Acrolein-induced MUC5ac expression in rat airways

MICHAEL T. BORCHERS,1 SUSAN E. WERT,2 AND GEORGE D. LEIKAUF1,3,4
Departments of 1Environmental Health, 3Molecular and Cellular Physiology, and 4Medicine, University of Cincinnati, Cincinnati 45267; and 2Division of Pulmonary Biology, Children’s Hospital Medical Center, Cincinnati, Ohio 45229-3039

Acrolein-induced MUC5ac expression in rat airways. Am. J. Physiol. 274 (Lung Cell. Mol. Physiol. 18): L573–L581, 1998.—Acrolein, a low-molecular-weight aldehyde found in photochemical smog and tobacco smoke, can induce mucus hypersecretion, inflammation, and airway hyperreactivity. To determine whether changes in steady-state mucin gene expression (MUC2 and MUC5ac) are associated with histological signs of mucus hypersecretion, rats were exposed to acrolein (3.0 parts/million, 6 h/day, 5 days/wk, 2 wk), and the trachea with the main stem bronchi was separated from the intrapulmonary airways (lung). The temporal expression of MUC2 and MUC5ac mRNA was determined by RT-PCR, and acidic mucin glycoproteins were detected by Alcian blue histochemical analysis. MUC5ac protein content in the airways was determined by immunohistochemical analysis. Tracheal MUC5ac mRNA increased within 2 days and was accompanied by an increase in MUC5ac immunostaining on the surface of the airways and in submucosal gland epithelium. By comparison, increases in lung MUC5ac mRNA and mucin glycoproteins were delayed and were elevated after exposures on days 5 and 9, respectively. Increased MUC5ac immunostaining was detected within the lumen and airway epithelium of the lung on day 12. In contrast, MUC2 mRNA levels were not significantly changed in the trachea or lung. These findings indicate that acrolein-induced mucus hypersecretion is due, in part, to increases in MUC5ac rather than to MUC2 gene expression. These findings suggest that aldehyde-induced increases in MUC5ac may play a role in chronic mucus hypersecretion, a pathognomonic feature of chronic obstructive pulmonary disease.

Keywords: aldehydes; mucin; hypersecretion

THE SURFACE RESPIRATORY EPITHELIUM is exposed continuously to the environment and is a primary target of airborne pollutants and pathogens. Mucus protects the airways and is secreted by surface epithelial cells of the trachea, bronchi, and bronchioles and by tracheal and bronchial submucosal glands. Although mucus protects the structure and function of the airway epithelium when secreted in moderate amounts, mucus hypersecretion is a hallmark feature in the pathogenesis of chronic obstructive pulmonary diseases including chronic bronchitis, asthma, and cystic fibrosis (36). Recent estimates indicate that 13 million people suffer from chronic bronchitis and 12 million suffer from asthma in the United States (6). Patients suffering from chronic bronchitis and asthma show histological signs of airway inflammation, submucosal gland enlargement, and excessive mucus secretions. Mucous cells that are present on the surface epithelium become more frequent in the large airways (hyperplasia) and are found in the small airways where they are normally not present (metaplasia) (49). Mucus hypersecretion can also be induced experimentally by exposure to respiratory tract irritants including tobacco smoke (14), ozone (25), sulfur dioxide (SO2) (33), and acrolein (37).

Mucin glycoproteins provide airway secretions with their characteristic adhesiveness, viscosity, and elasticity, which allow them to trap materials and be cleared by the mucociliary apparatus. These glycoproteins consist of very large and heterogeneous mucins (200–20,000 kDa), with ≤80% of the molecular mass consisting of carbohydrate side chains O-linked to serine and threonine residues of the peptide backbone (48). Nine distinct human mucin genes have been identified in the respiratory, gastrointestinal, and reproductive tracts (MUC1–4, MUC5ac, MUC5b, and MUC6–8). In the lung, MUC1–MUC5ac mRNAs have been detected in the surface epithelium, with MUC4, MUC5ac, and MUC8 mRNAs present in the submucosal glands (4, 17, 45). Of these, MUC5ac is the only mucin gene product isolated from normal human airway secretions and is therefore proposed to be a major airway secretory mucin (44).

