Inflammatory and Epithelial Responses during the Development of Ozone-Induced Mucous Cell Metaplasia in the Nasal Epithelium of Rats

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Rats repeatedly exposed to high ambient concentrations of ozone develop mucous cell metaplasia (MCM) in the nasal transitional epithelium (NTE). The present study was designed to determine the temporal relationships of ozone-induced inflammatory and epithelial responses and their correlation with subsequent MCM in the NTE of rats. Male F344/N rats were exposed to 0.5 ppm ozone, 8 h/day for 1, 2, or 3 days. Two h prior to sacrifice, all the rats were injected intraperitoneally with 5’-bromo-2-deoxyuridine (BrdU) to label epithelial cells undergoing DNA synthesis. Rats exposed to ozone for 1 or 2 days were killed 2 h after the exposure. Rats exposed to ozone for 3 days were killed 2 h or 1, 2, or 4 days after the exposure. Control rats were killed after a 7-day exposure to filtered air. One nasal passage from the anterior nasal cavity of each rat was fixed and processed for light microscopy to morphometrically determine the numeric densities of epithelial cells, neutrophils, and mucous cells, and the amount of intraepithelial mucosubstances in the NTE. The maxilloturbinate from the other nasal passage was processed for analysis of an airway mucin-specific gene (i.e., rMuc-5AC mRNA). Acute ozone exposure induced a rapid increase in rMuc-5AC mRNA levels prior to the onset of MCM, and the increased levels of rMuc-5AC mRNA persisted with MCM. Neutrophilic inflammation coincided with epithelial DNA synthesis and upregulation of rMuc-5AC, but was resolved when MCM first appeared in the NTE. The results of the present study suggest that upregulation of mucin mRNA by acute ozone exposure may be associated with the concurrent neutrophilic inflammation and epithelial hyperplasia in the NTE. Ozone-induced MCM may be dependent on these important pre-metaplastic responses (i.e., mucin mRNA upregulation, neutrophilic inflammation, and epithelial proliferation).

Key Words: ozone; mucous cell metaplasia; inflammation; mucin gene; nasal transitional epithelium; rat.

Ozone (O₃) is an irritating oxidant gas in photochemical smog, and one of the regulated criteria air pollutants for which national air quality standards have been designated under the Clean Air Act (Steinfeld, 1991). Controlled inhalation studies have demonstrated that acute ozone exposure induces cellular and biochemical changes in the pulmonary airways of human subjects (Leikau et al., 1995; Schelegle et al., 1991). Ozone-induced morphologic changes in the distal centriacinar regions of the lung in laboratory animals have been well documented in previous studies (Castleman et al., 1980; Stephens et al., 1974).

The airways of the upper respiratory tract, specifically the nose, are also susceptible to ozone toxicity. Nasal inflammation has been induced in human volunteers acutely exposed to high ambient concentrations (0.4–0.5 ppm for 2–4 h) of ozone (Graham et al., 1988; McBride et al., 1994). In addition, nasal epithelial lesions thought to be related to exposure to air pollution have been described in people living in ozone-polluted atmospheres of southwest metropolitan Mexico City (Calderon Garciduenas et al., 1992, 1995). Marked inflammatory and epithelial responses to near ambient concentrations of ozone have also been demonstrated in the nasal mucosa of both monkeys and rats (Harkema et al., 1987a,b; Hotchkiss et al., 1989).

We have previously reported that acute or chronic exposures of 0.5–1.0 ppm ozone cause epithelial proliferation and marked mucous cell metaplasia (MCM) in surface epithelial cells lining the lateral meatus of the proximal nasal airways (i.e., nasal transitional epithelium; NTE) in F344/N rats (Harkema et al., 1989, 1997; Hotchkiss et al., 1991). The ozone-induced MCM in rat nasal epithelium was similar in character to nasal epithelial changes previously reported by Harkema et al. (1987a) in macaque monkeys repeatedly exposed to 0.15 or 0.3 ppm ozone for 6 days or 13 weeks (6 h/day, 5 days/wk).

Though the ozone-induced morphological changes in the nasal epithelium have been well characterized, the cellular and molecular events preceding the onset of MCM and epithelial hyperplasia have not been thoroughly investigated. Previous studies have demonstrated that a transient neutrophilic inflammation is conspicuous in the nasal epithelium prior to the development of MCM (Hotchkiss et al., 1989, 1997). However, little is known about the relationship of neutrophilic
inflammation with the ozone-induced epithelial alterations in the nasal airway. In addition, the effects of ozone exposure on mucin gene expression as well as its relationship with the ozone-induced MCM in the NTE have not been previously investigated. To further understand how and when ozone induces nasal cell injury and reparative and adaptive changes (i.e., epithelial proliferation and MCM) in the NTE of rats, it is first important to clearly determine the temporal relationship of ozone-induced epithelial and inflammatory responses that occur early after the start of exposure and during the development of the mucous metaplastic changes.

