Human Airway Trypsin-Like Protease Increases Mucin Gene Expression in Airway Epithelial Cells

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Human airway trypsin-like protease (HAT) is a serine protease found in sputum of patients with chronic airway diseases and is an agonist of protease-activated receptor-2 (PAR-2). Results from this study show that HAT treatment also enhances mucus production by the airway epithelial cell line NCI-H292 *in vitro*. Histologic examination showed that HAT enhances mucous glycoconjugate synthesis, whereas the PAR-2 agonist peptide (PAR-2 AP) has no such effect. HAT, but not PAR-2 AP, enhances *MUC2* and *MUC5AC* gene expression 23-fold and 32-fold, respectively. The proteolytic activity of HAT is required to enhance *MUC5AC* gene expression; the addition of the inhibitors of trypsin-like protease activity of HAT, aprotinin and leupeptin, abolishes its enhancing effect. AG1478, anti-epidermal growth factor receptor (anti-EGFR)–neutralizing antibody, and anti-amphiregulin (AR)-neutralizing antibody all inhibited the stimulatory effect of HAT. Furthermore, HAT increases AR gene expression and subsequent AR protein release, whereas PAR-2 AP shows no such effects. These results indicate that HAT enhances mucin gene expression through an AR-EGFR pathway, and PAR-2 is not sufficient for or does not directly cause HAT-induced mucin gene expression. Thus, HAT might be a possible therapeutic target to prevent excessive mucus production in patients with chronic airway diseases.

Human airway trypsin-like protease (HAT) is a novel serine protease that, based on its protease activity, can be purified from the sputum of patients with chronic airway diseases such as chronic bronchitis and bronchial asthma (1). It exists in sputum as a monomer with a molecular size of 27 kD, estimated from gel filtration chromatography results. HAT cDNA has been cloned from a tracheal tissue cDNA library; analysis of this cDNA suggests that HAT is originally translated as a precursor with a molecular size of 48 kD and possesses a hydrophobic transmembrane domain near the *N*-terminus (2). Based on this derived structure, HAT is thought to be a member of the type-II transmembrane serine protease family that includes corin, enteropeptidase, MT-SP1 (also known as matriptase), and hepsin (reviewed in Ref. 3). Northern blotting results from 17 human tissues show that the HAT mRNA is most prominently expressed in tracheal tissue, which suggests that HAT is localized in the airway (2). Additionally, an HAT-specific, monoclonal antibody was used to conduct an immunohistochemical analysis of airway tissue from healthy subjects. The results show that the HAT protein is found specifically in ciliated epithelial cells but not in basal cells, goblet cells in the epithelium, or in submucosal gland cells (4). Therefore, it is thought that HAT might be responsible for regulating some biological processes in airway cells.

Protease-activated receptors (PARs) are thought to be candidate HAT target proteins for the following reasons. PARs are G-protein–coupled receptors that are activated by the cleavage of their *N*-terminal domain. The proteolytic cleavage of the *N*-terminal region of each PAR unmasks a new *N*-terminus. This newly unmasked terminus acts as a tethered ligand that binds to and autoactivates the receptor. PAR-1 and PAR-3 are activated by thrombin; PAR-2 is activated by trypsin and mast cell tryptase; and PAR-4 is activated by both thrombin and trypsin (reviewed in Ref. 5). In a previous study, HAT was shown to activate PAR-2 in human bronchial epithelial cells *in vitro* (6). It has been reported that activated PAR-2 mediates airway inflammation both *in vitro* (7, 8) and *in vivo* (9). Furthermore, it has been reported that the expression of PAR-2 mRNA in airway epithelium increases in patients with bronchial asthma (10). These observations suggest that HAT might mediate the airway inflammation through activation of PAR-2.

Not only airway inflammation, but also hypersecretion of airway mucus, is a typical sign of chronic obstructive airway diseases, including bronchitis, bronchial asthma, and cystic fibrosis (11, 12). This excessive mucus secretion causes airway obstruction, which contributes to the morbidity and mortality caused by these diseases (11, 12). Respiratory or airway mucus comprises two layers, a perciliary fluid layer (sol layer), and a mucus layer that consists of macromolecular glycoproteins called mucin. A high-molecular weight gel network consisting mainly of mucin is responsible for the gel formation of the mucus layer. It is possible that an increase in mucin gene expression, with a subsequent increase in mucin production, causes mucus hypersecretion in chronic obstructive airway diseases (COPD). To date, at least 12 mucin (*MUC*) genes have been identified in humans. Eight of these, *MUC1*, *MUC2*, *MUC4*, *MUC5AC*, *MUC5B*, *MUC7*, *MUC8*, and *MUC13*, are expressed in airway epithelium of healthy subjects (reviewed in Ref. 13). Of these

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Abbreviations: Alcian Blue and periodic acid-Schiff, AB-PAS; amphiregulin, AR; epidermal growth factor, EGF; epidermal growth factor receptor, EGFR; enzyme-linked immunosorbent assay, ELISA; human airway trypsin-like protease, HAT; heparin-binding EGF-like growth factor, HB-EGF; protease-activated receptor, PAR; PAR-2 agonist peptide, PAR-2 AP; heterotrimeric guanine nucleotide-binding protein, G-protein; reverse transcription–polymerase chain reaction, RT-PCR; serum-free medium, SFM; transforming growth factor-α, TGF-α.

