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$G\alpha_{13}$ regulates methacholine-induced contraction of bronchial smooth muscle via phosphorylation of MLC_{20}

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ABSTRACT

Reversible airway constriction is induced by an increase in airway smooth muscle contractility in response to methacholine likely as a bronchospastic stimulus. Despite the finding of $G\alpha_{12}$ and $G\alpha_{13}$ up-regulation in airway hyperresponsive animals, their functional role of contraction in airway smooth muscle has not been directly explored. This study investigated the differential regulatory role of $G\alpha_{12}$/$G\alpha_{13}$ in methacholine-induced contraction of trachea and bronchus in $G\alpha_{12}$ or $G\alpha_{13}$ gene knockout mice after ovalbumin sensitization and challenges. Organ bath assays and videomicroscopy revealed that $G\alpha_{13}$ deficiency delayed methacholine-induced contractile response of bronchiolar smooth muscle, but not that of tracheal smooth muscle. In primary bronchial smooth muscle cells, knockdown of $G\alpha_{13}$ blocked methacholine-induced phosphorylation of 20 kDa regulatory light chain of myosin II (MLC_{20}), a prerequisite step for the contractile initiation of actin and myosin. $G\alpha_{13}$-dependent MLC_{20} phosphorylation was confirmed in murine embryonic fibroblasts. After ovalbumin sensitization and challenges, wild type mice exhibited methacholine-induced bronchial contraction of lung tissue. Heterozygous absence of the $G\alpha_{13}$ gene abrogated methacholine-induced contractions, whereas homozygous absence of the $G\alpha_{12}$ gene failed to do so. Our findings indicate that $G\alpha_{13}$, but not $G\alpha_{12}$, specifically regulates cholinergic bronchial contraction in airway responsiveness via controlling phosphorylation of MLC_{20} by methacholine.

1. Introduction

Asthma is characterized by pathological features of airway remodeling and inflammation as a consequence of an outcome of complex interactions of multiple cell types. Airway hyperresponsiveness is induced by an increase in airway smooth muscle contractility in response to bronchospastic stimuli. In particular, smooth muscle cells serve the function of bronchoconstriction and inflammation in the airway system. Airway smooth muscle cells can proliferate, migrate and secrete substances such as chemokines, cytokines, extracellular matrix proteins and growth factors. Therefore, airway smooth muscle plays an active role in airway remodeling in inflammatory airway diseases. Moreover, the contractile phenotype of airway smooth muscle changes to hypercontractile phenotype. Because of the increase in the smooth muscle mass in an asthmatic disease process and the airway hyperreactivity, it is important to understand the molecular regulatory mechanics of asthmatic airway smooth muscle contractions.

G-protein-coupled receptors (GPCRs) regulate cell signaling pathways, and participate in the regulation of a variety of physiological processes via the heterotrimeric GTP-binding proteins (α, β and γ subunits) [1]. Activated GPCRs then couple to G-proteins defined by their α subunits. Gα subunits are divided into $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_q$ and $G\alpha_{12}$ [1]. Among the four G protein

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Abbreviations: GPCRs, G-protein-coupled receptors; G-proteins, GTP-binding proteins; MEF, murine embryonic fibroblast; BSMC, bronchial smooth muscle cell; WT, wild type; KO, knockout; KD, knockdown; MLC, myosin light chain; PCR, polymerase chain reaction; SMA, smooth muscle actin; siRNA, small interference RNA.

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families, $G\alpha_{12}$ family members, consisting of $G\alpha_{12}$ and $G\alpha_{13}$, are activated by the stimulation of thrombin, thromboxane A_2, lysophosphatidic acid, sphingosine 1-phosphate and thyroid-stimulating hormone receptors [2–5]. The $G\alpha_{12}$ and $G\alpha_{13}$ subunits regulate various intracellular effectors or cellular responses such as cytoskeletal rearrangement, actin-stress fiber formation [6], neurite retraction [7], platelet aggregation [3], gene induction [2,5] and apoptosis [8]. Although functions between $G\alpha_{12}$ and $G\alpha_{13}$ are mostly overlapping, the G-protein members seem to differ in their ability to couple to ligands and to recruit signaling pathways for physiological effectors [3,9]. Yet, the distinct or overlapping physiological or pathological functions that are regulated by $G\alpha_{12}/G\alpha_{13}$ and the associated downstream signaling pathways have not been completely identified.

A vast number of the acute inflammatory mediators released from the local environment (e.g. bradykinins, leukotrienes and histamine) regulate the contraction of airway smooth muscle via excitatory GPCRs [10,11]. In an animal model of airway hypersensitivity, cholinergic agonists induced contraction of bronchial smooth muscle presumably because of its proliferative and hypercontractile phenotypic changes of the smooth muscle cells [12]. Although it has been shown that the expression of $G\alpha_{12}$ and $G\alpha_{13}$ is increased in the bronchial smooth muscle of airway hyperresponsive rats [13], their functional roles in airway smooth muscle have not been explored yet.

This study investigated the distinct or overlapping regulatory role of $G\alpha_{12}$ and $G\alpha_{13}$ in the contraction of bronchial smooth muscle. In this study, we determined whether $G\alpha_{12}/G\alpha_{13}$ play(s) a role in a mouse model of ovalbumin sensitization and challenges producing airway remodeling and, if so, what the specific regulatory mechanism is for the change in airway responsiveness by $G\alpha_{12}$ and/or $G\alpha_{13}$. The molecular basis of $G\alpha_{13}$ function in the cholinomimetic contractile response of airway smooth muscle was also explored with particular reference to cholinergic myosin light $chain_{20}$ (MLC_{20}) phosphorylation.

