Lipopolysaccharide from *Pseudomonas aeruginosa* induces *MUC5AC* gene expression via Gαq-coupled P2Y2 receptor, and its pathway is regulated negatively by RGS4 in human airway epithelial cells

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Directed by Professor Joo-Heon Yoon

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Hyun Jun Kim

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The Graduate School
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In what feels like an instant, five semesters have passed since I began this journey. And now, as I prepare the final touches on my dissertation, I take a moment to recognize those who have helped me along the way. First, I would like to convey my deepest and most humble gratitude to my dissertation advisor, the esteemed Joo-Heon Yoon, whose boundless generosity and unwavering leadership has inspired me from beginning to end. I would like to thank Professor Jin Hee Cho, Professor Min-Koo Lee, Professor Jae Myun Lee, and Professor Won-Jae Lee for taking time from their busy schedules to provide invaluable guidance. I would also like to thank Professor KyungSeob Song, who never failed to express his encouragement and contribute timely advice during my research. And finally, I would like to share the joy of this occasion with my parents, my pillars of support throughout the journey and, indeed, throughout my life. Having learned so much from the experience, I am determined now to wield this individual achievement in the pursuit of even greater accomplishments. I will not disappoint all those who have believed in me. Thank you all once again.

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Lipopolysaccharide from *Pseudomonas aeruginosa* induces *MUC5AC* gene expression via Gαq-coupled P2Y2 receptor, and its pathway is regulated negatively by RGS4 in human airway epithelial cells

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Mucus hypersecretion by airway epithelium is a major characteristic of a number of respiratory diseases, including asthma, chronic bronchitis, and cystic fibrosis. Because the respiratory tract is highly exposed to many external stimulants such as bacteria or air pollution, it is important to clarify the mechanism by which major pathogens like *Staphylococcus aureus* and *Pseudomonas aeruginosa* increase mucus secretion. We examined the intracellular signal transduction pathway and regulation of toxin-induced mucin gene expression. First, lipopolysaccharide from *P. aeruginosa* induced intracellular ATP secretion, and secreted ATP is released extracellular to stimulate cells for inflammatory response. Moreover, we found that treatment with exogenous ATP resulted in the induction of *MUC5AC* gene expression in NCI-H292 cells, due to the activation of Gαq-coupled P2Y2 receptors. In

addition, the overexpression of dominant-negative mutants of PLCβ3, Akt, and either ERK1/2 or p38 signaling suppressed ATP-induced *MUC5AC* gene expression. Interestingly, regulator of G-protein signaling 4 (RGS4) inhibited ATP-induced *MUC5AC* gene expression by interacting between the Gαq and RGS domains in the RGS4 protein. RGS4 knocked down siRNA-enhanced ATP-induced activation of the P2Y2 receptor, suggesting that RGS4 may act as a suppressor of ATP-induced activation of the P2Y2 receptor. In conclusion, lipopolysaccharide from *P. aeroginosa* induces ATP secretion, and then ATP promotes *MUC5AC* gene expression via a sequential P2Y2/Gαq/PLCβ3/ Akt/ERK1/2 or p38 MAPK pathway, while RGS4 attenuates its signaling cascade by GTPase activation. These results give additional insights into the molecular mechanism of negative regulation of mucin gene expression and enhance our understanding of mucus hypersecretion during inflammation.

Key words : mucin, lipopolysaccharide, ATP, G protein coupled receptor, RGS proteins

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I. Introduction

Exposure of respiratory tract to noxious gases, air pollutants, bacteria, and viruses can lead to airway obstruction.[1] Endotoxins and other agents derived from microbes can cause occupational respiratory conditions and other diseases. Physiological responses to endotoxin after inhalation are well known.[2, 3] Irritation in the nose and airways and chronic airway inflammation with increased airway responsiveness can be related to the inflammatory properties of endotoxin. In addition, microbial cell wall agents (MCWAs) such as muramic acid, lipoteichoic acid, and peptidoplycans from gram-positive bacteria can also induce airway obstruction.[3] Moreover, unnecessary antibiotic treatment continues to contribute to the worldwide emergence of antibiotic-

resistant strains.[4] A full understanding of the molecular pathogenesis of endotoxin and MCWAs is an important part of developing novel therapeutic strategies for the diagnosis, prevention, and treatment of airway diseases.

The molecular pathogenesis of bacterial infections is still largely undefined. Interestingly, there is evidence that up-regulation of mucin production induced by bacteria could play an important role.[4] In addition, excessive secretion of mucus in the airways is an important cause of morbidity and mortality in diseases such as asthma, chronic obstructive pulmonary disease (COPD), and cystic fibrosis (CF).[5] Understanding the mechanisms that lead to increased mucus secretion in these diseases will be important for improving future therapies. Mucins are a family of large, heavily glycosylated proteins that comprise a significant portion of the secreted mucus and secretory granules.[6] The heavily glycosylated structures are believed to contribute to the highly viscoelastic property of secreted airway mucus. *MUC* genes encode the protein backbones of mucins. Twenty *mucin* genes have been identified, but it remains unclear which mucins are secreted in the various airway diseases. Mucins are usually subdivided into two groups based on domain: membrane-bound (MUC1, MUC3, MUC4, MUC11, MUC12, MUC13, MUC17, MUC18, and MUC20) and secreted (MUC2, MUC5AC, MUC5B, MUC6, MUC7, MUC9, and

MUC19). Some other mucin genes, including *MUC8,* have not yet been fully characterized.[7]

Nucleotides such as uridine-5′-triphosphate (UTP) and adenosine triphosphate (ATP) regulate mucin secretion by activating purinergic receptors (P2Y) in airway epithelial cells.[8, 9] Recently, we showed that purinergic receptors are expressed in cultured normal human middle ear epithelial (NHMEE) cells, and that UTP, an agonist, stimulates mucin secretion.[10] In addition, we also reported that stimulation of P2Y receptors directly activates the basolateral Na-K-2Cl^- cotransporter (NKCC) by Ca^2-dependent pathways in normal human nasal epithelial cells.[11] However, it remains unclear how P2Y, activated by specific agonists, can functionally induce and regulate *MUC5AC* gene expression in the airway epithelium.

Heterotrimeric G-proteins convey signals from G-protein coupled receptors (GPCR) to intracellular effectors. Hydrolysis of GTP to GDP by Gα returns the protein to its inactive conformation, promoting its association with Gβγ subunits. Regulators of G-protein signaling (RGS) constitute a large family of proteins that modulate the intensity and duration of cellular responses to G-protein activation.[12] Discovered as inhibitors of G-protein signaling, RGS proteins act as GTPase-activating proteins (GAP) for Gα subunits. A protein

domain known as the RGS box harbors 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. However, only Werry *et al*. have reported a relationship between P2Y2 and RGS2-regulated Ca^{2+} signaling in human embryonic kidney cells.[13] The regulation of *MUCIN* gene expression by interactions between P2Y receptors, PLCβ and mitogen-activated protein kinase (MAPK), and RGS characterization in the airway epithelium is unclear. Here, we demonstrate that lipopolysaccharide can induce intracellular ATP secretion. Secreted ATP binds to Gαq-coupled P2Y2 and activates PLCβ3/Akt/both ERK and p38 to induce *MUC5AC* gene expression. In addition, RGS4 promotes hydrolysis of GTP to GDP leading to stop G-protein signaling.

II. Materials and methods

1. Materials. PGN from *Staphylococcus aureus*, exotoxin A and LPS from *Pseudomonas aeruginosa*, and ATP were purchased from Sigma (St. Louis, MO). All antibodies were purchased from Cell Signaling (Beverly, MA), except RGS4, which came from Santa Cruz (Santa Cruz, CA). Constructs encoding P2Y2 and the RGSs were purchased from the UMR cDNA Resource Center (Rolla, MO). *pUSEamp-Akt1 K179M* was kindly provided by Dr. H.D. Um

(Korea Institute of Radiological and Medical Sciences, Seoul, Korea). All siRNAs were synthesized by Bioneer (Daejeon, Korea): P2Y2, GAGGAAGGUGGCUUACCAA(dTdT); RGS4, GGAUCAGCUGUGAAGA GUA (dTdT); and negative control CCUACGCCACCAAUUUCGU(dTdT).

2. Methods

A. Cell Cultures. The human lung mucoepidermoid carcinoma cell line (NCI-H292) was purchased from the American Type Culture Collection (CRL-1848; Manassas, VA) and cultured in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum in the presence of penicillin/streptomycin at 37 °C in a humidified chamber with 5% CO_2. For serum deprivation, confluent cells were washed twice with phosphate-buffered saline and recultured in RPMI 1640 with 0.2% fetal bovine serum.

