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Protease-Activated Receptor – 2 (PAR-2) is a weak enhancer of mucin secretion by human bronchial epithelial cells *in vitro*

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Abstract

PAR-2, a member of a family of G-protein-coupled receptors, can be activated by serine proteases via proteolytic cleavage. PAR-2 expression is known to be upregulated in respiratory epithelium subsequent to inflammation in asthma and chronic obstructive pulmonary disease (COPD). Since these diseases also are characterized by excessive mucus production and secretion, we investigated whether PAR-2 could be linked to mucin hypersecretion by airway epithelium. Normal human bronchial epithelial (NHBE) cells in primary culture or the human bronchial epithelial cell lines, NCI-H292 and HBE-1, were used. NHBE, NCI-H292, and HBE-1 cells expressed prominent levels of PAR-2 protein. Short term (30 min) exposure of cells to the synthetic PAR-2 agonist peptide (SLIGKV-NH₂) elicited a small but statistically significant increase in mucin secretion at high concentrations (100μM and 1000μM), compared to a control peptide with reversed amino acid sequence (VKGILS-NH₂). Neither human lung tryptase nor bovine pancreatic trypsin, both PAR-2 agonists, affected NHBE cell mucin secretion when added over a range of concentrations. Knockdown of PAR-2 expression by siRNA blocked the stimulatory effect of the AP. The results suggest that, since PAR-2 activation only weakly increases mucin secretion by human airway epithelial cells *in vitro*, PAR-2 probably is not a significant contributor to mucin hypersecretion in inflamed airways.

Keywords

PAR-2; mucin; airway; secretion

1. Introduction

Several proteases, including mast cell tryptase (MCT), matrix metalloproteinases (MMP), bacterial protease, and neutrophil elastase have been implicated in the pathogenesis of inflammatory airway diseases (Cairns & Walls, 1996; Kon et al., 1999; Nakamura, Yoshimura, McElvaney & Crystal, 1992; Ohno et al., 1997). Many of these diseases, such as asthma, chronic bronchitis, chronic obstructive pulmonary disease (COPD) and cystic fibrosis, are characterized by enhanced production and hypersecretion of mucin which contributes to airway obstruction and disease complications. Interestingly, many of the above proteases, whose levels

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may increase in these diseases, also can provoke production and secretion of mucin by airway epithelium. Mechanism(s) by which these proteases stimulate mucin secretion are not known.

Protease-activated receptors (PARs) are a family of G-protein-coupled receptors found in several cell types (Dery, Corvera, Steinhoff & Bunnett, 1998). Currently, four PAR family members have been identified, cloned and designated as PAR-1 to -4. PAR-2 is expressed in the respiratory and gastrointestinal tracts (Cocks & Moffatt, 2001; D'Andrea et al., 1998; Kawabata et al., 2001; Knight et al., 2001). It can be activated via proteolytic cleavage by serine proteases, especially trypsin and tryptase. Activation of PAR-2 has been implicated in pro-inflammatory processes in various tissues and cells (Miike, McWilliam & Kita, 2001; Nystedt, Ramakrishnan & Sundelin, 1996; Steinhoff et al., 2000), however, its potential role(s) in the pathogenesis of inflammatory airway diseases has not been elucidated. It is known that overexpression of PAR-2 may be induced under pathogenic circumstances, as enhanced PAR-2 expression is observed in respiratory epithelium of asthmatics and COPD patients (Cocks & Moffatt, 2001; Knight et al., 2001; Miotto et al., 2002). Although there are no reports linking PAR-2 activation to the actual secretory process, it has been shown that PAR-2 is involved in secretion of mucin by epithelium from the stomach and salivary glands in rodents (Kawabata et al., 2001; Kawabata et al., 2000; Kawabata, Nishikawa, Kuroda, Kawai & Hollenberg, 2000).