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genes, *MUC2* and *MUC5AC* are known to participate in the pathogenesis of mucus hypersecretion in patients with chronic airway diseases. Expression of *MUC2* mRNA increases in patients with cystic fibrosis (14). *MUC5AC* is a major mucin secreted from the goblet cells of the surface epithelium (15). *MUC5AC* mRNA is predominantly expressed in surface goblet cells (16) and is regarded as a marker for goblet cell metaplasia in murine airways (17). The number of goblet cells in airway tissue markedly increases in patients with bronchial asthma (12), and the *MUC5AC* mRNA level has been reported to increase in the bronchial tissue of patients with mild or moderate asthma (18). These observations strongly suggest that increased expression of the *MUC2* and *MUC5AC* genes contributes to mucus hypersecretion in patients with chronic airway diseases.

Consequently, because HAT was purified from the sputum of patients with airway mucus hypersecretion, it is possible that HAT is involved not only in airway inflammation, but also in mucus hypersecretion. Therefore, in this study, the effect of HAT on both mucin production and gene expression *in vitro* was examined in the mucin-producing airway epithelial cell line NCI-H292, which is often used as a model system for mucin production (reviewed in Ref. 19). Additionally, because little is known about the role of PAR-2 in airway mucin production, the PAR-2 agonist peptide (PAR-2 AP) was used to examine the contribution of PAR-2 to mucus hypersecretion. Moreover, involvement of epidermal growth factor (EGF) receptor was also evaluated, because EGFR has been shown to play an important role in induction of mucin gene expression or mucin production in NCI-H292 cells (20–25).

Materials and Methods

Materials

Recombinant human airway trypsin-like protease (60 U/mg of protein) was prepared as previously described (1, 2, 6). Briefly, HAT protein was expressed in insect cells infected with a recombinant baculovirus carrying the HAT cDNA (2). Benzamidine affinity chromatography was used to purify recombinant HAT from the cell lysate (6), and the specific activity of the purified protein was measured with Boc-Phe-Ser-Arg-MCA as a substrate, as previously described (1). Trypsin (from bovine pancreas, 6,600 U/mg protein) and aprotinin (from bovine lungs, 4 trypsin inhibiting U/mg protein) were purchased from Sigma (St. Louis, MO). Tryptase (from human lungs, 59.5 U/mg protein) and elastase (from human leukocytes, 16,000 U/mg protein) were obtained from Elastin Products (Owensville, MO). The PAR-2 agonist peptide (PAR-2 AP) SLIGKV-NH_2 (5) was purchased from Bachem AG (Bubendorf, Switzerland). Leupeptin was purchased from Wako Pure Chemicals (Osaka, Japan). AG1478 was from Biomol (Plymouth Meeting, PA). The mouse monoclonal neutralizing antibody against EGFR (clone LA1) was purchased from Upstate Biotechnologies (Lake Placid, NY). Human recombinant epidermal growth factor (EGF), the goat polyclonal neutralizing antibody against heparin-binding EGF-like growth factor (HB-EGF), the goat polyclonal neutralizing antibody against transforming growth factor-α (TGF-α) and nonimmune mouse IgG_1, used as a negative control, were obtained from R&D Systems Inc. (Minneapolis, MN). The mouse monoclonal neutralizing antibody against EGF (clone 10825.1), the mouse monoclonal neutralizing antibody against amphiregulin (AR; clone 31221.111), and enzyme-linked immunosorbent assay (ELISA) kit for AR were obtained from Genzyme (Minneapolis, MN).

Cell Culture

The human pulmonary mucoepidermoid carcinoma cell line NCI-H292 was purchased from American Type Culture Collection (Rockville, MD) and cultured in RPMI-1640 medium supplemented with 10% (vol/vol) fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin (Gibco BRL, Grand Island, NY) in a humidified incubator at 37°C in an atmosphere of 5% CO_2: 95% air. Before experiments, confluent NCI-H292 cells were cultured in serum-free medium (SFM; RPMI-1640 medium containing only 0.1% [wt/vol] bovine serum albumin [Sigma]) for 24 h, unless otherwise indicated. Human lung carcinoma cell line A549 cells were also obtained from ATCC and cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin (Gibco BRL) under the same conditions used for NCI-H292 cells.

Assessment of Cell Viability

Cell viability was determined by measuring metabolic activity of the cells using fluorogenic, oxidation-reduction indicator Alamar Blue (Biosource International, Camarillo, CA) after undergoing treatment with proteases. Serum-depleted NCI-H292 cells were treated for 24 h with HAT or other protease dissolved in SFM. The control group comprised cells treated with SFM that did not contain a protease. At the end of treatment, a volume of Alamar Blue equal to 1/10 the culture medium volume was added to the medium, and 2 h later, a CytoFluor 2300 fluorometer (Millipore, Bedford, MA) was used to measure fluorescence at 590 nm after excitation at 560 nm.

Measurement of Intracellular Ca^{2+} Mobilization

Three thousand NCI-H292 cells were seeded onto glass-bottom culture dishes (35 mm diameter; MatTek, Ashland, MA) and grown for 3 d. The cells were then incubated with 10 μM Fura-2 acetoxymethyl ester (Dojindo, Kumamoto, Japan) for 30 min at 37°C in basal salt solution (BSS) consisting of 20 mM HEPES pH 8.0, 130 mM NaCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 5.4 mM KCl, 5.5 mM glucose, 0.1% (wt/vol) bovine serum albumin. Cells were washed twice with BSS and immediately treated with HAT (200 nM) or PAR-2 AP (300 μM) dissolved in BSS. An ARGUS 50 system (Hamamatsu Photonics, Tokyo, Japan) was used to monitor the ratio of Fura-2 fluorescence at 510 nm after excitation, first at 340 nm, and then at 380 nm.