2. Materials and methods

2.1. Ovalbumin immunization

Animal experiments were conducted under the guidelines of the Institutional Animal Use and Care Committee at Seoul National University, Korea. Wild type (WT) and $G\alpha_{12}/G\alpha_{13}$ KO mice at the age of 8–10 weeks (25–30 g) ($G\alpha_{12}-/-$ and $G\alpha_{13}+/-$) were used for *in vivo* experiments [3]. WT and KO mice were supplied by Dr. M.I. Simon (Caltech, Pasadena, CA). To observe airway responsiveness by the challenge of methacholine, the mixture with 100 μg endotoxin-free ovalbumin (MP Biomedicals, Aurora, OH) dissolved in 100 μl each of PBS and alum (50 μl per mouse) (Pierce, Rockford, IL) was intraperitoneally injected to the animals on day 0. On day 14, the mice were intraperitoneally injected with 100 μg ovalbumin alone. The mice were exposed to aerosol of 1% ovalbumin for 30 min once a day on days 26, 27 and 28. Airway responsiveness was measured 24 h after the last dose of ovalbumin challenge, and then lung tissue was excised. To observe baseline airway response, WT and KO mice were intraperitoneally injected with 100 μl PBS on day 0 and day 14. The mice were exposed to aerosol of ovalbumin as described above. Airway responsiveness was measured 24 h after the last dose of ovalbumin nebulization.

2.2. Airway responsiveness

Mice were treated with varying concentrations of nebulized methacholine (Sigma–Aldrich, St. Louis, MO) for 3 min in a whole body chamber (Buxco Inc., Connecticut, MA) and then were placed in a whole body plethysmograph (Buxco Inc., Connecticut, MA). The mice were allowed to freely move in the chamber. The airflow rate was measured 3 min after the methacholine challenge with a pneumotachograph (Buxco Inc., Connecticut, MA). The airway resistance and compliance in response to methacholine were calculated with a pulmonary analyzer (Buxco Inc., Connecticut, MA).

2.3. Tracheal or bronchiolar contraction

Trachea was removed from vehicle-treated or ovalbumin-immunized mice. The whole tracheas (3 mm) excised from the mice were dissected free of adhering tissue using a stereoscope and were bathed at 37 °C within 20 ml organ baths containing Kreb's buffer solution. The tissues were given with resting tension of 1 g mass, first allowed to equilibrate for at least 30 min, and then six test concentrations of methacholine were applied to the tissues by an accumulation method without interval washout. The contractile activity was recorded using PowerLab recording systems (AD Instruments/8SP, Grand Junction, CO). After the methacholine test, the tissues were washed with fresh Kreb's buffer solution and were allowed to rest for 30 min. Contractile responses of the tissue to methacholine were normalized to those elicited by 80 mM KCl (Junsei Chemical Co., Tokyo, Japan).

The lung tissue was quickly removed from immunized mice after sacrifice. After washings, the lung tissue was dissected into about 2 mm of thickness by a tissue chopper (Mickle Laboratory Engineering Co., Surrey, UK) in a cold room (the concentrations of methacholine required for bronchiolar contraction were relatively higher in this assay because a 2 mm tissue chopper was used) and stabilized in Kreb's buffer solution comprising 115 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4 (Junsei Chemical Co., Tokyo, Japan), 10 mM glucose (Sigma–Aldrich, St. Louis, MO), and antifungal and antibiotic agents (Hyclone, Logan, UT) at 37 °C with 0.5% CO_2 supply for at least 2 h. The culture dish containing sliced lung tissue was mounted on the microscope with a camera and carbogen (5% CO_2/95% O_2) supply. Alveolar contraction was monitored in the lung tissue treated with varying concentrations of methacholine (50, 500 μM and 5 mM). The change of bronchiolar perimeter per minute was calculated and was represented as an airway contractile activity in response to methacholine. In this experiment, the lung tissues were incubated in the Kreb's buffer solution without serum and indomethacin, a cyclooxygenase inhibitor used for potentiating vasoconstriction [14].

2.4. Cell culture

Bronchial smooth muscle cells (BSMCs) were isolated from C57BL/6 mice according to the previously published method with slight modifications [15]. Briefly, lung tissues were excised and placed into an ice-cold Ca^{2+}-free Kreb's buffer solution. Bronchial tissues were dissected, cleaned of serosa, vasculature and epithelia at room temperature, and the isolated smooth muscle strip was washed four times in Hanks' balanced salt solution (Gibco BRL, Gaithersburg, MD) containing 100 mg/ml streptomycin and 100 U/ml penicillin (Hyclone, Logan, UT) under aseptic conditions. The muscle was then minced thoroughly with fine scissors and resuspended in Hanks' balanced salt solution (digestion buffer) containing 600 U/ml collagenase (Gibco BRL, Gaithersburg, MD), 8 U/ml type IV elastase (Sigma–Aldrich, St. Louis, MO), and 1 U/ml type XXVII Nagarse protease (Sigma–Aldrich, St. Louis, MO). The cells were isolated by serial digestions (3 stages, 45 min each) with vigorous shakings at 37 °C. The fractions were pooled, and were filtered through a 70 μm nylon mesh. Then the samples were diluted in a DMEM culture medium (Hyclone, Logan, UT)

containing 10% fetal bovine serum (Hyclone, Logan, UT). After estimation of cell number (ZBI Coulter counter), the cells were plated into 100-mm plastic culture dishes at a density of 5000 cells/cm^2 and were allowed to attach for 36 h. The cells were grown at 37 °C in a humidified atmosphere consisting of 5% CO_2. The medium was replaced with fresh one containing 10% fetal bovine serum and antibiotics every 72 h.

Murine embryonic fibroblasts (MEFs) were maintained in DMEM containing 10% fetal bovine serum, 50 units/ml penicillin and 50 μg/ml streptomycin at 37 °C in humidified atmosphere with 5% CO_2. The cells were plated at a density of 5×10^6/dish (10 cm-diameter) and were preincubated for 24 h at 37 °C. For all experiments, cells were grown to 80–90% confluency. After overnight serum-deprivation, the cells were scraped, transferred to microtubes, and allowed to swell by adding lysis buffer.

2.5. Polymerase chain reaction

DNA was extracted from the mouse tail and was amplified with specific primers. The sequences of the primers used were as follows: 5′-GTGCTCATCCTTCCTGGTTTCC-3′ and 5′-CGGGTCGCCCTTGAAATCTGG-3′ for $G\alpha_{12}$ wild type (441 bp); 5′-CGGGTCGCCCTTGAAATCTGG-3′ and 5′-GGCTGCTAAAGCGCATGCTCC-3′ for $G\alpha_{12}$ deletion mutant (314 bp); 5′-AGCAGCGCAAGTCCAAGGAGATCG-3′ and 5′-GATCACGTTGCTGTAGATGGTGGG-3′ for $G\alpha_{13}$ wild type (200 bp); and 5′-TAGGTCGCGTGAAGGTGACAGC-3′ and 5′-GATTCGCAGCGCATCGCCTTCTAT-3′ for $G\alpha_{13}$ deletion mutant (290 bp).