In this study, we hypothesized that proteases associated with airway inflammation, such as human lung tryptase and human neutrophil elastase (HNE), may provoke mucin secretion by human airway epithelium via activation of PAR-2 receptors on the cell surface. To look at this question, we examined whether or not direct activation of PAR-2 on human airway epithelial cells *in vitro* could stimulate mucin secretion. The results indicate that activation of PAR-2 receptors on airway epithelial cells enhances secretion of mucin, but only weakly and only in response to relatively high concentrations of PAR-2 agonists. Thus, it would seem that PAR-2 activation alone is not an important factor in enhanced mucin secretion in inflamed airways.

2. Materials and Methods

2.1 Materials

Normal human bronchial epithelial (NHBE) cells were purchased from Lonza (Walkersville, MD), as was bronchial epithelial cell growth medium (BEGM) and supplemental factors for NHBE cells. NCI-H292 cells were purchased from American Type Culture Collection (ATCC, CRL-1848, Manassas, VA). The immortalized normal bronchial epithelial cell line HBE1 was a gift from Dr. Reen Wu (University of California, Davis, CA). Transwell culture inserts were from Corning Inc. (Corning, NY), and rat tail collagen type I was from BD Biosciences (San Jose, CA). Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640 medium were from Mediatech, Inc. (Herndon, VA). Bovine serum albumin (BSA) was purchased from Millipore (Billerica, MA), nystatin from Amresco (Solon, OH), all-*trans* retinoic acid from Sigma (St. Louis, MO), and cholera toxin from List Biological Labs (Campbell, CA). Bovine pituitary extract (BPE) was prepared by a modification of the method of Bertolero (Bertolero, Kaighn, Gonda & Saffiotti, 1984) using pituitaries purchased from Pel-Freez (Rogers, AR). Trans-Blot transfer membranes were from Bio-Rad (Hercules, CA), goat polyclonal anti-PAR-2 antibody, mouse monoclonal anti-PAR2 (SAM11) antibody, HRP-conjugated donkey anti-goat IgG, and horseradish peroxidase (HRP) -conjugated goat anti-mouse IgG were from Santa Cruz (Santa Cruz, CA). An enhanced chemiluminescence detection system was purchased from Amersham Biosciences Inc. (Pittsburgh, PA). ChromPure goat IgG was from Jackson ImmunoResearch (West Grove, PA). DAB Chromogen was from Dako (Carpinteria, CA), Permunt from Fisher (Pittsburgh, PA), Versene from Invitrogen (Carlsbad, CA), modified Harris Hematoxylin from VWR (West Chester, PA), and Cytospin2 from Shandon (Pittsburgh, PA). The PAR-2 activating peptide AP (SLIGKV-NH₂) and the reverse peptide control RP (VKGILS-NH₂)

were purchased from Genemed Synthesis Inc. (South San Francisco, CA), and human lung trypsinase was purchased from U.S. Biologicals (Swampscott, MA). Endotoxin-free HNE purified from human sputum and the HNE inhibitor, chloromethyl ketone-modified tetrapeptide (CMK) were from Elastin Products Company (Owensville, MO). 17Q2 antibody was from Covance corporation (Berkeley, CA). Gastric MUC5AC mucin antibody (45M1) was from NeoMarkers (Fremont, CA). CytoTox 96 nonradioactive cytotoxicity assay kits were from Promega (Madison, WI). All other chemicals and reagents were from Sigma (St. Louis, MO).

