac1 signalling. The MLC phosphorylation induced by CCh was significantly attenuated by EHT1864 (Figure 2B and C). These data indicate that MLC phosphorylation in CCh-induced contractions in the BSM of mice is mediated by Rac1 signalling.

We observed that Rac1 signalling is involved in a Ca²⁺-independent pathway in rat BSM contractions. The PKC/CPI-17 pathway is known as one such pathway.²¹ We have previously

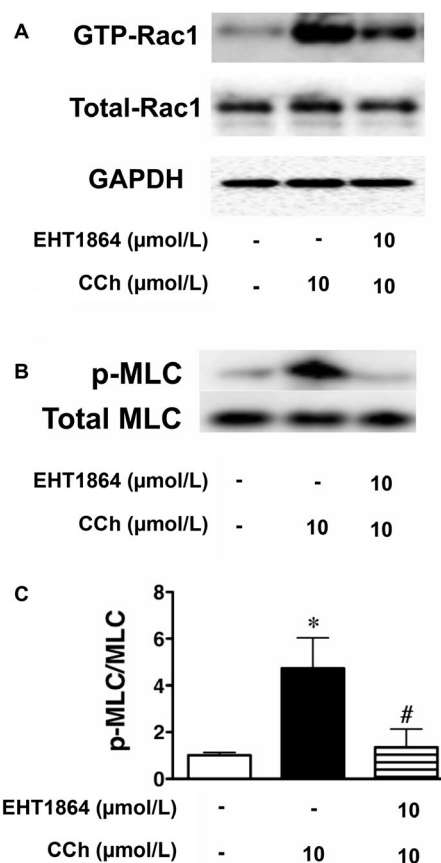


FIGURE 2 Effects of EHT1864 on CCh-induced Rac1 activation (GTP-Rac1) and myosin light chain (MLC) phosphorylation. The active form of Rac1, GTP-bound Rac1, in murine BSM was measured by a Rac1 pull-down assay (A). Photographs show typical results of Western blot analysis of MLC and phospho-MLC with EHT1864 (B). The data are summarized in panels (C). Values are depicted as mean $\pm$ SEM of 5 independent experiments, respectively. * $P < 0.05$ vs vehicle; # $P < 0.05$ vs CCh stimulation

found that PDBu-induced contractions increased contractions with high K⁺-depolarizing stimulation, although force development was not induced in murine BSM following the application of PDBu alone.²² This response was abolished by the subsequent application of the PKC inhibitor GF109203X (3 $\mu\text{mol/L}$; data not shown). High K⁺- and PDBu-induced contractions were not blocked by Rac1 inhibitor (Figure 3A). These data suggest that the inhibition of Rac1 does not inactivate the MLCP pathway via activation of the PKC/CPI-17 pathway. NaF, a trimeric G-protein activator,²³ is also known to be an important factor in MLCP pathways. NaF activates RhoA pathways and thus activates a Ca²⁺-independent pathway, thereby increasing MLC phosphorylation.²⁴ Rac1 inhibitor (10 $\mu\text{mol/L}$ EHT1864) significantly inhibited NaF-induced contractions (Figure 3B). It is possible that Rac1 itself and/or Rac1 signalling was activated by NaF stimulation.

Figure 4 demonstrates the CCh responsiveness of the BSMs isolated from sensitized control and repeatedly antigen-challenged mice. The contractile responsiveness to CCh of BSM