2.6. Immunoblot analysis

Cell lysates were prepared as described previously [2]. SDS-polyacrylamide gel electrophoresis and immunoblot analyses were performed according to previously published procedures [2]. Equal loading of proteins was verified by coomassie blue staining of gels and actin immunoblottings. Scanning densitometry of the immunoblots was performed with Image Scan & Analysis System (Alpha Innotech Corp., San Leandro, CA). The area of each lane was integrated using the software AlphaEaseTM version 5.5, followed by background subtraction.

2.7. Small interference RNA (siRNA) knockdown

MEFs or BSMCs were transfected with control siRNA or siRNA directed against $G\alpha_{13}$ (100 pmol) using Lipofectamine^{2000} (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Phosphorylated 20 kDa light chain of myosin II was immunoblotted in the lysates of cells treated with vehicle or methacholine. The target sequences of a commercially available $G\alpha_{12}$ siRNA mixture were 5′-CCAGUAAGCAAGACAUCCU-3′, 5′-GCAUCACAUCUAUCCUGUU-3′, and 5′-CUCUGCUGUUGAUCUGUAA-3′. The $G\alpha_{13}$ siRNA target sequences were 5′-GCAAGUUGUUAGCAUCAAA-3′, 5′-GGAAGGGCUGUUAGAGAAA3′, and 5′-CCAUACAUGCUGUCACUAA-3′ (SantaCruz Biotechnology, CA, USA).

2.8. Measurement of Rho activity

The activity of Rho kinase was assessed using the Rho binding domain of Rhotekin. After transfection of the control or $G\alpha_{13}$ siRNA (24–36 h), the cells were exposed to 1 μM methacholine for 3 min. Whole cell lysates were used to determine Rho GTPase activity. GTP-bound Rho protein was collected using the GST-RBD beads in a pull-down assay. Rho kinase activity represents the amount of GTP-bound Rho, which was determined by Rho immunoblotting. Rho protein in lysates represents loading control.

2.9. Measurement of Ca^{2+} influx

Intracellular Ca^{2+} was detected using the Fluo-4 NW (Molecular Probes, OR, USA), a fluorescent Ca^{2+} indicator. MEF cells were transfected with control or $G\alpha_{13}$ siRNA and then the growth medium was removed. After addition of the dye-loading solution (25 mM), the cells were treated with 1 μM methacholine for 1 min. Fluorescence was measured at the excitation and emission wave lengths of 494 nm and 516 nm, respectively.

2.10. Immunocytochemistry of myosin heavy chain

Standard immunocytochemical procedures using myosin heavy chain antibody (Santa Cruz Biotechnology, Santa Cruz, CA) were conducted to confirm the presence of myosin heavy chain protein in BSMCs, which was followed by counterstaining with propidium iodide (Sigma, St. Louis, MO).

2.11. Histochemical analyses

The mice were treated with ovalbumin for 8 weeks to induce airway remodeling. Tissue slices obtained from control or immunized mice were fixed in 10% buffered-neutral formalin for 6 h. Fixed lung tissue slices were processed and embedded in a paraplast automatic tissue processor, Citadel 2000 (Shandon Scientific, Cheshire, UK). Sections of 4 μm in thickness were subjected to Gomori's staining for the evaluation of collagen amount, periodic acid-schiff staining for goblet cells, and hematoxylin and eosin staining for general histological evaluation. Collagen accumulation and goblet cell hyperplasia were scored by the McMillan's method [16]. The degree of lymphocyte migration in the lung tissue was analyzed as described previously [17].

2.12. Statistical analysis

One-way analysis of variance (ANOVA) procedures were used to assess significant differences among treatment groups. For each significant effect of treatment, the Newman–Keuls test was used for comparisons of multiple group means. The criterion for statistical significance was set at $p < 0.05$ or $p < 0.01$. All statistical tests were two-sided.

3. Results

3.1. Methacholine-dependent bronchiolar contraction

The result of PCR assays confirmed specific disruption of the $G\alpha_{12}$ and/or $G\alpha_{13}$ gene in the mice (Fig. 1A). We first examined the effects of $G\alpha_{12}/G\alpha_{13}$ deficiency on tracheal smooth muscle contraction using an organ bath system. Methacholine treatment (1 nM–0.1 mM, 2 min) induced the contractile response of isolated tracheal rings from healthy control animals in a concentration-dependent manner (Fig. 1B), which was comparable to that obtained from the $G\alpha_{12}-/-$ or $G\alpha_{13}+/-$ mice. There was no significant difference observed between each genotype group. The ED_{50} values were 2.5–3.4 μM, which match with those in the previous reports (0.3–1.9 μM) [18–20], indicating that the time point and concentrations of methacholine used were appropriate. In this experiment, isotonic 80 mM KCl was used as a reference stimulus to normalize the contractile response.

Previous studies have shown that the mechanistic basis of the cholinergic effect on bronchial smooth muscle contraction may differ from that on tracheal smooth muscle contraction [21,22]. To find out whether $G\alpha_{12}$ and/or $G\alpha_{13}$ regulate(s) airway contraction, we next compared the effect of cumulative methacholine treatments on bronchiolar constriction using a phase-contrast