2.2 Cell Culture

NHBE cells—Expansion, cryopreservation, and culture of NHBE cells in air/liquid interface were performed as described previously (Krunkosky et al., 2000). Briefly, air/liquid interface culture was initiated by seeding passage-2 NHBE cells in Transwell® inserts coated with rat tail collagen type I at a density of 2×10^4 cells/cm². The culture medium was a 1:1 mixture of BEGM and DMEM containing 130 µg/ml BPE, 5×10^{-8} M all-*trans* retinoic acid, 1.5 µg/ml BSA, 20 U/ml nystatin, 0.5 ng/ml human recombinant epidermal growth factor (hEGF), 0.5 µg/ml hydrocortisone, 5 µg/ml insulin, 10 µg/ml transferrin, 0.5 µg/ml epinephrine, 6.5 ng/ml triiodothyronine, 50 µg/ml gentamicin, and 50 µg/ml amphotericin-B. Cells were cultured submerged in medium in a humidified 95% air, 5% CO₂ environment for 5–7 days until nearly confluent. At that time, an air/liquid interface was created by removing the medium in transwells and feeding cells only from the basolateral side. Medium was changed daily thereafter. Cells were cultured for an additional 14 days to allow full differentiation.

NCI-H292 cells—The NCI-H292 cell line, derived from a human lung mucoepidermoid carcinoma (Carney et al., 1985), was grown on plastic culture plates and cultured in RPMI 1640 medium with L-glutamine supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37°C in a humidified 5% CO₂ incubator until the cells reached confluence. For 24 h prior to experiments, cells were cultured in the same medium without serum.

HBE1 cells—HBE1, a papilloma virus-immortalized bronchial epithelial cell line, was cultured under air-liquid interface conditions as described above for NHBE cells (Yankaskas et al., 1993). The cells were maintained in a 1:1 mixture of Ham's F-12 and DMEM medium with 6 hormone supplemental factors: 5 µg/ml insulin, 5 µg/ml transferrin, 10 ng/ml EGF, 0.1 µM dexamethasone, 20 ng/ml cholera toxin, and 13 µg/ml BPE. 30 nM all-*trans* retinoic acid was added to induce cell differentiation after the air-liquid interface was created.

2.3 Expression of PAR-2 protein via immunoblotting

Total protein isolated from either NHBE, NCI-H292 or HBE1 cells was separated on a 4–12% SDS/PAGE gel before transfer to nitrocellulose Trans-Blot® transfer membranes. The membranes were then blocked with PBS containing 3% non-fat milk and incubated overnight at 4°C with a mouse monoclonal anti-PAR2 (SAM11) antibody at a concentration of 1 µg/ml in PBS/1% BSA. Membranes were washed with PBS/Tween 20 (0.1%) and then incubated with HRP-conjugated goat anti-mouse IgG at a 1:2000 dilution. Following further washes in PBS/Tween 20 (0.1%), epitopes on proteins recognized specifically by the antibodies were visualized with ECL detection system.

2.4 Identification of PAR-2 by immunohistochemistry and immunocytochemistry

Tissue slides of normal human bronchus (a gift from Dr. Scott Randell, UNC-Chapel Hill, NC) fixed in 10% neutral buffered formalin were re-hydrated, quenched with H₂O₂ and blocked for 1 h in PBS containing 5% (v/v) normal donkey serum. Sections were then washed four

times in PBS and incubated for 1 h at room temperature with primary goat anti PAR-2 antibody at 1: 50 dilution in PBS/0.1% BSA. After washing four times in PBS, samples were incubated with secondary HRP-conjugated donkey anti-goat antibody in PBS/0.1% BSA. Samples were again washed and the color developed with Dako DAB Chromogen. Samples were washed and counterstained with alcian blue to identify goblet cells, dehydrated, mounted with Permount and coverslipped. Control experiments in which ChromPure goat IgG was substituted for the primary antibody were routinely performed to verify the specificity of the labeling. For cytospin immunocytochemistry, well- differentiated NHBE cells were dissociated with Versene and collected in 1.5ml Eppendorf tubes. The cells were fixed in the tubes using fresh cold 3% formaldehyde. After fixation, cells were washed and suspended in PBS. Cytospin was performed at 1000 rpm for 5 min with Cytospin 2, and then cells were air-dried. Staining was performed as described previously, except samples were counterstained with modified Harris' Hematoxylin.

