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Suppression of prostaglandin E\textsubscript{2}-induced MUC5AC overproduction by RGS4 in the airway

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Song KS, Choi YH, Kim JM, Lee H, Lee TJ, Yoon JH. Suppression of prostaglandin E\textsubscript{2}-induced MUC5AC overproduction by RGS4 in the airway. Am J Physiol Lung Cell Mol Physiol 296: L684–L692, 2009. First published February 6, 2009; doi:10.1152/ajplung.90396.2008.—The mechanism by which E-prostanoid (EP) receptor is critically involved in PGE\textsubscript{2}-induced mucin 5AC (MUC5AC) gene expression in the airway has been unclear. Furthermore, there have been little reports regarding the negative regulatory mechanism and/or proteins that affect PGE\textsubscript{2}-induced MUC5AC overproduction. In the present study, we found that PGE\textsubscript{2} induced MUC5AC gene expression in a dose-dependent manner (EC\textsubscript{50} 73.31 ± 3.13 nM) and that the EP\textsubscript{2a}-specific agonist, misoprostol, increased MUC5AC mRNA level, whereas the EP\textsubscript{1a}-specific agonist, sulprostone, had no effect. Interestingly, the cAMP concentration (685.1 ± 14.9 pM) of the EC\textsubscript{50} value of EP\textsubscript{2}-mediated cAMP production was much higher than that of EP\textsubscript{2} (462.33 ± 23.79 pM), suggesting that EP\textsubscript{2} has higher sensitivity to PGE\textsubscript{2} compared with EP\textsubscript{2}. Moreover, PGE\textsubscript{2}-induced Muc5ac overproduction was much increased in regulator of G protein signaling (Rgs) 4 knockout (KO) mice compared with wild-type mice at both transcriptional and translational levels, and it was dramatically suppressed in Rgs4 KO mice that had been infected with lentivirus expressing RGS4 (lenti::RGS4) compared with lentivirus expressing enhanced green fluorescent protein (lenti::eGFP). Finally, we demonstrated that PGE\textsubscript{2} can induce MUC5AC overproduction via the EP\textsubscript{2} receptor and that RGS4 may have suppressive effects in controlling MUC5AC overexpression in the airway. These findings may provide a molecular paradigm for the development of novel drugs for respiratory diseases.

MUCINS are heavily glycosylated, high molecular weight glycoproteins produced by the epithelia of the respiratory, gastrointestinal, and reproductive tracts (25, 35). Twenty-one mucin genes have been identified; however, it has been unclear how mucins are regulated by various stimulants and are secreted in respiratory diseases (6). Of these, mucin 5AC (MUC5AC) is one of the major mucins in the human respiratory tract, and MUC5AC mRNA levels are known to be upregulated by proinflammatory cytokines, LPS, neutrophil elastase, cigarette smoking, or reactive oxygen species (15, 19, 31, 32, 36) via various signaling pathways. Nonetheless, there have been few reports on the negative regulatory mechanisms that affect stimulant-induced MUC5AC gene expression in the airway.

PGE\textsubscript{2}, one of the major prostaglandins produced, is a versatile eicosanoid that regulates key responses in numerous physiological and pathological processes, including angiogenesis, tumorigenesis, immune modulation, and inflammatory airway diseases (7, 33). PGE\textsubscript{2} exerts its biological functions through E-prostanoid (EP) cell surface receptors. EP\textsubscript{1,4} receptors couple to G\textsubscript{\textalpha} subunits, and G\textsubscript{\textbeta} subunits, respectively, and can activate or inhibit several signaling proteins resulting in the increased formation of secondary messengers. Recently, Gray et al. (14) reported EP\textsubscript{2} and EP\textsubscript{4} may be the major receptors for IL-1\textbeta-induced MUC5AC gene expression by producing PGE\textsubscript{2} in the normal human tracheobronchial epithelial cells, and Kim et al. (20) suggested that EP\textsubscript{4} is essential for PGE\textsubscript{2}-induced MUC5AC secretion in normal human nasal epithelial (NHNE) cells. However, the exact mechanism by which EP receptor is critically involved in PGE\textsubscript{2}-induced MUC5AC gene expression has been unclear. Furthermore, we wanted to find a negative regulator of PGE\textsubscript{2}-induced MUC5AC gene expression at the EP receptor level.

