Role of Hyaluronan and Reactive Oxygen Species in Tissue Kallikrein-mediated Epidermal Growth Factor Receptor Activation in Human Airways*

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In human airways, oxidative stress-induced submucosal gland cell hypertrophy and hyperplasia, histological features of chronic bronchitis, have been linked to epidermal growth factor receptor (EGFR) activation. To explore mechanisms of oxidative stress-induced EGFR activation and signaling, primary cultures of human tracheal submucosal gland (SMG) cells were used to assess EGFR ligand release, EGFR phosphorylation, p44/42 MAPK phosphorylation, and mucin 5AC synthesis in response to reactive oxygen species generated by xanthine/xanthine oxidase (X/XO). Exposure to X/XO increased release of epidemial growth factor (EGF) from these cells, thereby activating EGFR, phosphorylating MAPK, and increasing mucin 5AC production. The importance of EGF was confirmed by transfection of small interfering RNA inhibiting pro-EGF production, which resulted in inhibition of EGFR and MAPK phosphorylation despite X/XO exposure. Blocking signaling by using specific protease inhibitors showed that tissue kallikrein (TK) processed pro-EGF in response to X/XO. Airway TK is bound and inactivated by luminal hyaluronan (HA), and treatment of submucosal gland cells with X/XO induced HA depolymerization and TK activation. These events were blocked by reactive oxygen species scavengers and addition of exogenous excess HA and TK inhibitors. Thus, HA plays a crucial role in regulating airway TK activity and thereby TK-mediated release of active EGF from human SMG cells. Sustained HA depolymerization is expected to cause TK activation, EGF release, and EGFR signaling and to lead to SMG cell hypertrophy and hyperplasia as well as mucus hypersecretion with subsequent airflow obstruction.

Mucus hypersecretion and characteristic changes in epithelial cell morphology, including goblet cell hyperplasia in major airways and metaplasia in small airways as well as submucosal gland hypertrophy, are pathophysiological and histological hallmarks of chronic bronchitis (1, 2). Recent key observations have suggested that activation of the epidermal growth factor (EGF) receptor (EGFR) signaling cascade is responsible for at least some of the morphological changes in the airway epithelium (3–5, 47). Cigarette smoke, the major cause of chronic bronchitis in humans, has also been shown to induce mucin secretion and mucous cell hyperplasia, at least in part, via EGFR-mediated signaling (6). Because oxidative stress causes EGFR activation (7) and cigarette smoke is a major source of direct and indirect oxidative stress (8, 9), it is not surprising that many of the effects of cigarette smoke on mucin secretion and mucous cell hyperplasia can be blocked by anti-oxidants (6). However, the mechanisms by which oxidative stress stimulates EGFR signaling are not fully understood.

The classic EGFR, c-ErbB1, is activated by different ligands including EGF, transforming growth factor-α (TGF-α), heparin binding EGF-like growth factor (HB-EGF), amphiregulin, epiregulin, and betacellulin (10–14). EGFR ligand precursors are synthesized as transmembrane proteins that are cleaved to their active form by extracellular proteases (15). Whereas TGF-α, HB-EGF, amphiregulin, epiregulin, and betacellulin are processed by metalloproteases of a disintegrin and metalloprotease (ADAM) family (e.g. Ref. 16), pro-EGF is processed by a serine protease. This serine protease has been identified to be tissue kallikrein (TK) in salivary and mammary glands, and its activity has been found to be consistent with TK in the kidney (17–19). Although TK is present in the airway (20, 21), its potentially crucial role in EGFR precursor activation and EGFR-mediated signaling has not been examined. Bronchial TK is in fact uniquely regulated in the airway. Studies from this laboratory have shown that TK is secreted with hyaluronan (HA) from submucosal glands, which inhibits its catalytic activity (21). We have also shown that HA immobilizes inactive bronchial TK at the epithelial surface, creating a pool of readily available yet inactive TK on the airway surface (22) and that HA degradation results in a dramatic increase in TK activity (21). Such a TK/HA pool also exists within the lumen of submucosal gland

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1 The abbreviations used are: EGF, epidermal growth factor; SMG, submucosal gland; EGFR, epidermal growth factor receptor; ROS, reactive oxygen species; X/XO, xanthine/xanthine oxidase; TK, tissue kallikrein; MUC, mucin; siRNA, small interfering RNA; HA, hyaluronan; TGF-α, transforming growth factor-α; HB-EGF, heparin-binding EGF-like growth factor; ADAM, a disintegrin and metalloprotease; SBTI, soybean trypsin inhibitor; FMSF, phenylmethylsulfonyl fluoride; DMEM, Dulbecco’s modified Eagle’s medium; BSA, bovine serum albumin; TBS, Tris-buffered saline; FACE, fluorophore-assisted carbohydrate electrophoresis; AMAC, 2-aminoacridone; PI, peptide inhibitor; MAPK, mitogen-activated protein kinase; b-HABP, biotinylated HA-binding protein; PBS, phosphate-buffered saline; SOD, superoxide dismutase; ELISA, enzyme-linked immunosorbent assay; rTK, recombinant TK; pEGFR, phosphorylated EGFR.

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PRODUCTIVE PROCEDURES

Materials

All materials were purchased from Sigma unless otherwise specified.

