Molecules and Cells

PDZ Peptide of the ZO-1 Protein Significantly Increases UTP-Induced *MUC8* Anti-Inflammatory Mucin Overproduction in Human Airway Epithelial Cells

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https://doi.org/10.14348/molcells.2023.0107
www.molcells.org

Mucus hyperproduction and hypersecretion are observed often in respiratory diseases. MUC8 is a glycoprotein synthesized by epithelial cells and generally expressed in the respiratory track. However, the physiological mechanism by which extracellular nucleotides induce *MUC8* gene expression in human airway epithelial cells is unclear. Here, we show that UTP could induce *MUC8* gene expression through P2Y2-PLCβ3-Ca^{2+} activation. Because the full-length cDNA sequence of *MUC8* has not been identified, a specific siRNA-*MUC8* was designed based on the partial cDNA sequence of *MUC8*. siRNA-*MUC8* significantly increased TNF-α production and decreased IL-1Ra production, suggesting that MUC8 may downregulate UTP/P2Y2-induced airway inflammation. Interestingly, the PDZ peptide of ZO-1 protein strongly abolished UTP-induced TNF-α production and increased IL-1Ra production and *MUC8* gene expression. In addition, the PDZ peptide dramatically increased the levels of UTP-induced ZO proteins and TEER (trans-epithelial electrical resistance). These results show that the anti-inflammatory mucin MUC8 may contribute to homeostasis, and the PDZ peptide can be a novel therapeutic candidate for UTP-induced airway inflammation.

Keywords: airway inflammation, MUC8, P2Y2, PDZ peptide, UTP

INTRODUCTION

Mucus hypersecretion and hyperproduction are the hallmark of respiratory diseases because they are frequently detected in a number of respiratory diseases (Cha et al., 2015). For example, MUC5AC overproduction increases greatly (40-200 times higher than normal levels) during allergic mucous metaplasia of the epithelium in humans, and MUC5B overproduction increases moderately (3-10 times higher than normal levels) during allergic inflammation in mice (Fahy and Dickey, 2010). Another major feature of mucin proteins is the function as a glycoprotein, each with various physiological functions. Of the mucin proteins, only the partial cDNA sequence of MUC8 has been identified (Shankar et al., 1997). For this reason, the physiological function and signaling mechanisms by

Received July 3, 2023; revised August 29, 2023; accepted September 3, 2023; published online September 26, 2023

eISSN: 0219-1032

which MUC8 affects inflammation in the respiratory tract are unknown (Kim and Song, 2022). siRNA-*MUC8* significantly increased ATP/P2Y2-induced airway inflammation in human airway epithelial cells (Cha et al., 2015), suggesting MUC8 as an anti-inflammatory mucin. Nevertheless, the exact physiological mechanism and signaling pathway by which MUC8 regulates the inflammatory environment is not fully understood.

Nucleotides are known for their function as a universal energy currency and that in DNA replication and RNA transcription. However, extracellular nucleotides have a different role in mammalian cells, where they function as inflammatory signaling molecules through activation of nucleotide receptors (Idzko et al., 2014). Because P2YRs have critical roles in regulating immune responses, they are a pharmacological target for the treatment of inflammatory or infectious diseases (Idzko et al., 2014). Although ATP-P2Y2 or UTP-P2Y2 complex has potential as drug targets, only a few P2Y2R antagonists have been developed for various structural problems (Neumann et al., 2022). Therefore, a new novel paradigm of strategy for regulating UTP/P2Y2 signaling must be established.

Zonula occludens (ZO)-1 is one of the tight junction proteins (TJs) used to regulate cell-cell tension and permeability (Tornavaca et al., 2015). Disruption of TJs increases cell permeability, allowing extracellular pathogens such as microbes, viruses, and airborne particulate matter much easier entry into the cell-to-cell interspace. Such pathogens can pass into the blood stream through the basal membrane and can enter neighboring cells through the lateral membrane. Our previous studies (Kang et al., 2020; Lee et al., 2020) have shown ZO-1 to have an anti-inflammatory function and to increase cell-cell tension. In the domains of ZO-1, PDZ can bind the C-terminal peptides of different proteins and enforce the interaction with multiple protein complexes, targeting specific proteins and routing these proteins in signaling pathways (Kalyoncu et al., 2010). Nevertheless, because the ZO-1 protein is large (220 kDa), there are many technical limitations to handling the protein. Thus, peptides were synthesized with the critical core sequence in our system. The PDZ peptide significantly inhibited diesel particulate matter- or *Pseudomonas aeruginosa* lipopolysaccharide (LPS)-induced airway inflammation. However, their physiological mechanisms have not yet clearly understood. Specially, little information has been reported that PDZ can regulate UTP-induced airway inflammation.

In this study, we show that UTP increased *MUC8* gene expression through P2Y2- PLCβ3-Ca^{2+} activation. Because the partial cDNA sequences of *MUC8* have been identified (Shankar et al., 1997), a specific siRNA-MUC8 was designed based on the known partial cDNA sequence. siRNA-MUC8 significantly increased pro-inflammatory cytokine (TNF-α) and decreased anti-inflammatory cytokine (IL-1Ra). This suggests that MUC8 may serve as an anti-inflammatory mucin in UTP/P2Y2-induced airway inflammation. In addition, the PDZ peptide was designed based on the first PDZ domain of protein ZO-1. Interestingly, the wild-type (WT) PDZ peptide dramatically decreased TNF-α production, increased IL-1Ra and MUC8 production, and significantly decreased UTP/P2Y2-induced airway inflammation in human airway epithelial cells. In conclusion, MUC8 might downregulate UTP/P2Y2-induced airway inflammation, and the PDZ peptide may be a novel therapeutic candidate for airway inflammation.