2.5 Effects of PAR-2 peptides and PAR-2 agonists on mucin secretion

Confluent cells were treated with the human PAR-2-activating peptide (AP), or a reverse control peptide (RP), both between 0.1–1000 μ M, human lung trypsin (0.1, 1, 10 μ g/ml), or bovine pancreatic trypsin (1 and 10 μ g/ml) for indicated time periods, and then mucin secretion was measured as described previously (Li, Martin, Spizz & Adler, 2001). Briefly, the accumulated mucus at the apical surface of the cells was removed by washing with phosphate-buffered saline (PBS, pH 7.2). Cells were incubated with medium alone for “baseline” collection, and, after a 24 h. rest period, medium containing the selected reagents or appropriate controls for “test” treatments was added for the indicated time periods. Secreted mucin in the apical medium from “baseline” and “test” periods was collected and analyzed by ELISA utilizing the 17Q2 mouse monoclonal antibody that reacts with a carbohydrate epitope on human mucin (Lin, Carlson, St George, Plopper & Wu, 1989). The ratio of test/baseline mucin was used to quantify mucin secretion, allowing each culture dish to serve as its own control to minimize potential variability. Levels of mucin secretion were reported as percentage of control (medium only).

2.6 siRNA transfections

Chemically synthesized double-stranded siRNA oligos (PAR-2, 5'-GGAGUUACAGUUGAAACAGtt-3', and a control, non-targeting sequence) were purchased from Ambion (Austin, TX). NCI-H292 cells at approximately 40–50% confluence were transfected with 50nM of siRNA using the FuGene 6® transfection reagent (Roche Applied Science, Indianapolis, IN) following the manufacturer's instructions. Treatments and mucin collections were performed at 48 h after transfections. For HNE treatments, NCI-H292 cells were serum-starved for 24 hours before treatments. Ten μ M CMK and 1mM phenylmethyl sulfonyl fluoride (PMSF) were added to collected samples. Mucin samples were assayed by ELISA with 17Q2 antibody (PAR-2 AP treatments) or with MUC5AC antibody (HNE treatments) (Takeyama et al., 1999). Knock down of PAR-2 within these cells was confirmed by quantitative immunoblotting using a murine monoclonal anti-PAR-2 antibody.

2.7 Cytotoxicity Assay

Cytotoxicity of each reagent used was assessed using CytoTox 96 kits according to the manufacturer's instructions. All experiments were performed with reagents at non-cytotoxic concentrations.

2.8 Statistical analysis

Data are presented as mean $\pm$ S.E.M.. Significance was analyzed using one-way analysis of variance (ANOVA) with Bonferroni post-test corrections. $P < 0.05$ was considered significantly different between treatments.

3. Results

3.1 PAR-2 protein is expressed in human airway epithelial cells

To detect protein expression by immunoblot in cultured NHBE, NCI-H292 or HBE1 cells, we used a PAR-2 mouse monoclonal antibody (SAM11), which recognizes an epitope within amino acids 37–50 of human PAR-2. Relatively high expression of PAR-2 protein was seen in each of these human airway epithelial cell types (Fig. 1). PAR-2 protein bands are shown around 55 kDa. (the higher bands are un-cleaved PAR-2, the lower bands are cleaved PAR-2).

To confirm expression and localization of PAR-2 at the protein level, immunohistochemical methods were applied. PAR-2 receptors were identified in human bronchial epithelium via staining with PAR-2 antibody and counterstaining with alcian blue (to stain goblet cells). PAR-2 was seen in goblet cells of bronchial epithelium (Fig. 2A, left panel, indicated by arrows). Immunocytochemical staining of NHBE cytospin preparations indicated that PAR-2 receptors are localized on the surface of NHBE cells (Fig. 2B, left panel).