Alcian Blue–Periodic Acid-Schiff Staining to Visualize Mucous Glycoconjugates

Cells were seeded into each well of eight-well chamber slides (Nalge Nunc, Rochester, NY) and grown until confluent. The medium was changed to an SFM, cells were incubated for 24 more hours, and then treated with HAT (200 nM), EGF (1 ng/ml), or PAR-2 AP (300 μM) dissolved in SFM for an additional 24 h. Cells were fixed with 4% (wt/vol) paraformaldehyde for 1 h, and Alcian blue–periodic acid-Schiff (AB-PAS) staining was used to visualize the mucous glycoconjugates.

Reverse Transcriptase–Polymerase Chain Reaction to Detect PAR-2 mRNA

For each sample of cells, an RNeasy Mini Kit (QIAGEN, Hilden, Germany), which included a DNase I digestion step (RNase-free DNase set; QIAGEN), was used to extract total cellular RNA.

Approximately 0.5 μg of each total RNA preparation was reverse-transcribed with Omniscript RT (QIAGEN), and random hexamers were used to prime the reactions. These synthesized cDNA samples were separately diluted 5-fold with nuclease-free water, and 2 μl of each diluted cDNA solution was used as the template in a 20-μl Hotstar-*Taq* PCR master reaction mix, with specific primers and conducted according to the manufacturer's protocol (QIAGEN). The primers used for PAR-2 (Gene Bank Accession No.: U34038) mRNA detection were forward primer: 5′-TGGATGAGTTTTC TGCATCTGTCC-3′, and reverse primer: 5′-CGTGATGTTCAG GGCAGGAATG-3′. The primers used for detecting the β-actin (Gene Bank Accession No.: BC014861) internal control mRNA were forward primer: 5′-CAAGAGATGGCCACGGCTGCT-3′, and reverse primer: 5′-TCCTTCTGCATCCTGTCGGCA-3′. To ensure that the product resulted from reverse transcription, mock reactions that did not contain reverse transcriptase were conducted.

Real-Time Reverse Transcriptase–Polymerase Chain Reaction to Quantitate Mucin and Amphiregulin mRNA

A GeneAmp 5,700 sequence detection system (Applied Biosystems, Foster City, CA) to conduct real-time reverse transcriptase–polymerase chain reaction (RT-PCR) was used to measure *MUC2* and *MUC5AC* mRNA expression. Extraction of total cellular RNA and synthesis of cDNA were performed as described in the previous section. PCR was performed using a TaqMan Universal PCR Master Mix Kit (Applied Biosystems) and a fluorogenic probe (Applied Biosystems; Ref. 26) following the manufacturer's protocol. The Primer Express software program (Applied Biosystems) was used to design primers and probes. The sequences of the primers used for *MUC5AC* (Gene Bank Accession No.: AF015521) detection were forward: 5′-TCAACGGAGACTGCGAGTACAC-3′, reverse: 5′-TCTTGATGGCCTTGGAGCA-3′, and the FAM reporter dye-labeled hybridization probe: 5′-FAM-ACTCCTTTCG TGTTGTCACCGAGAACGTC-TAMRA-3′. The sequences of the primers used for *MUC2* (Gene Bank Accession No.: L21998) detection were forward: 5′-GCCCTGGCTTCGAACTCAT-3′, reverse: 5′-TCTTCGGGTCGCTCTTGAA-3′, and the FAM reporter dye-labeled hybridization probe: 5′-FAM-CACTGTATC ATCAAACGGCCCGACAA-TAMRA-3′. The sequences of the primers used for AR (Gene Bank Accession No.: M30704) were forward: 5′-AGGCCATTATGCTGCTGGAT-3′, reverse: 5′-TGT GGTCCCCAGAAAATGGT-3′, and the FAM reporter dye-labeled hybridization probe: 5′-ACCTCAATGACACCTACTCTGGGA AGCGT-3′. A Pre-Developed TaqMan Assay Reagent (Applied Biosystems) was used to measure β-actin mRNA according to the manufacturer's protocol. During PCR amplification using this assay system (26), the 5′ nucleolytic activity of *Taq* polymerase cleaves the probe separating the 5′ reporter fluorescent dye from the 3′ quencher dye. The threshold cycle, C_t, which correlates inversely with the level of the target mRNA, was measured as the cycle number at which the reporter fluorescent emission increased above the midpoint of the logarithmic increasing phase along the amplification curve. A standard curve relating C_t to a serial dilution of standard cDNA was used to compute the relative abundance of *MUC2*, *MUC5AC*, AR, and β-actin mRNA in each sample. In the quantitation of *MUC2*, *MUC5AC*, and β-actin mRNA, cDNA prepared from NCI-H292 cells treated with EGF (1 ng/ml for 24 h) was used as the standard, and in the quantitation of AR mRNA, cDNA prepared from NCI-H292 treated with HAT (200 nM for 2 h) was used as the standard. The relative abundance of β-actin mRNA in each sample of cells was used to normalize the *MUC2*, *MUC5AC*, and AR mRNA levels.

Determination of AR Protein

AR protein in the culture supernatant from NCI-H292 treated with vehicle, HAT, or PAR-2 AP were measured using a commercial, specific ELISA kit according to the manufacturer's instruction. The limit of assay sensitivity is 25.6 pg/ml.

Statistical Analysis

Data are presented as the mean ± SD of at least three separate experiments. For statistical analysis, Dunnett's two-tailed test was used. The commercial, statistical software Super ANOVA ver. 1.1 (Abacus Concepts Inc., Berkeley, CA) was used to perform the tests. Tests that returned $P < 0.05$ were regarded as showing statistically significant differences between results.