We thought a member of regulators of G protein signaling (RGS) proteins may play as a negative regulator of PGE\textsubscript{2}-induced MUC5AC gene expression since RGS proteins can stimulate the G protein GTPase activity and thereby produce desensitization (deactivation). RGS proteins are defined by a shared 120-amino acid domain that binds directly to activated G\textsubscript{\textalpha} subunits (3) and act as tightly regulated modulators and integrators of G protein signaling. A protein domain, known as the RGS box, harbors GTPase-accelerating proteins (GAP) activity and is the defining feature of RGS proteins. Outside the RGS box, the presence of other protein domains diversifies the range of protein interactions and regulatory activities of RGS proteins. Binding of RGS proteins to active G\textsubscript{\textalpha} (GTP-bound) can interfere with effector-binding proteins, thereby blocking activation and downstream signaling (16). In addition, the expression of RGS2, -3, -5, and -16 subtypes, mainly members of R4 RGS subfamily, has been detected in the lung (22, 26). In the airway, which RGS protein has a critical role and how the interplay between EP receptor and RGS leads to the alteration of MUC5AC overproduction remain poorly understood.

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In the present study, we examined the mechanism by which PGE2 increases MUC5AC overproduction at the receptor level. We show that the EP4 receptor plays as an important role in PGE2-induced MUC5AC overproduction in the airway. In addition, RGS4 suppressed PGE2-induced MUC5AC overproduction both in vitro and in vivo by binding to EP4-coupled Goα, depending on GTPγS.

MATERIALS AND METHODS

Materials. All chemical compounds were purchased from Cayman Chemical (Ann Arbor, MI). Goα, and Gβγ antibodies were purchased from Calbiochem (Merck; Darmstadt, Germany), RGS4 and actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and MUC5AC antibody was purchased from Lab Vision (Fremont, CA). The cDNA expression constructs encoding EPα, Goα, Goγ, Q227L, RG52, RGS3, RGS4, and RGS5 were purchased from the UM cDNA Resource Center (Rolla, MO). All of small interfering RNAs (siRNAs) were synthesized from Bioneer (Daejeon, Korea): EPα, 5'-UUCUUCAGCGACUAGUAU (dTdT)-3'; RGS4, 5'-GGAUCAGGCUGAGAAGAGU (dTdT)-3'; and negative control, 5'-CCUACCGCACCACAUUUUCGUU (dTdT)-3'.

Cell cultures. NHNE cells (2 × 10⁵ cells/well) were seeded in 0.5 ml of culture medium on Transwell clear culture inserts (24.5 mm, 0.45-μm pore size; Costar, Cambridge, MA). Cells were cultured in a 1:1 mixture of BEGM and DMEM containing all the supplements previously described (39). Human tissue was maintained in accordance with the guidelines and approval of the institutional review board of Yonsei University, Seoul, Korea. Consent from both patients and the local ethics committee was obtained for use of the specimens. The human lung mucociliary epithelial carcinoma cell line (NCI-H292) was purchased from the American Type Culture Collection (CRL-1848; Manassas, VA) and cultured in RPMI 1640 (Invitrogen; Carlsbad, CA) supplemented with 10% fetal bovine serum in the presence of penicillin-streptomycin at 37°C in a humidified chamber with 5% CO2.

Cell transfection. Cells were plated in 6-well plates 1 day before transfection with plasmid DNA (1 μg/well) or siRNA (100 pmol/well) using FuGENE 6 (Roche; Indianapolis, IN) according to the manufacturer’s instructions. Approximately 24 h after transfection, cells were maintained in 0.2% serum RPMI media for 16–18 h before treatment with PGE2 and then harvested 24 h after treatment.

