Ozone Exposure Enhances Endotoxin-Induced Mucous Cell Metaplasia in Rat Pulmonary Airways

James G. Wagner, Steven J. Van Dyken, Janelle R. Wierenga, Jon A. Hotchkiss, and Jack R. Harkema

Department of Pathobiology and Diagnostic Investigation, Michigan State University, East Lansing, Michigan 48824

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Coexposure to different airborne pollutants can be more toxic to airway epithelium than an inhalation exposure to a single pollutant. We have previously reported that coexposure to ozone, the primary oxidant gas in photochemical smog, and unique inflammatory biogenic substances such as allergens or bacterial endotoxin, results in augmented epithelial and inflammatory responses in rat nasal airways (M. V. Fanucchi et al., 1998, Toxicol. Appl. Pharmacol. 152, 1–9; J. G. Wagner et al., 2002a, Toxicol. Sci. 67, 284–294). In the present study, we investigated the toxic interaction of ozone and endotoxin on the respiratory epithelium in the pulmonary airways of laboratory rodents. F344 rats were intranasally instilled with 0, 2, or 20 μg endotoxin dissolved in sterile saline (150 μl/nasal passage). Six hours after instillation rats were exposed to air or 1 ppm ozone for 8 h. One day later, endotoxin and ozone exposures were repeated. Three days after the last exposure, rats were sacrificed, the lungs were lavaged with saline, and the collected bronchoalveolar lavage fluid (BALF) was analyzed for inflammatory cells and secreted mucosubstances (mucin 5AC). Lung tissues were processed for light microscopic examination and morphometric analysis of numeric density of epithelial cell populations and volume densities of intraepithelial mucosubstances (IM). Conducting airways were microdissected and analyzed by quantitative RT-PCR to determine steady-state mucin gene (rMuc5AC) mRNA levels in respiratory epithelium. Endotoxin instillation caused a dose-dependent increase in BALF neutrophils that was further increased twofold in ozone-exposed rats given 20 μg endotoxin. Mucin glycoprotein 5AC was elevated in BALF from rats exposed to 20 μg, but not 2 μg endotoxin. Exposure to ozone alone did not cause mucus hypersecretion, but ozone potentiated mucus secretion in rats given 2 or 20 μg endotoxin. Airways of rats exposed to air or ozone alone had scant amounts of IM. Endotoxin instillation induced a dose-dependent increase in IM in airway epithelium that was significantly increased (twofold) in rats that were also exposed to ozone. Expression of rMuc5AC was induced in axial pulmonary airways by 2 and 20 μg endotoxin, and was increased further by ozone-exposure in rats instilled with 20 μg endotoxin. These data demonstrate that ozone exposure potentiates neutrophilic inflammation and mucus production and secretion elicited by a biogenic substance in rat pulmonary airways.

Key Words: ozone; lipopolysaccharide; mucous cell metaplasia; mucin; neutrophil; inflammation.

Bacterial endotoxins are lipopolysaccharide-protein compounds derived from the cell wall of Gram-negative bacteria. Airway exposure of humans to endotoxin occurs by inhaling aerosols or contaminated dusts in agricultural, hospital, occupational, and domestic environments. Endotoxins are the principal etiologic agent responsible for acute inflammation during pneumonias and sepsis, and have been implicated in occupational diseases such as chronic grain dust-induced airway disease. Inhaled endotoxin causes nose and throat irritation in humans and the production in the pulmonary airways of soluble inflammatory mediators such as interleukin-8, tumor necrosis factor-α (TNF-α) and interleukin-1, and large infiltrates of inflammatory cells, particularly polymorphonuclear neutrophils (Michel et al., 1997; Wesselius et al., 1997). We have extended these observations in lungs of experimental animals wherein aerosolized or instilled endotoxin leads to airway remodeling (e.g., mucous cell metaplasia) and the overproduction and hypersecretion of mucus (Gordon and Harkema, 1994; Harkema and Hotchkiss, 1992; Steiger et al., 1995). Specifically, increases in stored mucosubstances, secretory cell hyperplasia, and secretory cell metaplasia occurred in the respiratory epithelium of airways that normally consist of ciliated and secretory cells.

Exposure of people to ozone, the primary oxidant gas in photochemical smog, is associated with altered pulmonary function and airway reactivity (Lippmann and Schlesinger, 2000), airway inflammation (Graham and Koren, 1990), and increased hospital admissions in people with preexisting airway diseases (Thurston et al., 1992; Wong et al., 2002). We have documented ozone-induced epithelial lesions and mucous cell metaplasia in the nasal epithelium of rats and primates (Harkema et al., 1987; Hotchkiss et al., 1991). However unlike endotoxin, ozone exposure has no effect on the mucous apparatus in axial, pulmonary airways in laboratory rodents (Harkema and Hotchkiss, 1993, Postlethwait et al., 2000).