Therefore, the present study was designed to test the hypotheses that (1) acute ozone exposure induces upregulation of mucin gene expression prior to the development of MCM, and (2) neutrophilic inflammation precedes, or is concurrent with, mucin gene over-expression and other pre-metaplastic events (e.g., hyperplasia). For this purpose, rats were exposed to 0.5 ppm ozone for 1–3 days (8 h/day). Some of the 3-day-exposed rats were held in air for an additional 1–4 days. We determined the time-dependent inflammatory and epithelial cell responses in the nasal epithelium of the ozone-exposed rats. We also determined the temporal expression of rMuc-5AC mRNA in the nasal tissues during and after ozone exposure. In addition, the temporal relationship of mucin gene upregulation with (1) neutrophilic inflammation, (2) epithelial proliferation (i.e., DNA synthesis and numeric density), and (3) onset of MCM was investigated. A better understanding of these exposure-related cellular and molecular events provides new insights into the pathogenesis of MCM caused by repeated ozone exposure.

MATERIALS AND METHODS

Animals and exposure. Fifty-six male F344/N rats (Harlan Sprague-Dawley, Indianapolis, IN), 10–12 weeks of age, were randomly assigned to one of 7 exposure groups (n = 8/group) based on their body weight. Male rats were chosen for all experiments to avoid hormonal changes during the estrous cycle. Estrous cycle-related changes have been shown to alter secretory cell proliferation in rodent airways (Hayashi et al., 1979).

Rats were housed, 2 per cage, in polycarbonate shoebox-type cages with Cell-Sorb Plus bedding (A&W Products, Inc., Cincinnati, OH) and filter caps. Water and food (Tek Lab 1640; Harlan Sprague Dawley, Indianapolis, IN) were available ad libitum. The rats were maintained on a 12-h light/dark cycle under controlled temperature (16–25°C) and humidity (40–70%).

Prior to the start of the inhalation exposure, rats were conditioned in whole-body exposure chambers (HC-1000, Lab Products, Maywood, NJ) supplied with filtered air for 1 day. The rats were individually housed in rack-mounted stainless-steel wire cages with free access to food and water prior to exposure. The room lights were set on a 12 h-light/dark cycle beginning at 6 am. The chamber temperature and relative humidity were maintained between 16 to 25°C and 40 to 70%, respectively.

Rats in one exposure group were exposed to 0 ppm ozone (filtered air) for 7 days (controls, n = 8). Rats in the other 7 exposure groups (n = 8/group) were exposed to 0.5 ppm ozone, 8 h/day, for 1, 2, or 3 days. The rats were exposed to ozone or filtered air in the whole-body ozone chambers from 6 A.M. to 2 P.M. in the Inhalation Toxicology Exposure Laboratory housed in the University Research Containment Facility at Michigan State University. Though food was removed, animals had free access to water during the exposure. Ozone was generated with an OREC Model O3VI-O ozonizer (Ozone Research and Equipment Corp., Phoenix, AZ) using compressed air (AGA Gas, Lansing, MI) as a source of oxygen. No NO gases have been detected by this method of generation, which uses uv light to convert oxygen to ozone (Sun et al., 1988). Dilation air was mixed with ozone and delivered to the chambers using teflon tubing. The total airflow through the exposure chambers was maintained at approximately 250 L/min (15 chamber air change/h). The chamber temperature and relative humidity remained the same as those during the animal conditioning period. The chamber ozone concentration was controlled by adjusting the intensity of uv radiation within the ozonizer. It was monitored throughout the exposure with Dasibi 1003 AH ozone monitors (Dasibi Environment Corp., Glendale, CA), and recorded by Linear 0141 strip chart recorders (Linear Instrument Corp., Reno, NV). The exposure-atmosphere sampling probes were positioned in the breathing zone of the rats, within the middle cage rack of the HC-1000 chambers. The mean chamber ozone concentrations during the 3-day exposure to 0.5 ppm ozone was 0.523 ± 0.006 (mean ± standard deviation). The chamber ozone concentration during 7-day exposure to filtered air remained below 0.05 ppm.