Measurement of cAMP level. Cells were plated in 6-well plates 1 day before transfection. Approximately 24 h after transfection, transfected cells were replated at 2,000/well in 96-well plates and maintained in 0.2% serum RPMI media for 16–18 h before treatment with PGE2. cAMP production was measured according to the manufacturer’s instructions (Promega; Madison, WI).

PCR. After reverse transcription, RT-PCR was performed to amplify DNA fragments. All of EP primers (18) were synthesized from Bioneer. PCR products were run for 35 cycles using the amplification conditions (18). Real-time PCR was performed using a Bio-Rad iCycler iQ Detection System (Bio-Rad, Hercules, CA) with iSYBR Green Superscript. The following primers were used: human MUC5AC, forward 5'-CACGACACGCTCCCTCAATA-3' and reverse 5'-ACCCGATTTCGGGCATCC-3'; β2-microglobulin, forward 5'-GCCTCCGGTGCCCTTACGC-3' and reverse 5'-GAGTACGCTGTGAGACCTCA-3'; mouse Muc5ac primers, forward 5'-CATTGAGACTTGCCAGGCAAGAAC-3' and reverse 5'-TTACTGGGAAAGGGCCGAGC-3'; and mouse GADPH primers, forward 5'-TGTGCCGTGCGATGCTG-3' and reverse 5'-CCCTTCCTTACACCATTTAG-3'. Parameters were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. The result was normalized against β2-microglobulin as an internal control (mouse GADPH for Muc5ac mRNA), and the relative quantity of MUC5AC mRNA was determined using a MUC5AC standard curve.

Western blot analysis, dot blot analysis, and immunoprecipitation. These methods were described previously (33). NCI-H292 cells were grown to confluence in 6-well plates. After treatment with PGE2, the cells were lysed with 2× lysis buffer [250 mM Tris·HCl (pH 6.5), 2% SDS, 4% 2-mercaptoethanol, 0.02% bromphenol blue, 10% glycerol]. Equal amounts of whole cell lysates from protein samples were resolved by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (PVDF; Millipore, Bedford, MA). For dot blot analysis, 125 μl of protein lysates were directly diluted with dot blot buffer (600 mM KSCN, 10 mM EDTA, pH 8.0) in 96-well plates. After serial dilution, lysates were applied by vacuum using a dot blot apparatus. The membrane were incubated with MUC5AC antibody as primary antibody and then incubated with anti-mouse horseradish peroxidase IgG. The signal was detected by enhanced chemiluminescence kit, and the relative fold increase was calculated by dividing stimulant-treated signal into control signal since there is no commercially available MUC5AC protein for standardization. The data were represented as means ± SD of triplicate cultures from the independent experiments. For immunoprecipitation, cells were transfected with either wild-type Goα, or dominant negative Goα, Q227L mutant and RGS4-expressing constructs using FuGENE 6. Following transfection, cells were washed with ice-cold PBS and harvested by scraping into lysis buffer [25 mM HEPES, 150 mM NaCl, 0.5% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, protease inhibitor tablet (Complete Mini; Roche)], sonicated (4 times each for 5 s), and centrifuged at 12,000 g for 15 min. Supernatant lysates (230 μl) were precleared with GammaBind G Sepharose (GE Healthcare; Uppsala, Sweden) for 30 min at 4°C. Following centrifugation, anti-Goα, antisera were added to precleared lysates, incubated for 14 h at 4°C, and added GammaBind G Sepharose.