Primary Cultures of Human Submucosal Gland Cells

Human tracheas and main bronchi from donor lungs rejected for transplantation were obtained through the University of Miami Life Alliance Organ Recovery Agency with approval from the local Institutional Review Board. The trachea and main bronchi were opened at the membranous portion, and the mucosa was dissected off the cartilage. Main bronchus strips were digested with 0.5% collagenase (type 1A, Difco)’s modified Eagle’s medium (DMEM; Invitrogen) and incubated overnight at 4 °C for 24 h to release ciliated epithelial cells (which were used for different experiments after shaking them off the strips). To release gland cells, the remaining tissue was incubated for another 24 h in 0.1% dispase in DMEM supplemented with penicillin (100 units/ml), streptomycin (0.25 µg/ml), amphotericin B (2.5 µg/ml), and gentamicin (50 µg/ml) (referred to as DMEM-AB). Each strip was scraped after digestion to release the remaining gland cells. Cells were washed once in DMEM-AB, resuspended in DMEM-AB containing 0.25% trypsin-EDTA, and trituated to dissociate the remaining “clumps” of gland acini. Digestion was stopped with soybean trypsin inhibitor (SBTI, 500 µg/ml), and glands were digested with 500 µg/ml aprotinin, pH 7.8 (lysis buffer), for 30 min at 37 °C. Supernatants and cell lysates were frozen at 4 °C in a 96-well plate. Then plates were washed three times with 20 mM of bicarbonate/carbonate buffer, pH 9.2, at 4 °C in a 96-well plate. Then plates were washed three times with 20 mM Tris, 500 mM NaCl, 50 mM HEPES, 1% Triton X-100, 50 mM NaF, 1 mM sodium orthovanadate, 5 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, pH 7.8 (lysis buffer), for 30 min at 4 °C. To remove insoluble material, cell lysates were centrifuged at 14,000 rpm for 5 min at 4 °C. Supernatants and cell lysates were frozen at −20 °C for later analysis.

In experiments designed to test the effects of reactive oxygen species (ROS) on EGF ligand processing and signaling, SMG cells were exposed to XNO (0.6 µm xanthine plus 10 µg/ml aminos) for 30 min in the absence or presence of the following: the ROS scavengers catalase (150 units/ml) or superoxide dismutase (SOD, 150 units/ml); sodium orthovanadate, 5 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, pH 7.8 (lysis buffer), for 30 min at 4 °C. To remove poorly soluble material, cell lysates were centrifuged at 14,000 rpm for 5 min at 4 °C. Supernatants and cell lysates were frozen at −20 °C for later analysis.

rTK was produced in Pichia pastoris as described (31). Briefly, P. pastoris (strain GS115) was transfected with a plasmid encoding rTK (pPIC9pro-TK) generously donated by Dr. Hedy Chan (Axsys Pharmaceuticals) using a Pichia Expression kit (Invitrogen) according to the manufacturer’s instructions. The plasmid contains the full coding sequence of human salivary pro-TK behind the alcohol dehydrogenase promoter and a signal sequence to allow induction of synthesis and secretion into the media. This pro-form is unstable and is converted to active kallikrein by autocalculation in the culture media. Cells were removed by centrifugation, and culture medium was dialyzed against 50 mM imidazole, pH 7.0. TK was purified using a benzamidine-Sepharose column (5 ml, Amersham Biotech) equilibrated in 50 mM imidazole, pH 7.0. Recombinant TK was eluted with a guanidine gradient (50–500 mM in 50 mM imidazole, pH 7.0) by using a Duo Flow high pressure liquid chromatography system (Bio-Rad). Purity of the protein was confirmed by PAGE and by specific activity. Purified fractions were dialyzed against sodium acetate, pH 7.0, and stored at −20 °C. The system yielded about 30 mg of rTK per liter of culture media.

TK Enzyme Activity

TK enzyme activity was determined using L-Val-Leu-Arg-p-nitroanilide as a substrate as described previously (21). In the presence of SBTI, this assay is highly specific for TK (29). Briefly, samples (100 µl) were incubated in an ultra low binding 96-well plate with 10 µl of trypsin (5 µg/ml) for 15 min at 37 °C to activate any pro-kallikrein. After adding 40 µl of SBTI (1 mg/ml) and 100 µl of rV-Val-Leu-Arg-p-nitroanilide (2 mM, ICN, Irvine, CA) in 200 mM Tris, pH 8.2, absorbance was monitored at 412 nm using a microplate reader (Molecular Devices, Sunnyvale, CA). Enzyme concentrations were calculated by interpolating activities from the rTK standard curve.

Quantitative Analysis of EGFF Lignoids

The apical media of cultured SMG cells were analyzed for the following soluble EGFF ligands: EGF, TGF-α, HB-EGF, amphiregulin, and betacellulin. EGF and TGF-α were measured using commercially available ELISA kits according to the manufacturer’s guidelines (Quantikine kit, R & D Systems, Minneapolis, MN, and Calbiochem, respectively). ELISAs were also used to measure HB-EGF, amphiregulin, and betacellulin. Briefly, 50 µl of sample (supernatant or standard) was incubated overnight with 50 µl of bicarbonate/carbonate buffer, pH 9.2, at 4 °C in a 96-well plate. Then plates were washed three times with 20 mM Tris, 500 mM NaCl, 0.05% Tween 20, pH 7.5 (TTBS), and blocked with 1% bovine serum albumin (BSA) in TTBS for 6 h at room temper-
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After washing, plates were incubated with 100 μl of monoclonal anti-HB-EGF antibody (R & D Systems, 10 μg/ml diluted into TTBS), goat polyclonal anti-α-macroglobulin antibody (R & D Systems, 5 μg/ml), or mouse monoclonal anti–beta-actin antibody (4 μg/ml). After 2 h, the wells were washed, and 100 μl of goat anti-mouse IgG (0.2 μg/ml) or mouse anti-goat IgG (0.5 μg/ml), both conjugated to alkaline phosphatase (Kirkegaard & Perry Laboratories, Gaithersburg, MD), were dispensed into each well and incubated for 2 h. Color was developed with p-nitrophenyl phosphate and stopped with 3 N NaOH. Absorbance was read at 410 nm. EGFR ligand concentrations were determined by interpolation from the standard curves.