MATERIALS AND METHODS

Materials

UTP and BAPTA-AM were purchased from Sigma-Aldrich (USA). The plasmids expressing WT P2Y2 construct were purchased from cDNA Resource Center (#P2Y0200000). The WT and mutant isotypes of PLCβ3 in the pcDNA3.1 overexpression plasmid were constructed from the cDNA of either WT or each of the mutated PLCβ3 isotypes. Each individual residue of the PDZ binding motif (NTQL) of PLCβ3 was mutated to an alanine codon (Song et al., 2008). The siRNA targeting P2Y2 and MUC8 was synthesized by Bioneer (Korea): P2Y2, GAGGAAGGUGGCUUACCAA (dTdT), MUC8, GCCUUUGUGUAAAUCAGAAUU, and negative control CCUACGCCACCAAUUUCGU (dTdT). Both WT and mutant PDZ peptides were synthesized by Peptron (Korea).

Real-time qPCR

Total RNA was isolated using TRIzol (Thermo Fisher Scientific, USA) from BEAS-2B cells treated with UPM (25 mg/ml). cDNA was synthesized by AccuPower CycleScript RT premix (dT20; Bioneer). Real-time PCR was performed using a QuantStudio 3 Real-Time PCR system (Thermo Fisher Scientific) with TOPreal™ SYBR Green qPCR MIX (Enzynomics, USA). All Reactions were carried out in a total volume of 20 μl which included 10 μl of 2× SYBR Green PCR Master Mix, 300 nM of each primer, and 1 μg of cDNA template. Real time RT-PCR was performed on a MiniOption Real-Time PCR Detection System (Bio-Rad, USA). The parameters were 95°C for 10 min, followed by 30-40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. All reactions were performed more than triplicate. The relative amount of mRNA was obtained using the comparative cycle threshold method and was normalized using β2-microglobulin as a loading control.

Cell cultures

The human lung mucoepidermoid carcinoma cell line (NCI-H292) was purchased from the American Type Culture Collection (CRL-1848) and cultured in RPMI-1640 (Invitrogen, USA) 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 re-cultured in RPMI-1640 with 0.2% fetal bovine serum.

Cell transfection

For transfection, cells were plated in six-well plates one day prior to transfection with plasmid DNA (1.0 μg/well) or siRNA (100 pmole/well) using FuGENE6 (Roche, Switzerland) according to the manufacturer's instructions. Approximately 24 h after transfection, cells were maintained in 0.2% serum RPMI media for 16 to 18 h before treatment with ATP and then harvested.

Western blot analysis

For Western blot analysis, NCI-H292 cells were grown to confluence in six-well plates. After UTP treatment, the cells were lysed with 2× lysis buffer (250 mM Tris-Cl [pH 6.5], 2% SDS, 4% β-mercaptoethanol, 0.02% BPB, and 10% glycerol). Equal amounts of whole cell lysates were resolved using 10% to 15% SDS-PAGE gels, and the proteins were transferred to a polyvinylidene difluoride membrane. The specific phospho-PLCβ3 antibody was used (Cell Signaling Technology, USA).

Trans-epithelial electrical resistance (TEER)

Before evaluation, the electrodes were sterilized and corrected according to the manufacturer's instructions (Merck, USA). The shorter tip was placed in the culture plate insert and the longer tip was placed in the outer well. The unit area resistance ($\Omega \times cm^2$) was calculated by multiplying the sample resistance (Ω) by the effective area of the membrane (4.2 cm^2 for 6-well Millicell inserts).

F-actin staining

F-actin staining was performed using ActinRed 555 ReadyProbe reagent (Molecular Probes, USA) following the manufacturer's instructions. Briefly, the cells were rinsed with phosphate-buffered saline (PBS), after which the ActinGreen 488 ReadyProbe reagent was added. The cells were incu-