RESULTS

Mice, genotyping, and tracheotomy. Six- to eight-week-old C57BL/6 mice and RGS4 knockout (KO) mice (B6-129P2-Rgs4<sup>−/−</sup>/J; The Jackson Laboratory; Bar Harbor, ME) were maintained in accordance with the guidelines and under approval of the Animal Care Committee of Yonsei University, Seoul, Korea. Mice were anesthetized with Zoletil (30 mg/kg). Genotyping of Rgs4<sup>−/−</sup> mice was performed by PCR on genomic tail DNA using an endogenous gene-specific primer (5'-GACATGAAACATCGGCTGGGGTTC-3') and targeted neo primer (5'-ACCGCATTTGACCATTTTT-3'), an endogenous and targeted gene-specific primer (5'-CCATCTTGACCCAATCTGGCAGC-3'), and targeted neo primer (5'-GGGCAGCTTATTCTCCTCCACTCAT-3'), which produced a 226-bp and a 484-bp band from the wild-type and the targeted allele, respectively. For intra-tracheal instillation, the trachea was surgically exposed after anesthetization by making an incision in the neck skin. A microsyringe carrying a 31-gauge needle filled with PGE2 solution was used for injection into the exposed trachea. PGE2 (50 μl; 0.5, 1.0, or 5.0 mg/kg) was injected into the lumen of each trachea. After injection, the skin was sutured. At 1 day after injection, the mouse was killed, and the trachea was removed (34).

Virus production and in vivo intranasal instillation for infection. Vector construction and virus production of Lentim1.2::RGS4-GFP, Lentim1.2::eGFP, and small hairpin (sh) Lentim3.4R::RGS4<sup>−/−</sup>-<sup>Q227L</sup> were performed by Macrogen (Seoul, Korea). Viruses [5 × 10⁶ transduction units (TU)/50 μl] were administered drop by drop to the right nostril of anesthetized mice using sequencing tips (27). Three days after infection, tracheotomy was performed.

Statistical analysis. The data represent the means ± SD for at least three independent experiments. Where appropriate, the statistical differences were assessed by Wilcoxon Mann-Whitney test. A P value <0.05 was considered statistically significant.