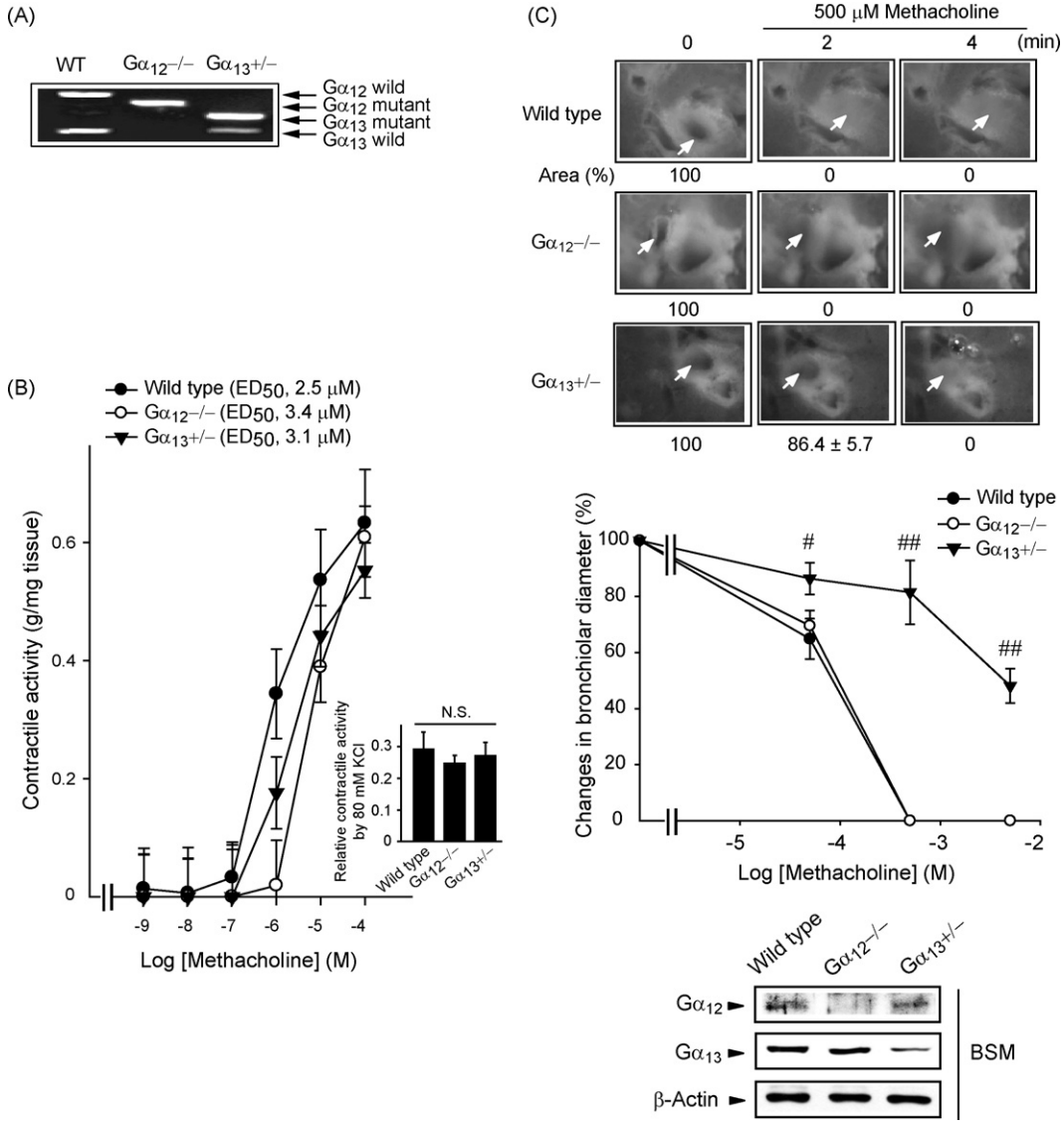

Fig. 1. Methacholine contraction of tracheal or bronchiolar smooth muscle. (A) Genotyping by PCR. DNA extracted from mouse tail was amplified with the primers specific for wild type or $G\alpha_{12}/G\alpha_{13}$ gene. (B) Methacholine-dependent contractile responses of isolated tracheal rings. Cumulative methacholine-induced tracheal smooth muscle contractions were constructed as described in Section 2. Data represent means ± S.E.M. of 6–8 animals. Isotonic high K^+ (80 mM)-induced contraction of isolated tracheal rings of WT mice was comparable to that of $G\alpha_{12}-/-$ or $G\alpha_{13}+/-$ mice. (C) Time- and concentration-dependent contractile reactivity of bronchiole. The phase-contrast microphotographs show the effects of 500 μM methacholine treatment on bronchiolar contraction in a lung slice prepared from WT mice or $G\alpha_{12}/G\alpha_{13}$ gene KO mice (upper). Bronchoconstriction was observed 2 min after cumulative treatments of increasing concentrations of methacholine (50 μM, 500 μM and 5 mM) in a lung slice prepared from WT or KO mice immunized and challenged with ovalbumin (middle). Relative bronchiolar areas were calculated by pixel summing after image analysis with Photoshop. The cross-sectional area quantified using digital video-microscopy (1024 × 768 pixels) was depicted as a function of concentration. Data represent means ± S.E.M. of 3 animals (significant compared to the respective methacholine treatment, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, N.S., not significant). Immunoblot analyses confirmed deficiency in $G\alpha_{12}$ or $G\alpha_{13}$ proteins in bronchial smooth muscle (lower).

microphotograph. Incubation of the lung tissue with methacholine (500 μM) began to decrease bronchiolar airway area as early as at 1 min, and caused complete constriction of bronchioles at 2 min (Fig. 1C, upper). Methacholine treatment did not change the vessel areas. We evaluated the concentration-responses of methacholine-induced bronchiolar contraction with the lung tissue of WT and $G\alpha_{12}/G\alpha_{13}$ KO mice. Digital video-microscopy allowed us to quantify the relative changes in the bronchiolar diameter of lung tissue after methacholine treatments. In the experiments, complete bronchiolar contraction was induced 2 min after 500 μM methacholine treatment in the immunized WT or $G\alpha_{12}$ KO mice. On the contrary, methacholine-induced contractile dose–response curve was shifted to the right in the immunized $G\alpha_{13}$ heterozygous KO mice and was not even complete at a high concentration of methacholine (5 mM) (Fig. 1C, middle). It is noteworthy that heterozygous KO of the $G\alpha_{13}$ gene notably inhibited methacholine-induced bronchiolar contraction. By contrast, the lack of $G\alpha_{12}$ failed to inhibit methacholine-induced bronchoconstriction, resulting in a decrease in bronchiolar airway area. Immunoblottings confirmed a deficiency in $G\alpha_{12}$ or $G\alpha_{13}$ protein in bronchial smooth muscle (Fig. 1C, lower). Moreover, no compensatory change was observed in the expression of $G\alpha_{12}$ or $G\alpha_{13}$. Taken together, our results indicate that $G\alpha_{13}$ may specifically regulate methacholine regulation of bronchiolar smooth muscle, but not tracheal smooth muscle.