Tissue selection and preparation for analyses. Two h prior to the designated sacrifice, each rat was injected intraperitoneally (ip) with 5′-bromo-2′-deoxyuridine (Brdu; 50 mg/Kg body wt.) to label cells undergoing DNA synthesis in the S-phase of the cell cycle. Rats exposed to ozone for 1, 2, or 3 days were killed 2 h after the end of exposure, to evaluate ozone-induced inflammatory and epithelial responses immediately after exposure and prior to the phenotypic expression of mucous cells in the NTE (premetaplastic effects). Additional rats that were exposed to ozone for 3 days were sacrificed 1 day, 2 days, or 4 days following the last day of exposure to examine the inflammatory and epithelial responses during the phenotypic transformation of the NTE (mucous cell metaplasia). These exposure and sacrifice times were chosen based on our previous observation that ozone-induced mucous cell metaplasia and epithelial hyperplasia in the NTE of rats can be induced with only 3 consecutive daily exposures to ozone (Hotchkiss, et al., 1991). Control rats were sacrificed after 7 days of exposure to filtered air. Rats were deeply anesthetized using 4% halothane in oxygen and killed by exsanguination via the abdominal aorta.

Immediately after death, the head of each rat was removed from the carcass. After the eyes, lower jaw, skin, and musculature were removed, the nasal airways were opened by splitting the nose in a sagittal plane adjacent to the midline. The maxilloturbinate from one nasal passage was excised by micro-dissection and immediately homogenized in Tri-Reagent (Molecular Research Center, Cincinnati, OH). The homogenate was snap frozen in liquid nitrogen and stored at –80°C until processed for isolation of total RNA and analysis of mucin mRNA.

The other nasal passage was immersed in a large volume of zinc formalin (Anatech, Ltd., Battle Creek, MI) for at least 24 h. The zinc formalin-fixed nasal tissues were decalcified in 13% formic acid for 4 days, and then rinsed in tap water for at least 2 h, as previously described by Harkema et al. (1988). A tissue block was removed from the proximal aspect of the nasal cavity by making 2 transverse cuts perpendicular to the hard plate. The first cut was immediately posterior to the upper incisor tooth (Fig. 1A), and the second cut was at the level of the incisive papilla. The tissue block was excised, embedded in paraffin, and 5-μm-thick sections were cut from the anterior face of the tissue block. One nasal tissue section from each animal was histologically stained with hematoxylin and eosin for morphological identification of epithelial cells. Another tissue section from each animal was immunohistochemically stained with anti-BrdU antibody (Becton Dickinson Immunocytometry Systems, San Jose, CA) to detect BrdU-labeled nuclei (Johnson et al., 1990), and counterstained with hematoxylin (Gill 3; Ricca Chemical Co., Arlington, TX). The other tissue section from the same block was stained with Avidin blue (pH 2.5)/periodic acid-Schiff sequence (AB/PAS) to identify acidic and neutral mucosubstances in the surface epithelium.

Morphometry of neutrophilic inflammation, epithelial cell numeric density and DNA synthesis. The NTE lining the maxilloturbinate of each animal was examined using computerized image analysis and standard morphometric

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basal lamina underlying the surface epithelium was calculated from the contour length of the digitized image of the basal lamina using a Power Macintosh 7100/66 computer and the public domain image analysis software (NIH Image; written by Wayne Rasband at the U.S. National Institutes of Health and available on the Internet at http://rib.info.nih.gov/ih/image/). The epithelial cell numeric density (i.e., epithelial nuclei/mm of basal lamina) was determined by counting the total number of epithelial cell nuclear profiles present in the NTE lining the maxilloturbinate, and dividing this number by the length of basal lamina. The epithelial cell-labeling index (LI) was determined as an indicator of the epithelial DNA synthesis. The number of BrdU-labeled NTE cell nuclei was counted, and divided by the total number of epithelial cell nuclei and multiplied by 100 (i.e., % BrdU-labeled epithelial cell nuclei).

**Morphometry of stored intraepithelial mucosubstances and mucous cells.** To estimate the amount of the intraepithelial mucosubstances in NTE lining the maxilloturbinate, the volume density (Vs) of AB/PAS-stained mucosubstances was quantified using computerized image analysis and standard morphometric techniques. The area of the AB/PAS-stained intraepithelial mucosubstances was calculated by the image analysis software program from the automatically circumscribed perimeter of the stained material. The length of the basal lamina underlying the surface epithelium was determined as described above. The volume of stored mucosubstances per unit of surface area of epithelial basal lamina was estimated using the method previously described in detail by Harkema et al. (1987a, 1989), and expressed as nL/mm$^2$ basal lamina.