Quantitative Analysis of HA
HA content of the supernatants was estimated using a biotinylated HA-binding protein (b-HABP) assay as described by Bray et al. (32). Briefly, the wells of a microtiter plate were coated overnight with 100 μg/ml of HA isolated from bovine vitreous humor (Worthington). Supernatants or HA standards (100 μl) were pre-incubated for 1 h at 37 °C with 16 μl of b-HABP (Seikagaku, 15 ng/ml) and 84 μl of PBS containing 2% BSA and 0.2% Tween 20. The pre-incubated supernatants/standards were added to the wells and incubated for 90 min at 37 °C. After washing with PBS, the wells were incubated with streptavidin coupled to alkaline phosphatase (1 μg/ml, Bio-Rad) for 2 h at room temperature. Color was developed with p-nitrophenyl phosphate and stopped with 3 N NaOH. Absorbance was read at 410 nm. Concentrations were determined by interpolation from a standard curve.

HA/TK Complex Disruption by X/XO
HA/TK complexes were generated in vitro by incubating HA (average molecular size 200 kDa, 40 μg/ml) with rTK (10 μg/ml) in 1 ml of PBS for 3 h at 37 °C. Complex formation was confirmed by determining inhibition of TK activity (resulting in 50% of baseline activity under these conditions) and by electrophoresis using an overlay membrane method to visualize enzyme activity and molecular distribution as described (21). Exposure to X/XO (0.6 mM xanthine plus 0.05 units of xanthine oxidase) resulted in 50% of baseline activity under these conditions. HA complexes were disrupted with 2% SDS and 0.5 M guanidine hydrochloride. HA/TK complexes were separated on 4–20% gradient sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) for 2 h at 150 V, fixed in 10% acetic acid, and transferred to a Hybond-C membrane (Amersham). The membranes were incubated with human monoclonal anti–HA antibodies (Prozyme, San Leandro, CA) at 1 μg/ml overnight at room temperature. The membranes were washed with 200 volumes of TTBS, blocked for 1 h at room temperature, and incubated with monoclonal anti–α-macroglobulin antibody (1 μg/ml, Kirkegaard & Perry Laboratories) at 15°C overnight. The membranes were washed with TTBS, blocked, and incubated with 100 μl of biotinylated HA fragments (1 μg/ml, Seikagaku) and 84 μl of PBS containing 2% BSA and 0.2% Tween 20. The pre-incubated membrane was washed with TTBS, and 100 μl of goat anti-mouse IgG conjugated to alkaline phosphatase (0.2 μg/ml, Kirkegaard & Perry Laboratories) was dispensed into each well. After 1 additional hour, plates were washed with TTBS, blocked, and incubated with 100 μl of goat anti-mouse IgG conjugated to alkaline phosphatase (0.2 μg/ml, Kirkegaard & Perry Laboratories) that was visualized using bromochloroindolyl phosphate/nitro blue tetrazolium as a blue precipitate. Developed blots were scanned, and the EGF to TK ratio was quantified using Quantity One analysis software (Bio-Rad).

Immunoblotting for p44/42 MAPK
Aliquots of cell lysates containing equal amounts of protein were suspended in Laemmli sample buffer and boiled for 5 min. Proteins were separated on 10–15% Tris-HCl Ready gels (Bio-Rad) and transferred to membranes as described above. The membranes were incubated with 1% gelatin in TBS containing 0.05% Tween 20 for 1 h followed by mouse monoclonal anti-phosphotyrosine antibody (PY99, 2 μg/ml; Santa Cruz Biotechnology) or anti-EGFR antibody. Secondary antibody was a mouse anti-goat IgG conjugated to alkaline phosphatase (0.5 μg/ml, Kirkegaard & Perry Laboratories) that was visualized using bromochloroindolyl phosphate/nitro blue tetrazolium as a blue precipitate. Developed blots were scanned, and the p44/42 MAPK to p44/42 MAPK ratio was quantified using Quantity One analysis software (Bio-Rad).

Immunoblot assay for MUC 5AC Protein
Cell lysates (50 μl) were incubated with bicarbonate-carbonate buffer, pH 9.2 (50 μl), for 1 h at 37 °C in a 96-well plate (Dynex, Chantilly, VA) or until dry. Plates were washed three times with PBS and blocked with 2% BSA for 1 h at room temperature. Plates were again washed three times with PBS and then incubated with 50 μl of monoclonal anti-MUC 5AC antibody (10 μg/ml, Chemicon International) diluted into PBS containing 0.05% Tween 20. After 1 h, wells were washed with PBS, and 1 μl of goat anti-mouse IgG conjugated to alkaline phosphatase (0.2 μg/ml, Kirkegaard & Perry Laboratories) was dispensed into each well. After 1 additional hour, plates were washed with PBS. Color was developed with p-nitrophenyl phosphate and stopped with 3 N NaOH. Absorbance was read at 410 nm. Results were expressed as % changes in A410 above the PBS control. This was necessary as pure MUC 5AC was not available to calibrate the assays.

Inhibition of Pro-EGF mRNA Expression
Double-stranded siRNA for the DNA target sequence of human pro-EGF (5’-AATCCTTATGGAGGATGCAGC-3’) and control, non-silencing RNA (5’-AATTCTCCGAACGTTCCACCT-3’) were bought from Qiagen-Xeragon (Germantown, MD) and transfected into human SMG cells with TransMessengerTM Transfection Reagent (Qiagen) according to the manufacturer’s instructions. Two days after transfection, the cells were exposed to X/XO in the absence or presence of catalase or PBS for 30 min. Cell lysates were analyzed for EGFR and p44/42 MAPK activation as described above.

To confirm that the siRNA silenced pro-EGF expression, experiments using siRNA and control RNA were repeated on SMG cells grown on coverslips. EGFR protein expression was assayed by immunofluorescence as described above. Sample volume was insufficient for determination of EGF by ELISA.

Statistical Analysis
Data were expressed as mean ± S.E. Differences between multiple groups were compared using a one-way analysis of variance followed by the Tukey Kramer honestly significant difference test. Levene test was used to analyze the homogeneity of variances. Significance was accepted at p < 0.05.