B. ATP measurement. The amount of ATP was measured using a CellTiter-Glo Luminescent Cell Viability assay kit (Promega; Madison, WI) according to the manufacturer's instructions. Briefly, 10,000 cells were plated into wells of a 96-well plate and then rendered quiescent for 24 h. After the cells were treated with several endotoxins, CellTiter Glo reagent was added in a volume equal to the cell culture media present in each well, and then incubated 2 min on an orbital

shaker. The cells were further incubated at room temperature for 10 min, and then assayed for luciferase activity.

C. Cell Transfection and Luciferase Assays. Hela cells were maintained in Dulbecco's modified Eagle's medium (DMEM) in the presence of 10% fetal bovine serum. For luciferase assays, cells were plated in 12-well plates one day before transfection and transfected with FuGENE6 (Roche; Indianapolis, IN) according to the manufacturer's instructions. Approximately 24 h after transfection, cells were treated with ATP for 6 h. For the SRE-promoter-driven luciferase assay, cells were maintained in serum-free DMEM for 16 - 18 h before treatment with ATP. Cells were harvested 6 h after ligand treatment, and luciferase activity in cell extracts was determined using a luciferase assay system according to the manufacturer’s instructions (Promega). The luciferase values were normalized by the β-galactosidase values. Transfection experiments were performed in duplicate and repeated at least three times.

D. RT-PCR. Real-time PCR was performed using a BioRad iQ iCycler Detection System (BioRad Laboratories, Ltd) with iQ SYBR Green Supermix. Reactions were performed in a total volume of 20 μL—including 10 μL 2x SYBR Green PCR Master Mix, 300 nM of each primer, and 1 μL of the previously reverse-transcribed cDNA template. The following primers were

used: *MUC5AC*, forward 5'-CAGCCACGTCCCCTTCAATA-3' and reverse 5'-ACCGCATTTGGGCATCC-3', and β_2-microglobulin (used as a reference for normalization), forward 5'-CGCTCCGTGGCCTTAGC-3' and reverse 5'-GAGTACGCTGGATAGCCTCCA-3'. Real time RT-PCR was performed on a MiniOption Real-Time PCR Detection System (Bio-Rad). Parameters were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. All reactions were performed in triplicate. The relative quantity of *MUC5AC* mRNA was obtained using a comparative cycle threshold method and was normalized using β_2-microglobulin as an endogenous control. Total RNA was isolated from NCI-H292 cells treated with ATP (10 μM) using TRIzol (Invitrogen). cDNA was synthesized with random hexamers (PerkinElmer Life Sciences and Roche Applied Science) using Moloney murine leukemia virus-reverse transcriptase (PerkinElmer Life Sciences). Oligonucleotide primers for PCR were designed based on the GenBank™ sequence of *MUC5AC* (GenBank™ accession number AJ001402, 5' primer CGACAACTACTTCTGCGGTGC; 3' primer GCACTCATCCTTCCTGTCG TT). PCR conditions consisted of 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and polymerization at 72 °C for 30 s. PCR products were run on a 1.5% agarose gel and visualized with ethidium bromide under a transilluminator.

E. Akt kinase assay. The lysate proteins (400 μg) were immunoprecipitated using anti-Akt IgG and resolved in 20 μL of a kinase buffer containing 20 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 20 mM β-glycerophosphate, 10 mM NaF, 1 mM DTT, 0.5 mM sodium orthovanadate, 50 μM ATP, and 10 μCi [γ-^{32}P]ATP. The kinase reactions were initiated by adding 2 μg of the recombinant GSK3α/β fusion protein (New England Biolaboratories, Beverly, MA). After incubation for 30 min, the reaction was quenched by adding boiled sample buffer, and the proteins were subsequently separated by 12% SDS-PAGE. The gels were dried, and a PhosphoImager using Tina 2.0 software visualized the radioactive bands.

F. Western Blot Analysis. NCI-H292 cells were grown to confluence in 6-well plates. After treatment with ATP, the cells were lysed with 2x lysis buffer (250 mM Tris-Cl (pH 6.5), 2% SDS, 4% β-mercaptoethanol, 0.02% bromphenol blue, 10% glycerol). Equal amounts of whole cell lysates were resolved by 10 − 15% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA). Membranes were blocked with 5% skim milk in Tris-buffered saline (50 mM Tris-Cl (pH 7.5), 150 mM NaCl) for 2 h at room temperature. This blot was then incubated overnight with primary antibody in TTBS (0.5% Tween 20 in Tris-buffered saline). After washing with TTBS, the blot was

further incubated for 45 min at room temperature with anti-rabbit or anti-mouse antibody (Cell Signaling) in TTBS and then visualized with the ECL system (GE Healthcare; Uppsala, Sweden).

G. Immunoprecipitation. NCI-H292 cells at 80 - 90% confluency in six-well plates were transfected with Gq or both Gq and RGS4 constructs using FuGene6. 24 h post-transfection cells were washed with ice-cold PBS and harvested by scraping into lysis buffer (25 mM HEPES, 150 mM NaCl, 0.5% NP-40, 1 mM EDTA, 1 mM EGTA, protease inhibitor tablet[Complete Mini; Roche]), sonicated (4 times each for 5 sec), and centrifuged at 12,000 x *g* for 15 min. 230 μL of supernatant lysates were pre-cleared with Gammabind G-Sepharose (GE Healthcare) for 30 min at 4 °C. Following centrifugation, anti-Gq antisera were added to pre-cleared lysates, incubated for 14 h at 4 °C, and microcentrifuged at 4°C. The pellet was washed with lysis buffer 3 times. Immunoprecipitated proteins were resuspended in 2X Laemmli buffer, placed in a boiling water bath for 5 min, and microcentrifuged prior to loading on denaturing 10% polyacrylamide gels for immunoblotting.

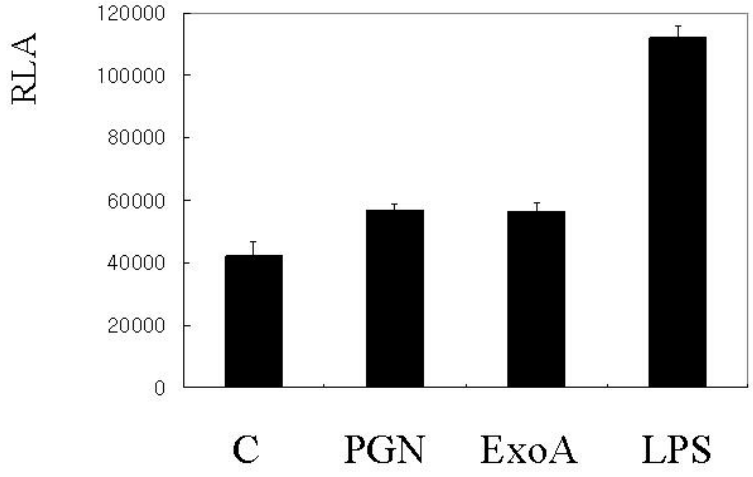
III. Results

1. Lipopolysaccharide (LPS) from *Pseudomonas aeruginosa* induces intracellular ATP secretion. *Staphylococcus aureus* and *P. aeruginosa* are major pathogens of the respiratory tract. The relative luciferase assay for secreted ATP was used to evaluate the induction of intracellular ATP secretion by bacterial toxins (Fig. 1A). We used three major toxins: peptidoglycan (PGN) from *S. aureus,* and both exotoxin A and LPS from *P. aeruginosa*. Of the three toxins, only LPS induced intracellular ATP secretion (Fig. 1A), which occurred in a dose-dependent manner with an EC_{50} value of 9.2 μg/mL (Fig. 1B). Moreover, to investigate whether these toxins could induce *MUC5AC* gene expression, real-time quantitative PCR was performed. Both PGN and LPS induced *MUC5AC* gene expression in NCI-H292 cells, but exotoxin A did not(Fig. 1C). These results suggest that PGN might induce *MUC5AC* gene expression independent of ATP. LPS from *P. aeruginosa* is an important inducer of ATP and *MUC5AC* gene expression, suggesting that the LPS induces ATP secretion, and ATP, in turn, induces *MUC5AC* gene expression. Interestingly, LPS did not induce expression of other secretory mucin genes (*MUC5B*, *MUC6*, *MUC7*, and *MUC19*) (Fig. 1D). Furthermore, we asked how ligation of LPS-increased ATP concentration leads to induction of *MUC5AC* gene expression. One possible mode of interaction between ATP and purinergic receptor would be through that

LPS stimulate the extra-cellular release of ATP, following by ATP binding to purinergic receptor on the same or adjacent cell to induce *mucin* gene expression. Indeed, ATP bioluminescence assay was performed with a luciferin-luciferase ATP system. ATP is released extracellularly in response to LPS, whereas glybenclamide, which is known to inhibit ATP release,[14] inhibited ATP release (Fig. 1D). This not only occur, but is required for *MUC5AC* gene expression (Fig. 1E), suggesting that ATP increased by LPS is released extra-cellular to stimulate the same cells or adjacent cells for inflammatory response.