The numeric cell densities of mucous cells (epithelial cells containing AB/PAS-stained mucosubstances) in the NTE lining the maxilloturbinate were also morphometrically determined. Only AB/PAS-positive epithelial cells with a nuclear profile were counted, and the data were expressed as the number of mucous cell nuclei/mm of basal lamina.

**Analysis for mucin mRNA in maxilloturbinate.** Total cellular RNA was isolated from the maxilloturbinate homogenate according to the method of Chomczynski and Sacchi, (1987). To avoid DNA contamination, the isolated RNA pellet was resuspended in nuclease-free water and treated with 10 units RNase-free DNase I (Boehringer Mannheim GmbH, Germany) in 5X Transcription Buffer (Promega, Madison, WI) at 37°C for 30 min. The RNA was sequenced by equal volumes of phenol/chloroform/isooamyl alcohol mixture (25:24:1) and chloroform/isoamyl alcohol mixture (24:1), and precipitated. The final pellet was washed with 75% ethanol, air dried, resuspended in nuclease-free water containing rRNasin (40 units/100 nl), and stored at –80°C.

The RNA was analyzed to determine the steady-state levels of muc5ac mRNA by quantitating the amount of muc5ac cDNA produced by reverse transcriptase polymerase chain reaction (RT-PCR). Cyclophilin is an abundant and ubiquitous cellular protein well known as a major intracellular receptor for immunosuppressant cyclosporin A, and considered as a putative molecular chaperone (Kern et al., 1994; Matouschek et al., 1995). Because cyclophilin mRNA expression was similar in all experimental groups, this housekeeping gene was used as an internal standard in this semi-quantitative RT-PCR analysis.

Primers specific for rat muc5ac cDNA and all-species cyclophilin cDNA sequences were synthesized and purified by the Macromolecular Structure Facility at Michigan State University. The sequences of the forward and reverse primers for cyclophilin are 5'-CTT GTC CAT GGC AAA TGC TG-3' and 5'-GTA ATC TTC TGT CTG TGC TTG-5', respectively. The sequences of the rat muc5ac forward and reverse primers are 5'-CAT CAT TCC TGT AGC AGT AGT GAG G-3' and 5'-GTT ACC CAG GTC TAC ACC TAC TCC G-3', respectively. The predicted amplified sizes of the cyclophilin cDNA and muc5ac cDNA products were ~190 bp and ~320 bp, respectively.

A 50 ng/μl working solution of each RNA sample was prepared and two μl (100 ng) aliquots were reverse transcribed into cDNA in a volume of 20 μl, each, containing PCR buffer (166 mM (NH$_4$)$_2$SO$_4$, 50 mM β-mercaptoethanol,
67 μM EDTA, 0.67 M Tris, pH 8.8, 0.8 mg/ml BSA) plus 5 mM MgCl₂, 1 mM each dNTP, 10 units RNasin, 125 ng oligo(dT)₁₅, and 50 units of Moloney Murine Leukemia Virus reverse transcriptase (M-MLV-RT; Gibco BRL, Gaithersburg, MD). A PCR master mix, consisting of PCR buffer, 4 mM MgCl₂, 6 pmol of each forward and reverse primers of MUC-5 mRNA and cyclophilin mRNA, and 1.25 units Taq DNA polymerase, was added to each cDNA sample for a final volume of 50 μl. The Taq was added only after the PCR master mix had been heated to 85°C for 5 min, in order to minimize primer dimer formation. RT-PCR was performed in 9600 Perkin Elmer Thermocycler, starting with a 3-min incubation at 95°C, followed by a 3-step temperature cycle, denaturation at 95°C for 30 s, annealing at 56°C for 1 min, and extension at 72°C for 1 min, for 25 cycles. A final extension step at 72°C for 10 min was included after the final cycle to complete polymerization. The number of cycles was chosen to ensure that amplification of cyclophilin (most abundant gene) did not reach a plateau level.

The abundance of mRNA was semi-quantitatively determined by densitometric analysis of ethidium bromide-stained agarose gels (3%, Nusieve:agarose = 3:1) using the Gel Doc 1000 analysis system (BioRad Laboratories, Inc., Hercules, CA) and Molecular Analyst Software Version 2.1 on a Power Macintosh 7100/80. The volume of the mMuc-5AC cDNA band was divided by the volume of the cyclophilin cDNA band. To minimize the variability of analysis, the quantitation for all the samples was performed at the same time.