1 To whom correspondence should be addressed at 212 Food Safety and Toxicology Building, Department of Pathobiology and Diagnostic Investigation, Michigan State University, East Lansing, MI 48824. Fax: (517) 353-9902. E-mail: harkema@msu.edu.

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these animals, ozone-induced pulmonary lesions are limited to
neutrophilic inflammation and minor epithelial injury in cen-
tricinar regions of the lung. Despite these relatively minor
responses, ozone exacerbates the severity of toxic responses of
pulmonary airways to such airborne pollutants as nitrogen
doxygen, and particulate matter (Bouthillier et al., 1998; Farman
et al., 1999; Madden et al., 2000). It is not known if ozone
would similarly worsen the toxic responses to endotoxin in
these airways.

Recent studies in our laboratory describe the toxic interac-
tion of endotoxin and ozone to produce enhanced alterations in
the nasal mucous apparatus (Fanucchi et al., 1998; Wagner et
al., 2001a). In pulmonary airways, ozone elicits neutrophilic
inflammation similar to endotoxin (Hotchkiss et al., 1989), and
we have previously shown that endotoxin-induced mucous cell
metaplasia in axial pulmonary airways of rats is dependent in
part on neutrophilic inflammation (Hotchkiss and Harkema,
1994). Using an endotoxin/ozone coexposure model, we hy-
pothesized that ozone exposure would exacerbate endotoxin-
induced mucous cell metaplasia in axial pulmonary airways of
rats. In the present study, we demonstrated, using histological,
morphological, biochemical, and molecular approaches, that
ozone exposure enhances endotoxin-induced alterations in the
mucus apparatus in rat lungs. Specifically, ozone enhanced
endotoxin-induced neutrophilic inflammation, mucin gene ex-
pression, and production and hypersecretion of mucin glyco-
proteins in rat airways.

MATERIALS AND METHODS

Animals. Thirty-six male F344/N rats (Harlan Sprague-Dawley,
Indianap-
ois, IN), 10–12 weeks of age, were randomly assigned to one of six experi-
mental groups (n = 6/group). Rats were free of pathogens and respiratory
disease, and used in accordance with guidelines set forth by the All-University
Committee on Animal Use and Care at Michigan State University. Animals
were housed two per cage in polycarbonate boxes on Cell-Sorb Plus bedding
(A&W Products, Cincinnati, OH) covered with filter lids, and had free access
to tap water and food (Tek Lad 22-5 Rodent Diet W, Harlan Sprague-Dawley,
Indianapolis, IN). Room lights were set on a 12-h light/dark cycle beginning at
21–24°C and 40–55%, respectively. During the inhalation portion of the study,
rats were housed individually in rack-mounted stainless steel wire cages in
whole-body inhalation exposure chambers (HC-100, Lab Products, Maywood,
NJ). During ozone exposure, food was removed, but water was available.

Ozone exposure. Rats were exposed to 1 ppm ozone for all studies.
National Ambient Air Quality Standards for ambient ozone are 120 ppb for 1 h
and 80 ppb for 8 h, which is exceeded during summer months in some areas
of the country where levels reach greater than 300 ppb (EPA, 2000) Dosimetry
studies suggest that rats require four- to fivefold higher doses of ozone than
humans to create an equal deposition and pulmonary inflammatory response
(Hatch et al., 1994). Therefore, 1 ppm is a reasonable exposure level from
which to make comparisons with humans.

Ozone was generated with an OREC model O3V1-O ozonizer (Ozone
Research and Equipment Corp., Phoenix, AZ) using compressed air as a source
of oxygen. Total airflow through the exposure chambers was 250 l/min
(15 chamber air changes/h). The concentration of ozone within the chambers
was monitored throughout the exposure using two Dasibi 1003 AH ambient air
ozone monitors (Dasibi Environmental Corp., Glendale, CA). Sampling probes
were placed in the breathing zone of rats within the middle of the cage racks.
The concentration of ozone during exposures was 1.0 ± 0.11 ppm (mean ±
SEM) for ozone chambers and less than 0.02 ppm for chambers receiving
filtered air.

Endotoxin instillation. Rats were anesthetized with 4% halothane in
oxygen, and 150 μl of endotoxin (lipopolysaccharide from Pseudomonas aerugi-
nosa, serotype 10), in pyrogen-free saline was instilled into each nasal passage
(total doses of 0, 2, or 20 μg). The highest dose of 20 μg elicits neutrophilic
inflammation and mucous cell metaplasia that is resolved by seven days
(Harkema and Hotchkiss, 1992; Steiger et al., 1995). Clinical human studies
use doses of 20–100 μg of inhaled endotoxin to elicit the same degrees of
pulmonary inflammation (Michel et al., 1997, 2000).