RESULTS

PGE2-induced MUC5AC gene expression was mediated via the EP4 receptor in NHNE cells. To determine whether PGE2 increases MUC5AC gene expression in NHNE cells, real-time
PCR analysis was performed. As seen in Fig. 1A, PGE_2 induced MUC5AC gene expression in a dose-dependent manner with an EC_50 value of 73.31 ± 3.13 nM. Since Kunikata et al. (21) reported that endogenous PGE_2 played an important feedback function in allergic inflammation, and we thought that newly synthesized endogenous prostaglandins by exogenous PGE_2 might influence the results, we wanted to rule out this effect. Accordingly, indomethacin, a nonselective cyclooxygenase-1 (COX-1) and COX-2 inhibitor, was employed to investigate the function of exogenous PGE_2. Cells were treated for 24 h with PGE_2 alone or pretreated indomethacin (3 μM) for 1 h before PGE_2 treatment. Indomethacin alone had no effect, and pretreatment of indomethacin did not affect PGE_2-induced MUC5AC gene expression (Fig. 1B). This result suggests that MUC5AC gene expression has been affected by exogenous PGE_2 but not endogenous PGE_2 in the present study. We next examined whether mRNA level of EP receptors was affected by PGE_2. All of EP receptors were expressed, and treatment with PGE_2 did not affect EP receptor gene expression in NHNE cells (Fig. 1C). To identify which EP receptors are involved in PGE_2-mediated MUC5AC gene expression, we performed with anti-HA antibody. No change was detected in EP_2, EP_4, or mock construct. Since these RGS subtype constructs have been excluded RGS16 from this study, and selected RGS2, -3, -5, and -16), containing only RGS domain, are expressed in the lung (22, 26). Of these RGS proteins, because RGS16 has been upregulated in germinal center and activated T cells, suggesting that it has a role in adaptive immunity (8), we have excluded RGS16 from this study, and selected RGS2, -3, -5, and -5 proteins. Since these RGS subtype constructs have been tagged with hemagglutinin (HA), Western blot analysis was performed with anti-HA antibody. No change was detected in the expression of HA in cells transfected with each RGS construct (Fig. 3A, top). Of the RGS proteins tested, only ectopic RGS4 expression inhibited PGE_2-induced MUC5AC gene expression (Fig. 3B, bottom). To determine a potential role for RGS4 in PGE_2-mediated MUC5AC gene expression, siRNA RGS4 was employed. Whereas wild-type RGS4 dramatically suppressed PGE_2-induced MUC5AC gene expression, siRNA RGS4 much increased PGE_2-induced MUC5AC gene expression compared with PGE_2 alone (Fig. 3B). This phenomenon is due to the inhibition of endogenous RGS4 expression. In addition, to investigate a potential role for the RGS4 in G_α_5-mediated signal processing, we asked whether RGS4 bound to G_α_5 as a component of the G protein complex. If so, we thought that RGS4 could negatively regulate MUC5AC gene expression by binding to the G_α_5, in vitro. We checked the inhibitory effects of RGS proteins containing GTPase activity on MUC5AC gene expression. We have reported that mainly R4 RGS family proteins (RGS2, -3, -5, and -16), containing only RGS domain, are expressed in the lung (22, 26). Of these RGS proteins, because RGS16 has been upregulated in germinal center and activated T cells, suggesting that it has a role in adaptive immunity (8), we have excluded RGS16 from this study, and selected RGS2, -3, -5, and -5 proteins. Since these RGS subtype constructs have been tagged with hemagglutinin (HA), Western blot analysis was performed with anti-HA antibody. No change was detected in the expression of HA in cells transfected with each RGS construct (Fig. 3A, top). Of the RGS proteins tested, only ectopic RGS4 expression inhibited PGE_2-induced MUC5AC gene expression (Fig. 3B, bottom). To determine a potential role for RGS4 in PGE_2-mediated MUC5AC gene expression, siRNA RGS4 was employed. Whereas wild-type RGS4 dramatically suppressed PGE_2-induced MUC5AC gene expression, siRNA RGS4 much increased PGE_2-induced MUC5AC gene expression compared with PGE_2 alone (Fig. 3B). This phenomenon is due to the inhibition of endogenous RGS4 expression. In addition, to investigate a potential role for the RGS4 in G_α_5-mediated signal processing, we asked whether RGS4 bound to G_α_5 as a component of the G protein complex. If so, we thought that RGS4 could negatively regulate MUC5AC gene expression by binding to the G_α_5, in vitro.
Fig. 1. PGE2 induces MUC5AC gene expression via the E-prostanoid (EP) receptor 4 (EP4) in normal human nasal epithelial (NHNE) and human lung mucoepidermoid carcinoma (NCI-H292) cells. A: NHNE cells were treated for 24 h with several concentrations of PGE2. Cell lysates were harvested for real-time quantitative PCR. Corresponding dissociation curves for standard (0.01 fg to 1 ng; Aa) and samples (Ab) were amplified using specific primers with SYBR Green dye. These curves were merged on the same panel (Ac).

B: NHNE cells were treated for 1 h with 3 μM indomethacin (indo), a cyclooxygenase-1 (COX-1) and COX-2 inhibitor, before treatment for 24 h with 100 nM PGE2. Cell lysates were harvested for real-time quantitative PCR. *P < 0.05 compared with control.

C: NHNE cells were treated with 100 nM PGE2 for 24 h. The presence of EP receptors mRNA was detected by RT-PCR with specific EP receptors primers, respectively.

D: NHNE cells were treated for 24 h with 100 nM PGE2, 10 μM EP1/3 agonist, sulprostone, or 10 μM EP2/4 agonist, misoprostol. Cell lysates were performed real-time quantitative PCR. *P < 0.05 compared with control.

