Note

Increased Rac1 Activation in the Enhanced Carbachol-Induced Bronchial Smooth Muscle Contraction of Repeatedly Antigen-Challenged Mice

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Recently, we demonstrated that Rac1 upregulation is involved in augmented bronchial smooth muscle (BSM) contractions of antigen-challenged mice. However, change in G protein-coupled receptor (GPCR)-induced Rac1 activation remains unknown in BSMs of repeatedly antigen-challenged (Chal.) mice. We here examined carbachol (CCh)-induced Rac1 activation in BSMs of Chal. mice. Gene expression levels of both *Rac1* and *Rac*-guanine nucleotide exchange factors (GEFs), such as *Tiam1* and *Trio*, were increased in BSMs of Chal. mice. Furthermore, CCh-induced *Rac1* activation was inhibited by pretreatment with Rac1-GEF inhibitor NSC23766 and *Rac1* inhibitor EHT1864 in BSMs of sensitized-control (S.C.) and Chal. mice. Compared with S.C. mice, CCh-induced *Rac1* activation was increased in BSMs of Chal. mice. In conclusion, we reported that increased CCh-induced *Rac1* activation via *Tiam1* and *Trio* upregulation, in addition to up-regulate *Rac1*, may be involved in increased CCh-induced BSM contractions in Chal. mice.

Key words bronchial smooth muscle; carbachol; asthma; Rac1; Rac-guanine nucleotide exchange factor (GEF)

INTRODUCTION

The Rho guanosine 5'-triphosphatase (GTPase) superfamily comprises 22 family involved Ras family of G proteins. Rho GTPases, particularly Cdc42, Rac1, and RhoA, have been linked to a large number of cellular functions, including reorganization of the cytoskeleton, such as formations of the filopodia, lamellipodia, and stress fiber.¹⁾ These GTPases cycle between a guanosine 5'-diphosphate (GDP)-loaded "ON" and a GDP-loaded "OFF" state.¹⁾ Regulators controlling these states include guanine nucleotide exchange factors (GEFs), GTPase activating proteins, and guanine nucleotide dissociation inhibitors.²⁾

Recently, we have demonstrated that Rac1 protein plays crucial role in G protein-coupled receptor (GPCR)-induced bronchial smooth muscle (BSM) contractions.³⁾ Further, we reported that *Rac1* upregulation may be involved in augmented BSM contractions of repeatedly antigen-challenged (Chal.) mice.⁴⁾ Rac1 activation is mediated by various GEFs, including *Tiam1* and *Trio*.^{5–8)} However, change in GPCR-induced *Rac1* activation in BSMs of Chal. mice remains unknown. We examined the gene expression of Rac1-GEFs (*Tiam1* and *Trio*) and carbachol (CCh)-induced *Rac1* activation in BSMs of Chal. mice.

MATERIALS AND METHODS

Animals Male BALB/c mice (7–8 weeks old) were purchased from the Tokyo Laboratory Animals Science Co., Ltd. (Tokyo, Japan) and housed in a pathogen-free facility. All animal experiments were approved by the Animal Care Committee of the Hoshi University, Tokyo, Japan (permission code: 30-091).

Sensitization and Antigenic Challenges Sensitization

and antigenic challenges in mice models of allergic bronchial hyper-responsiveness were performed as described previously.⁹⁾ Control (sensitized-control, S.C.) groups of mice received the same immunization procedure but inhaled saline aerosol instead of ovalbumin (OA; Sigma-Aldrich, MO, U.S.A.) challenge.

Functional Studies The suspension of left bronchi in organ baths were performed as described previously.⁴⁾ Fifteen minutes after the last wash, CCh (10 μ M) or K⁺ (60 mM) was applied. The BSM were treated with NSC23766 (100 μ M) or EHT1864 (10 μ M; Cayman Chemical, MI, U.S.A.) for 20 min before application of CCh or K⁺ to evaluate the effect of Rac1 inhibitors on CCh-induced BSM contractions. Force development was observed in response to K⁺ depolarization in the presence of atropine (1 μ M).