Coexposure protocol (days 1 and 2). Rats were first instilled with saline
or endotoxin, and 6 h later they were exposed to air or 1 ppm ozone for 8 h.
This dosing-exposure regimen was chosen so that endotoxin-elicted airway
neutrophils (which peak between 6 and 12 h), were present at the time of ozone
exposure. One day later, endotoxin instillation and ozone exposures were
repeated (Fig. 1).

Necropsy, lavage collection, and tissue preparation (day 5). Rats
were killed three days after the second endotoxin/ozone coexposure. Animals
were anesthetized with sodium pentobarbital (50 mg/kg), a midline laparotomy
was performed, and animals exanguinated by cutting the abdominal aorta.
Immediately after death, the trachea was exposed and cannulated, the heart and
lung were excised en bloc. The bronchus to the left lung was temporarily
closed with a hemostatic clamp, and 4 ml of sterile saline was instilled through
the tracheal cannula and withdrawn to recover bronchoalveolar lavage fluid
(BALF) from the right lung lobes. A second saline lavage was performed and
combined with the first.

After lavage, the right lung lobes were ligated and removed. The axial
conducting airway from the right caudal lobe was removed by microdissection
and homogenized in 0.5 ml Tri-Reagent (Molecular Research Center, Inc.,
Cincinnati, OH) using a post-mounted homogenizer with a 5-mm generator
(Model 250, Pro-Scientific, Inc., Monroe, CT). Samples were kept at –80°C
until further processing for RNA isolation.

The left lung was processed for histological analysis as follows. The clamp
was removed from the left bronchus, and the left lobe was inflated under
constant pressure (30 cm H2O) with zinc formalin (Anatech, Kalamazoo, MI)
for 2 h. The bronchial airway was ligated and the inflated lobe was stored in
a large volume of the same fixative for at least 24 h until further processing.
The intrapulmonary airways of the fixed left lung lobe from each rodent was
microdissected according to a modified version of the technique of Plopper et
al. (1983) and fully described in one of our previous publications (Harkema

![FIG. 1. Experimental design summary and treatment protocol for endo-
toxin instillation and ozone exposure. Animals were instilled with either saline
or endotoxin on days 1 and 2. Six h after each instillation, animals were
exposed to filtered air or 1 ppm ozone for 8 h. Animals were sacrificed on day
5, 72 h after the last endotoxin instillation and ozone exposure.](https://academic.oup.com/toxsci/article-abstract/74/2/437/1716359/2114079)
and Hotchkiss, 1992). Beginning at the lobar bronchus, airways will be split down the long axis of the largest daughter branches (i.e., main axial airway; large diameter conducting airway) through the twelfth airway generation. Tissue blocks that transverse the entire lung lobe at the level of the fifth and eleventh airway generation of the main axial airway were excised and processed for light microscopy and morphometric analyses. The tissue blocks were embedded in paraffin, and 5-6 μm thick sections were cut from the anterior surface. Lung sections were stained with hematoxylin and eosin (H&E) for routine histopathology or with Alcan Blue (pH 2.5)/Periodic Acid-Schiff (AB/PAS) to detect intraepithelial mucous substances.

**Bronchoalveolar Lavage**

**Cellularity.** Total leukocytes in BALF were enumerated with a hemocytometer, and fractions of neutrophils, macrophages, and lymphocytes were determined in a cytoospin sample stained with Diff-Quick (Dade Behring, Newark, DE).

**Secreted mucosubstances.** Secreted mucosubstances recovered in BALF fluid was determined by an ELISA for mucin glycoprotein 5AC using a mouse monoclonal antibody to the human MUC5AC protein (Mucin 5AC Ab-1, NeoMarkers, Fremont, CA) that has reactivity to the rat MUC5AC core protein. Fifty microliter aliquots of BALF were applied to a 96-well microtiter plate (Microfluor 2 Black, Dynex Technologies, Chantilly, VA) and dried overnight at 40°C. Plates were blocked with a solution of 1.5% horse serum and 2% rat serum in Automation Buffer Solution (ABS, pH 7.5; Biomedia Corp., Foster City, CA) for 30 min at 37°C. Plates were then incubated with anti-rMuc5AC antibody (1:400 in ABS containing 1.5% horse serum) for 1 h at 37°C and then washed three times with ABS. Bound primary antibody was detected with a biotinylated rabbit anti-mouse secondary antibody and quantitated using horse-radish-peroxidase-conjugated avidin/biotin complex (ABC Reagent; Vector Laboratories, Burlingame, CA) and a fluoroscent substrate (QuantaBlue; Pierce Chemical, Rockford, IL) using a fluorescence microplate reader (SpectraMax Gemini; Molecular Devices; 318 nm excitation/410 nm emission). Readings were taken at 3 min intervals for 24 min. Duplicate samples were averaged and the group data is represented as mean Vmax units/s.