3.2. $G\alpha_{13}$ regulation of methacholine-dependent MLC_{20} phosphorylation

Phosphorylation of MLC_{20} is a prerequisite step to initiate the contractile initiation of actin and myosin [23]. Cholinergic stimulation was shown to stimulate MLC_{20} phosphorylation. As

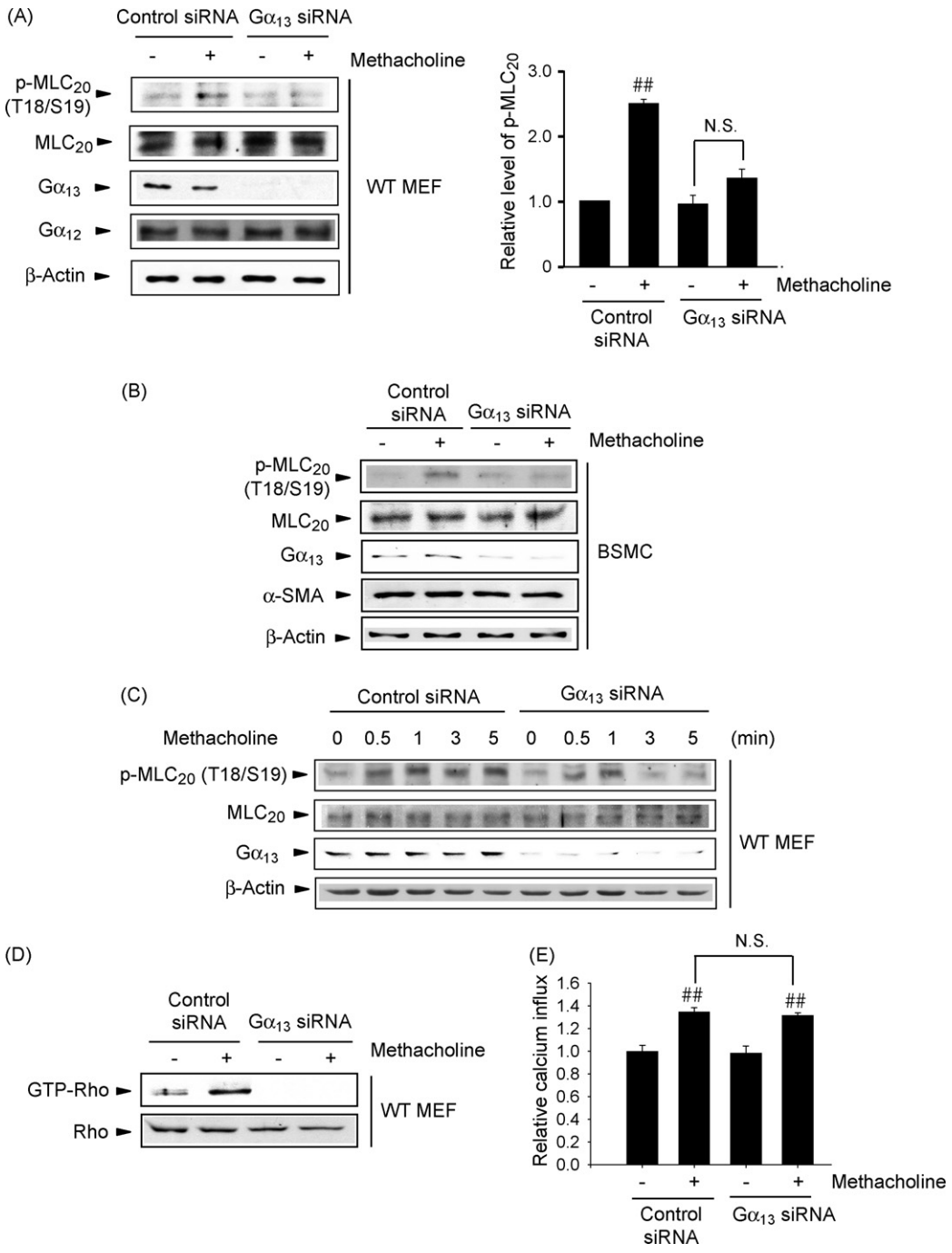

Fig. 2. $G\alpha_{13}$ regulation of methacholine-induced MLC_{20} phosphorylation. (A) Immunoblot analyses of phosphorylated MLC_{20} in MEFs. Wild type MEFs transfected with control or $G\alpha_{13}$ siRNA were incubated with 1 μM methacholine for 2 min, and the lysates were subjected to immunoblotting for phosphorylated MLC_{20} (Thr18/Ser19). Immunoblotting for $G\alpha_{13}$ confirms the siRNA knockdown effect. Data represent means ± S.E.M. of four separate experiments (significant compared to control, $^{\#\#}p < 0.01$, N.S., not significant). (B) Methacholine-induced MLC_{20} phosphorylation in primary cultured bronchial smooth muscle cells (BSMCs). BSMCs isolated from C57BL/6 mice were attached in dishes as described in Section 2, and cultured for 4 weeks. The cells were transfected with control or $G\alpha_{13}$ siRNA and then treated with 1 μM methacholine for 2 min. The lysates were used to determine the levels of phosphorylated MLC_{20}, total MLC_{20}, $G\alpha_{13}$, α-SMA and β-actin. Results were confirmed by repeated experiments. (C) Phosphorylated MLC_{20} was immunoblotted in the lysates of wild type MEFs treated with 1 μM methacholine for 0.5–5 min following transfection with control or $G\alpha_{13}$ siRNA. Equal loading of proteins was verified by probing the replicate blot for β-actin. (D) Rho activity. The activity of Rho was determined in MEFs treated with methacholine with or without $G\alpha_{13}$ knockdown. (E) Changes in Ca^{2+} influx. Intracellular Ca^{2+} levels were measured in MEFs treated as above. After transfection of control or $G\alpha_{13}$ siRNA, the cells were treated with vehicle or methacholine. Ca^{2+} fluorescence was monitored by using a microplate fluorescence reader.