RESULTS
X/XO Induces EGFR Activation and Signaling in Human Airway SMG Cells—To test if primary cultures of human SMG respond to oxidative stress as described for cell lines of epithelial origin (35–37), cultures were treated with X/XO and assessed for activation of the EGFR signaling cascade. X/XO treatment of SMG cell cultures (n = 12 cultures obtained from four different donors) induced EGFR activation as measured by EGFR phosphorylation and induced EGFR signaling as deter-
minded by MAPK phosphorylation (Fig. 1, a–d). The ROS scavengers, catalase and SOD, reduced X/XO-induced EGFR and p44/42 MAPK phosphorylation (Fig. 1, a–d). Functionally blocking anti-EGFR antibodies reduced EGFR phosphorylation and to a lesser degree p44/42 MAPK phosphorylation, possibly suggesting an alternative pathway of activating p44/42 MAPK. Although MUC5 AC amounts inside SMG cells did not change over this 30-min incubation time, exposure to X/XO for 18 h induced MUC 5AC significantly compared with PBS control (16.4 ± 3.1% above PBS; p < 0.05; not shown).

**EGF Is the Ligand Responsible for X/XO-induced EGFR Activation**—Because all EGFR ligands are synthesized as transmembrane precursors that need to be proteolytically cleaved to release active ligands, protease inhibitors were used to assess which proteases were involved in the activation of EGFR. The metalloprotease inhibitor GM6001 was used to inhibit the members of the ADAM family responsible for the activation of HB-EGF, TGF-α, amphiregulin, and betacellulin, whereas the serine protease inhibitors aprotinin and SBTI were used to inhibit the activation of EGF. In addition, HA was used, as it is the endogenous inhibitor of TK, an important serine protease in the airway. As seen in Fig. 1, a–d, aprotinin and HA but not SBTI or GM6001 inhibited EGFR and MAPK phosphorylation. Because aprotinin but not SBTI reduced activation, this inhibitory profile was consistent with TK being the enzyme responsible for the processing of the ligand that activates EGFR. Also, it suggests that EGF is the produced ligand because other ligands are processed by metalloproteases of the ADAM family that are sensitive to GM6001.

To confirm these findings, TK and EGF were measured in SMG cell culture media after X/XO treatment as described under “Experimental Procedures” (n = 12, from four different donors). Compared with PBS control, X/XO increased the concentration of active TK (4.3 ± 0.2 μg/mg protein versus 1.2 ± 0.4 μg/mg; p < 0.05) and of soluble EGF (4.4 ± 0.4 pg/mg protein versus 2.2 ± 0.4 μg/mg; p < 0.05). Catalase and SOD as well as aprotinin and an excess of HA brought TK activity and soluble EGF concentration back to base-line levels (Fig. 2). In contrast, X/XO did not modify the levels of TGF-α (10.4 ± 3.2 pg/mg cell lysate protein versus 11.4 ± 3.9 pg/mg; p > 0.05) or HB-EGF (1.6 ± 0.2 pg/mg cell lysate protein versus 1.8 ± 0.1 pg/mg; p > 0.05) in SMG cell culture supernatants. Amphiregulin and betacellulin were undetectable by our assays (<50 pg/ml and <1 ng/ml, respectively) in the cell supernatants under all experimental conditions. To confirm that the X/XO effect on pro-EGF processing was due to increased endogenous TK activity, a specific peptide inhibitor of TK was used. This peptide inhibitor (PI) blocked the X/XO-induced EGF release significantly (2.7 ± 0.1 pg/ml for PBS; 4.1 ± 0.2 pg/ml for X/XO, and 2.9 ± 0.3 pg/ml for PI, n = 4 obtained from two different lung donors, p < 0.05) and inhibited X/XO-induced EGFR and MAPK activation (Fig. 3).
X/XO Induces HA Depolymerization and TK Activation in Human SMG Cells in Vitro—Because bronchial TK is secreted by serous cells of airway submucosal glands together with HA, and because HA binds to and inhibits the bronchial enzyme activity of TK (21), TK-HA complexes generated in vitro, as described under “Experimental Procedures,” were exposed to X/XO to disrupt HA-TK association (by causing HA depolymerization), thereby releasing active TK. After 3 h of incubation with HA, TK showed a 50% inhibition of enzyme activity. X/XO exposure for 30 min restored TK activity to 100% (Fig. 4), whereas PBS did not (not shown). Additional experiments showed that X/XO alone did not affect purified rTK activity (16.8 ± 1.2 to 17.1 ± 2.7 milliunits/mg protein) or TK activity in cell supernatants previously treated with hyaluronidase (0.4 ± 0.03 (supernatants) to 1.9 ± 0.82 (+hyaluronidase) and 2.1 ± 0.3 (+hyaluronidase followed by X/XO) milliunits/mg protein.

ROS generated by X/XO during a 30-min exposure of the apical side of SMG cultures increased HA concentrations in apical cell supernatants when compared with PBS control (1.87 ± 0.31 µg/mg cell lysate protein versus 0.27 ± 0.05 µg/mg; p < 0.05), consistent with HA breakdown and release from the cell surface (Fig. 5a). Concurrent treatment with catalase prevented HA release (0.78 ± 0.05 µg/mg; p > 0.05 compared with control and p < 0.05 compared with X/XO). Analysis of HA molecular weight distribution in these samples showed that X/XO decreased the average molecular size from ~1,800 to ~300 kDa (increased mobility and decrease in HA size heterogeneity, Fig. 5b) and caused HA fragments to appear as seen in FACE (Fig. 5c). Together, these results suggested that X/XO induced HA degradation and caused active TK release from HA-TK complexes generated in vitro and in vivo on human tracheal SMG cells in culture.

EGF Is Present in Airway Submucosal Glands of Human Tracheal Tissue Sections—To confirm EGF protein expression by SMG (38), immunofluorescence of human tracheal sections was performed as described under “Experimental Procedures.” Immunostaining of tracheal sections showed that SMG cells synthesize proteins with an EGF domain both at their apical and basolateral membranes (Fig. 6).