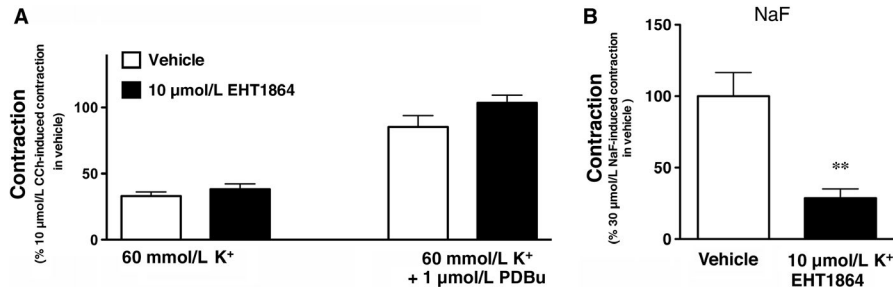


FIGURE 3 Effect of EHT1864 on PKC activation and NaF-induced contraction in the BSM of the mouse. Force development was not induced by the application of PDBu alone in murine BSM (data not shown). Nevertheless, PDBu-induced contraction induced force with high K^+ -depolarizing stimulation. High K^+ - and high K^+ + PDBu-induced contraction in the presence of EHT1864 or vehicle (A). Effects of EHT1864 on NaF-induced contractions in BSM of mouse. NaF-induced contraction in the presence of 10 EHT1864 $\mu\text{mol/L}$ (B) Values are depicted as mean $\pm$ SEM of 5 independent experiments, respectively. ** $P < 0.01$ vs vehicle

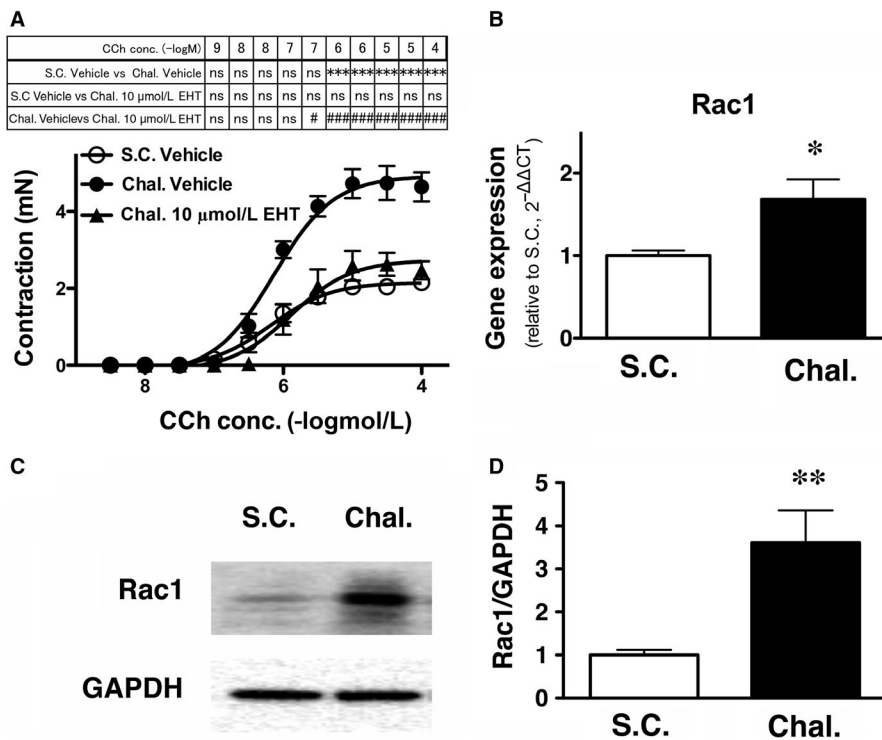


FIGURE 4 Effect of EHT1864 on CCh-induced contractions in the BSM of repeated antigen-challenged mice (A). The contractions are represented in mN of tension. Values are depicted as mean $\pm$ SEM of 5 independent experiments, respectively. *** $P < 0.001$ vs SC vehicle; # $P < 0.05$ and ### $P < 0.001$ vs Chal. vehicle. Changes in mRNA (B) and protein (D) levels of Rac1 in the BSM of repeated antigen-challenged mice (Chal.) and SC mice. Typical photograph of the protein expression of Rac1 (C). Values are depicted as mean $\pm$ SEM of 4 independent experiments, respectively. * $P < 0.05$ and ** $P < 0.01$ vs S.C.

from antigen-challenged mice (Chal.) was significantly increased compared with that of sensitized control (SC) mice (Figure 4A). Increased CCh-induced contractions in the BSM of AHR mice were inhibited to the levels of contractile response of the SC by EHT1864 treatment (Figure 4A). No significant differences in the high K^+ -induced BSM contractions were observed between the SC and repeatedly antigen-challenged mice. The contractions induced by high K^+ depolarization were not affected by antigen challenge or by EHT1864 treatment (data not shown).