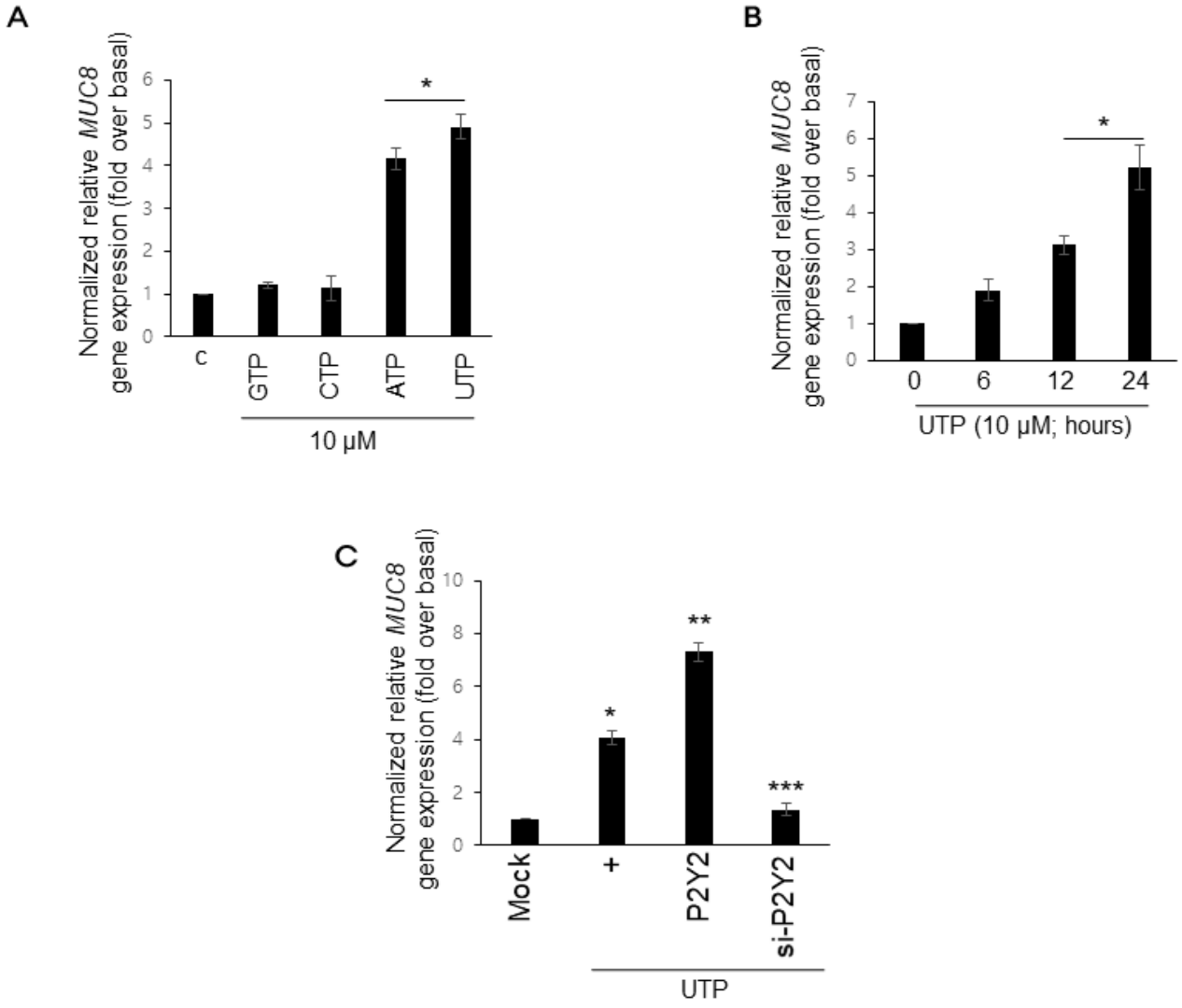

Fig. 1. UTP induces *MUC8* gene expression via a P2Y2-dependent manner in NCI-H292 cells. (A) The NCI-H292 cells were washed and serum-starved overnight. Each extracellular nucleotide (each 10 μM) was applied for 24 h, and the cell lysates were used for real-time quantitative PCR (qRT-PCR). $*P < 0.05$ compared to the control. (B) Confluent cells were treated with UTP for 6, 12, or 24 h, and cell lysates were harvested for qRT-PCR. $*P < 0.05$ compared to the control. (C) After transfection with a construct expressing wild-type P2Y2 or siRNA-P2Y2, the NCI-H292 cells were incubated with UTP for 24 h, and the cell lysates were used for qRT-PCR. The values shown are the mean ± SD of experiments performed in triplicate. $*P < 0.05$ compared to the control, $**P < 0.05$ compared to UTP only, and $**P < 0.05$ compared to UTP + P2Y2 transfection. Figures are representative of three independent experiments.

bated for 30 min. Next, the stain solution was removed, and the cells were rinsed with PBS. Images were obtained using a Eclipse 80i microscope (Nikon, Japan) with a 488 nm excitation filter and a 532 nm emission filter.

Statistical analysis

Data are presented as the mean ± SD of at least three independent experiments. Where appropriate, statistical differences were assessed by the Wilcoxon Mann–Whitney test. A *P* value less than 0.05 was considered statistically significant.

RESULTS

UTP/P2Y2 signaling mediated MUC8 gene expression in human airway epithelial (NCI-H292) cells

Because extracellular nucleotides can induce inflammation in mammalian cells, we used several extracellular nucleotides to induce *MUC8* gene expression in NCI-H292 cells (Fig. 1A). Only extracellular ATP and UTP could induce *MUC8* gene expression, and UTP induced *MUC8* gene expression in a time-dependent manner (Fig. 1B). Because P2Y2 purinergic GPCR is the receptor of UTP, and because it was sensitive and increased the activity of P2Y2-coupled Gαq. To know whether P2Y2 might essential for UTP-induced *MUC8* gene expression, the P2Y2 overexpression construct was used (Fig. 1C). UTP-induced *MUC8* gene expression was significantly increased about two-fold, in cells transfected with P2Y2, whereas no corresponding change was found in cells transfected with siRNA-P2Y2, suggesting that P2Y2 is critical for UTP-induced *MUC8* gene expression. These results show that the gene expression of UTP-induced *MUC8* is mediated in a P2Y2-dependent manner.

UTP-induced MUC8 gene expression is mediated for the Gαq-PLCβ3-Ca^{2+} signaling cascade

Because P2Y2 is a Gαq-coupled receptor in the activation of Ca^{2+}, we used BAPTA-AM that could chelate Ca^{2+} to examine whether P2Y2-mediated Ca^{2+} was essential for UTP-induced *MUC8* gene expression (Fig. 2A). BAPTA-AM dramatically inhibited UTP-induced *MUC8* gene expression in a dose-dependent manner, suggesting that Ca^{2+} activation is critical for UTP/P2Y2-induced *MUC8* gene expression. Next, we