Enzyme with the Biochemical Characteristics of TK Processes Pro-EGF in Human SMG Cells under Resting Conditions—To define the protease responsible for pro-EGF activa-
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Fig. 4. ROS induces TK activation from TK-HA complexes. HA inhibited TK activity to 50%, while a 30-min exposure to X/XO reverted the inhibition of TK by HA (n = 4). * indicates p < 0.001 TK versus TK-HA; #, p < 0.001 TK-HA versus TK-HA + X/XO.

Fig. 5. ROS induced depolymerization and release of HA. Apical compartments of human SMG cells cultures (n = 6 from three different lung donors) were exposed for 30 min to PBS or X/XO alone or with catalase. Soluble HA was measured and normalized to the simultaneously recovered cell lysate protein (a). * indicates p < 0.01 compared with PBS (control); ** indicates p < 0.01 compared with X/XO. Agarose electrophoresis followed by transblotting using a b-HABP and FACE analysis (n = 4 from two different lung donors) shows that X/XO exposure induced a decrease in HA average molecular weight (b) and appearance of HA fragments (c) in culture media, y axis in b shows standards in kDa, as estimated using DNA standards, and in c are AMAC-derivatized disaccharides.

FIG.5

Exposure induced a decrease in HA average molecular weight (b) and calibration of HA fragments (c) in culture media. FACE analysis (Agarose electrophoresis followed by transblotting using a b-HABP, aprotinin (50 µg/ml) and GM6001 (4 µM) were added to the culture media. p indicates p < 0.05 versus control (Fig. 7).

None of the inhibitors modified low base-line release of HB-EGF by SMG cells (1.5 ± 0.1 pg/mg cell lysate protein). SMG cells also released TGF-α (70.4 ± 7.9 pg/mg cell lysate protein). The TGF-α release was significantly decreased by GM6001 (to 45.7 ± 9.4 pg/mg, p < 0.05 versus control) but not by SBTI (61.4 ± 10.2 pg/mg, p > 0.05 versus control). Surprisingly, aprotinin also decreased TGF-α release (to 25.4 ± 6.5 pg/mg, p < 0.05 versus control) suggesting that, under unstimulated conditions, TK plays a role in pro-TGF-α processing. Amphiregulin and betacellulin remain undetectable in our assays under baseline conditions. The sensitivity of our assay was borderline in detecting betacellulin at the EC50 levels (1.4 nM, see Ref. 39); however, a significant role of betacellulin in EGF release after stimulation with X/XO was excluded by the inefficiency of GM6001 to block EGF release and signaling. Because the affinity of EGF for amphiregulin is lower (1 nM) than for EGF or TGF-α (1.2 nM) (11), it is unlikely that amphiregulin is involved in EGF release under these conditions. In addition, its lower affinity, amphiregulin cannot dimerize EGFR unless other accessory molecules (such as heparin) are present to stabilize its functional association with EGFR (11).

In summary, these data support the hypothesis that a TK-like enzyme is the endogenous activator of pro-EGF in human SMG cells under resting conditions, similar to the findings in the kidney as well as salivary and mammary glands (17–19).

Recombinant TK Cleave Pro-EGF, Releases EGF, and Activates EGFR in Human SMG Cells

To confirm that TK cleaves pro-EGF from SMG and to test that the release of mature EGF activates EGFR signaling, SMG cells were incubated for 18 h with PBS (control) or rTK in the absence or presence of protease inhibitors (aprotinin, SBTI, or GM6001). HA, or functionally blocking anti-EGF antibodies (R-Ab) (each n = 12, cells from four different donors). Whereas EGF was quantified in apical culture supernatants, cell lysates were probed for tyrosine phosphorylation of EGFR and p44/42 MAPK. Cell lysates were also used for MUC 5AC quantification. rTK increased EGF release from SMG cells compared with baseline (18.5 ± 1.0 pg/mg cell lysate protein versus 10.5 ± 1.2 pg/mg; p < 0.05; Fig. 8). EGF release induced by rTK was blocked by aprotinin (3.1 ± 0.9 pg/mg; p < 0.05 compared with rTK and PBS) and HA (7.0 ± 1.3 pg/mg; p < 0.05 compared with rTK and PBS), whereas SBTI and GM6001 had no effect (16.6 ± 2.6 and 19.7 ± 2.7 pg/mg, respectively; both p > 0.05 compared with rTK). In addition, incubation with functionally blocking antibodies increased EGF levels in the incubation media (31.7 ± 4.5 pg/mg), probably due to inhibition of EGF binding to its receptor by the blocking antibody.

In parallel to the EGF increase in culture media, EGFR as well as p44/42 MAPKs were phosphorylated upon exposure of SMG cells to rTK. Aprotinin as well HA reduced EGF phosphorylation, whereas SBTI and GM6001 did not (Fig. 9). A similar profile of stimulation and inhibition was evident in downstream signaling as evidenced by p44/42 MAPK phosphorylation (Fig. 9) and MUC 5AC protein expression (Fig. 10).
MUC 5AC protein expression in cell lysates increased by 20.3 ± 3.0% above base line in response to rTK (p < 0.05; Fig. 10). The rTK-induced increase in intracellular MUC 5AC was inhibited by aprotinin, HA, and functionally blocking anti-EGFR antibodies (decreased by 4.0 ± 4.0%, 1.7 ± 6.5%, and 6.2 ± 3.9% below base line, respectively; all p < 0.05 compared with rTK stimulation and all p > 0.05 compared with base line) but not by SBTI or GM6001 (22.0 ± 4.9% and 11.2 ± 3.2 above base line, respectively; all p < 0.05 compared with base line). These results confirm that TK induces EGF release from SMG cells and that this release results in EGFR activation and downstream signaling.