an effort to verify $G\alpha_{13}$ regulation on methacholine response, the knockdown effect of $G\alpha_{13}$ on MLC_{20} phosphorylation was examined in MEFs and BSMCs. In WT MEFs, MLC_{20} phosphorylation was decreased by a deficiency in $G\alpha_{13}$ (Fig. 2A), but not by that in $G\alpha_{12}$ (data not shown). Consistently, $G\alpha_{13}$ knockdown attenuated methacholine-induced MLC_{20} phosphorylation in primary-cultured bronchial smooth muscle cells (Fig. 2B). In this experiment, α-SMA was used as a marker of smooth muscle cells. Immunocytochemistry confirmed the presence of myosin heavy chain, another marker of BSMCs (Fig. S1). A time-course study indicated that methacholine treatment led to sustained phosphorylation of MLC_{20} at early times (i.e., 0.5–5 min), which was inhibited by siRNA knockdown of $G\alpha_{13}$ (Fig. 2C). It is known that the signaling of $G\alpha_{12}/G\alpha_{13}$ regulates Rho activity [1]. We verified

that methacholine treatment (1 min) increased the activity of Rho, which was antagonized by $G\alpha_{13}$ knockdown (Fig. 2D). Because methacholine stimulates $G\alpha_q$ and PLCβ and thereby causes an increase in cellular Ca^{2+} [19], we examined the possible role of $G\alpha_{13}$ in Ca^{2+} influx, which affects MLC_{20} phosphorylation. $G\alpha_{13}$ knockdown did not inhibit the ability of methacholine to elicit Ca^{2+} influx (Fig. 2E), suggesting that MLC_{20} phosphorylation regulated by the $G\alpha_{13}$ pathway may not involve a change in cellular Ca^{2+}. Taken together, our findings demonstrate that $G\alpha_{13}$ regulates methacholine-induced MLC_{20} phosphorylation.

3.3. $G\alpha_{13}$-specific regulation of airway responsiveness

In the next *in vivo* experiments, we examined airway responsiveness in response to varying doses of methacholine following ovalbumin sensitizations. After immunization and inhalational exposures to ovalbumin, WT mice exhibited methacholine-induced bronchial contractions (Fig. 3A), as evidenced by the dose-dependent increases of Penh (index for airway resistance) values. It is noteworthy that heterozygous absence of the $G\alpha_{13}$ gene significantly abrogated methacholine-induced contraction. In ovalbumin-sensitized homozygous $G\alpha_{12}$ KO mice, airway responsiveness was the same as in ovalbumin-sensitized WT mice. In this experiment, we could not assess airway responsiveness in homozygous $G\alpha_{13}$ KO animals because the mutation resulted in intrauterine death [24]. As controls, additional experiments were conducted using sham groups exposed to ovalbumin only (Fig. 3B). No difference in baseline resistance was observed in $G\alpha_{13}$ KO animals compared to WT or $G\alpha_{12}$ KO ones. Our findings indicate that, of the two $G\alpha_{12}$ members, $G\alpha_{13}$ might be responsible for the regulation of cholinergic airway smooth muscle contraction.

3.4. Histological alterations after ovalbumin sensitization

Smooth muscle proliferation, goblet cell hyperplasia, cell migration to bronchiole, and collagen accumulation characterize the asthmatic condition of lung tissue [25]. In our experimental model, ovalbumin sensitization and challenges increased subepithelial deposition of collagen, as evidenced by increases in Gomori-staining intensity of lung tissue in WT or $G\alpha_{12}$ or $G\alpha_{13}$ KO mice (Fig. 4A; Fig. S2). Periodic acid-Schiff (PAS) and H&E stainings confirmed that exposures of the mice to repeated ovalbumin stimuli provoke airway inflammation (Fig. 4B and C; Figs. S3 and S4). Moreover, ovalbumin immunization and airway challenges resulted in goblet cell hyperplasia with lymphocytes recruitment to the site of bronchial injury in WT mice. The degrees of goblet cell hyperplasia and lymphocytes infiltrations in the airways of sensitized $G\alpha_{12}$ or $G\alpha_{13}$ KO mice were comparable to those in WT mice (Fig. 4B and C). Our results indicate that the chronic airway inflammatory process of asthma was not notably affected by a deficiency in $G\alpha_{12}$ or $G\alpha_{13}$.