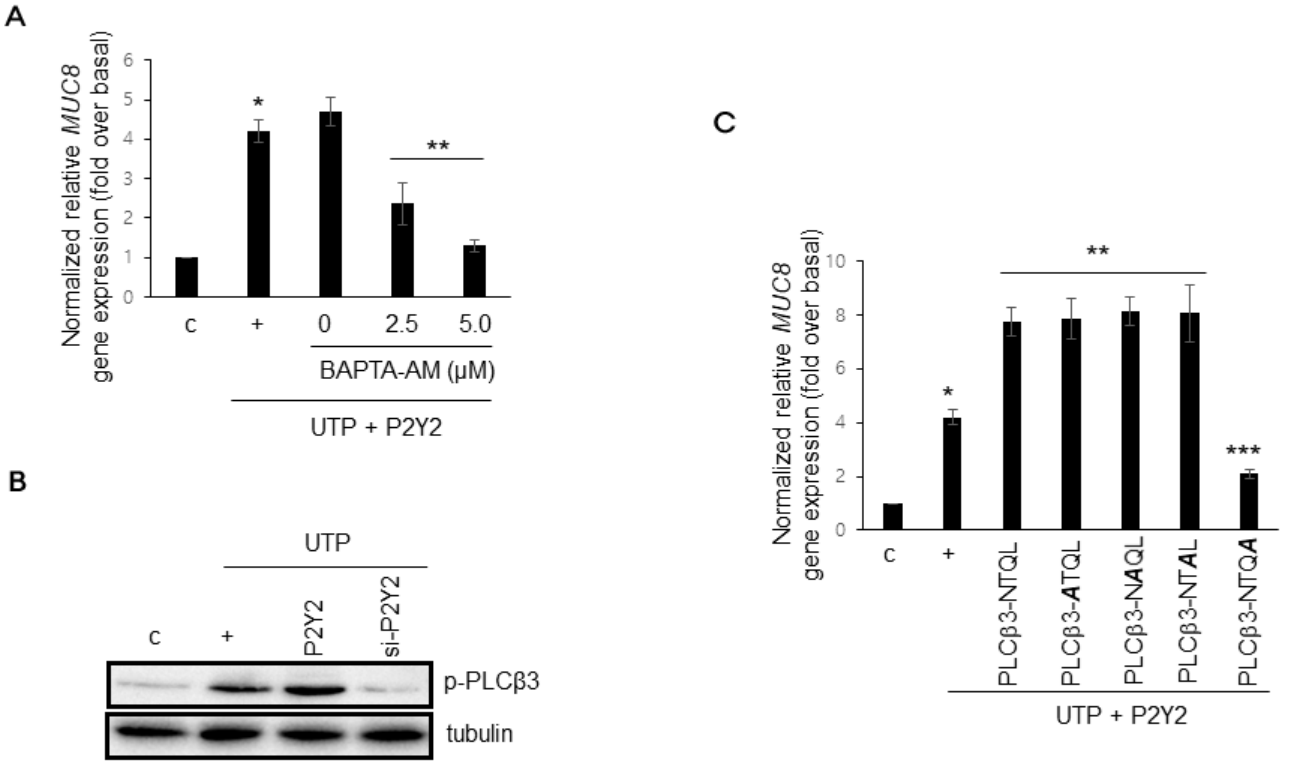

Fig. 2. Ca^{2+} and PLCβ3 are essential for UTP-induced *MUC8* gene expression. (A) After transfection with a construct expressing wild-type P2Y2, confluent cells were pretreated for 4 h with 2.5 or 5.0 μM BAPTA-AM and then stimulated for 24 h with UTP prior to collection of total cell lysates for real-time quantitative PCR (qRT-PCR). $*P < 0.05$ compared to the control, $**P < 0.05$ compared to UTP + P2Y2 transfection. (B) After cells were transfected with a wild-type P2Y2 or siRNA-P2Y2 construct, they were treated with UTP for 10 min prior to collection of cell lysates for Western blot analysis. Tubulin was used as a total protein loading control. (C) After transfection with wild-type P2Y2 construct, cells were transiently transfected with wild-type (^{1231}NTQL^{1234}) or a dominant-negative PLCβ3 ATQL (N1231A), NAQL (T1232A), NTAL (Q1233A), or NTQA (L1234A) construct. Each of the individual residues of the PDZ-binding motif (NTQL) of PLCβ3 were mutated to Ala. Cells were serum-starved overnight and then treated with UTP for 24 h, after which cell lysates were harvested for qRT-PCR. $*P < 0.05$ compared to the control, $**P < 0.05$ compared to UTP + P2Y2 transfection, and $**P < 0.05$ compared to UTP, P2Y2, and wild-type PLCβ3 transfection. Figures are representative of three independent experiments.

assessed PLCβ3 activation as an upstream signaling mediator of Ca^{2+} signaling. The UTP/P2Y2 complex activated PLCβ3 phosphorylation but not siRNA-P2Y2 (Fig. 2B). In the C-terminus of PLCβ3, the postsynaptic density-95/disc large/ZO-1 (PDZ)-binding motifs have short consensus sequences that consist of the amino acids -X(S/T)X(V/L)-COOH (Kornau et al., 1995; Song et al., 2008). This PDZ domain of PLCβ3 can perform critical physiological functions in a PDZ-PDZ interaction or a PDZ-non PDZ interaction (Kornau et al., 1995; Song et al., 2008). To examine whether the PDZ domain of the C-terminus of PLCβ3 can play a critical function in UTP-induced *MUC8* gene expression, the last four amino acid residues of PLCβ3 (^{1231}NTQL^{1234}-COOH) were mutated to Ala (Hwang et al., 2000). UTP-induced *MUC8* gene expression was significantly decreased in cells transfected with PLCβ3 NTQA (L1234A) compared to cells transfected with the WT PLCβ3 construct. However, the cells transfected with the construct expressing the dominant-negative mutant