We evaluated Rac1 expression in the BSM of AHR mice. Rac1 expression was significantly increased by antigen challenge. The protein and mRNA levels of Rac1 were also up-regulated in the antigen-challenged group (Chal.) (Figure 4B-D).

MLCP holoenzyme activity is decreased by the phosphorylation of its regulatory subunit MYPT1 at two sites: Thr696 and Thr853.²⁵ Therefore, we examined the effect of Rac1 inhibition on the phosphorylation of these inhibitory sites. The phosphorylation of MYPT1 by CCh at Thr853, a Rho kinase-specific site,²⁶ but not at Thr696, was significantly increased in the BSM of the Chal. group compared with the SC groups. This increased phosphorylation was attenuated by EHT1864 in both the groups (Figure 5A, B and C). As well as phosphorylation of MYPT1 at Thr853, phosphorylation of MLC is increased in the BSM of the Chal. group compared with that of the SC group (Figure 5D). These data suggest that Rac1 affects RhoA/Rho kinase signalling in BSM contractions.

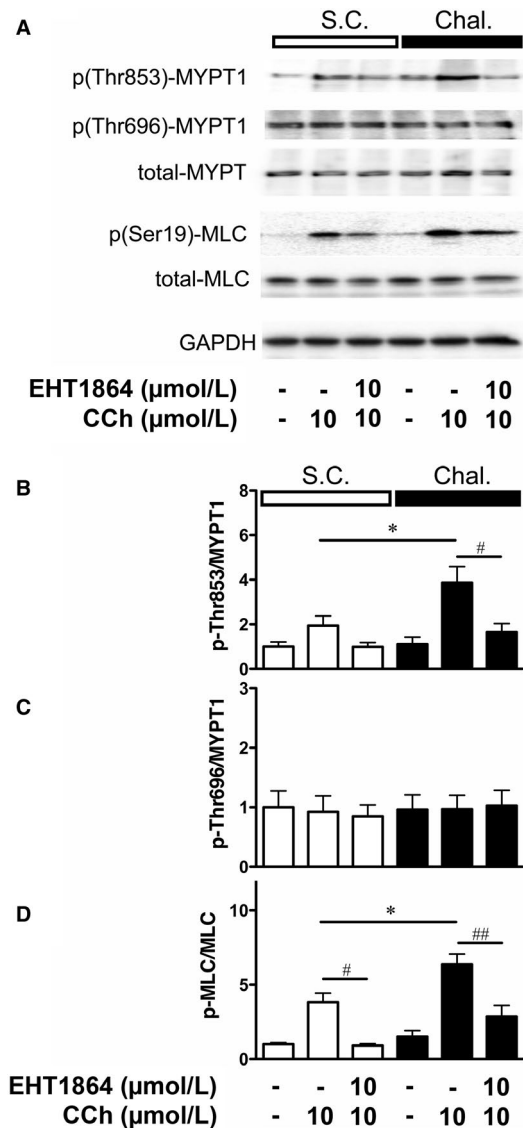


FIGURE 5 Effect of EHT1864 on CCh-induced phosphorylation of myosin phosphatase targeting protein (MYPT) and MLC on repeated antigen-challenged mice. Typical photograph of the phosphorylation of MYPT at Thr853, Thr696, MLC and GAPDH in the BSM of repeated antigen-challenged (Chal.) and SC mice (A). These data are summarized in B-C. Values are depicted as mean $\pm$ SEM of 5 independent experiments, respectively. * $P < 0.05$ (vs SC), # $P < 0.05$ and ## $P < 0.01$ (CCh vs EHT + CCh)

4 | DISCUSSION

Our results greatly extend our understanding of the role of Rac1 in murine BSM contractions. The Rac1 pathway appears to modulate CCh-induced BSM contractions. Conversely, we found that contractions induced by high K^+ depolarization were not attenuated by the Rac1 inhibitor EHT1864. Moreover, contractions induced by the activation of PKC were not attenuated by EHT1864. These results suggest that the inhibition of Rac1 does not inhibit the Ca^{2+} /CaM/MLCK and PKC/CPI-17/MLCP pathways (Figure 6). Increased

CCh-induced contractions in the BSM of AHR mice were inhibited by EHT1864. The mRNA and protein expressions of Rac1 were also increased in AHR mice.