3.2 Effects of PAR-2 – related peptides on mucin secretion

To investigate the relationship between PAR-2 activation and mucin secretion, secretion in response to the PAR-2 activating peptide (AP) was measured, with the reverse peptide (RP) as a control. In NCI-H292 cells, AP enhanced mucin secretion significantly only at the highest concentration tested (100 μ M), while the control peptide RP had no effect at this concentration (Fig. 3A). Similar results were seen with HBE1 cells (Figure 3B). In well-differentiated NHBE cells, AP stimulated secretion significantly at 100 and 1000 μ M, while lower concentrations had no effect, nor did the control peptide RP (Fig. 4A). The secretory response was seen only at 30 min, as secretion measured at 15 or 60 min was not enhanced (Fig. 4B).

3.3 Effects of physiological PAR-2 agonists on mucin secretion

To investigate the response to physiologically-relevant PAR-2 agonists, effects of either human lung trypsin or bovine pancreatic trypsin on mucin secretion by NHBE cells were assessed. NHBE cells were exposed to trypsin at concentrations between 0.1– 10 μ g/ml for 30 min, and mucin samples collected and assayed by 17Q2 ELISA. As illustrated in Fig. 5, neither trypsin nor trypsin appeared to affect mucin secretion by NHBE cells at these concentrations.

3.4 Effects of knock down of PAR-2 by small interfering RNA

An siRNA against PAR-2 and a control siRNA were synthesized and transiently transfected into NCI-H292 cells. To quantify the effects of siRNA on PAR-2 protein expression, immunoblot analysis was performed 48 h after transfection using a mouse monoclonal anti-PAR-2 antibody. Band density was analyzed using LabWorks 4.0 software, and was normalized to β -actin protein expression (loading control). As shown in Figure 6A, compared to FuGene 6® transfection control (CTL) and an irrelevant siRNA control (siCTL), siRNA against PAR-2 decreased PAR-2 protein expression about 65% in NCI-H292 cells.

We then investigated the effects of knocked down PAR-2 expression in NCI-H292 cells on mucin secretion after treatment with PAR-2 AP or HNE. At 48 h after transfection of PAR-2 or control siRNA, cells were exposed to 100 μ M PAR-2 AP for 30 min or 1 μ M HNE for 15

min. Mucin samples were then collected and assayed by ELISA. The results showed that knock down of PAR-2 expression via siRNA decreased the amount of secreted mucin by 25 % in response to PAR-2 AP stimulation (Fig. 6B), although the difference did not reach statistical significance. Transfection of the irrelevant control siRNA did not affect mucin secretion. Knock down of PAR-2 did not affect the mucin secretory response to HNE (Fig. 6C).

4. Discussion

The results of this study indicate that PAR-2 protein is expressed in human primary airway epithelial cells and two epithelial cell lines (NCI-H292 and HBE1). PAR-2 appears localized at apical regions of airway epithelium in intact human bronchial tissue, and appears associated with the plasma membrane on the surface of NHBE cells when cytospin preparations of these cells are assessed by immunocytochemistry. Activation of PAR-2 on airway epithelial cells by higher concentrations of a PAR-2 active peptide (AP; 100 and 1000 μ M) stimulated mucin secretion significantly but not dramatically after 30 min incubation. Lower concentration of AP (0.1, 1, or 10 μ M) did not affect mucin secretion. Neither tryptase nor trypsin, known physiological agonists of PAR-2, affected mucin secretion in these cells. Knockdown of PAR-2 protein by small interfering RNA appeared to attenuate somewhat mucin secretion enhanced by PAR-2 AP, but did not affect secretion stimulated by exposure of the cells to HNE. Our findings suggested that PAR-2 activation is weakly related to mucin secretion in human airway epithelium, and that HNE, an endogenous protease and known mucin secretagogue, does not enhance mucin secretion through a PAR-2-related pathway of stimulation.