E: NHNE cells were treated for 1 h with either 10 or 50 μM EP2 antagonist, AH6809, or 10 or 50 μM EP4 antagonist, GW627368X, before treatment for 24 h with 100 nM PGE2. Cell lysates were harvested for PCR. *P < 0.05 compared with PGE2 alone and **P < 0.05 compared with overexpressed EP2 or EP4. All data shown are representative of 3 independent experiments. Tm, melting temperature; β2M, β2-microglobulin; WT, wild-type; siEP2, siRNA EP2; sicon, siRNA control.

F: NCI-H292 cells transfected with construct encoding wild-type EP2 or EP4, small interfering RNA (siRNA) EP2 or EP4, or siRNA control were treated for 24 h with 100 nM PGE2. Cell lysates were harvested for PCR. *P < 0.05 compared with PGE2 alone and **P < 0.05 compared with overexpressed EP2 or EP4. All data shown are representative of 3 independent experiments.

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was surgically exposed under general anesthesia and aseptic conditions, and 50 μL of 0.1, 1.0, and 5.0 mg/kg PGE2 was intratracheally instilled with a 31-gauge needle. After 24 h, PCR and dot blotting for Muc5ac expression were performed using the trachea from the killed mice (Fig. 4, A and B). PGE2 increased MUC5AC expression in vivo at both the transcriptional and translational levels. These results were consistent with the in vitro results. In addition, to investigate whether RGS4 has an effect on PGE2-induced Muc5ac expression in vivo, Rgs4 KO mice were employed. After Rgs4<sup>tm1Dgen/tm1Dgen</sup> mice were genotyped by PCR with genomic tail DNA using specific primers, which produced a 226-bp and a 484-bp band from the wild-type and the targeted allele, respectively (data not shown), tracheotomy was performed for PGE2 instillation. Twenty-four hours later, total RNA and tissue lysates were purified from the trachea of Rgs4 KO mice. PGE2-induced Muc5ac expression was much increased in Rgs4 KO mice compared with wild-type mice at both the transcriptional and translational levels (Fig. 4, C and D), indicating that RGS4 probably has a suppression effect on PGE2-induced Muc5ac overproduction in the airway. We thought that when lent::RGS4 virus administrate into Rgs4 KO mice, it may restore lost RGS function in PGE2-induced Muc5ac overproduction in the airway. Lentivirus expression system was used. Viruses (5 × 10<sup>5</sup> TU/50 μL) were administrated in the right nostril of anesthetized mice drop by drop using sequencing tips. Three days after infection, the trachea was surgically exposed to instill PGE2. RGS4 was well-expressed in Rgs4 KO mice that had been infected with lent::RGS4-eGFP (Fig. 4E, top). PGE2 instillation to the trachea of Rgs4 KO mice that had been infected with lent::RGS4-eGFP resulted in a fourfold decrease of Muc5ac expression compared with lent::Mock-eGFP (Fig. 4E, bottom), indicating that administration of RGS4 to Rgs4 KO mice airway restores its suppressive function in Rgs4 KO mice. These results were consistent with in vitro results (Fig. 3B), and an inhibitory effect of RGS4 on Muc5ac overproduction was revealed by knocking out RGS4. Next, to investigate whether EP4 is responsible for PGE<sub>2</sub>-induced Muc5ac gene expression in vivo, wild-type mice were injected with the EP4-specific agonist, PGE1-OH, and expression level of Muc5ac mRNA was determined. After the mice were preinjected with 1.0, 2.5, and 5.0 mg/kg GW627368X (ip) for 1 h, PGE1-OH or PGE2 was instilled intratracheally. The EP4 agonist increased Muc5ac mRNA level, whereas the mRNA level strongly inhibited GW627368X in a dose-dependent manner, and PGE2-induced mRNA level was also inhibited by GW627368X (Fig. 4F), indicating that EP<sub>4</sub> receptor is essential for PGE<sub>2</sub>-induced Muc5ac overproduction. These results suggest that PGE<sub>2</sub> binds to the EP<sub>4</sub> receptor thereby inducing MUC5AC overproduction, and RGS4 may be a key negative regulator during airway mucosal inflammation.
DISCUSSION