Consistent with the autocrine release of ATP, exogenous nucleotides had additive or greater effects than additive effects with agonist.[14] Indeed, we next tested whether exogenous ATP can induce *MUC5AC* gene expression. As shown in Figure 1F, exogenous ATP induced *MUC5AC* gene expression in a dose-dependent manner with an EC_{50} value of 1.24 μM. On the basis of these results, an ATP concentration of 10 μM was used for the subsequent experiments. The EC_{50} of LPS, which represents the concentration of ATP secreted after stimulation by LPS, was approximately 6.18 μM, according to the ATP standard curve, indicating that 10 μM ATP may be sufficient to induce inflammation in airway cells.

A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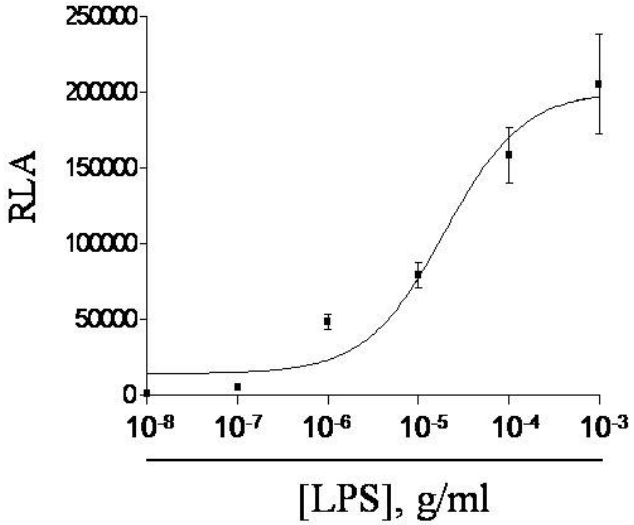
C
PGN
ExoA
LPS

B


RLA
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150000
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10-7
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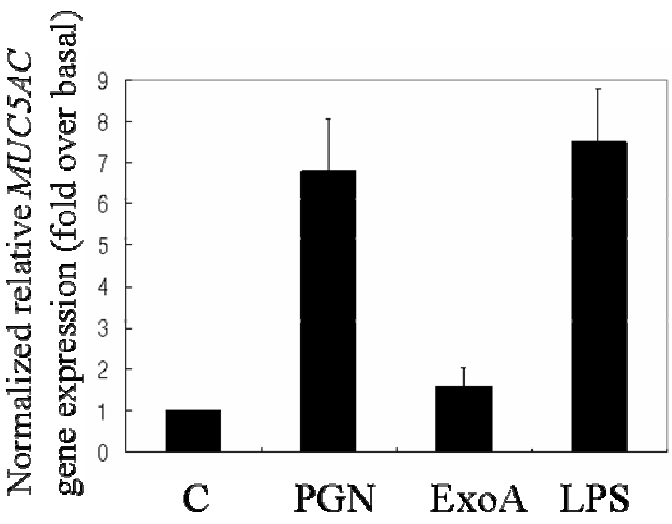
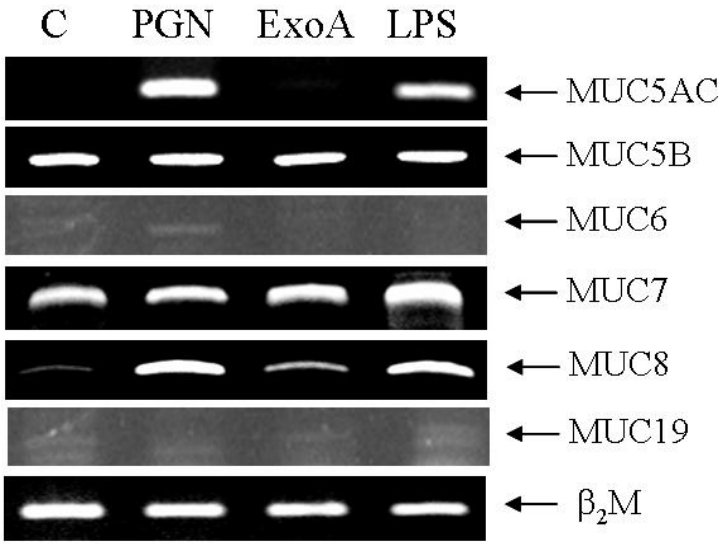
[LPS], g/ml

C


Normalized relative MUC5AC gene expression (fold over basal)
0
1
2
3
4
5
6
7
8
9
C
PGN
ExoA
LPS

D


C
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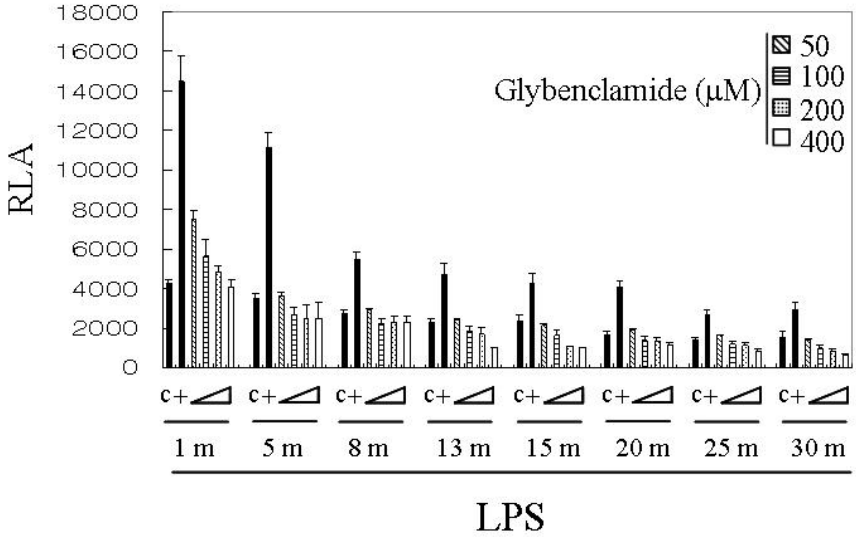
LPS
MUC5AC
MUC5B
MUC6
MUC7
MUC8
MUC19
β2M

E


18000
16000
14000
12000
10000
8000
6000
4000
2000
0
RLA
Glybenclamide (μM)
50
100
200
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c+
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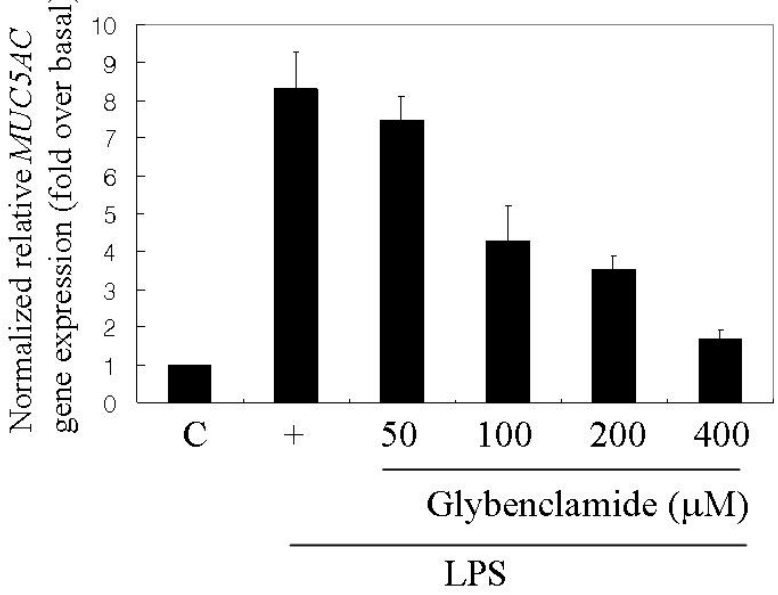
5 m
8 m
13 m
15 m
20 m
25 m
30 m
LPS