Acrolein is a low-molecular-weight, α,β-unsaturated aldehyde found in high concentrations (>4.0 parts/million (ppm)) in tobacco smoke and is a potent irritant component of photochemical smog (2, 5). Acrolein is absorbed primarily in the upper respiratory tract but has been demonstrated to induce responses characteristic of bronchitis, including epithelial damage, mucous cell metaplasia, mucus hypersecretion, and mucus plugging in the airways of animals exposed subchronically to ≤4.0 ppm (16, 19).

The mechanisms controlling mucous hypersecretion in response to inhaled irritants are likely attributable to conversion of nonsecretory cells to mucus-secreting phenotypes (mucous cell metaplasia) (33). Rats exposed to SO2, for example, demonstrate an increased number of mucous cells without an increase in mitotic rate, suggesting that hypersecretion can occur without hyperplasia. Similar observations have been reported in response to tobacco smoke (14) and ozone (25).

Limited in vivo and in vitro evidence suggest a mechanism mediating irritant-induced mucus hypersecretion that involves increased mucin steady-state mRNA. MUC2 mRNA is increased in virus-infected rat airways after SO2 exposure (29) and in a human pulmonary carcinoma cell line in response to tumor necrosis factor-α (TNF-α) (35). MUC5ac mRNA is increased in rat tracheal epithelial cell cultures in response to edermal growth factor and retinoic acid (RA) (22, 24). Currently, sequence data for murine homologues of secreted mucins are available only for MUC2 and MUC5ac (22, 40), and it is not known which, if either, is synthesized, stored, or secreted in vivo.
The purpose of this study was to determine the contribution of MUC2 and MUC5ac to the pathogenesis of irritant-induced mucus hypersecretion. This was investigated by exposing rats subchronically to selected concentrations of acrolein and measuring airway mucin steady-state mRNA levels. These findings were compared with histological examination using Alcian blue or MUC5ac antibody staining.

**METHODS**

Experimental design. To determine whether increases in mRNA levels and mucous cell metaplasia occurred within the lungs of rats exposed to acrolein, Sprague-Dawley rats (male, 250–300 g; Harlan) were exposed to 3.0 ppm of acrolein for 4 h/day, 5 days/wk, for up to 12 days. After the exposures, the animals were killed (n = 6/group), and the trachea and lung were removed. Mucous cell metaplasia was assessed by enumerating airway mucous cells, and airway mucin (MUC2 and MUC5ac) mRNA levels were measured by RT-PCR. To determine the effects of acrolein concentration on mRNA expression, rats were exposed daily to 0.3, 0.75, 1.5, or 3.0 ppm of acrolein (up to day 9), and steady-state levels of MUC2 and MUC5ac were measured. To determine whether increases in mRNA levels and mucous cell metaplasia were accompanied by an increase in MUC5ac protein, sections of lung tissue (3.0 ppm, day 12) or trachea (3.0 ppm, day 2) were stained with an MUC5ac antibody.

Acrolein exposure. The rats were exposed to acrolein as previously described (34). Acrolein vapor was generated by passing nitrogen gas (3–15 ml/min) over a 3-mL reservoir of liquid acrolein (J. Ansen Chimica, New Brunswick, NJ). This mixture was diluted with high-efficiency particle-filtered room air (400 ml/min) and introduced into a 0.32-m3 stainless steel chamber. Acrolein concentrations were determined in the chamber atmosphere from the 3 generations 11–16 correspond to airways 0.8 by 10.220.33.6 on October 19, 2017 http://ajplung.physiology.org/ Downloaded from