Statistical analyses. All data was expressed as the mean group value ± the standard error of the mean (SEM). The natural logarithms of the data were used for statistical analysis to make the variances approximately equal for all groups. The data were first analyzed using one-way analysis of variance (ANOVA) to identify the time-response of inflammation and epithelial events induced by ozone exposure. Significant differences between air control groups and ozone-exposed groups were evaluated using Dunnett’s Method. Statistical analyses were performed using a commercial statistical analysis package (SigmaStat; Jandel Scientific Software, San Rafael, CA). The level of statistical significance was set at p ≤ 0.05.

RESULTS

Histopathology of Nasal Mucosa

Exposure-related nasal lesions were present only in rats exposed to 0.5 ppm ozone. No lesions were observed in the nasal airways of filtered air (0 ppm ozone)-exposed rats (controls). In all of the rats exposed to 0.5 ppm ozone, regardless of the duration of the exposure, the nasal lesions were restricted to the mucosa containing the NTE that lined the lateral meatus in the proximal nasal cavity. The character and severity of these site-specific mucosal lesions, however, varied according to the number of ozone exposures and the length of time of postexposure. Figure 2 contains light photomicrographs of representative maxilloturbinate that depict time-dependent changes of inflammatory and epithelial cell responses in the NTE. Time-dependent progression of MCM in the NTE is illustrated in Figure 3, which contains photomicrographs of maxilloturbinates stained with AB/PAS to detect stored intraepithelial mucusubstances.

After one day of exposure to 0.5 ppm ozone, the principal
morphologic alterations in the NTE were epithelial degeneration and atrophy due to individual cell necrosis and exfoliation (Fig. 2B). These ozone-induced alterations in the NTE were most noticeable in the dorsolateral aspect of the maxilloturbinate, the lateral ridge of the nasoturbinate and the dorsal recess of the lateral wall. Concurrent with the epithelial lesions was a transient mild-moderate inflammatory response in the affected mucosa that was greatest in animals sacrificed 2 h after 1 or 2 days of ozone exposure. The inflammatory lesion was characterized by endothelial margination of neutrophils in the large capacitance vessels of the lamina propria and by an influx of neutrophils in the adjacent interstitial tissue of the lamina propria, also extending into the NTE (Fig. 2B).

After 2 days of ozone exposure, the neutrophilic inflammatory response in the nasal mucosa was similar to that observed after one day of exposure to ozone with conspicuous accumulations of neutrophils in both the lamina propria and NTE. However, the epithelial degeneration and atrophy of the NTE observed in the one-day-exposed rats was replaced by a regenerative, basophilic epithelium in the rats exposed to ozone for 2 consecutive days. The principal features of the regenerative NTE were mild hyperplasia of basal cells and the appearance of widely scattered mitotic figures. In addition, necrotic epithelial cells, a common feature in the NTE of the 1-day ozone-exposed animals, were infrequently observed in the NTE of the 2-day-exposed rats.

Epithelial hyperplasia was the principal feature in the NTE of rats exposed for 3 consecutive days and sacrificed at the end of the exposure (Fig. 2C). The hyperplastic NTE in these ozone-exposed rats was approximately 3–4 cells in thickness compared to 1–2 cells in the NTE of control rats exposed only to filtered air. Only a mild influx of neutrophils was present in the nasal mucosa containing hyperplastic NTE after 3 days of ozone exposure.

In rats exposed to 0.5 ppm ozone for 3 days and sacrificed 1 day after the end of the exposure, the NTE remained hyperplastic. However, there were also a few widely scattered AB/PAS-stained mucous cells in the NTE, indicating the onset of a mild MCM. Only a few widely scattered neutrophils were present in the NTE or lamina propria of these repeatedly exposed rats.

Ozone-induced MCM in the NTE was even more marked in rats exposed to 0.5 ppm ozone for 3 days and sacrificed 2 or 4 days postexposure. The 3-day-ozone-exposed rats that were sacrificed 4 days after the end of the exposure had the most severe MCM compared to the other 3-day-ozone-exposed rats that were sacrificed 1 or 2 days postexposure (Fig. 3). Epithelial hyperplasia was also a conspicuous feature of the NTE in rats sacrificed 2 or 4 days postexposure. However, no associated inflammatory cell influx was present in the nasal mucosa with the hyperplastic and metaplastic NTE of the 3-day-ozone-exposed rats sacrificed 2 or 4 days after the end of the exposure.
Morphometric Quantitation