F


Normalized relative MUC5AC gene expression (fold over basal)
0
1
2
3
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5
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7
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9
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C
+
50
100
200
400
Glybenclamide (μM)
LPS

G

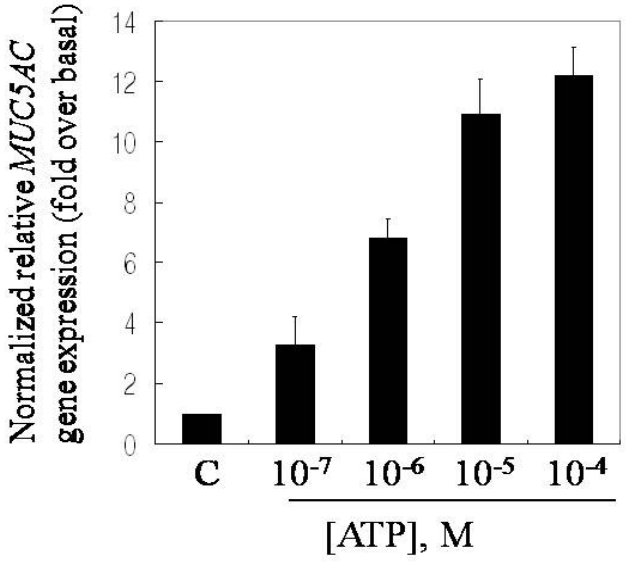

Normalized relative MUC5AC gene expression (fold over basal)
0
2
4
6
8
10
12
14
C
10-7
10-6
10-5
10-4
[ATP], M

Fig. 1. *Pseudomonas aerusinosa* LPS induced intracellular ATP and MUC5AC gene expression. (a) Confluent and quiescent cells were treated for 5 min with several endotoxins: peptidoglycan (PGN; 10 μg/mL) from *Staphylococcus aureus,* and both exotoxin A (ExoA; 500 ng/mL) and LPS (10 μg/mL) from *P. aeruginosa*. ATP concentration was measured using an ATP assay kit (Promega) according to the manufacturer's instructions. (b) Cells were treated for 5 min with the indicated concentrations of LPS and ATP was quantified. (c) Confluent and quiescent cells were treated for 24 h with several endotoxins, and cell lysates were harvested for real-time quantitative PCR. (d) Cells were treated with ATP and glybenclamide, and ATP concentration was measured. (e) Cells were treated with LPS and glybenclamide, and cell lysates were harvested for real-time quantitative PCR. (f) Cells were treated for 24 h with the indicated concentrations of extracellular ATP, and cell lysates were harvested for real-time quantitative PCR.

2. *Gαq-coupled* P2Y2 receptor is essential for ATP-induced *MUC5AC* gene expression. Many reports have indicated that ATP is an agonist for purinergic receptors,[15-17] and that purinergic agonists regulate mucin secretion in airway epithelial cells.[18] However, it is unclear how ATP induces *MUC5AC* gene expression. We hypothesized that ATP binds to the P2Y2 purinergic receptor

and thus activates its coupled Gαq. To verify this hypothesis, first, the ligand selectivity of ATP was examined using an SRE-promoter-driven luciferase assay.[19, 20] For the P2Y2 assay, we chose the SRE-driven luciferase system because it is more sensitive than the Ca^{2+} or inositol phosphate production assay system.[21] We used Hela cells for this study because they have diverse G-protein and effector repertoires that can be used to measure downstream signaling activity.[22-24] Hela cells were transiently transfected with both human P2Y2-expressing construct and SRE-luciferase construct. As shown in Figure 2A, ATP induced SRE-luciferase activity in a dose-dependent manner with a low EC_{50} (0.735 μM), indicating that P2Y2 has a high sensitivity to ATP and that ATP increases Gαq activity in a dose-dependent manner. Next, to investigate whether P2Y2 is essential for ATP-induced *MUC5AC* gene expression, we used a P2Y2 wild-type construct and siRNA, so that P2Y2 was increased in the P2Y2 wild-type construct and decreased in the siRNA. *MUC5AC* gene expression was greater with the wild-type P2Y2 activated by ATP compared to treatment with ATP only. In contrast, *MUC5AC* gene expression was significantly suppressed with the P2Y2 siRNA, indicating that the P2Y2 receptor may be closely related to ATP-induced *MUC5AC* gene expression.

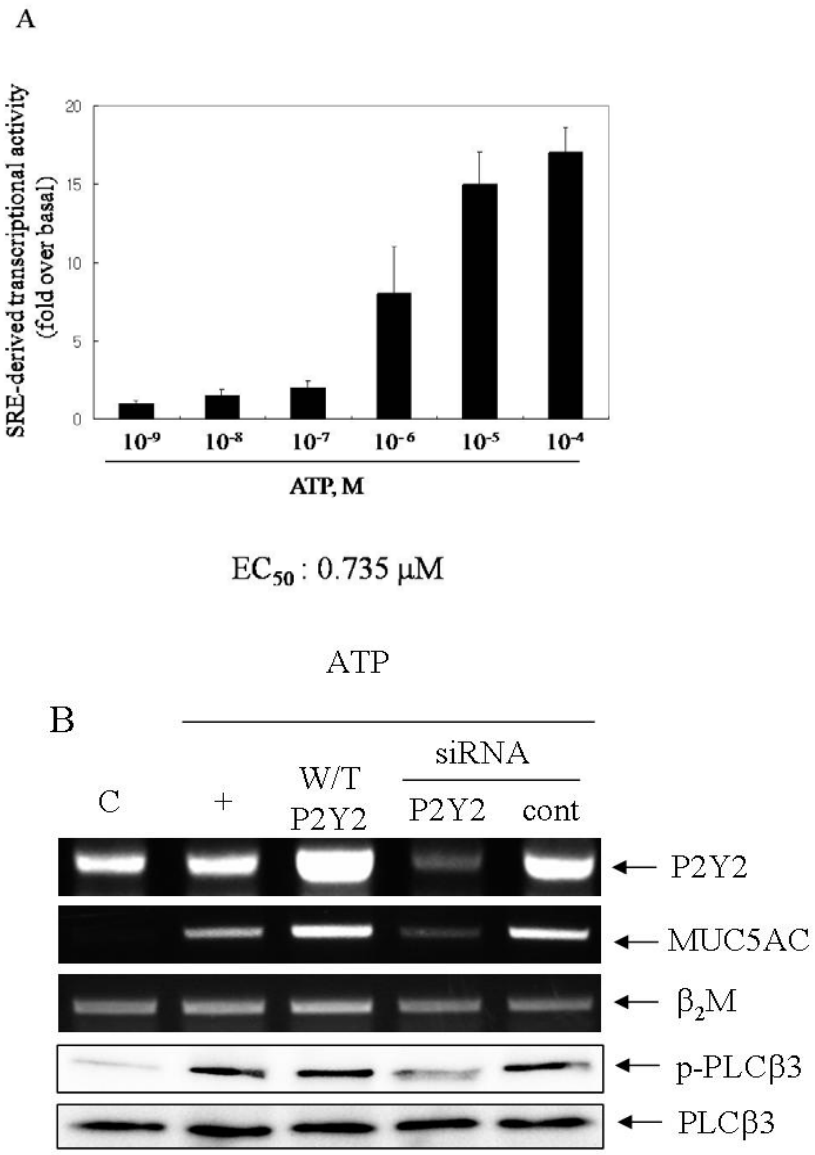


Fig. 2. P2Y2 is essential for ATP-induced MUC5AC gene expression through

Gαq activation. (a) Cells were transiently transfected with *pCMV-P2Y2*, *pSRE-Luc* and *pCMV-gal* reporters. Cells were serum-starved overnight and then treated with indicated concentrations of ATP for 6 h, after which the luciferase activity was measured and normalized to the X-Gal activity. The values shown are mean ± S.D. of experiments performed in triplicate. (b) Cells were transiently transfected with *pCMV-P2Y2*, *siRNA-P2Y2*, or *siRNA-control* constructs. Cells were serum-starved overnight and then treated with ATP (10 μM) for 24 h, followed by PCR. In addition, after ATP treatment for 5 min, cell lysates were harvested for western blot analysis using phospho-specific PLCβ3 antibody or total PLCβ3 antibody (used as a loading control).