The purity was estimated by measuring the 260- to 280-nm absorption ratio with a spectrophotometer. Purified RNA was stored at −70°C until analysis by RT-PCR. For RT-PCR, primers were selected from published sequences of rat MUC5ac (22), rat MUC2 (40), and murine β-actin (50). Primer sequences were as follows: MUC5ac 5′- primer, 5′-GCC CTC CGG ACA GAA GCA GCC TTG-3′; MUC5ac 3′- primer, 5′-CAG GAG TTT CTC ATG ACC ACC ACC TTC CTA CTT-3′; MUC2 5′- primer, 5′-ACC CTC CTC CTA CCA CAT TG-3′; β-actin 5′- primer, 5′-CAG GAT GCC GTG AGG GAG AGC-3′; and β-actin 3′- primer, 5′-AAG GTG TGA TGG TGG GAA TGG-3′. MUC5ac and β-actin were reverse transcribed in a 10-µl mixture of 250 ng of total RNA with 25 units of Superscript II reverse transcriptase (GIBCO BRL, Life Technologies, Grand Island, NY) in buffer containing 10 mM diithiothreitol, 1 mM deoxynucleotide triphosphates (Promega, Madison, WI), 30 units of RNAse inhibitor, and 0.2 µM oligonucleotide primer. Rat MUC2 was reverse transcribed under the same conditions with a short-sequence-specific primer (5′-TGG TAG CAG G-3′) located downstream from the 3′ primer used in the amplification procedure (41). First-strand synthesis consisted of primer annealing (25°C, 10 min) and template extension (42°C, 45 min) (Biometra Thermocycler, Tampa, FL). Newly synthesized cDNA was amplified by PCR in 50 µl with 0.75 units of Taq polymerase (GIBCO BRL) in Taq buffer (GIBCO BRL) containing 1.5 mM MgCl2 and 0.2 µM 5′ primer. The amplification reaction for MUC2 also contained 0.2 µM 3′ primer. The amplification conditions were 20 s at 94°C, 30 s at the annealing temperature specific for each primer pair (5′-3′), and 30 s plus 1 s/cycle for n number of cycles (MUC5ac Ta5′ = 61.5°C, n = 32; MUC2 Ta5′ = 57°C, n = 32; β-actin Ta5′ = 58°C, n = 18). The specificity of the PCR products was confirmed by dye terminator sequencing with an ABI PRISM cycle-sequencing kit (Perkin-Elmer, Foster City, CA).

Quantitation of PCR products. PCR products were quantitated by densitometry measurement. DNA (10 µl) was electrophoresed on a 2% agarose gel containing 0.5 µg/ml of ethidium bromide in 90 mM Tris-phosphate-2 mM EGTA buffer. The DNA bands in the gel were illuminated with ultraviolet light and photographed (type 665 black and white film, Polaroid). Band images were scanned and analyzed by an image-processing computer.
analysis software program (Mocha, Jandel Scientific), and the total intensity (average intensity × total pixels) was measured. All messages examined amplified exponentially (until saturation) according to the amount of target mRNA in the sample. The relationship between mRNA level and band intensity was determined by a curve-fitting software program (SigmaPlot, Jandel Scientific) and found to be y = a(1 – exp(–bx)) + c, where a is the amplitude of the exponential, b is the rate constant, and c is the zero intercept. For each RT-PCR, a serial dilution (1,000–32 ng) of total rat lung mRNA was amplified and included on each gel to obtain an internally consistent reference curve. All samples were analyzed in the linear portion of the curve. The relative amount of mRNA was determined by comparing the total intensity of each sample against the standard curve. Mucin mRNA levels are expressed as multiples of increase over the control values after normalization to β-actin.