Results

HAT Activates PAR-2 in NCI-H292 Cells

Because the expression of PAR-2 by NCI-H292 has not been reported, RT-PCR was used to test whether these cells express PAR-2 mRNA. As shown in Figure 1A, PAR-2 mRNA expression was detected in NCI-H292 cells, as well as in A549 cells, which are known to express PAR-2 mRNA (7). Additionally, no PCR product was detected in reactions run without reverse transcriptase, ruling out the possibility of genomic DNA contamination. Thus, these results demonstrate that both NCI-H292 and A549 cells express PAR-2 mRNA. Next, the presence of functional PAR-2 was confirmed by measuring intracellular Ca^{2+} mobilization in response to PAR-2 agonist peptide (PAR-2 AP) in these cells. Not only PAR-2 AP (Figure 1B) but also HAT (Figure 1C) evoked intracellular Ca^{2+} mobilization in NCI-H292 cells. Furthermore, desensitization of the PAR-2 pathway

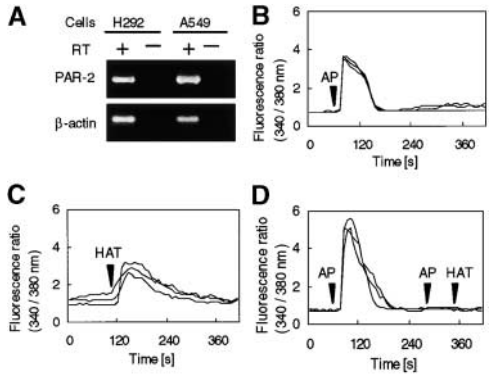

Figure 1. Expression of PAR-2 mRNA and PAR-2–mediated Ca^{2+} mobilization in NCI-H292 cells. Total cellular RNA was extracted from NCI-H292 and A549 cells, and RT-PCR was used to determine the amounts of PAR-2 or β-actin (*A*). cDNA reactions in which the RT was omitted (− *lanes*) were used as the negative control for each cell line and mRNA tested. Intracellular Ca^{2+} mobilization was induced by treatment with (*B*) 300 μM PAR-2 AP (AP) or (*C*) 200 nM HAT. (*D*) Desensitization of intracellular Ca^{2+} mobilization to treatment with 200 nM HAT caused by repeated application of 300 μM PAR-2 AP at the times indicated by *arrows*. The Ca^{2+} concentration was determined by monitoring fluorescence changes in cells loaded with Fura-2 AM.

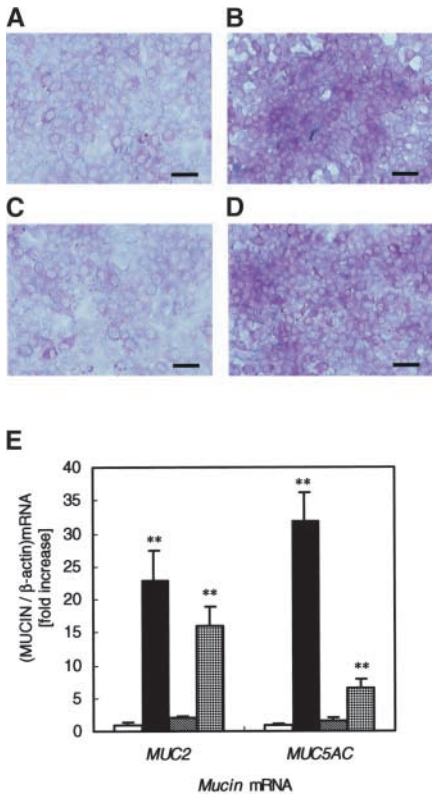

Figure 2. HAT, not PAR-2 AP, induces mucus production and mucin gene expression in NCI-H292 cells. Confluent, serum-depleted NCI-H292 cells were treated with (*A*) the vehicle alone, (*B*) 200 nM HAT, (*C*) 300 μM PAR-2 AP, or (*D*) 1 ng/ml EGF for 24 h. AB-PAS staining was then used to determine the presence of mucus. The results shown are representative photographs of three separate experiments. *Scale bar:* 50 μm. (*E*) Total RNA was extracted from NCI-H292 cells treated with the vehicle alone (*open bars*), HAT (200 nM, *solid bars*), PAR-2 AP (300 mM, *striped bars*), or EGF (1 ng/mL, *hatched bars*) for 24 h. Quantitative real-time RT-PCR (TaqMan) analysis was used to determine the amount of mucin and β-actin mRNA. The results are expressed as the mean ± SD ($n = 3$). $^{**}P < 0.01$, compared with vehicle-treated cells, Dunnett's test.

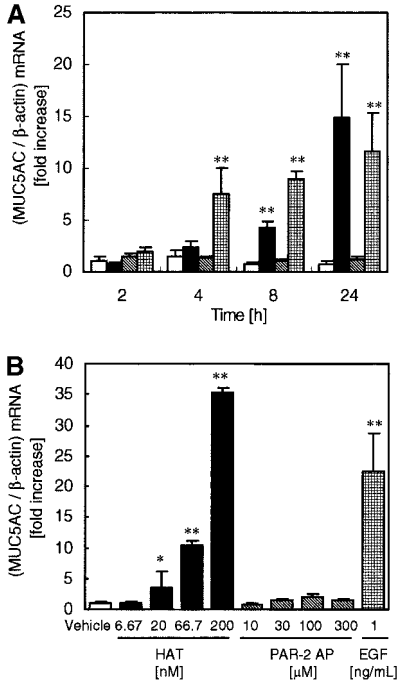

Figure 3. Kinetic analysis of HAT-induced *MUC5AC* gene expression in NCI-H292 cells. (*A*) Confluent, serum-depleted NCI-H292 cells were treated with the vehicle alone (*open bars*), HAT (200 nM; *solid bars*), PAR-2 AP (300 μM; *striped bars*), or EGF (1 ng/ml; *hatched bars*) for 2, 4, 8, or 24 h. (*B*) NCI-H292 cells were treated with a range of HAT or PAR-2 AP concentrations for 24 h. Total RNA was then extracted, and quantitative real-time RT-PCR (TaqMan) analysis was used to determine the amounts of *MUC5AC* and β-actin mRNA. The results are expressed as the mean ± SD ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, compared with vehicle-treated cells at the same time point, Dunnett's test.

by brief exposure to PAR-2 AP before HAT treatment completely disrupted HAT-induced intracellular Ca^{2+} mobilization (Figure 1D). These results demonstrate that functional PAR-2 is expressed by NCI-H292 cells, and that HAT also can activate PAR-2 in these cells.