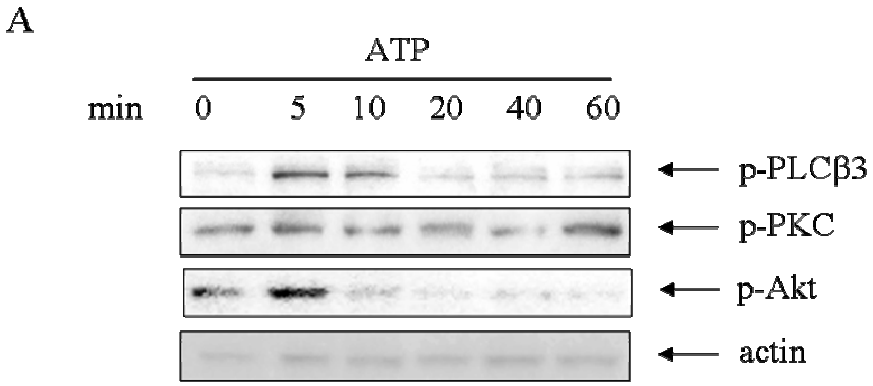
3. *PLCβ3 and Akt are down-stream signaling mediators of Gq in the induction of MUC5AC gene expression*. To determine which molecules are involved in the down-stream signaling of Gq in ATP-induced *MUC5AC* gene expression, we investigated the phosphorylation of PLCβ3, PKC, and Akt, using western blotting. The phosphorylation of PLCβ3 by ATP reached a maximum at 5 min and decreased at 10 min. Whereas PKC phosphorylation was not altered by ATP, Akt phosphorylation reached a maximum at 5 min (Fig. 3A). These results suggest that PKC is not involved in this pathway.

As expected, when treated with ATP, cells that were transfected with constructs

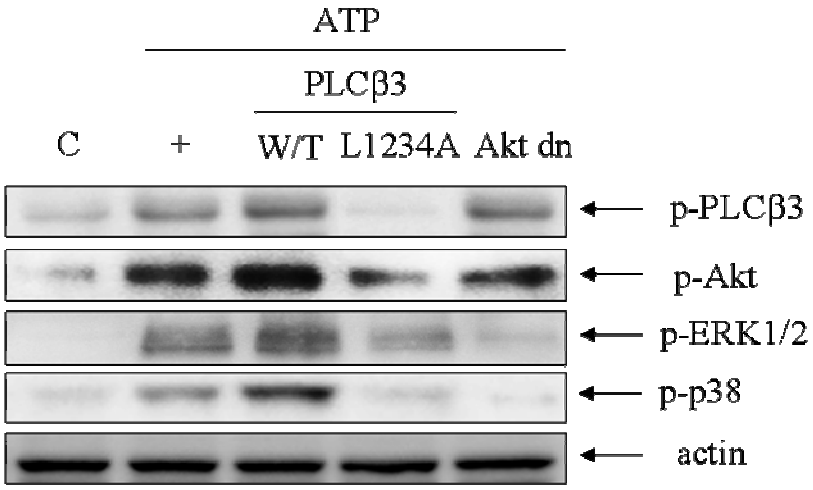
expressing wild-type PLCβ3 showed an increase of phosphorylated PLCβ3, whereas the cells that were transfected with constructs expressing PLCβ3 L1234A, but not N1231A, T1232A, or Q1233A , dominant-negative mutant showed a dramatic suppression of phosphorylated PLCβ3 (Fig. 3B). Interestingly, when treated with ATP, cells that were transfected with the Akt dominant-negative vector did not show a suppression of phosphorylated PLCβ3. Phosphorylated Akt was suppressed in cells transfected with PLCβ3 L1234A dominant-negative vector, but, on the other hand, phosphorylated PLCβ3 was not affected when cells were transfected with Akt dominant-negative vector. Taken together, this evidence suggests that PLCβ3 may be an upstream signaling mediator of Akt.

In addition, phosphorylation of both ERK 1/2 and p38 was suppressed when cells were transfected with either the PLCβ3 or Akt dominant-negative vector. Interestingly, over-expression of Akt dominant-negative did not result in a suppression of phosphorylated Akt (second panel from top in Fig. 3B). However, over-expression of Akt dominant-negative did suppress the phosphorylation of recombinant GSK3α/β protein *in vitro* (Fig. 3C), suggesting that although over-expression of the Akt dominant-negative mutant did not inhibit phosphorylation of Akt, it did inhibit kinase activity. Furthermore, to examine whether PLCβ3 and Akt are required for ATP-induced *MUC5AC* gene expression, we utilized

two dominant-negative constructs: L1234A for PLCβ3 and K179M for Akt (Upstate; Lake Placid, NY, USA). As shown in Figure 3D, ATP-induced *MUC5AC* gene expression was significantly suppressed in cells transfected with the dominant-negative constructs. This result indicates that PLCβ3 and Akt are required for ATP-induced *MUC5AC* gene expression in human airway epithelial cells. In other words, ATP induces *MUC5AC* gene expression via the PLCβ3-Akt cascade.



B


ATP
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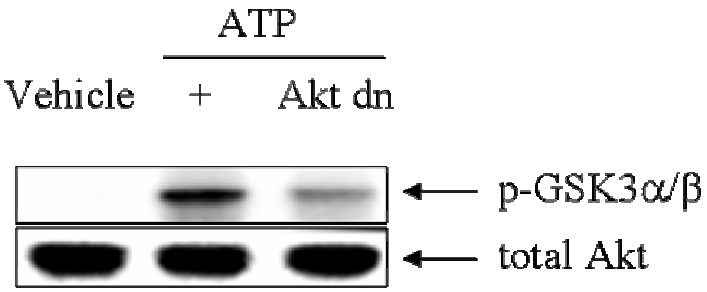
actin

C


ATP
Vehicle
+
Akt dn
p-GSK3α/β
total Akt

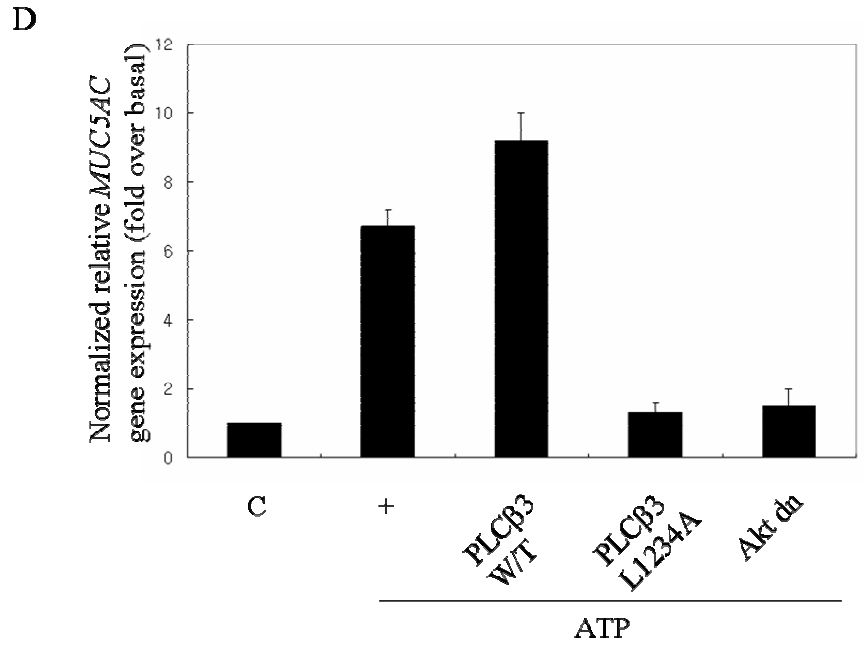
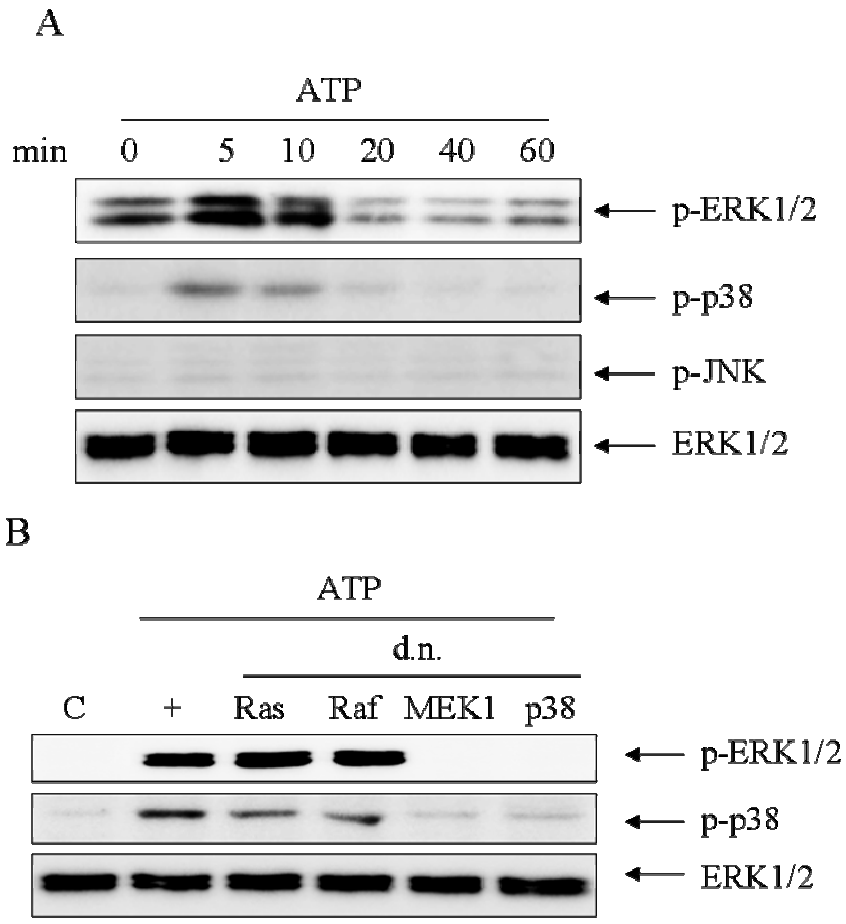