Inhibition of Pro-EGF mRNA Expression Is Associated with a Decrease of EGFR and MAPK Phosphorylation in Response to Oxidative Stress—To confirm that activation of EGFR in response to ROS was due to endogenous processing of pro-EGF by TK, we inhibited pro-EGF mRNA translation using siRNA. RNA with 16/21-base overlap with a Thermotoga maritime gene and no known homology to any other mRNA in human sequence data bases was used as a non-silencing RNA control. As shown in Fig. 11, transfection of human SMG cells with pro-EGF siRNA blocked EGF protein expression in these cells as measured by immunofluorescence. In contrast to the non-silencing RNA transfection and PBS control, XOXO treatment did not result in EGFR phosphorylation and MAPK activation (Fig. 12) in cells transfected with siRNA. These data confirm that EGF and not other EGFR ligands are responsible for EGFR activation and signaling in response to oxidative stress in human SMG, and support the hypothesis that ROS-induced HA depolymerization and TK activation are responsible for initiation of EGFR signaling in these cells.

DISCUSSION

The data presented here support the hypothesis that oxidative stress-induced EGFR activation of human airway submucosal gland cells is due, at least in part, to a cascade of events initiated by depolymerization of HA, activation of TK, and release of mature EGF. The data show the importance of intact HA and its inhibition of TK in the airway lumen. They also show that TK processing of pro-EGF plays a role in regulating the EGF signaling pathway in airway submucosal glands and provides a possible direct mechanistic link between sustained airway ROS production during inflammation and the development of gland hyperplasia in the airways.
HA/TK interaction, previously demonstrated in sheep and human airways (21, 22), is shown here to be functionally relevant in regulating EGF release from the cell membrane with subsequent EGFR activation in these cells. The integrity of HA is sensitive to cleavage by ROS (23), and the tracheobronchial tree is exposed to increased levels of ROS either by inhalation of exogenous sources (e.g., tobacco smoke) or by stimulation of endogenous production by airway epithelial and inflammatory cells (40). ROS generated by X/XO increased active TK availability by HA degradation, thereby leading to EGF release and EGFR signaling. The fact that EGF up-regulates HA synthase 2 expression (41) suggests that negative feedback might exist to control pro-EGF activation (by controlling TK activity with increased HA), thereby inhibiting uncontrolled cell growth in airway submucosal glands leading to gland cell hypertrophy and hyperplasia. Chronic HA depolymerization by oxidants might disrupt this control mechanism.

FIG. 7. Human SMG cells release TK and EGF constitutively. Human SMG cells (n = 12 cultures from four different donors) were incubated in RPMI media in the basolateral compartment and with PBS alone or PBS containing either aprotinin, SBTI, or GM6001 in the apical compartment. Supernatants were analyzed for TK activity (top; μg/mg cell lysate protein) and EGF levels (bottom; pg/mg cell lysate protein). TK activity levels correlated with EGF release (r² = 0.91). TK was inhibited by aprotinin but not SBTI or GM6001. * indicates p < 0.05 compared with PBS control.

FIG. 8. Recombinant TK increases EGF release from SMG cells. Human SMG cultures were exposed for 18 h to PBS or rTK. rTK in the presence of aprotinin (Apr), SBTI, GM6001, HA, or functionally blocking anti-EGFR antibody (RAb) (n = 12, four lungs). EGF was measured and expressed as pg/mg cell lysate protein. * indicates p < 0.01 compared with PBS (control); ** indicates p < 0.01 compared with rTK, and *** indicates p < 0.01 compared with both PBS and rTK.

FIG. 9. Treatment with recombinant TK induced EGFR and MAPK activation in SMG cells. Human SMG cultures were exposed to PBS or rTK, in the absence or presence of aprotinin (Apr), SBTI, GM6001, or HA. rTK increased the pEGFR-EGFR ratio (a and b) and MAPK phosphorylation (c and d).

FIG. 10. MUC5 AC content is induced by rTK and specifically blocked by TK inhibitors. Human SMG cells were exposed to rTK for 18 h in the presence or absence of aprotinin, HA, SBTI, GM6001, or functionally blocking anti-EGFR monoclonal antibodies (RAb). MUC 5AC levels were expressed relative to PBS control. rTK increased MUC 5AC levels, and this effect could be blocked with aprotinin, HA, and functionally blocking anti-EGFR monoclonal antibodies (RAb), whereas SBTI or GM6001 had no effect. * indicates p < 0.05 compared with PBS; ** indicates p < 0.05 compared with both PBS and rTK.

EGF receptor tyrosine kinase activation (c-ErbB 1) can be achieved by multiple EGFR ligands as follows: EGF, TGF-α, HB-EGF, amphiregulin, epiregulin, and betacellulin, although affinities and specificities for the different Erb receptors vary between these ligands (14, 38, 39, 42). All of these ligands are synthesized as transmembrane precursors and are cleaved to
their active form by proteases. TK is responsible for release of mature EGF in mammary and salivary glands as well as the kidney (17–19) and, as shown here, in airway submucosal gland cells. Cleavage of all other EGFR ligands involves matrix metalloproteases of the ADAM family (16). ADAM17/TACE cleaves pro-TGF-β, amphiregulin, and HB-EGF (16), whereas ADAM9, ADAM10, and ADAM12 have been implicated in HB-EGF shedding (37, 43, 44). Oxidative stress is likely to increase the availability of a specific subset of these EGFR ligands depending on the anatomical location or cell type investigated. For instance, in rat gastric epithelial cells, oxidative stress has been shown to increase gene expression of the ligands HB-EGF and amphiregulin (10); on the other hand, only amphiregulin but not HB-EGF has been reported to be released in response to cigarette smoke in a human pulmonary muco-epidermoid carcinoma cell line (NCI-H292) via oxidative stress (5, 47). Our study suggests that EGFR activation in the airway, at least in experiments using primary cultures of human submucosal gland cells, is mediated by EGF, which is cleaved from its precursor by TK, an enzyme produced and secreted locally. In this system, we did not find measurable amounts of amphiregulin and betacellulin either at base line or upon ROS exposure, and the low levels of HB-EGF were not modified by ROS. The high concentrations of TGF-β1 under resting conditions (after 18 h of incubation) and the decreased levels after aprотinin treatment are very intriguing. It has been shown that EGFR activation by EGF can increase pro-TGF-β1 synthesis and cleavage by an unknown mechanism (45), possibly partially explaining these findings.