Induction of Mucous Glycoconjugate Production and Mucin Gene Expression

To evaluate the possibility that HAT enhances mucin production, the effect of HAT on mucous glycoconjugate production in NCI-H292 cells was examined. As Figure 2A shows, few vehicle-treated cells were stained by AB-PAS staining. However, the number of AB-PAS–positive cells and the intensity of AB-PAS staining in each cell clearly increased after treatment with either the test protein HAT (Figure 2B), or the positive control EGF (Figure 2D; Ref. 25). In contrast, no change was observed in PAR-2 AP-treated cells (Figure 2C). Because EGF has been reported to increase *MUC2* and *MUC5AC* gene expression (20), this increase in mucus production is thought to be accompanied by enhancement of mucin gene expression. To determine whether HAT increases mucin gene expression, the effect of HAT on *MUC2* and *MUC5AC* mRNA level was examined using real-time RT-PCR (Figure 2E). HAT treatment induced a 23-fold increase in *MUC2* mRNA level and a 32-fold increase in *MUC5AC* mRNA level. EGF treatment also increased the *MUC2* mRNA level 16-fold and the *MUC5AC* mRNA level 6-fold in this study, in accordance with a previous observation (20). In contrast, PAR-2 AP treatment did not significantly change basal *MUC2* and *MUC5AC* mRNA levels. These results show that both HAT and EGF increase mucin gene expression, and together with the staining experiments suggest that increased mucin gene expression leads to increased production of MUC2 and MUC5AC proteins.

Time Course and Dose Response to HAT Treatment

Because the *MUC5AC* is one of the mucin genes most active in patients with chronic airway diseases, and because it is thought to be a marker for goblet cell metaplasia (17, 18), the effect of HAT on *MUC5AC* gene expression was examined in detail. As Figure 3 shows, HAT increased the *MUC5AC* mRNA levels in NCI-H292 cells in a time-dependent (Figure 3A) and concentration-dependent (Figure 3B) manner. In the HAT-treated cells, *MUC5AC* mRNA levels significantly increased to five times greater than the basal level by 8 h after the onset of treatment and continued to increase steadily, until the last time point at

TABLE 1
Effect of HAT on viability of NCI-H292 cells

	Cell viability (*% of vehicle-treated group*)					
Protease	—	3 nM	10 nM	30 nM	100 nM	300 nM
Vehicle	100.0 ± 8.4					
HAT		96.1 ± 0.5	100.1 ± 7.3	104.4 ± 4.0	107.3 ± 5.5	114.8 ± 2.4*
Elastase		95.3 ± 5.7	98.8 ± 8.7	95.4 ± 2.6	103.3 ± 1.4	100.4 ± 5.0
Tryptase		91.9 ± 3.9	98.2 ± 3.6	92.4 ± 3.5	95.7 ± 3.2	93.9 ± 3.9

Definition of abbreviation: HAT, human airway trypsin-like protease.
Results are expressed as mean ± SD ($n = 3$).
*$P < 0.05$, compared with vehicle-treated group, Dunnet's test.

24 h after the onset of treatment. EGF also increased the *MUC5AC* mRNA level in a time-dependent manner. In contrast to the HAT results, the *MUC5AC* mRNA level in the EGF-treated cells started to increase significantly faster (after treatment for only 4 h) than in HAT-treated cells (Figure 3A). As Figure 3B shows, a significant HAT-induced increase of *MUC5AC* mRNA levels, compared with vehicle-treated cells, was observed at an HAT concentration as low as 20 nM. Table 1 shows the effect of HAT on the viability of NCI-H292 cells. HAT, at concentrations of 3, 10, 30, and 100 nM did not significantly reduce the cell viability; indeed, the results suggest that 300 nM HAT slightly enhances cell viability. These results indicate that HAT increases *MUC5AC* gene expression without exerting a cytotoxic effect.

To clarify whether or not PAR-2 AP induces increased expression of the *MUC5AC* gene, the time- and concentration-dependent effects of PAR-2 AP on *MUC5AC* mRNA levels were also examined. However, as Figure 3A and 3B show, PAR-AP did not significantly induce *MUC5AC* gene expression at any time from 2 to 24 h following the beginning of treatment, or at any concentration tested (10–300 μM). These results indicate that HAT, but not PAR-2 AP, potently stimulates *MUC5AC* gene expression. The PAR-2 AP used in the present study is thought to be biologically active, because it evoked intracellular Ca^{2+} mobilization in NCI-H292 cells (Figures 1B and 1D). These results suggest that activation of PAR-2 alone is neither sufficient nor directly involved in the regulation of *MUC5AC* gene expression.