Rac1 Activity Pull-Down Assay *Rac1* activation assays were performed using a commercially available *Rac1* activation assay kit (Thermo Pierce #16118), according to the manufacturer's protocol.

Quantitative (q)RT-PCR Analysis qRT-PCR was performed as described previously.⁴⁾ The following PCR primer sets were used: for *Tiam1*, 5'-CAAGGTCGCCAGTCA CAT CA-3' and 5'-TCTCCCGTCTGCTCAGCAAT-3' were used to generate a 90 bp PCR product; for *Trio*, 5'-CGGGATGCC ATCGATATCAT-3' and 5'-TCC TCCGTA TCG AAGTCA TTC A-3' were used to generate an 83 bp PCR product; for *Rac1*, 5'-AGTTGCGTTGTGCTGAGAACA-3' and 5'-CTCCAG AAGCTGCGGTCTTC-3' were used to generate an 80 bp PCR product; and for *Gapdh*, 5'-CCTCGTCCCCTGACAAA ATG-3' and 5'-TCTCCACTTGGCCACTGCAA-3' were used to generate a 100 bp PCR product.

Immunoblotting Immunoblotting was performed as described previously.⁴⁾ The polyvinylidene difluoride membranes were incubated with the following primary antibodies: rabbit

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Fig. 1. Effects of NSC23766 and EHT1864 on Carbachol (CCh) and High K⁺ Depolarization-Induced Contractions in the Bronchial Smooth Muscles (BSMs) of Sensitized-Control (S.C.) and OA-Challenged (Chal.) Mice

The 10 μ M CCh and 60 mM high K⁺ responsiveness in the absence or presence of 100 μ M NSC23766 and 10 μ M EHT1864 (A). Each column represents the mean $\pm$ S.E.M. of 4–8 independent experiments. * p < 0.05 and ** p < 0.01 vs. CCh only (S.C.). ### p < 0.01 and ### p < 0.001 vs. CCh only (Chal.). Gene expression changes of *Trio*, *Tiam1*, *Rac1*, and *Chrm3* in BSMs of S.C. and Chal. mice (B–E). Each column represents the mean $\pm$ S.E.M. from four experiments. * p < 0.05 vs. S.C.

Fig. 2. Protein Levels of Tiam1, Trio, Rac1, and Chrm3 and CCh-Induced Rac1 Activation in BSMs of S.C. and Chal. Mice

Representative photographs showing bands for *Tiam1*, *Trio*, *Rac1*, *Chrm3*, and *Gapdh* (A). Levels of Tiam1, Trio, Rac1, and Chrm3 expressed as the ratios of the intensities of Tiam1, Trio, Rac1, and Chrm3 to Gapdh protein bands (B–E). Each column represents the mean $\pm$ S.E.M. from four experiments. * p < 0.05 and *** p < 0.001 vs. S.C. The active form of *Rac1*, GTP-bound *Rac1*, in BSMs of mice was measured using *Rac1* pull-down assay (F and G). Representative photographs showing bands for pull-down *Rac1*-GTP (upper) and Gapdh in total protein (lower). Each column represents the mean $\pm$ S.E.M. of 3–4 independent experiments. * p < 0.05: Vehicle (S.C.) vs. CCh (S.C.). ### p < 0.001: Vehicle (Chal.) vs. CCh (Chal.). ^{ss} p < 0.01: CCh (S.C.) vs. CCh (Chal.). ^{†††} p < 0.001: CCh (Chal.) vs. NSC + CCh (Chal.) or EHT + CCh (Chal.).

anti-Rac1 (1:1000; Merck Millipore, MA, U.S.A.), Tiam1 (1:1000; Bethyl Laboratories, Inc., TX, U.S.A.), Trio (1:1000; Aviva System Biology, CA, U.S.A.), *Chrm3* (1:1000; Santa Cruz Biotechnology, Inc., TX, U.S.A.), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:5000; Cell Signaling Technology Japan, K.K., Tokyo, Japan).