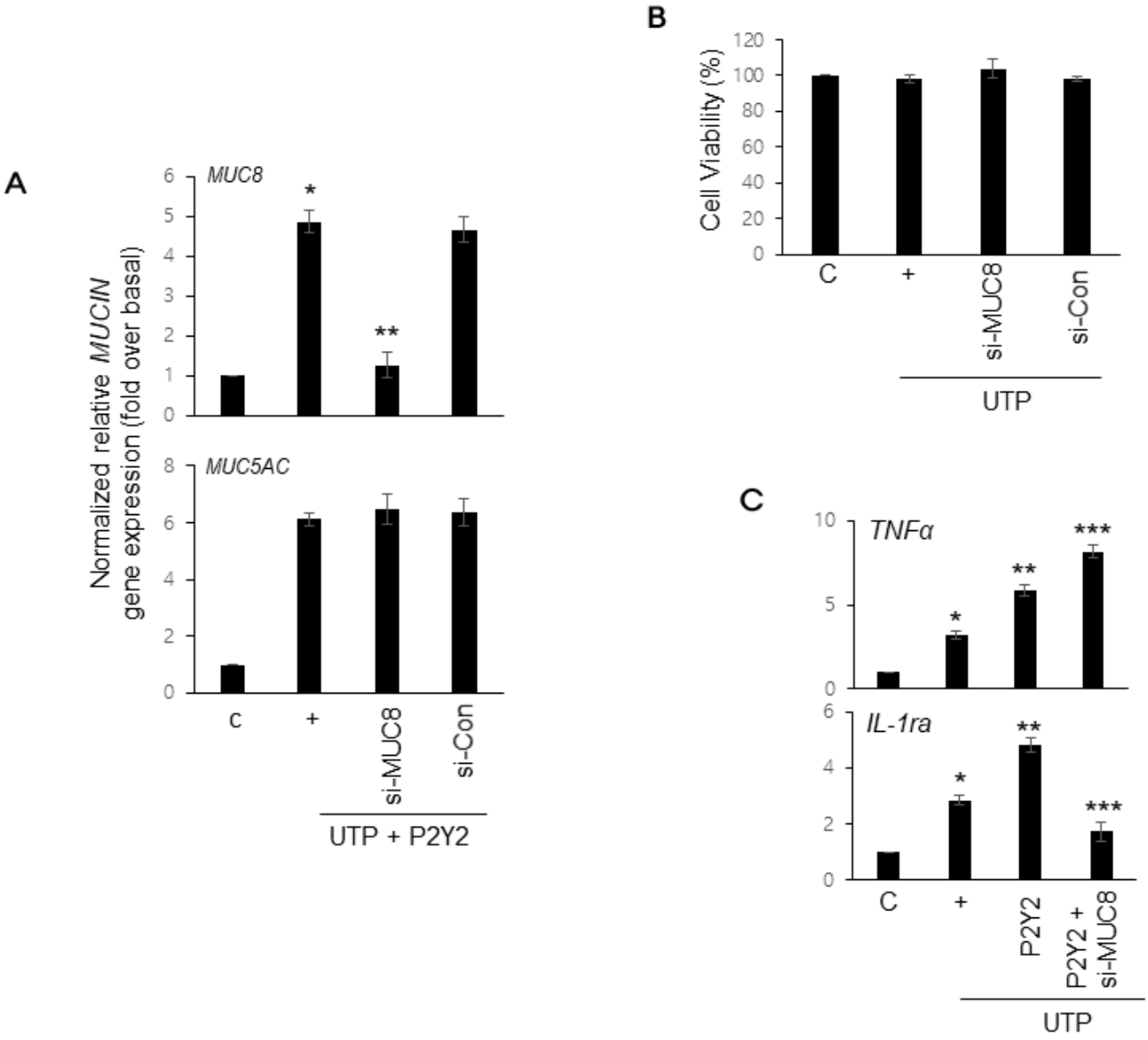

Fig. 3. siRNA-mediated silencing of *MUC8* up-regulates UTP-induced airway inflammation. (A) The cells were cotransfected with both a construct driving the expression of wild-type P2Y2 and either siRNA-*MUC8* or control siRNA. Transfected cells were treated with UTP for 24 h. *MUC5AC* gene expression was used as the negative control. $*P < 0.05$ compared to the control, $**P < 0.05$ compared to UTP + P2Y2 transfection. (B) Five thousand transfected cells were incubated for 24 h with UTP. Cell proliferation was measured using a Cell-Counting Kit-8 according to the manufacturer's instructions (Dojindo Laboratories). (C) The cells were cotransfected with constructs driving the expression of wild-type P2Y2 and of siRNA-*MUC8*. Transfected cells were treated with UTP for 4 h, and the cell lysates were used for real-time quantitative PCR for cytokine production. $*P < 0.05$ compared to the control, $**P < 0.05$ compared to UTP only, and $**P < 0.05$ compared to UTP + P2Y2 transfection. Figures are representative of three independent experiments.

PLCβ3, ATQL (N1231A), NAQL (T1232A), or NTAL (Q1233A) showed a dramatic increase in *MUC8* gene expression (Fig. 2C). This result suggests that the last "Leu1234" residue of the PDZ domain in the C-terminus of PLCβ3 is critical for binding to/recruiting other signaling/target molecule(s) to activate UTP-induced *MUC8* gene expression.

MUC8 increased by UTP suppresses the pro-inflammatory cytokine but activates the anti-inflammatory cytokine

To date, the full-length cDNA and promoter sequences for *MUC8* have not been identified. Nevertheless, based on known sequences (Shankar et al., 1997), many scientific analyses have been performed using primers for RT-PCR, peptides for an antibody, and probes for FISH (fluorescence *in situ* hybridization). Due to scientific limitations, only loss-of-function studies of MUC8 could be performed. In our previous study (Cha et al., 2015), we designed and synthesized the siRNA sequence to specifically silence *MUC8* expression and then determined the effects of siRNA-mediated *MUC8* silencing on cells treated with ATP. We examined whether siRNA-*MUC8* could inhibit UTP-induced *MUC8* gene expression (Fig. 3A). siRNA-*MUC8* inhibited specific *MUC8* gene expression but not *MUC5AC* gene expression as a negative control. We did not observe nonselective cytotoxicity of siRNA-*MUC8* (Fig. 3B). Interestingly, siRNA-*MUC8* dramatically increased P2Y2/UTP-induced TNF-α expression compared with cells treated with UTP and overexpressing P2Y2, suggesting that MUC8, conversely, may decrease TNF-α expression. However, the expression of IL-1Ra, an anti-inflammatory cytokine, was robustly decreased by siRNA-*MUC8* (Fig. 3C). These results suggest that MUC8 acts as an anti-inflammatory mucin against UTP/P2Y2-inflammatory signaling.

The PDZ peptide of ZO-1 protein increases the expression of MUC8 and anti-inflammatory cytokine gene

In our previous studies (Kang et al., 2020; Lee et al., 2020), the PDZ peptide of ZO-1 dramatically suppressed LPS- or PM2.5-induced airway inflammation in BEAS-2B cells. The PDZ peptide consisting of 22 amino acids and an additional TAT (GRKKRRQRRR) sequence to enable cell permeability was tagged with FITC for monitoring (Fig. 4A). To determine whether this PDZ peptide can affect the UTP-induced airway