HAT Protease Activity Is Required for Increased *MUC5AC* Gene Expression

Some proteases are known to express their function through nonproteolytic actions in addition to their function through proteolysis (27). To clarify whether the stimulatory effect of HAT on *MUC5AC* gene expression is caused by its proteolytic activity alone, the effect of serine protease inhibitors on HAT-induced *MUC5AC* gene expression was examined. As shown in Figure 4A, both leupeptin and aprotinin, which are potent inhibitors of HAT trypsin-like activity (1), markedly reduced the HAT-induced increase of *MUC5AC* mRNA levels, whereas they showed no significant effect on either basal *MUC5AC* mRNA levels or on the EGF-induced increase of *MUC5AC* levels (Figure 4A). Moreover, heat inactivation of HAT by boiling almost completely destroyed its ability to increase *MUC5AC* mRNA levels (Figure 4A). These results indicate that HAT-induced *MUC5AC* gene expression is caused by the proteolytic activity of HAT.

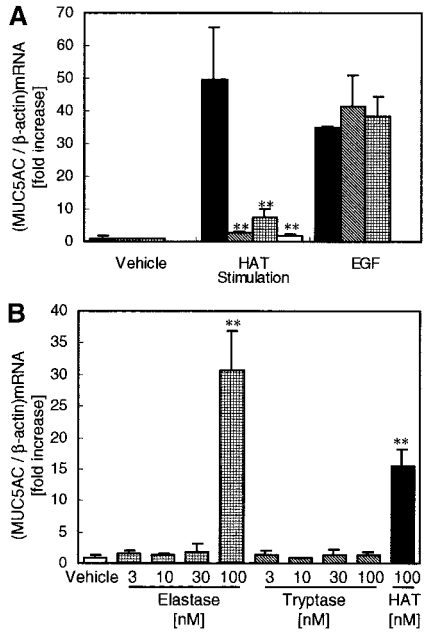

Figure 4. HAT protease activity is required to induce *MUC5AC* gene expression. (*A*) HAT (200 nM) or EGF (1 ng/ml) were separately preincubated in the absence (*solid bars*) or presence of aprotinin (1 μM, *striped bars*) or leupeptin (1 μM, *hatched bars*) for 30 min at 4°C. After warming to 37°C, these mixtures were added to separate confluent, serum-depleted NCI-H292 cell cultures. HAT was also heat-inactivated by incubation at 99°C for 10 min before addition to the cells (*open bars*). (*B*) In another experiment, separate NCI-H292 cell cultures were treated with the indicated concentration of human leukocyte elastase, human lung tryptase, or HAT. After treatment for 24 h, total RNA was extracted, and quantitative real-time RT-PCR (TaqMan) analysis was used to determine the amounts of *MUCAC* and β-actin mRNA. The results are presented as the mean ± SD ($n = 3$). **$P < 0.01$, compared with (*A*) cells treated with HAT in the absence of protease inhibitors, or (*B*) vehicle-treated cells, Dunnett's test.

Because *MUC5AC* gene expression is modulated by HAT, the ability of other serine proteases to induce *MUC5AC* gene expression was examined (Figure 4B). Neutrophil elastase, an inflammatory mediator present at high concentration in airway tissue from patients with bronchitis, has been reported to increase *MUC5AC* mRNA levels in the A549 carcinoma cell line and primary human bronchial epithelial cells taken from healthy subjects (28); it has also been reported to increase MUC5AC protein production in the NCI-H292 cells used in this study (21). As expected, leukocyte elastase at concentration of 100 nM increased *MUC5AC* mRNA levels in NCI-H292 cells. In contrast, lung mast cell tryptase, a major trypsin-like protease found in airway tissue, did not affect *MUC5AC* mRNA levels. These results indicate that HAT, like leukocyte elastase, is an important serine protease that regulates *MUC5AC* gene expression during the course of chronic airway diseases.

The Effect of EGFR Inhibitors and Neutralizing Antibodies to EGFR Ligands on HAT-Induced *MUC5AC* Gene Expression

The cellular mechanism responsible for HAT-induced *MUC5AC* gene expression was examined. Activation of epidermal growth factor receptor (EGFR) is one of the best known mechanisms responsible for regulation of mucin production and mucin gene expression in NCI-H292 cells (20–25). The results of the present study showed that both EGF and HAT increase expression of the *MUC2* and the *MUC5AC* genes; in addition, HAT increases viable cell number, indicating that HAT enhances growth of the cells. These observations strongly suggest that HAT activates EGFR. To test whether HAT-induced *MUC5AC* gene expression is mediated by activation of EGFR, AG1478, a potent and selective chemical inhibitor of EGFR tyrosine kinase activity, and an anti-EGFR neutralizing antibody were used to block EGFR signal transduction. As expected, pretreatment (20 min) with either AG1478 or the anti-EGFR neutralizing antibody inhibited the EGF-induced increase of *MUC5AC* mRNA levels (Figure 5A). Furthermore, the effect of HAT was also completely disrupted by pretreatment with these EGFR inhibitors. Because anti-EGFR neutralizing antibody used in this study binds to the extracellular domain of EGFR and competes for binding of ligands to the EGFR on cells (29), HAT-induced *MUC5AC* gene expression seems to depend on binding of certain ligands to the EGFR. Six EGF-like mammalian gene products including amphiregulin (AR), betacellulin, EGF, epiregulin, heparin-binding EGF-like growth factor (HB-EGF) and transforming growth factor-α (TGF-α) are known to activate EGFR directly (30). Because four of these, AR, EGF, HB-EGF, and TGF-α have been reported to be expressed in NCI-H292 cells (31), the effect of neutralizing antibodies to these EGFR ligands on the enhancing effect of HAT were determined next. As shown in Figure 5B, the effect of HAT was almost completely inhibited by anti-AR neutralizing antibody, and partially inhibited by anti–TGF-α neutralizing antibody, and not affected by anti-EGF neutralizing antibody and anti–HB-EGF neutralizing antibody. These results suggest HAT-induced *MUC5AC* gene expression was regulated by both the AR-EGFR and TGF-α EGFR pathway. In addition, although both anti-AR neutralizing antibody and anti–TGF-α neutralizing antibody are effective, anti-AR neutralizing antibody almost completely blocked the effect of HAT, whereas anti–TGF-α antibody blocked only part of the effect of HAT, suggesting that AR acts as an initial activator of EGFR in HAT-stimulated NCI-H292 cells.