Fig. 3. PLCβ3 and Akt act as down-stream signaling mediators of Gαq to induce ATP-mediated *MUC5A*C gene expression. (a) Confluent and quiescent cells were treated for 5 min with ATP, then cell lysates were harvested for Western blot analysis using indicated phospho-specific antibodies. (b) Cells were transiently transfected with *pCMV-PLCβ3, pCMV-PLCβ3 L1234A*, or *pUSE-Akt1 K179M* constructs. Cells were serum-starved overnight and then treated with ATP (10 μM) for 5 min, after which cell lysates were harvested for western blot analysis using phospho-specific antibodies or for real-time

quantitative RT-PCR (d). Actin was used as a total protein loading control. (c) Cells were transiently transfected with *pUSEamp* or *pUSE-Akt1 K179M* constructs. The transfectants were washed and treated with ATP for 5 min. Akt activity was analyzed by an *in vitro* kinase assay using GSK3α/β as the substrate. Akt was used as a total protein loading control (bottom panel).

4. *Both ERK1/2 and p38 MAP kinases are essential for ATP-induced MUC5AC gene expression via a Ras-Raf independent pathway*. In a similar way, we investigated whether the ERK1/2 or p38 pathways play an important role in ATP-induced *MUC5AC* gene expression. The phosphorylation of ERK1/2 and p38 peaked at 5 min and then began to decrease by 10 min after ATP treatment (Fig. 4A). No change in phosphorylation of JNK was observed. To examine whether the Ras-Raf pathway participates in ATP-induced *MUC5AC* gene expression, we utilized dominant-negative constructs for each molecule: N17 for Ras (a kinase-defective form of Raf1), K97R for MEK1, and T180A-Y182F for p38. As shown in Figures 4B and C, ATP-induced signaling pathways and *MUC5AC* gene expression were significantly suppressed in cells transfected with MEK1 and p38 dominant-negative constructs, but Ras N17 and Raf1 DN did not affect the transfected cells. Interestingly, inhibition of either ERK1/2 or p38 suppressed ATP-induced *MUC5AC* gene expression. These results suggest

that ERK1/2 and p38 may be closely related to the ATP-induced signaling pathway and that cross-talk between ERK1/2 and p38 might lead to the induction of *MUC5AC* gene expression.



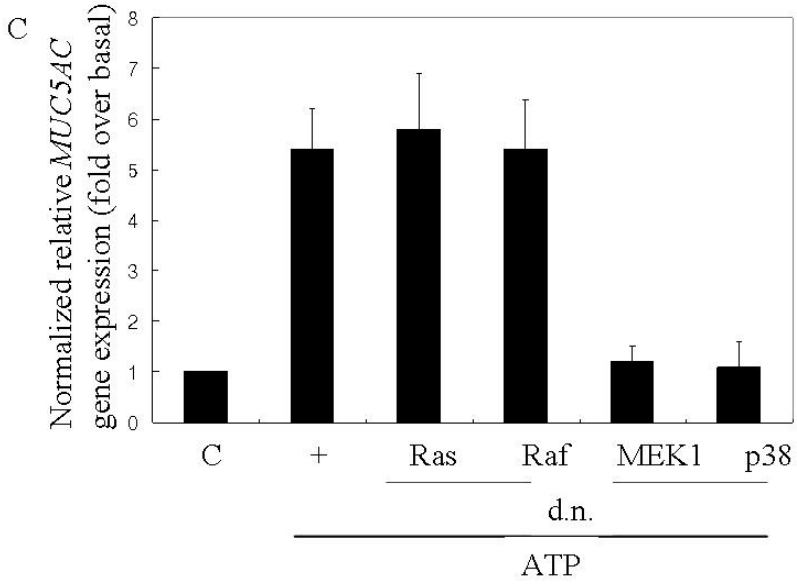


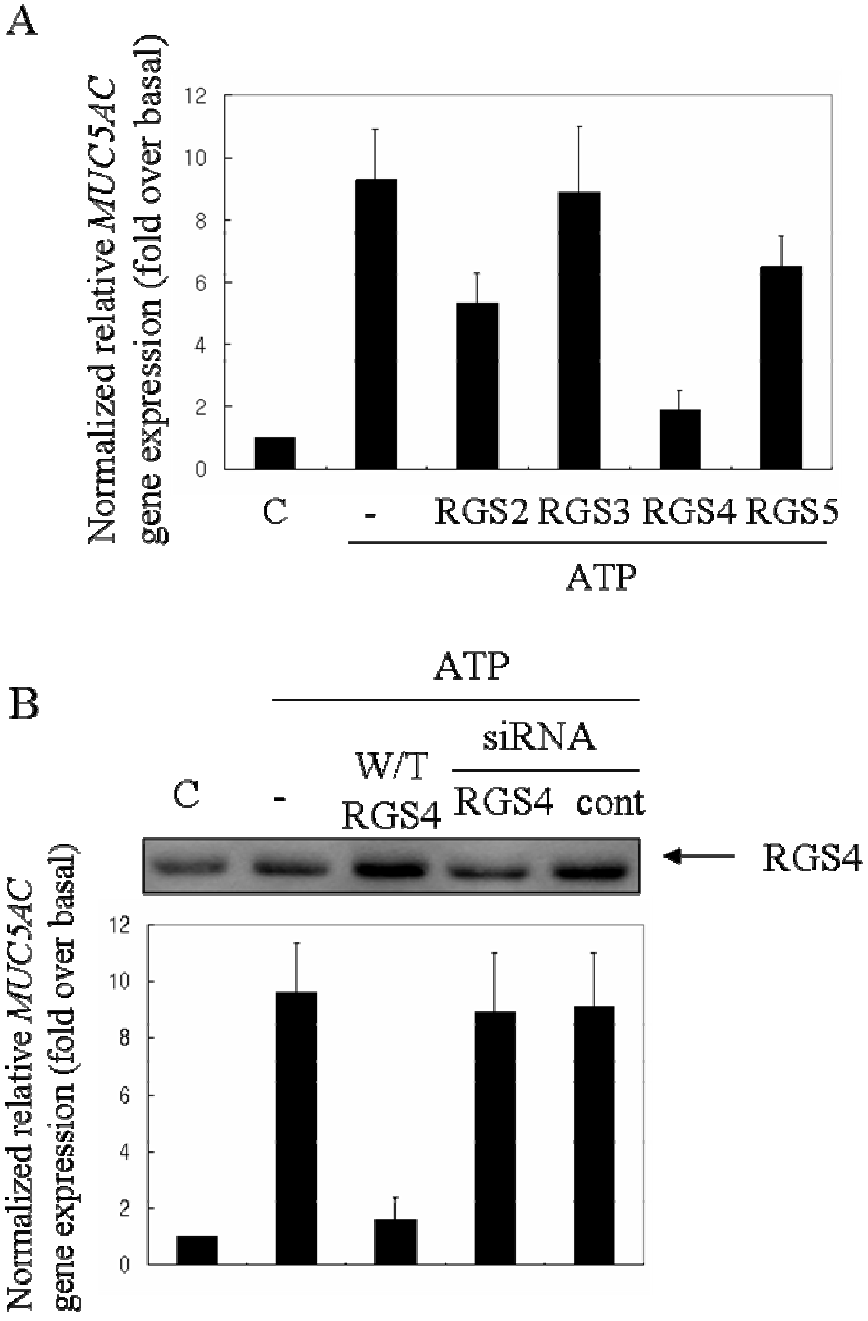
Fig. 4. ATP-induced *MUC5AC* gene expression is mediated by both ERK1/2 and p38 MAP kinases. (a) Confluent cells were treated with ATP for the indicated time, after which cell lysates were harvested for western blot analysis. Representative western blots using phospho-specific antibodies show transient activation of ERK1/2 and p38 but not of JNK, with the maximum effect at 5 min. The figures shown are representative of three independent experiments. ERK1/2 was employed as an internal control. (b) Cells were transiently transfected with *pCMV-Ras, craf1* (a kinase-defective form of Raf-1), *pcDNA5-MEK1DN,* or *pcDNA3-p38AGF* constructs. Cells were serum-starved overnight and then treated with ATP (10 μM) for 5 min, after which cell lysates were