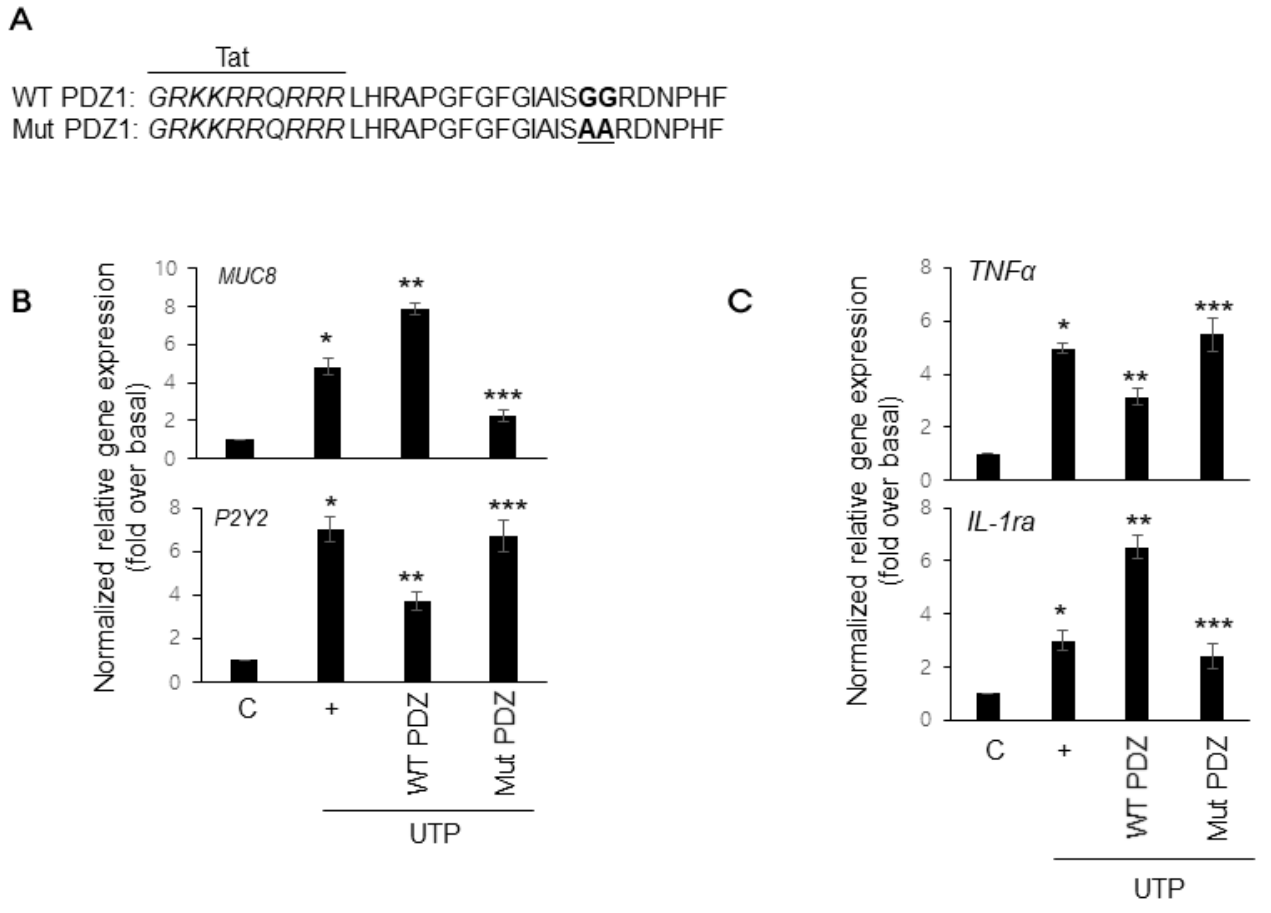

Fig. 4. Effect of PDZ peptide on UTP-induced airway inflammation. (A) Wild-type (WT) PDZ peptide was synthesized with a Tat region (italic amino acids) based on the first PDZ domain sequence of ZO-1 protein, and the mutant PDZ peptide was changed from ^{25}GG^{26} to ^{25}AA^{26}. The cells were treated with WT PDZ or mutant PDZ peptide for 24 h and then incubated with UTP for either 24 h (B) or 10 h (C), after which real-time quantitative PCR was performed. $*P < 0.05$ compared with the control, $**P < 0.05$ compared with UTP alone, and $***P < 0.05$ compared with WT PDZ peptide treatment.

microenvironment in NCI-H292 cells, we performed *MUC8* and *P2Y2* real-time qPCR. Whereas WT PDZ peptide significantly increased UTP-induced *MUC8* and decreased *P2Y2* receptor, mutant PDZ induced the opposite results (Fig. 4B). The WT PDZ peptide decreased TNF-α gene expression and significantly increased IL-1Ra gene expression, while mutant PDZ had also the opposite effects (Fig. 4C). These results show that the PDZ peptide increased UTP-induced *MUC8* gene expression to inhibit an inflamed condition, suggesting that PDZ can be considered as a therapeutic candidate for UTP-induced airway inflammation.

The PDZ peptide increases UTP-induced membrane permeability by up-regulating the expression of TJs

We hypothesized that PDZ peptide affects UTP-induced airway inflammation to maintain homeostasis. To support this hypothesis, either WT or mutant PDZ was treated to the cells. As expected, the PDZ peptide significantly increased UTP-induced the production of ZO-1, ZO-2, and ZO-3 after treatment of UTP at 24 h; this increase was not observed with the mutant PDZ peptide (Fig. 5A). TEER is a widely useful quantitative technique used to measure the integrity of TJ dynamics in cell culture models of epithelial monolayers. Since the production of ZO proteins was robustly increased by PDZ peptide, TEER was performed to determine whether PDZ can affect membrane integrity during UTP-induced airway inflammation. We found that TEER was reinstated by WT PDZ peptide but not by mutant PDZ (Fig. 5B). More interestingly, F-actin was generated by UTP. In the cells exposed to UTP and PDZ peptide, F-actin production, apical extension, and protrusion were all decreased compared to those in the cells exposed to UTP only (Fig. 5C). However, there were no such changes in the cells treated with mutant PDZ peptide, suggesting that the PDZ peptide reduces UTP-induced airway inflammation, removing the need for F-actin formation. These findings suggest that the PDZ peptide can control the inflammatory microenvironment by regulating the production of ZO proteins and the membrane permeability after UTP treatment.