**Neutrophilic influx.** Exposure to 0.5 ppm ozone resulted in a transient influx of neutrophils within the NTE lining the maxilloturbinates (Fig. 4). Significant increases in the number of intraepithelial neutrophils were evident in rats exposed to 0.5 ppm ozone for 1 and 2 days (38- and 47-times greater than air-exposed controls, respectively). The number of neutrophils in the NTE of rats exposed to ozone for 3 days was slightly attenuated (45% fewer neutrophils) compared to rats exposed to ozone for 1 or 2 days. One day after 3 days of ozone exposure, the number of intraepithelial neutrophils was still 10-fold greater than air-exposed controls. However, the intraepithelial neutrophil numbers returned to control levels in rats sacrificed 2 and 4 days postexposure.

**Epithelial cell DNA synthesis.** Only a few widely scattered BrdU-labeled NTE cells were present in air-exposed control rats. There was a marked, but transient, increase in the number of BrdU-labeled cells in rats exposed to ozone for 2 days (143-fold increase) or 3 days (112-fold increase), compared to controls (Fig. 5). The numbers of BrdU-labeled cells in rats exposed to 0.5 ppm ozone for 3 days and sacrificed 1, 2, or 4 days postexposure were similar to those of air-exposed control rats.

**NTE cell numeric density.** Ozone exposure altered the number of NTE cells lining the maxilloturbinates (Fig. 6). Rats exposed to ozone for 1 day had 9% fewer NTE cells than air-exposed control rats. However, rats exposed to ozone for 2 days had NTE cell numeric densities that were not significantly different from those of controls. After 3 days of ozone exposure, rats had significantly more NTE cells (36% increase in NTE cell numeric density) compared to air-exposed controls. The ozone-induced NTE cell hyperplasia persisted in rats sacrificed 2 and 4 days postexposure (i.e., 52 and 37% more NTE cells than those in controls, respectively).

**Amount of stored intraepithelial mucosubstance.** Little AB/PAS-stained intraepithelial mucosubstances were present in the NTE of control rats or rats exposed to ozone for 1 or 2 days (Fig. 7). A trend toward increased amounts of stored mucosubstances was first detected in the NTE of rats exposed to ozone for 3 days and sacrificed 2 h later. A significant increase (24-fold, compared to controls) of the intraepithelial mucosubstances was observed in rats sacrificed 1 day after the 3-day ozone exposure. The amount of stored intraepithelial mucosubstances increased with time postexposure. Compared to air-exposed control animals, rats sacrificed 2 days or 4 days following 3 days of ozone exposure had 30- and 40-fold more stored mucosubstances in their NTE, respectively.
Mucous cell numeric density. Control animals exposed to filtered air (0 ppm ozone) had no or only a few mucous cells in the NTE. A significant increase (22-fold more than air-exposed controls) in the number of mucous cells was first detected in 3-day ozone-exposed rats sacrificed 1 day postexposure (Fig. 8). The numeric density of mucous cells in the NTE of ozone-exposed animals increased with time postexposure. Rats sacrificed 4 days after the 3-day-ozone exposure had 43-fold more mucous cells than air controls.

Mucin mRNA (rMuc-5AC) Abundance

Figure 9 depicts an agarose gel with representative RT-PCR cDNA products from each exposure group indicating the abundance of rMuc-5AC and cyclophilin mRNA. In Figure 10, the mean steady-state levels of rMuc-5AC mRNA in maxilloturbinate isolates from rats 2 h following 1, 2, or 3 days of ozone exposure (Ozone exposure) or 1, 2, or 3 days following 3 days of ozone exposure (Postexposure) are presented. Maxilloturbinates of control animals, which normally have few mucous cells, had low, but detectable levels of rMuc-5AC mRNA. Exposure to ozone resulted in marked increases of rMuc-5AC mRNA in these nasal tissues (Fig. 10). There were 132% (1 day), 140% (2 day) or 126% (3 day) more rMuc-5AC mRNA in ozone-exposed rats, compared to air-exposed controls. The elevation of rMuc-5AC mRNA persisted in rats sacrificed 2 days following the end of the 3-day-ozone exposure (70 % more, compared to controls).

**DISCUSSION**

The results of the present study demonstrate that a single 8-h exposure to 0.5 ppm ozone rapidly induces an increase in rMuc-5AC mRNA in the nasal mucosa of rats. This ozone-induced increase in rMuc-5AC mRNA preceded the morphologic appearance of increased numbers of mucous cells in the NTE by three days. This is the first report, to our knowledge, indicating that a single or repeated inhalation of a high ambient concentration of ozone can induce an increase in the steady-state levels of a mucin-specific mRNA in the nasal airways of laboratory animals. In addition, this study is the first to demonstrate that this ozone-induced increase in rMuc-5AC mRNA precedes, by several days, the onset of MCM in the affected nasal epithelium.