PAR-2 is a G-protein coupled receptor containing seven transmembrane domains, and it can be activated by proteolytic cleavage (Dery, Corvera, Steinhoff & Bunnett, 1998; Nystedt, Emilsson, Larsson, Strombeck & Sundelin, 1995). The N-terminal extracellular domain of the receptor contains specific site SKGR↓SLIG that is recognized and cleaved by exogenous or endogenous proteases, such as trypsin, mast cell tryptase, and coagulation factors VIIa and Xa (Bohm et al., 1996a; Camerer, Huang & Coughlin, 2000; Molino et al., 1997). Following cleavage, the newly-formed N-terminal sequence (SLIGKV) acts as a tethered ligand and binds to the second extracellular loop of the receptor. This binding activates the receptor and induces downstream cellular signaling events. It has been suggested that PAR-2 may interact with G_q/G_{11} and possibly with G_o/G_i after being activated (Schultheiss, Neumcke & Richter, 1997). Signaling downstream of the receptor may involve PLC, PKC, JNK and/or p38 MAPK activation, with PAR-2-related signaling pathways directing proliferation, differentiation, depolarization, mitogenic responses, or cytoskeletal changes in cells (Berger, Tunon-De-Lara, Savineau & Marthan, 2001; Kanke et al., 2001; Okamoto et al., 2001). Activation of PAR-2 also may increase cytosolic calcium ion concentrations (Bohm et al., 1996a), and also may activate ERK-related pathways and enhance production of cytokines (Page, Strunk & Hershenson, 2003) and the prostanoid, prostaglandin E_2 (PGE_2). Indeed, in airway smooth muscle cells, PAR-2-mediated bronchorelaxation activated by trypsin is cyclooxygenase-dependent and mediated by PGE_2 (Cocks et al., 1999). After a series of rapid signaling events, PAR-2 receptors are desensitized by interaction with β -arrestins (DeFea et al., 2000; Dery, Thoma, Wong, Grady & Bunnett, 1999), and endocytosis of PAR-2 is then mediated by the GTPases dynamin and Rab5a (Roosterman, Schmidlin & Bunnett, 2003).

Allergic asthma and chronic bronchitis are associated with increased PAR-2 expression in the bronchial epithelium, suggesting a potential role of PAR-2 in the development of respiratory disease (Knight et al., 2001; Miotto et al., 2002). Coagulation factor Xa, a PAR-2 agonist, regulates amphiregulin expression and collagen deposition in secretion in remodeling airways of asthmatic mice (Shinagawa, Martin, Ploplis & Castellino, 2007). PAR-2 also regulates Na^+ absorption, luminal Cl^- and basolateral K^+ channels, and release of prostaglandin E_2 by airway epithelium (Kunzelmann et al., 2005). Airway epithelial cells also secrete a numerous

proinflammatory agents, including cytokines, chemokines, prostanoids, and nitric oxide, all of which can contribute to respiratory inflammation (Knight et al., 2001), and activation of PAR-2 in airway epithelial cells also is associated with release of IL-6 and IL-8 (Kauffman, Tomee, van de Riet, Timmerman & Borger, 2000), matrix metalloproteinase (MMP)-9 (Vliagoftis et al., 2000), and platelet-derived growth factor (PDGF) (Shimizu et al., 2000), all of which can contribute to the inflammatory and/or profibrotic milieu in the airways. PAR-2 activation also stimulates airway epithelial cells to release some eosinophil survival-promoting factors (e.g. GM-CSF) (Vliagoftis, Befus, Hollenberg & Moqbel, 2001). Thus, PAR-2 may be an important component of airway inflammation.