Statistical Analysis Indicators of statistical significance were generated using GraphPad Prism 5 for Max OS X (GraphPad Software, Inc., CA, U.S.A.). Results are expressed as mean $\pm$ standard error of the mean (S.E.M.). Statistical significance of difference was determined by unpaired Student's *t*-test or two-way ANOVA with *post hoc* Bonferroni or Newman-Keuls for differences among individual groups. $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Previously, we have reported that muscarinic agonist CCh-induced maximal contraction may be augmented by repeated OA challenges in BSMs of mice and found that 10 μ M CCh-induced contraction was the maximum response in BSM contractions of mice.⁴⁾ We examined the effect of *Rac1* inhibitors (NSC23766 and EHT1864) on 10 μ M CCh-induced BSM contraction in both S.C. and Chal. mice. Reportedly, *Tiam1*- or *Trio*-induced *Rac1* activation is specifically inhibited by NSC23766; however, activation of other small G proteins, such as Cdc42 and Rho, remains unaffected even by 100 μ M NSC23766,¹⁰⁾ whereas *Rac1* downstream signaling and guanine nucleotide displacement are selectively inhibited by EHT1864; however, activation of other small GTPase remain unaffected even by 50 μ M EHT1864.¹¹⁾ Compared with S.C. mice, 10 μ M CCh-induced BSM contraction was increased in Chal. mice. This CCh-induced contraction was attenuated by pretreatment with 100 μ M NSC23766 or 10 μ M EHT1864; however, 60 mM high K⁺ depolarization-induced contraction was not different in both S.C. and Chal. mice. Moreover, the high K⁺-induced contraction was not inhibited by *Rac1* inhibitors, NSC23766 and EHT1864 (Fig. 1A). These findings are consistent with those of our previous reports.^{3,4)} Recently, we have reported that *Rac1* upregulation may be involved in augmented BSM contractions of Chal. mice.⁴⁾ As mentioned above, NSC23766 inhibited *Rac1* binding and activation by the *Rac1*-specific GEFs, *Trio* or *Tiam1*.¹⁰⁾ The muscarinic acetylcholine receptor M₃ (*Chrm3*) mediates contractile responses in the airway smooth muscle.^{12,13)} Therefore, we examined the gene expressions of *Rac1*, *Tiam1*, *Trio*, and *Chrm3* in BSMs of Chal mice. Interestingly, although the gene expression level of *Chrm3* remained unchanged, that of *Rac1*, *Tiam1*, and *Trio* was increased in BSMs of Chal mice (Figs. 1B–E). Further, we investigated the protein levels of *Tiam1*, *Trio*, *Rac1*, and *Chrm3* in BSMs of S.C. and Chal. mice. In addition to gene expression changes, protein levels of *Rac1*, *Tiam1*, and *Trio* were increased in BSMs of Chal. mice (Figs. 2A–E). In summary, *Rac1* upregulation as well as activation may be involved in augmented BSM contractions of Chal. mice. Therefore, we examined CCh-induced *Rac1* activation in BSMs of Chal. mice. CCh induced *Rac1* activation (*Rac1*-GTP), and this CCh-induced *Rac1* activation was inhibited by pretreatment with NSC23766 and EHT1864 in BSMs of S.C. and Chal.

mice. Compared with S.C. mice, the CCh-induced *Rac1*-GTP was increased in BSMs of Chal. mice and the activation of *Rac1* was attenuated by NSC23766 and EHT1864 (Figs. 2F, G). However, further research is needed to determine the detailed activations of *Rac1*-GEFs, such as *Tiam1* and *Trio*, by CCh/*Chrm3*.

CONCLUSION

In conclusion, we reported that not only upregulated *Rac1* but also CCh-induced *Rac1* activation, *via* upregulation of *Tiam1* and *Trio*, may be involved in increased CCh-induced BSM contractions of Chal. mice. However, further studies on the *Rac1* signaling are needed to examine new treatment targets in asthma.

Conflict of Interest The authors declare no conflict of interest.

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