MCM in rat airway epithelium is also a prominent morphologic response to exposure to other airway irritants, such as sulfur dioxide (Jany et al., 1991), tobacco smoke (Lamb and Reid, 1969), bacterial endotoxin (Harkema and Hotchkiss, 1993; Shimizu et al., 1996), 3-methylcholanthrene (Rehm and Kelloff, 1991), acrolein (Borchers and Leikauf, 1997), siloxane (Burns-Naas et al., 1998), and chlorine (Wolf et al., 1995). However, only a few previous studies have demonstrated irritant-induced alterations in mucin gene expression associated with MCM in airway epithelium of rats. Repeated exposure to sulfur dioxide induced early upregulation of mucin mRNA that persisted throughout the development of MCM in rat tracheobronchial epithelium (Jany et al., 1991). Elevated rMuc-2 mRNA expression concurrent with MCM was also reported in tracheobronchial and pulmonary airway epithelium of rats after repeated exposure to acrolein (Borchers and Leikauf, 1997). These previous studies have reported elevated airway mucin mRNA levels in rat airways after repeated long-term (i.e., >1 week) inhalation exposure. The results of the present study suggest that ozone-induced alteration in mucin mRNA abundance is an early molecular predictor of mucous cell metaplastic changes. This increase in mRNA probably plays a crucial role in the development of the phenotypic expression of mucous (goblet) cells in the nasal epithelium (i.e., MCM).

Our present observations are supported by studies conducted in vitro that examined mucous differentiation of normal airway epithelial cells induced by retinoic acid, a major regulator of
mucous cell differentiation (Guzman et al., 1996) or by methotrexate, an inhibitor of nucleic acid metabolism (Lesuffleur et al., 1993). In these studies, mucin messages were strongly expressed only in cultures that had undergone mucous cell differentiation. In addition, there was a time-lag (2–7 days) between the first detection of mucin gene expression and that of mucus production during the in vitro mucous cell differentiation. Little is known about the kinetics of mucus biosynthesis in either normal or metaplastic mucous cells in airway epithelium. However, it is assumed that during the ozone-induced mucous metaplastic differentiation in the nasal epithelium, the mucin gene is activated in premetaplastic cells, and it takes time for (1) the synthesis of mucin core protein from the abundant mucin mRNA, (2) its glycosylation, and (3) the storage of the glycosylated mucin molecules into secretory granules of fully differentiated mucous cells. Further studies are needed to determine the kinetics of rMuc-5AC mRNA accumulation, apomucin (protein core) synthesis, and glycosylation. Results from such studies will further our understanding of the underlying molecular mechanisms of ozone-induced MCM.

In the present study, we determined the time-dependent relationships of pre-metaplastic inflammatory and epithelial events (i.e., neutrophilic inflammation, epithelial injury, regeneration, and proliferation) in the nasal epithelium induced by single and repeated exposures to ozone. The coincidence of the onset of increased steady-state rMuc-5AC mRNA levels and the transient neutrophilic influx into the NTE, prior to the development of MCM, suggest that the early neutrophilic inflammatory response may be involved in the upregulation of mucin mRNA levels in the NTE and in the initiation of MCM. Neutrophils, as well as airway epithelial cells, are significant sources of soluble mediators that can initiate or amplify inflammatory responses in airway tissues. We hypothesize that neutrophils play an essential role in the ozone-induced mucin gene upregulation, and ultimately in the pathogenesis of the MCM, by releasing distinctive soluble mediators or by stimulating other resident cells (e.g., epithelial cells) to release inflammatory mediators. It has been shown that soluble inflammatory mediators can rapidly modulate various cellular genes (e.g., genes for secondary mediators like cytokines) during airway injury and repair induced by inhaled toxicants including ozone (Leikauf et al., 1995; Levine, 1995). Recently, several studies have focused on the role of inflammatory mediators in the expression of airway mucin-specific genes. Cytokines such as TNF-α (Levine et al., 1995), IL-6 (Levine et al., 1994) and IL-4 (Rankin et al., 1996; Temann et al., 1997), or neutrophil proteases, specifically elastases (Voynow et al., 1997), have been reported to induce mucin mRNA upregulation in airway epithelial cells in vivo or in vitro. Neutrophil elastase is a well-known mucous secretagogue and induces MCM in the airways of laboratory animals (Breuer et al., 1985; Kim et al., 1987). Both soluble TNF-α and IL-6 induce mucin hypersecretion in airway epithelial cells in vitro at concentrations that also cause mucin gene upregulation (Levine et al., 1994, 1995). Transgenic mice that overexpress IL-4 or IL-5 have MCM (Lee...
et al., 1997; Rankin et al., 1996) and mucin hypersecretion (McBride et al., 1994) with mucin gene upregulation in tracheobronchial or pulmonary airways. However, the precise roles of these inflammatory mediators in mucin gene expression or mucous cell differentiation are not known.