harvested for western blot analysis using phospho-specific antibodies or treated with ATP for 24 h for real-time quantitative RT-PCR (c).

5. *RGS4 attenuates ATP-induced MUC5AC gene expression by binding to Gαq*. We investigated the inhibitory effect of RGS proteins on Gαq-mediated *MUC5AC* gene expression under the same conditions. Of the four known subtype groups of the RGS protein, we selected the simplest group with only one RGS motif: RGS2 to RGS5. *MUC5AC* gene expression was not affected by either RGS3 or 5, but was slightly lower after transfection with RGS2, and was dramatically suppressed by RGS4. Accordingly, we selected RGS4 for the next experiment because of its strong inhibitory effect. To examine whether RGS4 acts as a negative regulator in ATP-induced *MUC5AC* gene expression, we used RGS4 siRNA. RGS4 siRNA recovered ATP-induced *MUC5AC* gene expression that was significantly diminished by the RGS4. Irrelevant siRNA, used as a control for the RGS4 siRNA, did not affect ATP-induced *MUC5AC* gene expression (Fig. 5B). To determine a potential role for RGS4 in ATP-mediated signal processing, we examined whether RGS4 was a component of the Gαq complex of scaffold proteins. The specificity of interactions between Gαq and RGS4 was addressed by cotransfection of cells with Gαq and RGS4 constructs.

Transfected RGS4 and Gαq were coimmunoprecipitated by addition of GTPγS, and this reaction was disrupted by the addition of GDP. However, Gβγ was dissociated with the Gαq-RGS4 complex in transfected cell lysates (Fig. 5C). These results indicate that RGS4 may act as an attenuator in a GTP-dependent manner, because RGS4 bound to Gαq to switch G-protein signaling off.

To determine whether RGS4 affects Gαq activity, we performed a Gαq-functionality assay using wild-type expressing RGS4 construct and RGS4 siRNA. Whereas wild-type RGS4 attenuated Gαq activity in a dose-dependent manner, RGS4 siRNA increased Gαq activity in a dose-dependent manner (Fig. 5D). These results show that RGS4 may operate as a negative regulator in ATP-induced *MUC5AC* gene expression via Gαq, and that RGS4 may directly negatively regulate the dynamic of the heterotrimeric G-protein signaling complex.


A
Normalized relative MUC5AC gene expression (fold over basal)
12
10
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RGS2
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ATP
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ATP
siRNA
C
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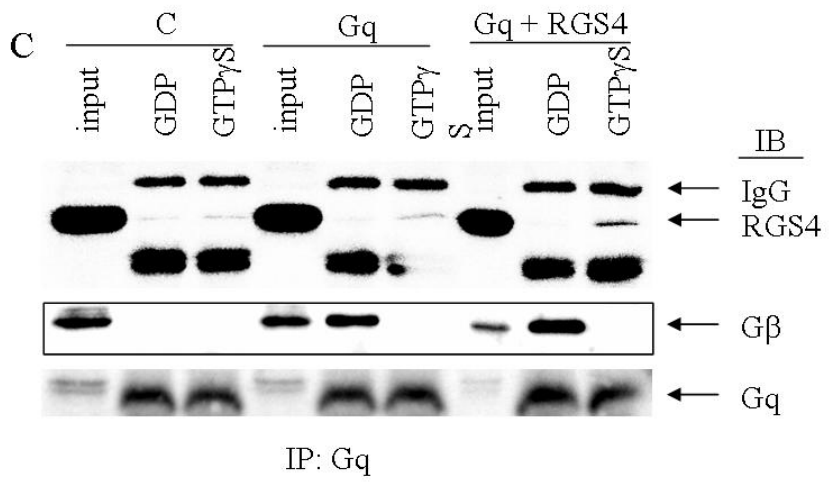

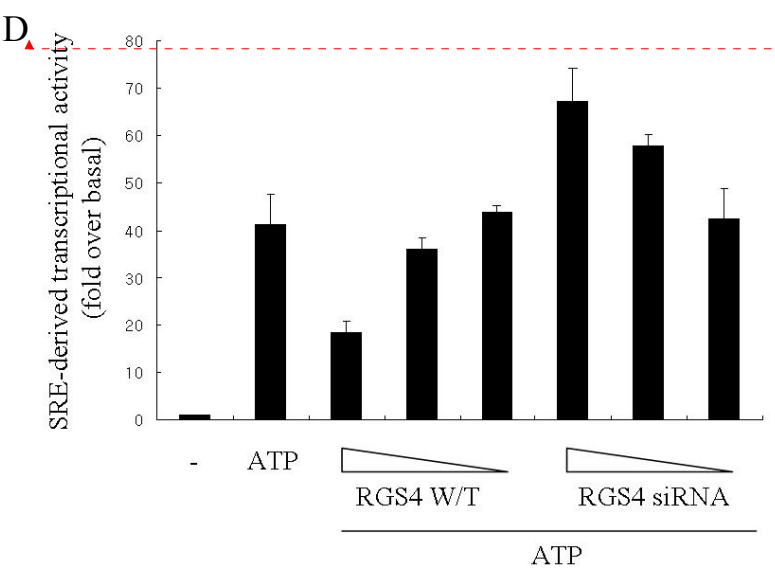

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Fig. 5. RGS4 acts as an attenuator in ATP-induced *MUC5AC* gene expression

by interacting with Gαq. (a) Cells were transiently transfected with *pCMV-RGS2, 3, 4, or 5* constructs. Cells were serum-starved overnight and then treated with ATP for 24 h, followed by PCR. (b) Cells were transiently transfected with *pCMV-RGS4*, *siRNA-RGS4*, or *siRNA-control* constructs. Cells were serum-starved overnight and then treated with ATP (10 μM) for 24 h, after which cell lysates were harvested for western blot analysis with anti-RGS4 antibody and for real-time quantitative RT-PCR (c). (d) After transfection with *pCMV-Gαq, pCMV-RGS4*, or both constructs, cell lysates were preincubated with 100 μM GDP or 100 μM GTPγS and 25 mM $MgCl_2$ at 24 °C for 30 min prior to immunoprecipitation. Membrane transfers were first blotted with RGS4 antisera and then stripped and sequentially reprobed with sera recognizing Gαq and Gβ. The input lane contains one-tenth of the lysate volume used for immunoprecipitation. The data are representative of three experiments. IP – immunoprecipitation; IB – immunoblot.

IV. Discussion

Mucus hypersecretion by airway epithelium is a major characteristic of a number of respiratory diseases, including asthma, chronic bronchitis, and cystic fibrosis. Because the respiratory tract is highly exposed to many external

stimulants such as bacteria or air pollution, it is important to clarify the mechanism by which major pathogens like *S. aureus* and *P. aeruginosa* increase mucus secretion.