Immunohistochemistry. MUC5ac protein was detected in paraffin sections of the rat airways with a chicken polyclonal antibody to mouse gastric mucin (a generous gift from Dr. Samuel Ho) as previously described (28). This antibody recognizes the peptides sequence QTSPPN9GKTS1S1T coded by the MUC5ac mRNA found in gastric and lung tissues (46). MUC5ac antigen-antibody complexes were detected with the Vectastain ABC Peroxidase Elite Goat IgG Kit (Vector Laboratories, Burlingame, CA). The enzymatic reaction product was enhanced with nickel cobalt to give a black precipitate. Endogenous peroxidase was quenched (15 min, 22°C) with 3% 

\[
H_2O_2 \text{ in methanol. Sections were incubated in 2% normal goat}
\]

serum in PBS with 0.2% Triton X-100 (blocking solution; 2 h, 22°C) and were then incubated (20 h, 4°C) with primary antibody (1:15,000 dilution). The sections were washed (6 × 5 min) in PBS with 0.2% Triton X-100 and incubated (30 min, 22°C) with biotinylated goat anti-chicken antibody (1:2,000 dilution). Sections were treated (15 min, 22°C) with the avidin-biotin blocking kit (Vector Laboratories) to block endogenous biotin in the tissue. Sections were incubated with the avidin-biotin-peroxidase complex diluted in blocking solution (30 min, 22°C), nickel diaminobenzidine in 0.1 M acetate buffer (4 min, 22°C) for development of a colored reaction product, and Tris cobalt (4 min, 22°C) and counterstained with 0.1% nuclear fast red (2 min, 22°C). Positive control staining was detected in the rat stomach. Negative controls included 1) omission of the primary antibody, 2) incubation with chicken preimmune serum, and 3) staining of a pathogen-free adult mouse lung.

Data analysis. To assess changes in mucous cell number, two nonadjacent sections from each animal (n = 3/group) were scored and are presented as the means ± SE. Statistical analysis was performed with the Kruskal-Wallis one-way analysis of variance and Dunn’s method for comparison of groups. Differences from control values were considered significant at P < 0.05.

Mucin mRNA levels were determined in duplicate from six animals per group and are presented as the means ± SE. Statistical analysis was performed with the Kruskal-Wallis one-way analysis of variance and Dunn’s method for comparison of groups. Differences from control values were considered significant at P < 0.05.

RESULTS

Histological changes after acrolein exposure. As previously described (31), acrolein exposure induced mucous cell differentiation and mucus hypersecretion in rat lungs. Morphological changes in both the large and small intrapulmonary airways progressed with time in rats exposed to 3.0 ppm of acrolein. After the first week of exposure, all intrapulmonary airways appeared normal. Examination on days 9 and 12, however, revealed prominent changes in the surface epithelium (Fig. 1). The small-airway epithelium showed signs of hyperplasia, with fewer ciliated cells. Mucous cells increased, and several airways contained excessive mucus.

Both larger and smaller airways exhibited significantly increased in the percentage of mucous cells along the surface epithelium. To further describe this response, histological changes were quantified in both large (>0.8-mm-diameter) and small (=0.8-mm-diameter) intrapulmonary airways. Mucous cell differentiation developed earlier in the larger airways (day 9) than in the smaller airways (day 12) (Fig. 2). Although the percentage of mucous cells increased to approximately the same level in both airways, the magnitude of the effect compared with that in the control cells was greater in the small airways where mucous cells were rare or absent (small-airway control cells = 0.02%; large-airway control cells = 0.23%). The increase over the control value was 270- and 26-fold for small and large airways, respectively.

The trachea of control animals possessed an intact epithelial surface consisting primarily of ciliated, serous, and basal cells. After exposure on days 1–3, examination of the trachea revealed sloughing of the epithelium accompanied by excessive mucus and inflammatory cells in the lumen. Cells lining the submucosal glands in the trachea were enlarged compared with those in the control glands and demonstrated increased MUC5ac staining (see Fig. 5, E and F). After exposure on days 5–12, the surface epithelium appeared squamous and demonstrated increased MUC5ac staining on the airway surface compared with the control cells (data not shown).