EGFR signaling can be specifically regulated at a cellular level either by the expression of a subset of specific ligand precursors or by the expression of a subset of proteases, specific for the cleavage of ligand precursors. These mechanisms of specificity make it possible that different stimuli induce cleavage of different ligand precursors from the same cells. In NCI-H292 cells for instance, lipoteichoic acid from Gram-positive bacteria activates ADAM10 to process HB-EGF precursors resulting in EGFR activation and increased mucin synthesis (37), whereas tobacco smoke extracts activate ADAM17 to cleave amphiregulin precursors, again resulting in EGFR signaling (5, 47). Thus, EGFR activation pathways cannot be predicted from immunohistochemical analysis alone nor can they be extrapolated from experiments done in other cell types. Here we show that ROS generated by X/XO stimulates EGFR activation in SMG cells via TK-mediated cleavage of EGF precursors. Although EGF was functionally important in SMG cells, TGF-β1 and HB-EGF were also expressed in vitro, consistent with a previous report (38) using immunohistochemistry. Despite their expression, these EGFR ligands did not appear to play a role in ROS-induced EGFR activation and signaling in SMG cells because the metalloprotease inhibitor GM6001 (effective in blocking the activity of all members of the ADAM family) did not modify X/XO-induced EGFR activation and because blocking pro-EGF mRNA expression with siRNA resulted in inhibition of EGFR and MAPK phosphorylation. In our experiments, however, we also found that functionally blocking EGFR antibodies did not completely inhibit MAPK phosphorylation, suggesting that an additional EGFR-independent signaling pathway in response to ROS may also be present in our cells.

We used MUC 5AC synthesis as the final end point of assessing the X/XO/TK-mediated EGF activation of EGFR signaling. Although MUC 5AC is not the major mucin produced in SMG cells, it is produced by the mucous cells in SMG (46, 48). We chose to study this mucin to compare our results using primary cells with the results reported by others using cell lines, and we showed that MUC 5AC content was also increased in SMG cells upon EGFR and MAPK activation. As expected for gene induction, the increase in MUC 5AC synthesis was observed after 18 h of rTK treatment of SMG cells and was not seen after only 30 min of stimulation. These data confirm other findings that EGFR signaling leads to MUC 5AC induction, although the stimulus used in our experiments was TK-induced EGF release as opposed to TGF-β1 (4) or ligand-independent EGFR activation induced by oxidative stress (7).
In conclusion, our results suggest that TK plays an important role in the airways by processing pro-EGF to mature EGF in airway submucosal gland cells. The activity of TK is regulated by HA; TK is inhibited in the presence of native HA in the airway and in the submucosal gland duct. However, upon depolymerization of HA by ROS, active TK is released to cleave EGF precursors expressed at the cell surface. The released mature EGF interacts with EGFR to initiate signaling, including MAPK kinase phosphorylation and increases of gene and protein expression of MUC 5AC. The novel recognition of the central role of HA in this signaling pathway could potentially be used for new therapeutic approaches in airway disease associated with hypersecretory states.

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REFERENCES
1. Trevisani, L., Sartori, S., Bevelenta, M. R., Mazzoni, M., Pazzi, P., Putinati, S., and Pataia, A. (1992) *Respiration* 59, 136–144
2. Spurr, J. R., Thompson, A. B., Daughton, D. M., Mueller, M., Linder, J., and Rennard, S. I. (1991) *Chest* 100, 389–393
3. Nadel, J. A. (2000) *Chest* 117, S262S–S266
4. Takeyama, K., Dabbagh, K., Lee, H. M., Agusti, C., Lauzier, J. A., Ueki, I. F., Grattan, K. M., and Nadel, J. A. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 3081–3086
5. Basbaum, C., Li, D., Gensch, E., Gallup, M., and Lemjabbar, H. (2002) *Novartis Found. Symp.* 248, 171–176
6. Takeyama, K., Jung, B., Shim, J. J., Burgel, P. R., Dao-Pick, T., Ueki, I. F., Proin, U., Kreuchel, P., and Nadel, J. A. (2001) *Am. J. Physiol.* 280, L165–L172
7. Takeyama, K., Dabbagh, K., Jeong Shim, J., Dao-Pick, T., Ueki, I. F., and Nadel, J. A. (2000) *J. Immunol.* 164, 1546–1552
8. Chow, C. K. (1998) *Ann. N. Y. Acad. Sci.* 886, 289–298
9. MacNee, W. (2001) *Novartis Found. Symp.* 234, 169–185
10. Miyazaki, Y., Shinomura, Y., Tsutsui, S., Yasunaga, Y., Zushi, S., Higashiyama, S., Taniguchi, N., and Matsuzawa, Y. (1996) *Biochem. Biophys. Res. Commun.* 226, 542–546
11. Neelam, B., Richter, A., Chamberlin, S. G., Puddicombe, S. M., Wood, L., Murray, M. B., Nandgopal, K., Niyogi, S. K., and Davies, D. E. (1998) *Biochemistry* 37, 4884–4891
12. Hisaka, T., Yano, H., Hara, T., and Kojo, M. (1999) *Int. J. Oncol.* 14, 453–460
13. Garach-Jehoshua, O., David, A., Liberman, U. A., and Koren, R. (1999) *Endocrinology* 140, 713–721
14. Strachan, L., Murison, J. G., Prestidge, R. L., Sleeman, M. A., Watson, J. D., and Kumble, K. D. (2001) *J. Biol. Chem.* 276, 18265–18271
15. Massague, J., and Pandiella, A. (1995) *Annu. Rev. Biochem.* 62, 515–541
16. Sunnarborg, S. W., Hinkle, C. L., Stevenson, M., Russell, W. E., Baska, C. S., Fishen, J. J., Castner, B. J., Gerhart, M. H., Paxton, R. J., Black, B. A., and Lee, D. C. (2002) *J. Biol. Chem.* 277, 12828–12845
17. Jorgensen, P. E., Nexo, E., Poulsen, S. S., Almendingen, M., and Berg, T. (1994) *Growth Factors* 11, 113–123
18. Jahnke, G. D., Chao, J., Walker, M. P., and Diasurgiune, R. P. (1994) *Endocrinology* 135, 2922–2929
19. Jorgensen, E., Nexo, E., and Poulsen, S. S. (1991) *Biochem. Biophys. Acta* 1074, 264–268
20. Proos, D., and Vio, C. P. (1993) *Am. J. Respir. Cell Mol. Biol.* 8, 16–19
21. Forteza, R., Lauredo, I., Abraham, W. M., and Conner, G. E. (1999) *Am. J. Physiol.* 21, 666–674
Tissue Kallikrein Activates EGF in Human Airways