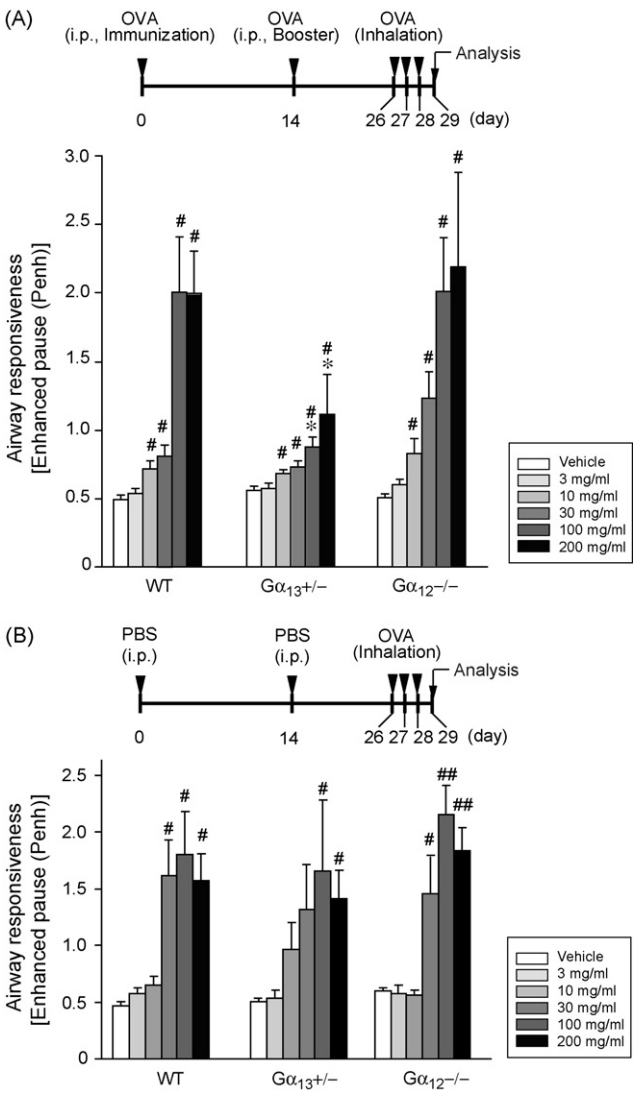

Fig. 3. Airway responsiveness in wild type or $G\alpha_{12}/G\alpha_{13}$ KO mice (immunized and challenged with ovalbumin). (A) Airway responses were evaluated by challenging increasing doses of methacholine to the mice immunized with ovalbumin. The animals were exposed to varying doses of nebulized methacholine for 3 min in a whole body chamber. The airflow rate was measured using a pneumotachograph, and airway resistance and compliance were calculated with a pulmonary analyzer. Data represented means ± S.E.M. with 10 mice per group (significant compared to the respective vehicle nebulization, $^{\#}p < 0.05$; significant compared to the same dose of methacholine nebulization in immunized WT mice, $^{*}p < 0.05$). (B) Baseline resistance was monitored in WT and $G\alpha_{12}/G\alpha_{13}$ KO mice without ovalbumin sensitization. The experiment was performed as described in panel A. Data represented means ± S.E.M. with 5 mice per group (significant compared to the respective vehicle nebulization, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$).

4. Discussion

The increased airway reactivity results from the multiple mechanisms including airway inflammation, airway smooth muscle proliferation, increased cholinergic sensitivity of bronchoconstriction and IgE-mediated allergic responses [16,26]. Cholinergic stimulation triggers bronchial hyperreactivity in asthma [12]. Because the receptor responsible for bronchial smooth muscle contraction is the muscarinic M_3 receptor, activation of cholinergic pathway participates in generating asthmatic symptoms as a result of muscarinic activation on the cell surface membrane [27,28].

$G\alpha_{12}$ family members play a role in the signaling pathways linked to the essential biological processes of cell proliferation, development and survival [1,29]. In the present study, we hypothesized that the G protein member(s) may function as a regulator required for the process of respiratory disease. This study investigated the role of $G\alpha_{12}/G\alpha_{13}$ for the contraction of airway smooth muscle in a murine model of ovalbumin sensitization and challenges, and explored their differential regulatory role in bronchial contraction. Our data clearly indicate the specific regulation of cholinergic bronchial smooth muscle contraction by $G\alpha_{13}$, but not by $G\alpha_{12}$. Both $G\alpha_{12}$ and $G\alpha_{13}$ can interact with specific Rho guanine nucleotide exchanging factor (RhoGEFs) leading to small GTPase RhoA activation [7,30]. In this study, we confirmed that a deficiency of $G\alpha_{13}$ decreased the activity of Rho. Hence, the identified regulation of airway smooth muscle contraction by $G\alpha_{13}$ may involve the functional linkage of the

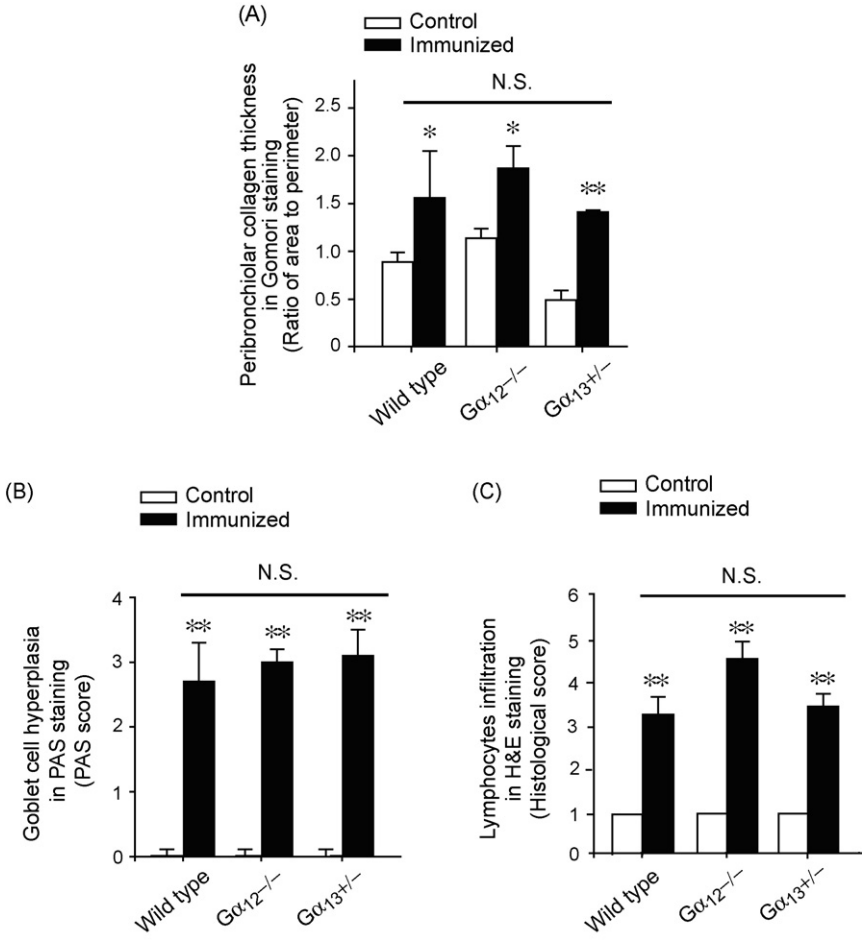

Fig. 4. Histological changes in murine lung following ovalbumin sensitization and challenges. (A) Peribronchiolar collagen thickness. Peribronchiolar collagen thickness in Gomori-stained bronchial tissues was scored by the McMillan's method. (B) Goblet cell hyperplasia and (C) lymphocytes infiltrations. Goblet cell hyperplasia and lymphocytes infiltration were assessed by counting the cells stained with PAS and H&E in the airway tissue of mice. Data represent means ± S.E.M. of 7–10 animals (significant compared to the respective vehicle nebulization, $^{*}p < 0.05$, $^{**}p < 0.01$, N.S., not significant).

membrane receptor-$G\alpha_{13}$-Rho pathway in bronchial tissue. It is worth noting that $G\alpha_{13}$ deficiency failed to antagonize methacholine-induced tracheal contraction. Our data show the greater extent of $G\alpha_{13}$ regulation for bronchiolar smooth muscle than for tracheal smooth muscle.