Rac1 reportedly regulates smooth muscle contractions by inactivating the MLCP pathway in rabbit femoral arterial smooth muscle¹⁰ and rat BSM.¹¹ In the rabbit femoral artery, Rac1 inhibition decreases the PKC-related phosphorylation and hence the activation of CPI-17.¹⁰ However, PKC activation-induced BSM contractions were not inhibited by EHT1864 in the present study. These observations are consistent with the pattern of results observed in BSM contractions in rats,¹¹ that is they strongly support the notion that Rac1 signalling has a different pathway in arterial smooth muscle and BSM. NaF-induced contractions were attenuated by a Rac1 inhibitor, EHT1864. In the present study, the CCh-induced phosphorylation of MYPT1 at Thr853, a Rho kinase-specific site in murine BSM, was increased in the Chal. group. The phosphorylation was inhibited by EHT1864 (Figure 6). These results indicate that Rac1 signalling contributes to RhoA/Rho kinase signalling; however, further studies are warranted to clarify the signalling mechanisms in detail.

Our previous studies indicated that the contractile GPCR agonist-induced Ca^{2+} sensitization of contraction is strongly augmented in the BSMs of animal models of antigen-induced AHR. In the BSMs of these animal models, both RhoA and CPI-17 protein expression were markedly increased, resulting in the augmentation of Ca^{2+} sensitizing pathways (Figure 6).⁵ It has been demonstrated that incubation of BSM cells with asthma-associated cytokines, such as interleukin-13 (IL-13), IL-17 and tumour necrosis factor- α (TNF- α), caused up-regulations of RhoA in BSM cells of naive animals and cultured human BSM cells.⁵ Since it is not clear how Rac1 expression is up-regulated in BSM of asthmatic state, further research is necessary.

Recently, André-Grégoire *et al*¹² demonstrated that Rac1 activation is caused by CCh stimulation in murine and human airway smooth muscle cells. The Rac1 activation induced by CCh acts via phospholipase (PLC) β 2 to induce intracellular Ca^{2+} signalling and hence contraction. This report summarizes our understanding of the mechanisms of that essential role of Rac1 in the regulation of intracellular Ca, contraction of airway smooth muscle cells and the development of AHR. However, we have demonstrated that Ca^{2+} -induced contraction does not produce changes in BSM contractions in an antigen-induced AHR rodent model.⁵ Although it is unknown whether these effects occur in humans, treatments targeting not only RhoA and CPI-17 but also Rac1 signalling may be effective against asthma (Figure 6).

In conclusion, we here have suggested that Rac1 regulates the contractions of murine BSM, and Rac1 signalling was involved in increased BSM contraction of AHR mouse, although further research of Rac1 signalling will be necessary