DISCUSSION

In patients with respiratory diseases like asthma or chronic obstructive pulmonary disease (COPD), mucus hyperproduction and hypersecretion are frequent (Kim and Criner, 2013; Tian and Wen, 2015). The main causes of these pulmonary diseases are overexpression and hypersecretion of mucin proteins (Hauber et al., 2006; Singanayagam et al., 2022; Song, 2010). The mucus blanket consists of many mucin proteins and other secretions that accumulate in the respiratory tract. Although the physiological functions of many mucus proteins in respiratory diseases have been identified, the mechanism of anti-inflammatory mucins through a specific signaling pathway or regulatory molecules is unclear. Of the mucins, Of 13 mucins expressed in the lung, MUC8 has been as a secreted mucin (Voynow, 2002). Mucin secretion is essential for lung health, because deletion of Muc5b (mouse mucin 5b) in mice showed a severe loss of mucociliary clearance and accumulation of particulate debris within lung (Roy et al., 2014). In addition, mucin should be secreted to build up extracellular mucus, thus, mucin secretion is critical for lung health. Nevertheless, the exact physiological mechanism and signaling pathway by which MUC8 regulates the inflammatory environment are not fully understood.

Extracellular UTP can secreted PGE2 through P2Y2 receptors in normal human bronchial epithelial (NHBE) cells, while ATP secreted IL-6 via ionotropic P2X receptors (Kountz et al., 2021). The commonality with our results is the resulting respiratory inflammation. In cells, the concentration of ATP is up to six times higher than that of UTP (3,152 ± 1,698 μM vs 567 ± 460 μM) (Traut, 1994). Although the physiological mechanism by which UTP could induce *MUC8* gene expression in airway epithelial cells has been unclear, UTP itself significantly increased *MUC8* gene expression in human airway epithelial cells. We showed that UTP-induced *MUC8* gene expression was essential for the intracellular of Ca^{2+}, thus PLCβ3 phosphorylation was activated, sequentially. Importantly, the PDZ domain of the C-terminus of PLCβ3 might be part of a critical sequence in the regulation of *MUC8* gene expression (Fig. 2C). PDZ domains have a crucial role in protein-protein interaction in various species (Kennedy, 1995). These PDZ-PDZ interactions have many physiological functions, especially in mediating signaling network complexes (Spaller, 2006). Despite the increasing knowledge of the PDZ domain, little is known regarding its role in regulating either *MUC8* gene expression or MUC8-mediated physiological actions.

The ZO-1 TJ is highly expressed in normal human lung tissue, whereas its expression is significantly decreased in inflammatory lung tissue (Lee et al., 2020), suggesting that ZO-1 acts as a protective protein to maintain homeostasis and hinder inflammation. The WT PDZ peptide (based on PDZ domain of ZO-1) dramatically increased UTP-induced *MUC8* gene expression but decreased *P2Y2* gene expression. Mutant PDZ peptide showed the opposite results (Fig. 4B), suggesting that PDZ peptides can reduce UTP-induced airway inflammation by increasing the expression of *MUC8*. In addition, whereas PDZ peptide significantly increased TJs protein (ZO-1, -2, and -3), it dramatically decreased TEER and F-actin polymerization. This is a worthy investigation about the anti-inflammatory function of PDZ peptide as a therapeutic candidate. Because the amount of F-actin varies from cell to cell, the anti-inflammatory effects of F-actin may vary from cell to cell. Nevertheless, PDZ peptide is essential for anti-inflammation of UTP using the dynamics of F-actin and TJs. However, there is little information available regarding how the PDZ peptide affects/regulates airway inflammation. We hypothesized that (1) the Tat sequence of PDZ allows the peptide to enter easily into mitochondria to regulate reactive oxygen species (ROS) generation (data not shown). The PDZ peptide inhibited iNOS and eNOS activities in human bronchial epithelial cells (BEAS-2B) (data not shown), but the mutant PDZ peptide did not. In addition, PDZ peptide strongly inhibited ROS production from kidney mitochondria after treatment with LPS-induced systemic inflammation in mice (unpublished data). (2) As mentioned above, PDZ interacts with itself to regulate biochemical functions in mammalian cells (Lee and Zheng, 2010; Lee et al., 2006; Gisler et al., 2008). After pull-down assay with GST::PDZ (the same as PDZ peptide sequence) fusion protein in the human lung cell lysates,