Recently we demonstrated that an anti-inflammatory steroid, fluticasone propionate, decreased neutrophilic inflammation and MCM in the nasal epithelium of rats exposed to ozone (Hotchkiss et al., 1998). Similarly, another steroid, dexamethasone, has been shown to attenuate rat tracheal MCM induced by neutrophil lysates or elastase (Lundgren et al., 1988). In addition, Kai et al. (1996) reported that dexamethasone suppressed the mucin mRNA expression and stored mucous product in airway epithelial cells in culture. These studies suggest a role for inflammatory cells or their secreted products in the induction of MCM, the upregulation of airway mucin genes, and the overproduction of mucins in airway epithelium.

The present study was also designed to examine the kinetics of epithelial injury, regeneration, and proliferative adaptation (i.e., hyperplasia), and the time-dependent relationships of these ozone-induced epithelial changes with mucin gene expression and MCM. Interestingly, the severity and temporal pattern of nasal epithelial cell loss, subsequent burst of DNA synthesis, and cell proliferation leading to epithelial repair during single and repeated daily exposure to ozone were similar to those observed following a single ozone exposure in a previous study reported by Hotchkiss et al. (1997). In that study, rats were exposed to 0.5 ppm ozone once for 8 h, and the epithelial responses were examined 2–36 h postexposure. Even though rats in our study received repeated ozone exposures, the temporal relationship of epithelial DNA synthesis and the proliferation of injured NTE cells observed after 2 and 3 days of exposure were similar to those observed in the previous study at 24 and 36 h after the single exposure to ozone. This suggests that the repeated ozone exposures, in the present study, did not interfere with the cellular repair responses initiated by ozone-induced NTE cell injury incurred during the first exposure. The epithelial regeneration after 2 days of exposure and subsequent hyperproliferative response (i.e., epithelial hyperplasia) were concurrent with increased levels of rMuc-5AC mRNA in the NTE. It is plausible that new epithelial cells with abundant mucin message repopulate the injured epithelium, and are responsible for the observed increase in mucin mRNA in the regenerative and hyperplastic epithelium. It is also possible that NTE cells that survive the initial ozone exposure are stimulated by ozone to up-regulate their normally low-constitutive levels of mucin mRNA. The exact cellular mechanisms responsible for ozone-induced upregulation of rMuc-5AC mRNA in the NTE cannot be determined from the results of our present study. Further studies, using in situ hybridization and immunohistochemistry techniques, are needed to identify the NTE cells that express rMuc-5AC mRNA and produce mucin protein during regeneration and hyperproliferation after ozone exposure.

In our study, neutrophilic inflammation preceded both the hyperplastic and metaplastic responses in the ozone-exposed NTE. Though the role of neutrophils in the repair and hyperproliferation of NTE is unknown, other studies in the literature suggest that these inflammatory cells are important in airway epithelial repair following ozone-induced injury in the lungs of laboratory animals (Hyde et al., 1992; Pino et al., 1992).

In conclusion, acute ozone exposure induced increased levels of rMuc-5AC mRNA in the NTE within hours after the start of exposure. This ozone-induced upregulation of the airway mucin gene was observed several days before the phenotypic expression and the intraepithelial production and storage of mucosubstances (i.e., MCM). Mucin gene upregulation occurred concurrently with ozone-induced neutrophilic inflammation in the NTE, but remained elevated even after the initial neutrophilic inflammation was resolved 2 days later. Although temporal correlations of epithelial and inflammatory responses in the present study do not prove causality, our results suggest that (1) upregulation of mucin mRNA by acute ozone exposure may be associated with the concurrent neutrophilic inflammation and epithelial hyperplasia in the NTE, and (2) ozone-induced MCM may be dependent on these important pre-metaplastic responses (i.e., mucin mRNA upregulation, neutrophilic inflammation and epithelial proliferation).

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