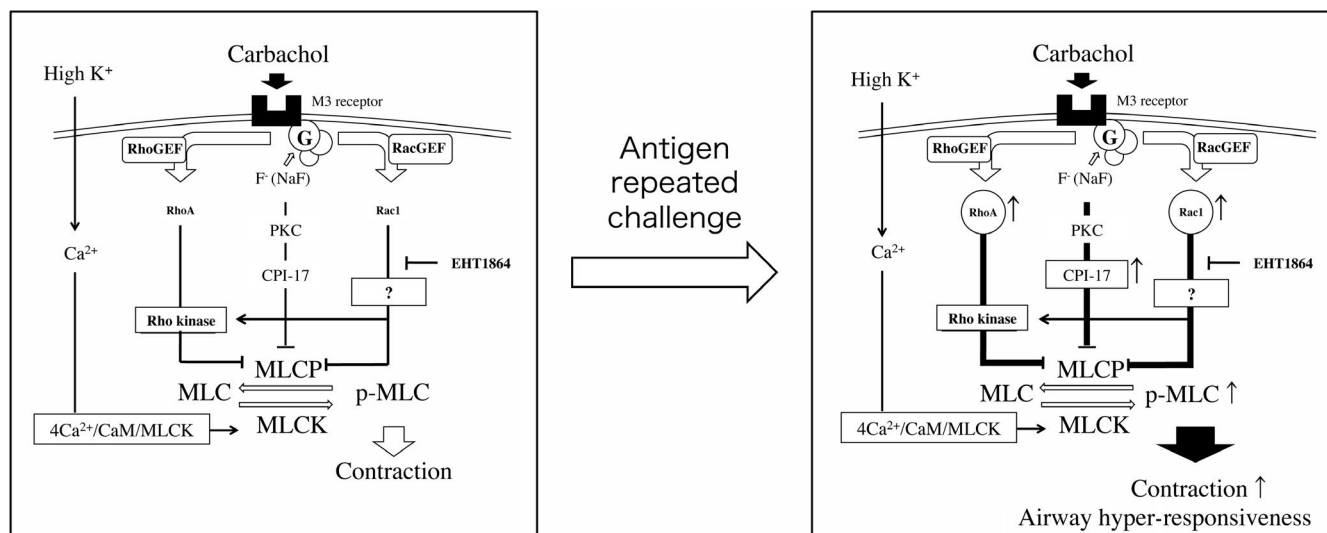


FIGURE 6 Simplified schematic illustration of the up-regulated Rac1 signalling pathway in carbachol-induced BSM contraction of repeated antigen-challenged mice. These pathways are discussed in the Discussion section

to validate a novel therapeutic target for obstructive respiratory diseases such as asthma.

ACKNOWLEDGEMENTS

This work was supported by the MEXT-Supported Program for the Strategic Research Foundation at Private Universities (S1411019). The authors would like to thank Enago (www.enago.jp) for the English language review.

CONFLICT OF INTEREST

The authors declare no conflict of interest. H. Sakai, Ph.D., has compiled a full disclosure statement that accurately reflects what each author has disclosed.

REFERENCES

- Wright DB, Tripathi S, Sikarwar A, Santosh KT, Perez-Zoghbi J, Ojo OO, et al. Regulation of GPCR-mediated smooth muscle contraction: implications for asthma and pulmonary hypertension. *Pulm Pharmacol Ther.* 2013;26:121-131.
- Hart MJ, Jiang X, Kozasa T, Roscoe W, Singer WD, Gilman AG, et al. Direct stimulation of the guanine nucleotide exchange activity of p115 RhoGEF by Gα13. *Science* 1998;280:2112-2114.
- Walter H, Sadeque-Iqbal F, Ulysse R, Castillo D, Fitzpatrick A, Singleton J. The effectiveness of school-based family asthma educational programs on the quality of life and number of asthma exacerbations of children aged five to 18 years diagnosed with asthma: a systematic review protocol. *JBI Database System Rev Implement Rep.* 2015;13:69-81.
- Chapman DG, Irvin CG. Mechanisms of airway hyper-responsiveness in asthma: the past, present and yet to come. *Clin Exp Allergy.* 2015;45:706-719.
- Sakai H, Suto W, Kai Y, Chiba Y. Mechanisms underlying the pathogenesis of hyper-contraction of bronchial smooth muscle in allergic asthma. *J Smooth Muscle Res.* 2017;53:37-47.
- Sadok A, Marshall CJ. Rho GTPases: masters of cell migration. *Small GTPases.* 2014;5:e29710.
- Seko T, Ito M, Kureishi Y, Okamoto R, Moriki N, Onishi K, et al. Activation of RhoA and inhibition of myosin phosphatase as important components in hypertension in vascular smooth muscle. *Circ Res.* 2003;92:411-418.
- Kandabashi T, Shimokawa H, Miyata K, Kunihiro I, Kawano Y, Fukata Y, et al. Inhibition of myosin phosphatase by upregulated rho-kinase plays a key role for coronary artery spasm in a porcine model with interleukin-1β. *Circulation* 2000;101:1319-1323.
- Sasaki Y, Suzuki M, Hidaka H. The novel and specific Rho-kinase inhibitor (S)-(+)-2-methyl-1-[(4-methyl-5-isoquinoline)sulfonyl]-homopiperazine as a probing molecule for Rho-kinase-involved pathway. *Pharmacol Ther.* 2002;93:225-232.
- Shibata K, Sakai H, Huang Q, Kamata H, Chiba Y, Misawa M, et al. Rac1 regulates myosin II phosphorylation through regulation of myosin light chain phosphatase. *J Cell Physiol.* 2015;230:1352-1364.
- Sakai H, Kai Y, Sato K, Ikebe M, Chiba Y. Rac1 modulates G-protein-coupled receptor-induced bronchial smooth muscle contraction. *Eur J Pharmacol.* 2018;818:74-83.
- Andre-Gregoire G, Dilasser F, Chesne J, Braza F, Magnan A, Loirand G, et al. Targeting of Rac1 prevents bronchoconstriction and airway hyperresponsiveness. *J Allergy Clin Immunol.* 2018;142(824-33):e3.
- Chiba Y, Tanoue G, Suto R, Suto W, Hanazaki M, Katayama H, et al. Interleukin-17A directly acts on bronchial smooth muscle cells and augments the contractility. *Pharmacol Rep.* 2017;69:377-385.
- Shutes A, Onesto C, Picard V, Leblond B, Schweighoffer F, Der CJ. Specificity and mechanism of action of EHT 1864, a novel small molecule inhibitor of Rac family small GTPases. *J Biol Chem.* 2007;282:35666-35678.