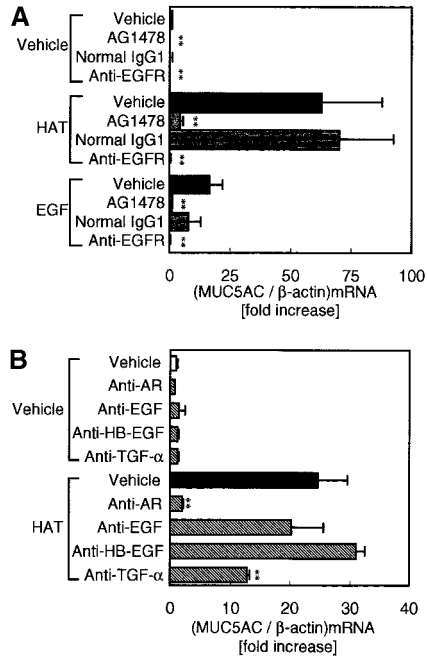

Figure 5. HAT increases *MUC5AC* gene expression mediated by AR-EGFR and TGF-α–EGFR pathway. (*A*) Confluent, serum-depleted NCI-H292 cells were pretreated with the vehicle, 100 nM AG1478, 3 μg/ml anti-EGFR neutralizing antibody, or 3 μg/ml nonimmune mouse IgG_1 for 20 min, and then stimulated with the vehicle, 200 nM HAT, or 1 ng/ml EGF for 24 h in the presence of EGFR inhibitor. (*B*) In a separate experiment, cells were treated with 10 μg/ml anti-AR, anti-EGF, anti–HB-EGF, or anti–TGF-α neutralizing antibody for 20 min, and then stimulated with the vehicle or 200 nM HAT for 24 h in the presence of neutralizing antibody. Total RNA was extracted, and quantitative real-time RT-PCR (TaqMan) analysis was used to determine the amounts of *MUCAC* and β-actin mRNA. The results are presented as the mean ± SD ($n = 3$). $^{**}P < 0.01$, compared with cells not treated with EGFR inhibitor or neutralizing antibody in vehicle-treated, HAT-stimulated, or EGF-stimulated groups, Dunnett's test.

Induction of Protein Release and Gene Expression of AR

Finally, induction of protein release and gene expression of AR by HAT was examined. As Figure 6A shows, HAT caused release of AR from NCI-H292 cells in a concentration-dependent manner, over a 24-h period. A significant HAT-induced increase of release of AR, compared with vehicle-treated cells, was observed at concentrations as low as 20 nM, and this result is in good agreement with the observation from the evaluation of the HAT-induced *MUC5AC* gene expression (Figure 3B). To clarify the mechanism of HAT-induced release of AR, the effect of HAT

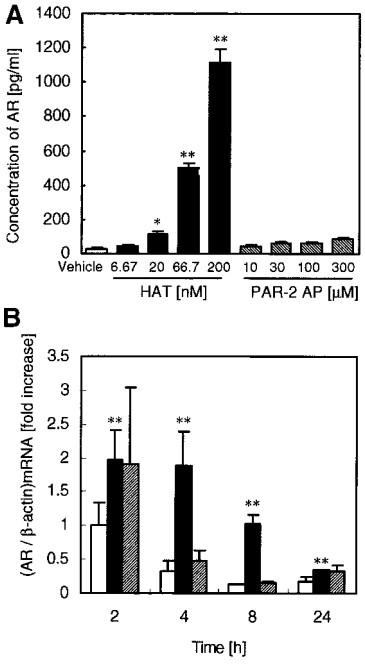

Figure 6. Induction of protein release and gene expression of AR by HAT. (*A*) Confluent, serum-depleted NCI-H292 cells were treated with a range of HAT or PAR-2 AP concentrations for 24 h. Concentration of AR in supernatants was determined by ELISA. (*B*) NCI-H292 cells were treated with the vehicle alone (*open bars*), HAT (200 nM; *solid bars*), or PAR-2 AP (300 μM; *striped bars*) for 2, 4, 8, or 24 h. Total RNA was then extracted, and quantitative real-time RT-PCR (TaqMan) analysis was used to determine the amounts of AR and β-actin mRNA. The results are expressed as the mean ± SD ($n = 3$). $^*P < 0.05$, $^{**}P < 0.01$, compared with vehicle-treated cells at the same time point, Dunnett's test.

on AR gene expression was next examined. As shown in Figure 6B, in HAT-treated cells, the AR mRNA level significantly increased 2.5 times greater than the basal level by 2 h after the onset of HAT treatment and returned to the basal level 24 h later. To confirm the involvement of PAR-2 in HAT-induced *MUC5AC* gene expression mediated by EGFR, the effect of PAR-2 AP was also examined. In agreement with the results from the evaluation of the effect of PAR-2 AP on *MUC5AC* gene expression, PAR-2 AP showed a significant effect on neither release of AR nor gene expression of AR at any time during 2–24 h following the beginning of treatment or at any concentration tested (10–300 μM). These results suggest that HAT stimulates gene expression, which results in subsequent production and release of AR protein, and activation of PAR-2 alone is neither sufficient nor directly involved in the regulation of AR. These observations indicate that HAT activates EGFR subsequent to *MUC5AC* gene expression through gene expression and release of AR.