PAR-2 has been shown to be involved in other secretory systems. Activation of PAR-2 in pancreatic duct and kidney epithelium induces a Cl^- secretory response that may function to flush bacterial toxins or microorganisms out of the system (Bertog et al., 1999; Nguyen et al., 1999). In the gastrointestinal system, PAR-2 activation triggers the cytoprotective secretion of gastric mucus by stimulating the release of calcitonin gene-related peptide (CGRP) and tachykinins from sensory neurons (Kawabata et al., 2001). Pretreatment with the PAR-2 agonist SLIGRL-NH₂, significantly attenuates the gastric injury caused by HCl/ethanol or indomethacin in rats. In a rat intestinal epithelial cell line, activation of PAR-2 stimulates an increase in intracellular Ca^{2+} concentration and amylase secretion by pancreatic acini (Bohm et al., 1996b). PAR-2 also mediates salivary exocrine secretion and this secretory response appears to be independent of capsaicinsensitive sensory neurons (Kawabata et al., 2001). Salivary glands also express PAR-2, and activation of PAR-2 triggers *in vitro* secretion of N-acetylneuraminic acid, an indicator of salivary mucin, by rat sublingual glands via activation (at least in part) of a tyrosine kinase (Kawabata et al., 2000). Human trypsin-like protease (HAT), an agonist of PAR-2, regulates lung fluid clearance via regulation of ion transport in pulmonary epithelium (Chokki et al., 2004) and also increases mucin gene expression in airway epithelial cells (Chokki et al., 2004).

In this study, we used a synthetic peptide (SLIGKV-NH₂) to activate PAR-2 in bronchial epithelial cells. This peptide is known to activate PAR-2 in bronchial epithelial cell lines, including NCI-H292, A549 cells, and 16HBE, and also in primary human bronchial epithelial cells (Chokki et al., 2004; Dulon et al., 2003; Miki et al., 2003). Our results indicate that PAR-2 activation by AP in airway epithelial cells stimulates a relatively small amount of mucin secretion after exposure for 30 min. However, trypsin and tryptase, physiologically-relevant PAR-2 agonists, did not cause the same effects. This discrepancy could be due to our addition of protease inhibitors in experiments with these proteases. Since trypsin and tryptase are potent proteases, they may still have enough activity to digest the collected mucin samples and thereby influence the ELISA results.

Human neutrophil elastase (HNE), a serine protease, is a potent endogenous mucin secretagogue (Park et al., 2005) that also may cause goblet cell metaplasia and increased production of mucin (Breuer, Christensen, Lucey, Stone & Snider, 1985; Kim et al., 1987). HNE may be an important stimulus for mucin hypersecretion in obstructive airway diseases, such as asthma and COPD, since high levels of airway HNE are observed in patients with these conditions (Vignola et al., 1998). PAR-2 activation also enhances neutrophil adhesion to alveolar type II epithelial cells and induces actin reorganization in these cells, suggesting a possible relationship between PAR-2 and neutrophil elastase (Yagi et al., 2006). Here we investigated whether or not HNE induces mucin hypersecretion via a signaling pathway involving PAR-2. Our results showed that, after knockdown of PAR-2 expression by siRNA in NCI-H292 cells, elastase still induced a similar amount of mucin secretion as control cells. This suggests that mucin hypersecretion stimulated by elastase does not involve PAR-2. Interestingly, it has been shown that, although elastase derived from *Pseudomonas*

aeruginosa cleaves the N-terminal domain of PAR-2, PAR-2 is not activated by this elastase (Dulon et al., 2005) not by neutrophil elastase (Dulon et al., 2003).

In summary, our results show that PAR-2 activation weakly enhances mucin secretion by airway epithelium, in contrast to more potent mucin secretagogues, such as HNE or phorbol 12-myristate 13-acetate (PMA). Thus, PAR-2 probably is not a major molecule involved in mucin secretion in the airways under normal or inflammatory conditions.

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Fig. 1.

Detection of PAR-2 protein in airway epithelial cells via immunoblotting. Total protein was isolated from NHBE, NCI-H292, and HBE1 cells. Protein samples were then analyzed by SDS-PAGE gel electrophoresis and immunoblotting with mouse monoclonal anti-PAR-2 (SAM11) antibody. Lane 1: NHBE cells; lane 2: NCI-H292 cells; lane 3: HBE1 cells. The bands at approximately 55 kDa represent PAR-2 protein.