PGE2 is frequently observed at high concentrations at the sites of inflammation and plays physiological roles in the regulation of mucosal immunity (9). Secreted PGE2 stimulates epithelial cells in an autocrine and paracrine fashion. Epithelial cells thereby increase mucin secretion and mucin gene expression, including MUC5AC and MUC8 (20). Interestingly, even though the importance of PGE2 in human airway epithelium is well-established (29), the molecular mechanism by which PGE2-induced MUC5AC overproduction is regulated is still unclear. The focus of the present study was to identify molecular mechanism(s) and regulatory molecule(s) for PGE2-induced MUC5AC overproduction.

In the present study, we showed that PGE2 induced MUC5AC mRNA expression in NHNE cells, whereas endogenously produced or endocrinal PGE2 did not affect MUC5AC gene expression during exogenous PGE2 treatment (Fig. 1B). Interestingly, even if the half-life of PGE2 is very short (10), PGE2 had an effect in the early stage during the 24-h treatment period. The fact that MUC5AC gene expression increases at 12 h after the beginning of PGE2 exposure can be attributed to PGE2 binding to its receptors in the early stage. It seems that the kinetics of PGE2 in the early stage is sufficient to induce MUC5AC gene expression because activated receptor propagates specific signaling to amplify physiological PGE2 signaling. The biological actions of PGE2 are mediated by seventh transmembrane EP receptors (23). Using RT-PCR, we found that all EP receptor mRNAs were expressed and PGE2 treatment did not affect EP receptor gene expression in NHNE cells (Fig. 1C). In the airway, EP2 and EP4 receptors have been known as major receptors for MUC5AC gene expression and secretion (14, 20). Consistent with this result, the EP2/4-selective agonist (misoprostol) had a significant effect on MUC5AC gene expression (Fig. 1D), whereas the EP1/3-selective agonist (sulprostone) had no effect. These results suggest that either the simultaneous induction of the two different G coupled PGE2 receptors plays a pivotal role for PGE2 during airway mucosal inflammation or both EP2 and EP4 may cooperatively work as a compensatory system for MUC5AC gene expression.

Among these receptors, however, the EP4-specific antagonist has much more inhibited PGE2-induced MUC5AC gene expression than the EP2 antagonist (Fig. 1E). Even if the pharmacokinetics of the two different drugs has been considered deeply, this suggests that EP4 may be more closely related to PGE2 signaling at least in part in NHNE cells. Consistent with the pharmacological data, knockdown of EP4 suppressed PGE2-induced MUC5AC gene expression (Fig. 1F). We wondered what is different between EP2 and EP4 despite them both...
being the same G_\alpha_\text{q/-} coupled receptors. One reason may be due to a difference in their ligand selectivity of PGE_2. Despite almost the same mRNA expression levels of the receptors, cAMP production of the EC_50 value of EP_4-mediated cAMP production was about 685.1 \pm 14.9 \text{ pM} (Fig. 2A), whereas that of EP_2-mediated cAMP production was 462.33 \pm 23.79 \text{ pM} following PGE_2 treatment (Fig. 2B). This indicates that the higher cAMP concentration of the EC_50 value of EP_4-mediated cAMP production is because of high selectivity of PGE_2 to convey signaling efficiently to coupled signaling pathways. Consistent with our results, Fujino et al. (11, 12) reported that EP_4 receptor strengthens an association of the PGE_2 to induce T cell factor and early growth response factor-1 in inflammation. Another possible reason that there is a difference in
biological actions of EP2 and EP4 may be due to posttranslational modifications, such as glycosylation, hydroxylation, methylation, nucleotideylation, and phosphorylation. Our thinking was supported by several studies that a charged residue(s) in the seventh transmembrane domain and posttranslational modification sites were involved in receptor activation and desensitization (1, 2, 13, 24).