Discussion

In the present study, the results indicate that HAT induces increased expression of the *MUC2* and *MUC5AC* genes in the airway epithelial cell line NCI-H292, which results in subsequent mucous glycoconjugate production. HAT significantly enhances *MUC5AC* gene expression in NCI-H292 cells at a concentration of 20 nM (equivalent to 32.4 mU/ml), and this concentration is similar to the HAT concentration observed in mucoid sputum from patients with either chronic bronchitis (23.46 ± 18.03 mU/ml) or bronchial asthma (46.96 ± 43.96 mU/ml; Ref. 31). Although HAT is originally translated as a precursor possessing a hydrophobic transmembrane domain near the *N*-terminus (2), most HAT molecules in the sputum from patients with chronic airway diseases exist as a mature form lacking the transmembrane domain (1). It is thought that the HAT precursor biosynthesized in airway epithelial cells is released by proteolytic cleavage, both into the extracellular spaces of the epithelial layer and into the airway lumen. Because neither secretory leukocyte protease inhibitor nor α1-antitrypsin, which are the main serine protease inhibitors in airway mucus, inhibit HAT at the levels found in airway mucus (1), the released mature form of HAT probably acts on target cells without loss of its trypsin-like activity. Indeed, results from a previous study indicate that the greatest trypsin-like enzyme activity found in mucoid sputum from patients with either chronic bronchitis or bronchial asthma was derived from HAT (32). From these observations, it is possible that HAT exists at a sufficient level to enhance mucin production in the mucoid sputum from patients with chronic airway diseases and is involved in airway mucus hypersecretion.

The next question addressed was that of the target of HAT activity. In a previous study, HAT was shown to activate PAR-2 in primary bronchial epithelial cells (6). Although the functional PAR-2 is expressed in NCI-H292 cells, PAR-2 AP could not induce either mucin production or mucin gene expression in these cells, during the present study (Figures 2 and 3). Results from control experiments show that this batch of PAR-2 AP was biologically active; it initiated intracellular Ca^{2+} mobilization in NCI-H292 cells (Figures 1B and 1D). In addition, tryptase, which is known to activate PAR-2 (5), did not show any effect on *MUC5AC* mRNA levels in NCI-H292 cells (Figure 4B). These results indicate that HAT activation of PAR-2 alone cannot account for stimulation of mucin production and mucin gene expression caused by HAT. Further investigation was needed to clarify the target protein of HAT.

To help determine the HAT target protein, involvement of EGFR on HAT-induced *MUC5AC* gene expression was examined in NCI-H292 cells, because EGFR has been shown to play an important role in induction of mucin gene expression or mucin production in NCI-H292 cells (25). Cigarette smoke extract (23), hydrogen peroxide (22), and elastase (21) stimulate *MUC5AC* protein production mediated by EGFR, and the effect of a *Pseudomonas aeruginosa* extract that stimulates transcriptional activation of the *MUC2* gene is also mediated by EGFR (24). Pharmacologic inhibition of EGFR almost completely disrupted the effect of HAT; furthermore, anti-AR neutralizing antibody and anti–TGF-α neutralizing antibody also inhibited the effect of HAT. These observations suggest that HAT-induced *MUC5AC* gene expression is mediated by both AR-EGFR and TGF-α−EGFR pathway. Moreover, results show that both anti-EGFR neutralizing antibody and anti-AR neutralizing antibody almost completely inhibited the enhancing effect of HAT; this suggests that HAT-induced activation of EGFR is completely dependent on AR. Because gene expression of TGF-α has been reported to increase following activation of EGFR (33), the results from this study showing that neutralizing antibody to TGF-α only partially inhibited the effect of HAT suggest that TGF-α

is induced following activation of EGFR by AR, and that it prolongs the effect of HAT. From these observations, AR seems to act as an initial activator of EGFR, whereas TGF-α seems to act a secondary activator in HAT-stimulated NCI-H292 cells. Finally, the mechanisms of HAT-induced release of AR were examined, and the results showed HAT-induced AR gene expression accompanied by enhanced release of AR protein. In NCI-H292 cells, two distinct mechanisms causing release of AR have been reported, including proteolytic cleavage of its transmembrane precursor by tumor necrosis factor-α–converting enzyme (34) and induction of gene expression subsequent to release of protein (33). Although the effect of HAT on proteolytic cleavage of transmembrane precursor of AR was not determined in this study, such mechanisms that cause an immediate activation of EGFR are probably not responsible for HAT-induced release of AR, because the time-course analysis results of the present study show that the HAT-mediated increase of *MUC5AC* mRNA levels occurs later than that of EGF (Figure 3A). A study to delineate in detail the mechanism by which HAT induces AR gene expression is now under way. Although results from the present study suggest that PAR-2 AP does not affect mucin production, it has been reported that PAR-2 AP stimulates secretion of mucin in the rat stomach (5, 35). This observation suggests that PAR-2 might regulate mucin secretion from, rather than mucin production by airway tissue, and that HAT might have a dual action in airway mucin regulation: increasing mucin production through the AR-EGFR pathway, and enhancing mucin secretion through a PAR-2–mediated pathway. Further studies are necessary to examine biochemical and morphologic changes in the airway tissue of HAT-treated animals and to clarify roles of HAT in the pathogenesis of chronic airway diseases such as chronic bronchitis and bronchial asthma.

In conclusion, the results of this study show that HAT regulates mucin gene expression in the NCI-H292 airway epithelial cell line, through an AR-EGFR pathway. In addition, results of previous studies suggest that HAT is involved in airway inflammation mediated by PAR-2. The results of the present study, together with those results, show that excess HAT activity might directly lead to the pathogenesis of chronic airway diseases, by controlling PAR-2 and AR-EGFR signaling pathways. Consequently, HAT inhibitors are possible new therapeutic agents to treat chronic airway diseases.

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