Fig. 2. Immunohistochemical and immunocytochemical localization of PAR-2 in human bronchial epithelial cells. (A) Human bronchial tissue was stained with a specific goat polyclonal anti-PAR-2 antibody, and counterstained with alcian blue. PAR-2 appears localized to goblet cells of bronchial epithelium (left panel, indicated by arrows); right panel stained with control antibody (ChromPure goat IgG). (B) Cytospin preparations of NHBE cells were stained with goat polyclonal anti-PAR-2 antibody. PAR-2 appears to be localized on the surfaces of the epithelial cells (indicated by arrowheads). Left panel, goat anti-PAR-2 antibody; right panel, control antibody (ChromPure goat IgG). (Original magnification $\times 200$)

Fig. 3.

Effects of PAR-2-activating peptide on mucin secretion by human airway epithelial cell lines. (A) NCI-H292 cells were exposed to the PAR-2-activating peptide (AP: 0.1, 10, 100 μ M) for 30 min. Mucin released by the cells in response was collected and assayed by ELISA. There was a significant increase in secretion in response to 100 μ M AP ($p < 0.01$), while the control RP did not affect secretion. (B) HBE1 cells were exposed to 100 μ M AP for 30 min., and collected mucin samples were assayed by 17Q2 ELISA. The PAR-2 AP significantly enhanced mucin secretion in HBE1 cells ($p < 0.05$) at this concentration. Data are presented as mean $\pm$ SEM. ($n = 6$ at each point). *, significantly different from medium control.

Fig. 4.

Effects of synthetic PAR-2-activating peptide on mucin secretion by well-differentiated primary NHBE cells. (A) NHBE cells were exposed to the PAR-2-activating peptide AP (0.1, 1, 10, 100, 1000 μ M) for 30 min. Mucin released by the cells in response was collected and assayed by ELISA. There was a significant increase in secretion in response to 100 and 1000 μ M AP, while the control RP had no effect. (B) Time course; NHBE cells were exposed to the PAR-2 activating peptide (AP; 100 μ M for 15, 30 or 60 min), and released mucin assayed by 17Q2 ELISA. There was a significant increase in secretion only at 30 min. Data are presented as mean $\pm$ SEM (n= 6 at each point). *, significantly different from medium control ($p < 0.05$).

Fig. 5.

Effects of human lung trypsinase or bovine pancreatic trypsin, known PAR-2 physiological agonists, on mucin secretion by NHBE cells. NHBE cells were exposed to either human lung trypsinase (A) or bovine pancreatic trypsin (B) between 0.1– 10 $\mu\text{g/ml}$ for 30 min, and mucin secretion was measured by 17Q2 ELISA. Neither of these two PAR-2 agonists affected mucin secretion significantly at these concentrations. Data are presented as mean $\pm$ SEM ($n=6$ at each point).

Fig. 6.

Effects of siRNA on PAR-2 protein levels and mucin secretion by NCI-H292 cells. (A) PAR-2 protein expression was detected by immunoblotting. Cells were untransfected (CTL) or transfected (siPAR2: PAR-2 siRNA 50nM; siCTL: control siRNA 50nM) for 48 h, and proteins were detected by immunoblotting using a mouse monoclonal anti-PAR-2 antibody, with β -actin as a control. Treatment with the PAR-2 siRNA knocked down expression of PAR-2 protein in these cells by approximately 65%. (B) and (C) Effects of siRNA knock down of PAR-2 expression on mucin secretion. Cells were either untransfected (bars 1 and 2), transfected with PAR-2 siRNA (bar 3), or transfected with control siRNA (bar 4). After treatment with 100 μ M PAR-2 for 30 min (B) or with 1 μ M elastase for 15 min (C), mucin

samples were collected and measured by ELISA (B: 17Q2 antibody; C: MUC5AC antibody). Knock down of PAR-2 expression attenuated the secretory response to the PAR-2 AP, but had no effect on secretion stimulated by HNE. Data are expressed as mean $\pm$ SEM (n = 3). *, significantly different from medium control (p < 0.05).