22. Forteza, R., Lieb, T., Ask, T., Savani, R. C., Conner, G. E., and Salathe, M. (2001) *FASEB J.* **15**, 2179–2186

23. Agren, U. M., Tammi, R. H., and Tammi, M. I. (1997) *Free Radic. Biol. Med.* **23**, 996–1001

24. Christiansen, S. C., Proud, D., and Cochrane, C. G. (1987) *J. Clin. Investig.* **79**, 188–197

25. Zhang, M., Peng, B., Niehus, J., Baumgarten, C. R., Brunee, T., Thalhofer, S., Dorow, P., and Runkel, G. (1997) *Eur. Respir. J.* **10**, 1747–1753

26. Balazs, E. A., Watson, D., Duff, I. F., and Roseman, S. (1967) *Arthritis Rheum.* **10**, 357–376

27. Matsumura, G., Herp, A., and Pigman, W. (1966) *Radiat. Res.* **28**, 735–752

28. Halliwell, B., Hoult, J. R., and Blake, D. R. (1988) *FASEB J.* **2**, 2867–2873

29. Geiger, R., and Miska, W. (1988) *Methods Enzymol.* **163**, 102–115

30. Cagliari, C. I., De Caroli, F. P., Nakahata, A. M., Araujo, M. S., Sampaio, M. U., Sampaio, C. A., and Oliva, M. L. (2003) *Biochem. Biophys. Res. Commun.* **311**, 241–245

31. Chan, H., Springman, E. B., and Clark, J. M. (1998) *Protein Expression Purif.* **12**, 361–370

32. Bray, B. A., Hsu, W., and Turino, G. M. (1994) *Exp. Lung Res.* **20**, 317–330

33. Lee, H. G., and Cowman, M. K. (1994) *Anal. Biochem.* **219**, 278–287

34. Calabris, A., Hascall, V. C., and Midura, R. J. (2000) *Glycobiology* **10**, 283–293

35. Basbaum, C., Lemjabbar, H., Longphre, M., Li, D., Gensch, E., and McNamara, N. (1999) *Am. J. Respir. Crit. Care Med.* **160**, S44–48

36. Lemjabbar, H., Li, D., Gallup, M., Siddhu, S., Drori, E., and Basbaum, C. (2003) *J. Biol. Chem.* **278**, 26202–26207

37. Lemjabbar, H., and Basbaum, C. (2002) *Nat. Med.* **8**, 41–46

38. Polissa, R., Prosperini, G., Leir, S. H., Holgate, S. T., Luckie, P. M., and Davies, D. E. (1999) *Am. J. Respir. Cell Mol. Biol.* **20**, 914–923

39. Jones, J. T., Akita, R. W., and Sliwkowski, M. X. (1999) *FEBS Lett.* **447**, 227–231

40. Comhair, S. A., and Erruzum, S. C. (2002) *Am. J. Physiol.* **283**, L246–L255

41. Pienimaki, J. P., Rilla, K., Fulop, C., Sironen, R. K., Karvinen, S., Pasonen, S., Lammi, M. J., Tammi, R., Hascall, V. C., and Tammi, M. I. (2001) *J. Biol. Chem.* **276**, 20428–20435

42. Hunter, T., and Cooper, J. A. (1981) *Cell* **24**, 741–752

43. Asakura, M., Kitakaze, M., Takashima, S., Liao, Y., Ishikura, F., Yoshinaka, T., Ohmoto, H., Node, K., Yshino, K., Ishiguro, H., Asanuma, H., Sanada, S., Matsumura, Y., Takeda, H., Beppu, S., Tada, M., Hori, M., and Higashiyama, S. (2002) *Nat. Med.* **8**, 35–40

44. Izumi, Y., Hirata, M., Hasuwa, H., Iwanoto, R., Umata, T., Miyado, K., Tamai, Y., Kurisaki, T., Sehara-Fujisawa, A., Ohno, S., and Mekada, E. (1998) *EMBO J.* **17**, 7260–7272

45. Baselga, J., Mendelsohn, J., and Pandiella, A. (1996) *J. Biol. Chem.* **271**, 3279–3284

46. Davies, J. R., Herrmann, A., Russell, W., Svitacheva, N., Wickstrom, C., and Carlstedt, I. (2002) *Novartis Found. Symp.* **248**, 76–88

47. Basbaum, C., Li, D., Gensch, E., Gallup, M., and Lemjabbar, H. (2002) *Novartis Found. Symp.* **180**, 277–282

48. Davies, J. R., Herrmann, A., Russell, W., Svitacheva, N., Wickstrom, C., and Carlstedt, I. (2002) *Novartis Found. Symp.* **93**, 277–282
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