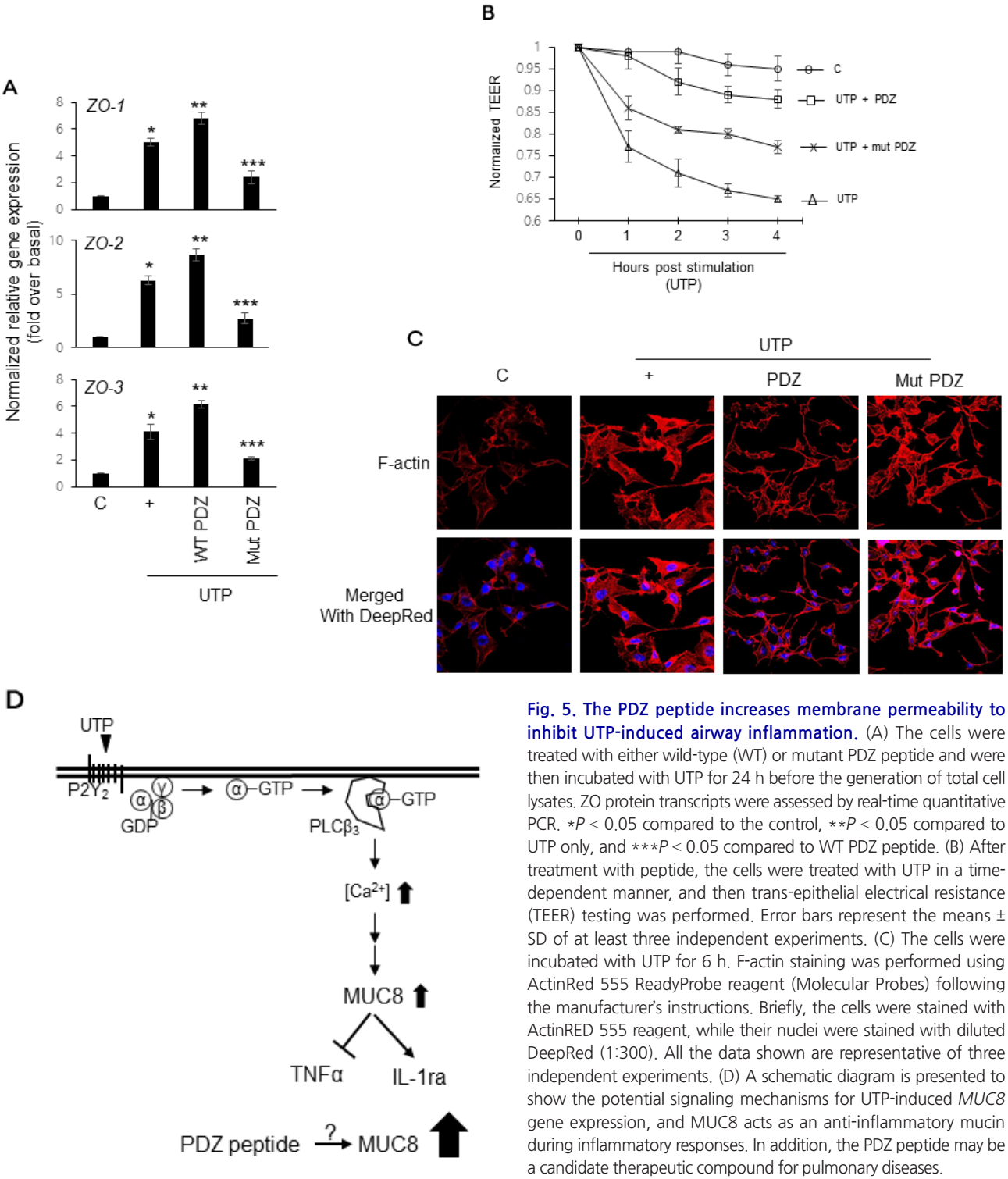

Fig. 5. The PDZ peptide increases membrane permeability to inhibit UTP-induced airway inflammation. (A) The cells were treated with either wild-type (WT) or mutant PDZ peptide and were then incubated with UTP for 24 h before the generation of total cell lysates. ZO protein transcripts were assessed by real-time quantitative PCR. $*P < 0.05$ compared to the control, $**P < 0.05$ compared to UTP only, and $***P < 0.05$ compared to WT PDZ peptide. (B) After treatment with peptide, the cells were treated with UTP in a time-dependent manner, and then trans-epithelial electrical resistance (TEER) testing was performed. Error bars represent the means ± SD of at least three independent experiments. (C) The cells were incubated with UTP for 6 h. F-actin staining was performed using ActinRed 555 ReadyProbe reagent (Molecular Probes) following the manufacturer's instructions. Briefly, the cells were stained with ActinRED 555 reagent, while their nuclei were stained with diluted DeepRed (1:300). All the data shown are representative of three independent experiments. (D) A schematic diagram is presented to show the potential signaling mechanisms for UTP-induced *MUC8* gene expression, and MUC8 acts as an anti-inflammatory mucin during inflammatory responses. In addition, the PDZ peptide may be a candidate therapeutic compound for pulmonary diseases.

PDZ-binding protein was identified by LC-MS/MS (data not shown). This PDZ-binding protein could regulate HIPPO signaling to control PDZ-mediated the suppression of airway inflammation (data not shown). (3) PDZ peptide activated Regulator of G-protein Signaling (RGS) protein to shut off the GPCR signaling. LPS-induced IL-8 secretion activated CXCR2 (IL-8 receptor) to augment LPS-induced airway inflammation. However, PDZ peptide activated RGS12 expression to diminish IL-8/CXCR2 signaling (Lee et al., 2020). Thus, PDZ peptide may contribute to maintain intracellular homeostasis by regulating RGS proteins. Lastly, (4) PDZ peptide can promote the interaction of TJs proteins to prevent the intracellular invasion.

PDZ peptide increased ZO-1, -2, and -3 protein (Fig. 5A). Because ZO protein orchestrated the tuning cell-to-cell tension, migration, and barrier formation (Tornavaca et al., 2015), PDZ peptide-induced ZO proteins expression can strengthen cell-to-cell interaction, preventing antigen invasion, and thus inhibiting inflammation at the source.

In summary, UTP could increase *MUC8* gene expression via P2Y2 receptors. MUC8 acts as an anti-inflammatory mucin after treatment with UTP or ATP. The physiological function of MUC8 is controversial (Lee et al., 2004), but it may act as an anti-inflammatory mucin in humen (Cha and Song, 2018; Cha et al., 2015). In addition, treatment with PDZ peptide and an extracellular nucleotide such as UTP or ATP for acute infection downregulated the inflamed microenvironment by upregulating the expression of TJs and anti-inflammatory responses (Fig. 5D). Therefore, we suggest the PDZ peptide as a therapeutic candidate for UTP-induced airway inflammation.

ACKNOWLEDGMENTS

This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Korean government (NRF-2021R1A4A1031380 to H.J.C. [Hee-Jae Cha], J.K. [Jeongtae Kim], and K.S.S.).

AUTHOR CONTRIBUTIONS

H.S., K.C.L., and K.S.S. conceived and designed the experiments. H.S., H.C.L., J.K. (Jiwook Kim), D.K. (Doosik Kim), and H.J.C. (Hyung-Joo Chung) performed the experiments. D.K. (Donghee Kang), H.J.C. (Hyung-Joo Chung), J.K. (Jeongtae Kim), and K.S.S. analyzed the data. H.J.C. (Hee-Jae Cha) provided reagents, materials, and analysis tools. H.S. and K.S.S. wrote the paper.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to disclose.

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