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Note

Increased Rac1 activation in the enhanced carbachol-induced bronchial smooth muscle contraction of repeatedly antigen-challenged mice

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Abstract

Recently, we demonstrated that Rac1 upregulation is involved in augmented bronchial smooth muscle (BSM) contractions of antigen-challenged mice. However, change in 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 upregulate Rac1, may be involved in increased CCh-induced BSM contractions in Chal. mice.

Keywords: bronchial smooth muscle; carbachol; asthma; Rac1; RacGEF
INTRODUCTION

The Rho 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. These GTPases cycle between a GTP-loaded “ON” and a GDP-loaded “OFF” state. Regulators controlling these states include guanine nucleotide exchange factors (GEFs), GTPase activating proteins, and guanine nucleotide dissociation inhibitors.

Recently, we have demonstrated that Rac1 protein plays crucial role in GPCR-induced bronchial smooth muscle (BSM) contractions. Further, we reported that Rac1 upregulation may be involved in augmented BSM contractions of repeatedly antigen-challenged (Chal.) mice. Rac1 activation is mediated by various GEFs, including Tiam1 and Trio. 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. Control (sensitized-control, S.C.) groups of mice received the same immunization procedure but inhaled saline aerosol instead of ovalbumin (OA; Sigma Aldrich, MO, USA) challenge.

Functional studies

The suspension of left bronchi in organ baths were performed as described previously. 15 minutes after the last wash, CCh (10 μM) or K+ (60 mM) was applied. The BSM were treated with NSC23766 (100 μM) or EHT1864 (10 μM; Cayman Chemical, MI, USA) 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 μM).

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.

qRT-PCR analysis

qRT-PCR was performed as described previously. 4) The following PCR primer sets were used: for Tiam1, 5′-CAAGGTCGCCAGTCATCA-3′ and 5′-TCTCCGTCTGCTCAGCAAT-3′ were used to generate a 90 bp PCR product; for Trio, 5′-CGGGATGCCATCGATATCAT-3′ and 5′-TCCTCCGTATCGAAGTCATTCA-3′ were used to generate an 83 bp PCR product; for Rac1, 5′-AGTTGCCTTTGTGCTGGAACA-3′ and 5′-CTCCAGAAGCTCGGCTTC-3′ were used to generate an 80 bp PCR product; and for Gapdh, 5′-CCTCGTCCCGTAGACAAAATG-3′ and 5′-TCTCCACTTTGCCCAGGTCA-3′ were used to generate a 100 bp PCR product.
**Immunoblotting**

Immunoblotting was performed as described previously. The polyvinylidene difluoride membranes were incubated with the following primary antibodies: rabbit anti-Rac1 (1:1000; Merck Millipore, MA, USA), Tiam1 (1:1000; Bethyl Laboratories, Inc., TX, USA), Trio (1:1000; Aviva System Biology, CA, USA), Chrm3 (1:1000; Santa Cruz Biotechnology, Inc., TX, USA), and 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, USA). Results are expressed as mean ± SEM. Statistical significance of difference was determined by unpaired Student’s t-test or two-way analysis of variance (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 μM 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 μM 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
μM 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 μM EHT1864. 11) Compared with S.C. mice, 10 μM CCh-induced BSM contraction was
increased in Chal. mice. This CCh-induced contraction was attenuated by pretreatment with 100 μM
NSC23766 or 10 μM 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 (Fig. 1B–1E). 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 (Fig. 2A–2E). 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 (Fig. 2F and 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.
REFERENCES

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 μM CCh and 60 mM high K\(^+\) responsiveness in the absence or presence of 100 μM NSC23766 and 10 μM EHT1864 (A). Each column represents the mean ± SEM 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 ± SEM 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 ± SEM 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 ± SEM of 3–4 independent experiments. *p < 0.05: Vehicle (S.C.) vs. CCh (S.C.). ###p < 0.001: Vehicle (Chal.) vs. CCh (Chal.). $$p<0.01$: CCh (S.C.)
vs. CCh (Chal.), †††p<0.001; CCh (Chal.) vs. NSC+CCh (Chal.) or EHT+CCh (Chal.).