15. Rahman A, Davis B, Lovdahl C, Hanumaiah VT, Feil R, Brakebusch C, et al. The small GTPase Rac1 is required for smooth muscle contraction. *J Physiol*. 2014;592:915-926.
16. Sidarala V, Veluthakal R, Syeda K, Kowluru A. EHT 1864, a small molecule inhibitor of Ras-related C3 botulinum toxin substrate 1 (Rac1), attenuates glucose-stimulated insulin secretion in pancreatic beta-cells. *Cell Signal*. 2015;27:1159-1167.
17. Sakai H, Hirano T, Chiba Y, Misawa M. Acetylcholine-induced phosphorylation and membrane translocation of CPI-17 in bronchial smooth muscle of rats. *Am J Physiol Lung Cell Mol Physiol*. 2005;289:L925-L930.
18. Chiba Y, Danno S, Suto R, Suto W, Yamane Y, Hanazaki M, et al. Intranasal administration of recombinant progranulin inhibits bronchial smooth muscle hyperresponsiveness in mouse allergic asthma. *Am J Physiol Lung Cell Mol Physiol*. 2018;314:L215-L223.
19. Tveden-Nyborg P, Bergmann TK, Lykkesfeldt J. Basic & Clinical Pharmacology & Toxicology Policy for Experimental and Clinical studies. *Basic Clin Pharmacol Toxicol*. 2018;123:233-235.
20. Karaki H, Sato K, Ozaki H. Different effects of verapamil on cytosolic Ca²⁺ and contraction in norepinephrine-stimulated vascular smooth muscle. *Jpn J Pharmacol*. 1991;55:35-42.
21. Perrino BA. Calcium sensitization mechanisms in gastrointestinal smooth muscles. *J Neurogastroenterol Motil*. 2016;22:213-225.
22. Sakai H, Kurihara Y, Hashimoto Y, Chiba Y, Misawa M. Phorbol 12,13-dibutyrate-mediated contraction of the stimulated bronchial smooth muscles of mice. *Respir Physiol Neurobiol*. 2010;173:120-124.
23. Kanaho Y, Moss J, Vaughan M. Mechanism of inhibition of transducin GTPase activity by fluoride and aluminum. *J Biol Chem*. 1985;260:11493-11497.
24. Jeon SB, Jin F, Kim JI, Kim SH, Suk K, Chae SC, et al. A role for Rho kinase in vascular contraction evoked by sodium fluoride. *Biochem Biophys Res Commun*. 2006;343:27-33.
25. Barman SA, Zhu S, White RE. RhoA/Rho-kinase signaling: a therapeutic target in pulmonary hypertension. *Vasc Health Risk Manag*. 2009;5:663-671.
26. Ito M, Nakano T, Erdodi F, Hartshorne DJ. Myosin phosphatase: structure, regulation and function. *Mol Cell Biochem*. 2004;259:197-209.

How to cite this article: Kai Y, Motegi M, Suzuki Y, et al. Up-regulation of Rac1 in the bronchial smooth muscle of murine experimental asthma. *Basic Clin Pharmacol Toxicol*. 2019;125:8–15. <https://doi.org/10.1111/bcpt.13204>