Previous studies have shown that endothelin-1-induced vascular smooth muscle contraction is mediated by MLC_{20} phosphorylation through Ca^{2+}-dependent stimulation of myosin light chain kinase and Rho/Rho kinase-mediated inhibition of myosin phosphatase [31], and that the $G\alpha_{12}/G\alpha_{13}$-mediated Rho/Rho kinase-dependent signaling pathway regulates MLC_{20} phosphorylation by vasoconstrictors in smooth muscle cells [32]. Our findings presented here in this study clearly demonstrate that, of the two $G\alpha_{12}$ members, only $G\alpha_{13}$ is involved in the regulation of MLC_{20} phosphorylation in response to cholinomimetic stimulation. Hence, this study identifies for the first time the concept that $G\alpha_{13}$, but not $G\alpha_{12}$, is responsible for MLC_{20} phosphorylation in bronchial smooth muscle cells, which matches well with our *in vivo* result showing the regulation of airway response by $G\alpha_{13}$ in an asthma model. In this study, we observed that a deficiency in $G\alpha_{13}$ did not inhibit the ability of methacholine to elicit Ca^{2+} influx. However, a preliminary study from this laboratory showed that the lack of $G\alpha_{13}$, but not $G\alpha_{12}$, blocked lysophosphatidic acid-induced increase in intracellular Ca^{2+} in MEF cells (Cho and Kim, unpublished data). Therefore, we cannot completely rule out the possibility that the G-protein-dependent signaling pathway may be coupled with Ca^{2+} sensitization.

A previous study has shown that repeated antigen challenges increase the expression of $G\alpha_{12}/G\alpha_{13}$ in the bronchial smooth muscle of an airway hyper-responsive animal model [13], which adds support to the regulatory role of $G\alpha_{13}$ in the contraction of bronchial smooth muscle to muscarinic stimuli. Our KO and knockdown results presented here showed that partial loss of $G\alpha_{13}$ might be sufficient to block methacholine-induced contractile response of bronchial smooth muscle. In this study, the result obtained from Penh measurement of bronchial resistance was dose-dependent and statistically significant. Moreover, ex-vivo videomicroscopy was used to directly show morphological changes of bronchial contractions after methacholine treatments, which was reproducible by repeating another set of experiments [in ex-vivo videomicroscopy study, we used a 2 mm tissue chopper. Because our bronchial samples were several fold thicker than 250 μM samples, the methacholine concentrations required for contraction were higher, which may account for the difference in ED_{50} values (85 μM vs. 0.7 μM) [33]]. In addition, the regulatory role of $G\alpha_{12}/G\alpha_{13}$ in bronchial smooth muscle contraction was also determined in cellular models, which confirms the results obtained *in vivo*. All of these data strongly support the conclusion that $G\alpha_{13}$ regulates airway smooth muscle contraction. Inhibition of the contraction of bronchial smooth muscle by heterozygous deficiency of $G\alpha_{13}$ renders the possibility that the G protein serves as a potential target since pharmacological inhibition would be usually achieved at the dose range of IC_{50}. A previous gene KO experiment indicated that the homozygous KO of $G\alpha_{13}$ resulted in impaired vasculogenesis and

intrauterine death, whereas that of $G\alpha_{12}$ gene did not do so [24]. Because of this lethality by the complete absence of $G\alpha_{13}$, we could not examine the effect of homozygous KO of $G\alpha_{13}$.

Airway structural changes occur in response to persistent inflammation, leading to airway remodeling [34]. In our experimental model, ovalbumin sensitization and challenges also increased sub-epithelial deposition of collagen and provoked airway inflammation. Our result confirmed airway wall thickening, subepithelial fibrosis and hyperplasia of mucus glands. The lack of difference in the degree of airway remodeling between WT and $G\alpha_{12}/G\alpha_{13}$ KO mice after repeated antigen challenges indicates that either homozygous $G\alpha_{12}$ deficiency or heterozygous $G\alpha_{13}$ deficiency was insufficient to alter the characteristic features of chronic airway inflammation and remodeling. However, the possibility still exists that certain events such as inflammatory process of asthma may require $G\alpha_{13}$ because the $G\alpha_{13}$ gene was not completely knocked out in this experimental model.

Previously, we found that homozygous KO of $G\alpha_{12}$ or double heterozygous KO of $G\alpha_{12}/G\alpha_{13}$ decreased the production of IgG1, IgG2a and IgG2b subclasses, as compared to control. Also, mice deficient in $G\alpha_{12}$ or partially deficient in $G\alpha_{13}$ or $G\alpha_{12}/G\alpha_{13}$ showed low production of IgG2b in response to TNP-LPS. Hence, both $G\alpha_{12}$ and $G\alpha_{13}$ are involved in thymus-dependent and independent productions of IgG [35]. In addition, $G\alpha_{12}$ deficiency decreased ovalbumin-specific IgE level, whereas heterozygous KO of $G\alpha_{13}$ failed to do so (Lee and Kim, unpublished data). However, the increase in IgE level after ovalbumin immunization was minor. After antigen sensitization, Th_2-dependent antigen-specific antibodies mediate airway hyper-responsiveness by recruiting blood-borne cells (e.g., eosinophils) into the injured airway tissues [36]. We found that the levels of IL-4, IL-5 and IL-13, representing the activity of Th_2 lymphocytes that participate in asthma pathogenesis, were increased after ovalbumin challenges regardless of $G\alpha_{12}$ or $G\alpha_{13}$ KO (Lee and Kim, unpublished data). Our data might support eosinophil survival. The lack of changes in the production of IL-4, IL-5 and IL-13 in conjunction with the fact that airway contraction regulated by $G\alpha_{13}$ (but not by $G\alpha_{12}$) did not match with the humoral response regulation by $G\alpha_{12}$ and/or $G\alpha_{13}$ excludes the possibility that $G\alpha_{13}$-specific regulation of hyper-reactivity of bronchial contraction results from changes in the lymphocyte-directed response.

In conclusion, heterozygous absence of the $G\alpha_{13}$ gene abrogated methacholine-induced contractions of airway, whereas homozygous absence of the $G\alpha_{12}$ gene failed to do so. Our data may provide important new information to increase understanding of cholinergic bronchial contraction in airway responsiveness. As future research provides us with chemical and biological tools for a target of $G\alpha_{13}$, their physiological and pharmacological potential as a target for intervention can be explored.

Competing interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2009.01.016.

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