Time course of mucin mRNA expression. Morphological changes in the lung were accompanied by an increase in MUC5ac steady-state mRNA levels. Acrolein exposure increased lung MUC5ac mRNA levels in a time-dependent manner, preceding an increase in mucous cells by 3–4 days. Maximal increases in expression of approximately threefold over the control value occurred on day 9 (Fig. 3A). MUC2 mRNA levels increased slightly but were not significantly different from the control level. The effect of acrolein exposure on mucin mRNA expression in the trachea differed from the lung. MUC5ac mRNA levels in the trachea increased rapidly and remained elevated for several days in response to 3.0 ppm of acrolein. MUC5ac mRNA levels then decreased but remained elevated over the control level on days 9 and 12 (Fig. 3B). Again, MUC2 mRNA levels did not significantly increase in response to acrolein exposure in the trachea.

Concentration-dependent effects in lung tissue. Lung MUC5ac steady-state mRNA levels were regulated in a concentration-dependent manner in response to acrolein (Fig. 4). Increased expression was maximal at 3.0 ppm (day 9) and elevated at concentrations as low as 0.75 ppm. Lung MUC2 mRNA levels were not significantly altered at any of the concentrations tested. Rats
exposed to 0.75 and 1.5 ppm of acrolein (day 9) showed an increase in mucous cells (≅0.2%) compared with control rats. Rats exposed to 0.3 ppm of acrolein did not demonstrate an increase in mucous cells (<0.02%) or mucin gene expression compared with control rats.

MUC5ac immunohistochemistry. MUC5ac staining was faint or absent throughout the lungs of the control animals but was readily observed in the lungs of rats exposed to 3.0 ppm of acrolein (day 12). MUC5ac staining was present in mucous cell granules, on surface epithelium, and in airway mucus secretions (Fig. 5). The presence of MUC5ac staining corresponded with the appearance of mucous cells along the airways and increased MUC5ac steady-state mRNA levels. MUC5ac staining in the trachea appears minimal in the surface epithelium and gland epithelium in control rats. After 2 days of exposure, intense staining is observed in the gland epithelium (Fig. 5, E and F) and on the surface epithelium.

DISCUSSION

Several investigators (3, 22, 24) have examined the role of MUC5ac and MUC2 gene expression in the...
process of airway mucous cell differentiation. We demonstrated that, in vivo, acrolein exposure induces mucous cell metaplasia accompanied by increased MUC5ac mRNA levels and MUC5ac immunoreactivity. Previous in vitro studies reported increased MUC5ac mRNA in cultured rat tracheal epithelial cells in response to epidermal growth factor (24) and RA (22) and in human tracheobronchial epithelial cells treated with RA (23). In RA-treated rat airway epithelium, 3 days elapsed between the time of maximum message induction and mucin secretion (22). Similarly, acrolein-exposed rats exhibited peak MUC5ac gene expression in the lung (Fig. 3A) that preceded mucous cell differentiation by ~4 days.

In contrast, the tracheae of acrolein-exposed rats demonstrated a rapid increase in MUC5ac mRNA levels (Fig. 3B). This differs from in vitro studies that required 13 days to attain maximal MUC5ac expression (22). This discrepancy could be explained by the difference in target cell populations examined. The in vitro study examined the response of a homogenous population of surface epithelial cells, whereas this study analyzed the intact tissue including the submucosal glands. In vivo, rat tracheal surface epithelium consists of <1% mucous cells, and the predominant source of mucus is therefore believed to be the submucosal glands (30). This suggests that increased MUC5ac expression in the trachea is due to stimulation of resident mucous cells in the submucosal glands, leading to mucin synthesis and secretion, as opposed to the later events in the lung involving nonmucous cells undergoing differentiation. This is supported in the present study by histological analysis of exposed tracheae that exhibited epithelial cell sloughing after day 2 but...
enlarged MUC5ac-positive staining glandular epithelium concurrent with increased MUC5ac mRNA expression. After additional exposures (days 5–12), MUC5ac mRNA levels in the trachea decreased. This was accompanied by squamous cell metaplasia and the persistence of MUC5ac immunostaining on the surface epithelium. These data suggested that the acute effects of acrolein exposure on the trachea caused the submucosal glands to rapidly secrete and synthesize MUC5ac to protect the injured surface epithelium. However, due to the injury observed in the trachea, data from this tissue were difficult to interpret. Acrolein is highly water soluble (210 g/l), and most of the exposure was absorbed in the upper respiratory tract. Therefore, high concentrations were used to examine effects in the more distal airways.