The main finding of this study is that RGS4 suppressed PGE2-induced G\textsubscript{\textalpha}RGS mediated signaling in vitro and in vivo by enhancing GTPase acceleration. Until now, there has been no report of a relationship between PGE2 receptors and RGS in mucin-related literature. Despite that the characterized main function of RGS proteins is GTPase-activating proteins for G\textsubscript{\textalpha}i/o, G\textsubscript{\textalpha}q, or G\textsubscript{12/13} classes of G proteins, no interaction between RGS and G\textsubscript{\textalpha} has been found (40). Recently, Castellone et al. (4) reported that the G\textsubscript{\textalpha}-GTP bound axin protein contained an RGS domain, and thus PGE2 was able to promote colon cancer cell growth. In addition, Zheng et al. (40) suggested that the RGS domain of RGS-PX1 specifically interacted with G\textsubscript{\textalpha}, thereby accelerating its GTP hydrolysis, which leads to an attenuation of G\textsubscript{\textalpha}-mediated signaling in bovine brain lysates. In the present study, RGS4 bound G\textsubscript{\textalpha}-GTP, thereby attenuating PGE2-induced MUC5AC overproduction. In addition, the dynamics of heterotrimeric G protein signaling complex are regulated by RGS4 protein in GTP-dependent manner (Fig. 3C). To address the role of RGS4 protein in the pathogenesis of anti-inflammation in the airway, a gain/loss-of-function study in an animal model must be considered. PGE2 instillation into Rgs4/ KO mice much increased Muc5ac overproduction compared with that in wild-type mice (Fig. 4, C and D), and Rgs4 function has been restored and Muc5ac gene expression was suppressed after administration of RGS4-expressing lentivirus into Rgs4 KO mice (Fig. 4E). These results demonstrate that RGS4, a classical R4 RGS superfamily lacking any additional well-defined domains (3), negatively regulates the G\textsubscript{\textalpha}-mediated signaling that induced Muc5ac production during airway mucosal inflammation. These results, however, raise an interesting question. Why does only RGS4 function in the airway epithelium? We just thought that specificity may be mediated in part through selectivity of RGS proteins for particular G protein substrates (17) or that posttranslational modification of NH\textsubscript{2}-terminal domain except palmitoylation may be required for the plasma membrane association of RGS4. In fact, although RGS2 does not have an inhibitory function (Fig. 3A), it is similar in its NH\textsubscript{2}-terminal structure to RGS4, including an amphipathic \alpha-helical membrane targeting domain (5, 17). In addition, although it has been known that RGS4 and RGS2 are the most likely to be involved in regulation of signaling by receptors coupled to G\textsubscript{\textalpha}q pathways (28), RGS4 inhibited G\textsubscript{\textalpha}q pathway in the present study. These findings support our hypothesis that either the difference of the physiological functions of RGS2 and RGS4 may due to posttranslational modifications on NH\textsubscript{2}- or COOH-terminal domain, excluding RGS box, or that some mediating (bridging) protein(s) may regulate the ability of RGS4 protein like a small switching molecule when it (they) senses the multiplicity of different signaling pathways during airway inflammation. In the present study, we used mice trachea to detect differences in Muc5ac overproduction in Rgs4\textsuperscript{+/+} and Rgs4\textsuperscript{−/−} mice but not lung and selected 24 h as Muc5ac overproduction peaked at 24 h. Although 24 h may be sufficient to express Muc5ac in goblet cells before inducing inflammatory cell infiltration or mucous metaplasia, it may be not enough to induce inflammatory cell infiltration or mucous metaplasia in the airway.

Taken together, these findings suggest that PGE2 may interact directly with EP\textsubscript{2} receptor, resulting in increased MUC5AC overproduction. The ability of RGS4 proteins to interact with G\textsubscript{\textalpha} is important to negatively regulate PGE2-induced MUC5AC overproduction in the airway. As more mechanistic insights are obtained, identification of an RGS accessory molecule that regulates RGS function may be considered.

GRANTS

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