We found that LPS from *P. aeruginosa* regulated *MUC5AC* gene expression, depending on the concentration of intracellular ATP when the cells were exposed to the bacteria. *P. aeruginosa* reportedly induces *MUC5AC* gene expression via epidermal growth factor receptor,[25] while macrolide antibiotics such as azithromycin decrease *P. aeruginosa*-induced *MUC5AC* gene expression by interfering with ERK1/2 MAP kinase.[26] In this study, LPS from *P. aeruginosa* induced *MUC5AC* gene expression by increasing intracellular ATP concentration (Fig. 1) in the early phase (under 5 min), suggesting that LPS from *P. aeruginosa* could induce *MUC5AC* gene expression through several signaling pathways. Exotoxin A from *P. aeruginosa*, known to be an important virulence factor, did not induce *MUC5AC* gene expression (Fig. 1A), suggesting that *mucin* gene expression may result from the presence of LPS (Fig. 1C with *MUC8* gene expression also increased). Boucher *et al*. reported that ATP released at the apical surface of airway epithelium by *S. aureus* in primary human bronchial epithelial cells reached levels necessary for P2Y2 receptor activation.[27]

Our functional studies demonstrated that ATP increases Gαq activity in a dose-dependent manner (Fig. 2A), and that similar concentrations of ATP can also increase *MUC5AC* gene expression (Fig. 1D). Taken together, these results show that LPS from *P. aeruginosa* may regulate the dynamics of the P2Y2 receptor by ATP. This hypothesis is also supported by a report that ATP enhances LPS-induced TNF-α production via P2Y receptors in astrocytes to modulate inflammatory responses.[28] Recently, Babaum *et al*. reported that *P. aeruginosa* increases the autocrine release of ATP through asialoGM1 (ASGM1), a glycolipid receptor that binds to G-protein coupled receptors (GPCR), especially P2Y1, P2Y2, and (or) P2Y11, to induce *MUC2* gene expression in HM3 cells.[14] However, they were unable to provide direct evidence that secreted ATP directly binds to P2Y2, or that ASGM1 can induce steady-state levels of *MUC2* mRNA expression. In addition, they did not determine how GPCR activation leads to ERK1/2 after PLCβ3 activation.

To investigate whether P2Y2 is essential for ATP-induced *MUC5AC* gene expression, we utilized wild-type and siRNA constructs (Fig. 2B). The knock down of P2Y2 expression affected ATP-induced *MUC5AC* gene expression, suggesting that the P2Y2 receptor may be involved in this pathway. The studies described in this report arose from the observation that exposure of human

airway epithelium to *P. aeruginosa* leads to an alteration in the pathways that mediate cell-cell communication, shifting the mode of intercellular calcium wave transmission from a predominantly gap junction-dependent mechanism to a P2 receptor-dependent mechanism.[29] Our findings support the conclusion that extracellular ATP signaling through P2 receptors may form an important regulatory mechanism controlling inflammation during bacterial infection. Our hypothesis is consistent with several reports that P2 receptor blockage down regulates LPS-induced *iNOS* expression and nitrite production in murine macrophages,[30, 31] and that P2 receptor both potentiates IL-1β-induced activation of transcription factors NF-κB and AP-1 and differentially regulates expression of chemokines IL-8 and IP-10, early response molecules involved in the initiation of the inflammatory cascade.[32] In this study, we showed that the P2 receptor, P2Y2, when activated by extracellular ATP, induced Gα q activity with the end result of increased *mucin* gene expression.

We also found that downstream signaling through the PLCβ3-Akt-ERK1/2 and p38 MAP kinase pathways was responsible for ATP-dependent *MUC5AC* gene expression. Interestingly, despite the fact that Akt was involved in ATP-dependent *MUC5AC* gene expression, PKC was not. These results indicate that the Akt pathway likely plays some significant role in ATP-enhanced *MUC5AC*

gene expression. We also found that either ERK1/2 or p38 inhibited ATP-induced *MUC5AC* gene expression, indicating that these MAP kinases may be essential for this process. This finding is in agreement with our previous work.[6] More than one MAP kinase may be necessary for ATP-induced *MUC5AC* gene expression in cells (Figs. 4C), suggesting that the signaling pathways leading to *MUC5AC* gene expression vary depending on the type of stimuli and cell lines used.

The main finding of this study is that RGS4 negatively regulated ATP-induced Gαq- mediated signaling *in vitro* by enhancing GTPase acceleration. RGS2 and RGS3 markedly inhibited Gαq-mediated PLCβ activation in the absence of GTPase acceleration, suggesting that GTPase-activating protein (GAP)-independent mechanism(s) (such as effector antagonism) are sufficient to mediate their inhibitory effect. In contrast, RGS5 and RGS16 did not inhibit Gαq-induced PLCβ activation in a setting where they could not exert any GAP effect.[33] Up to now, there has been no report of a relationship between purinergic receptors and G-proteins with RGS in mucin-related literature. We wondered if RGS4 directly regulates Gαq function or whether Gαq is simply docked, awaiting an incoming signal from receptor-mediated guanine nucleotide exchange factor. Our results indicate that RGS4 might directly

regulate the dynamics of the heterotrimeric G-protein signaling complex, which is supported by the observation that the interaction of RSG4 with Gαq in the heterotrimeric G-protein complex was GTPγS-sensitive (Fig. 5C).

We also demonstrated that RGS4 attenuated ATP-stimulated SRE activation (Fig. 5D). RGS4 is a small protein composed primarily of just the RGS domain, lacking the additional protein-protein interaction motifs found in larger RGS (12).[12] Thus, the observed attenuation is most likely contributed by the Gαq GAP activity of the RGS protein. Perhaps the presence of RGS4 in a heterotrimeric G-protein complex provides a mechanism for the down-regulation of ATP-induced *mucin* gene expression by forming a ternary complex.

V. Conclusion

In summary, our results reveal the existence of an autocrine or paracrine nucleotide loop responsible for mediating host cell response to *P. aeruginosa* infection. *P. aeruginosa* stimulation induced ATP secretion in NCI-H292 cells. The signaling events involved a Gαq-coupled P2Y2 receptor and were initiated by the binding of secreted ATP to P2Y2 receptors. The Gαq signaling needed to induce *MUC5AC* gene expression required the PLCβ3-Akt-ERK1/2 and p38 MAP kinases pathways. These results give additional insights into the

molecular mechanism of negative regulation of mucin gene expression and enhance our understanding of mucus hypersecretion during inflammation.

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Lipopolysaccharide from *Pseudomonas aeruginosa* induces *MUC5AC* gene expression via Gαq-coupled P2Y2 receptor, and its pathway is regulated negatively by RGS4 in human airway epithelial cells

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기도 상피에서 점액 과분비는 천식, 만성 기관지염, cystic fibrosis 등의 수많은 호흡기계 질환의 공통적인 특징이다. 호흡기계는 박테리아나 공해 등의 많은 외부 자극에 노출되어 있기 때문에 *Staphylococcus aureus*이나 *Pseudomonas aeruginosa*과 같은 주요 병원균에 의한 점액 분비 기전을 밝히는 것은 매우 중요하다. 본 연구에서는 독소에 의한 점액 유전자의 발현의 세포내 신호 전달 경로와 조절에 대해 연구하였다. 먼저, *P. aeruginosa*로부터 추출한 lipopolysaccharide는 세포내 ATP분비를 유도하였으며, 분비된 ATP는 세포외로 이동하여 세포에 염증반응을 자극하였다. 또한, 외부의 ATP의 유입은 NCI-H292 cells에서 Gαq-coupled P2Y2 수용체를 통하여 *MUC5AC* 유전자 발현을 증가시켰다. PLCβ3와 Akt 그리고, ERK1/2 혹은 p38 중 하나의 dominant-negative mutants에서는 ATP에 의한

MUC5AC 유전자 발현을 억제하였다. 흥미롭게도 regulator of G-protein signaling 4 (RGS4)는 Gαq와 RGS4 단백질에 있는 RGS domains과 상호작용을 하여 ATP에 의한 *MUC5AC* 유전자 발현을 억제하였다. RGS4 knocked down siRNA에서는 ATP에 의한 P2Y2 수용체의 활성화를 억제하였으며, 이는 RGS4가 ATP에 의한 P2Y2 수용체의 활성화를 억제하는 작용을 하는 것으로 추정되었다. 결론적으로, *P. aeroginosa*로부터 추출한 lipopolysaccharide는 ATP분비를 증가시키며, 분비된 ATP는 P2Y2/Gαq/PLCβ3/Akt/ERK1/2 혹은 p38 MAPK 경로를 통해 *MUC5AC* 유전자 발현을 증가시키며, RGS4는 GTPase 활성화를 통하여, 신호 전달을 억제하였다. 이 결과는 점액 유전자 발현의 억제 조절의 기전에 새로운 지식을 제공하며, 염증 반응에서 점액 과분비의 이해를 증진시킬 것으로 생각된다.

핵심되는 말 : 점소, lipopolysaccharide, ATP, G 단백질 결합 수용체, RGS 단백질