Regulation of MUC2 expression has also been demonstrated in vitro and in vivo. After infection and SO$_2$ exposure, rats developed mucous cell metaplasia accompanied by increased mucin gene expression as indicated by Northern hybridization of rat lung mRNA to a human intestinal mucin probe (29). MUC2 mRNA is also increased in vitro in response to TNF-$\alpha$ and RA (23, 36). In this study, however, changes in MUC2 mRNA in either the trachea or lung were less than those observed for MUC5ac and were not coincident with mucus hypersecretion (Fig. 3). This is consistent with recent reports (3, 43) demonstrating that, in vitro,
mucous cell differentiation of rat and monkey tracheobronchial epithelium does not correlate with increased MUC2 mRNA levels.

Studies conducted with cultured surface epithelial cells may involve mechanisms distinct from those cells lining the smallest airways. This is significant because the functional effects of mucus hypersecretion (increased airway obstruction and decreased lung function) in the smallest airways are presumably greater due to the increased number of airways and total cross-sectional area affected. Greater than 90% of rat airways are ≤0.8 mm in diameter, and these account for 70% of the total cross-sectional area in the rat airways (54). Mucous cell differentiation was greatest in these airways in response to acrolein exposure, suggesting that acrolein exposure may contribute to the pathogenesis of obstructive airway diseases.

Several cell types have been identified in the rat small intrapulmonary airways, with mucous cells being the least abundant in pathogen-free animals (30). In control animals, mucous cells were rare or absent (<0.1%) in airways ≤ 0.8 mm in diameter but accounted for >5% of the cell population after 12 days of exposure to acrolein. The discrepancy between the large increase in mucous cell number and the smaller increase in MUC5ac steady-state levels could exist for several reasons. Increased MUC5ac levels persist for several days (as opposed to hours for most mRNAs), which could lead to the accumulation of a substantial amount of protein and, subsequently, mucous granules (cells). Additionally, other mucin genes not examined may exhibit increased expression and contribute to elevated mucous cell numbers. However, exposed airway epithelial cells stain positively with the MUC5ac antibody (Fig. 5), supporting a relationship between induction of MUC5ac mRNA and subsequent protein synthesis. This finding is important for several reasons: 1) several mucin mRNAs have been detected in airway epithelial cells (4), with potentially more to be identified; 2) mucin mRNA has been detected in epithelial cell types that do not synthesize or store mucin glycoprotein (17); and 3) the relative contribution of known mucins to mucous hypersecretion remains to be established. A role for MUC5ac as a predominant airway mucin is difficult to speculate on whether each mucin gene will be regulated by a similar mechanism.

In summary, this study demonstrates that acrolein-induced MUC5ac expression is accompanied by mucous cell differentiation and airway obstruction, with the greatest effects observed in the smallest airways. These effects were accompanied by time- and dose-dependent increases in MUC5ac mRNA levels and increased MUC5ac antibody staining in epithelial cells and the airway lumen. These results suggest that exposure to environmental irritants may contribute to the pathogenesis of obstructive airway diseases by inducing the production of MUC5ac. This study prompts the investigation of both the mechanisms of acrolein-induced mucin gene expression and the functional effects of acrolein exposure in hypersecretory conditions.

We thank Dr. Samuel Ho (Veterans Affairs Medical Center, Minneapolis, MN) for the generous gift of the MUC5ac antibody and Sherri Profitt, Scott Wesselkamper, and Dr. Marian Miller for helpful advice and technical assistance.
REFERENCES