**Lavaged elastase.** Airway elastase recovered in BALF was determined by an ELISA for elastase using a rabbit monoclonal antibody to the human elastase (Calbiochem, La Jolla, CA). Fifty microliter aliquots of BALF were applied to a 96-well microtiter plate (Microfluor 2 Black, Dynex Technologies, Chantilly, VA) and dried overnight at 40°C. Plates were blocked with a solution of 1.5% goat serum and 2% rat serum in Automation Buffer Solution (ABS, pH 7.5; Biomedia Corp., Foster City, CA) for 30 min at 37°C. Plates were then incubated with anti-elastase antibody (1:400 in ABS containing 1.5% goat serum) for 1 h at 37°C and then washed three times with ABS. Bound primary antibody was detected with a biotinylated goat anti-rabbit secondary antibody and quantitated using horse-radish-peroxidase-conjugated avidin/biotin complex (ABC Reagent; Vector Laboratories, Burlingame, CA) and a fluoroscent substrate (QuantaBlue; Pierce Chemical, Rockford, IL) using a fluorescence microplate reader (SpectraMax Gemini; Molecular Devices; 318 nm excitation/410 nm emission). Readings were taken at 3 min intervals for 24 min. Duplicate samples were averaged and the group data is represented as mean Vmax units/s.

**Morphometry of stored intraepithelial mucosubstances.** To estimate the amount of the intraepithelial mucosubstances (IM) in respiratory epithelium lining axial airways, the volume density (Vs) of AB/PAS-stained mucosubstances was quantified using computerized image analysis and standard morphometric analyses. The area of AB/PAS stained mucosubstance was calculated from the automatically circumscribed perimeter of stained material using a Power Macintosh 7100/66 computer and the public domain NIH Image program (written by Wayne Rasband, U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). The length of the basal lamina underlying the surface epithelium was calculated from the contour length of the digitized image of the basal lamina. The volume of stored mucosubstances per unit of surface area of epithelial basal lamina was estimated using the method described in detail by Harkema et al. (1987). The Vs of intraepithelial mucosubstances is expressed as nanoliters of intraepithelial mucosubstances per mm² of basal lamina.

**Morphometry of epithelial cell numeric density.** The numeric epithelial cell density was determined by counting the number of epithelial cell nuclear profiles in the surface epithelium and dividing by the length of the underlying basal lamina. The length of the basal lamina was calculated from its contour length in a digitized image using the NIH image system described above.

**RNA isolation.** Total RNA was isolated from microdissected, homogenized axial airways by following the method of Chomczynski and coworkers (Chomczynski and Mackey, 1995; Chomczynski and Sacchi, 1987). Isolated RNA pellets were resuspended in nuclease-free water and incubated with DNase solution (100 units rRNasin [Promega, Madison, WI], 100 mM DTT [Life Sciences Technology Inc., Island, NY], and 10 units DNase I [Boehringer Mannheim, Indianapolis, IN] in 5X transcription buffer [Promega]) for 45 min at 37°C. The RNA was extracted sequentially with equal volumes of phenol/chloroform/isooamy alcohol (25:24:1) and chloroform/isoamy alcohol (24:1), and precipitated with 10 M ammonium acetate and isopropanol. The pellet was washed with 75% ethanol, air dried, and resuspended in nuclease-free water containing rRNAs (40 units/100 μl). RNA concentrations were determined with a fluorescence RNA-binding assay (Ribogreen; Molecular Probes, Eugene, OR), using a SpectraMax GEMINI spectrophuorometer (Molecular Devices Corp., Sunnyvale, CA).

**Quantitative RT-PCR (reverse transcriptase polymerase chain reaction).** Steady state levels of rMuc-5AC mRNA were determined in rat airway from ozone-exposed rats using a quantitative RT-PCR technique. Muc5AC is a specific protein for secretory mucin glycoproteins that is expressed in secretory epithelial cells and not in other cells of the airway. As such, RT-PCR of airway RNA was used to estimate the rMuc-5AC mRNA that is present in epithelium. The quantitative RT-PCR technique employs a recombinant competitor RNA (rcRNA), used as an internal standard (IS), that is reverse transcribed and amplified in the same tubes as the target sequence (rMuc-5AC). The IS rcRNA was synthesized as described previously (Fanucchi et al., 1999). The IS contains the same sequences recognized by the amplification primers for rMuc-5AC, but has a different-sized intervening sequence and therefore yields a different-sized RT-PCR product. The concentration of rMuc-5AC mRNA was estimated by adding increasing, known amounts of IS (in numbers of molecules) to the RT-PCR mixtures that contain a constant, unknown amount of sample RNA. Because both the IS and sample RNA are amplified at the same rate, this procedure results in an absolute experimental readout (molecules of target gene mRNA per unit sample).

RT-PCR for rMuc-5AC was performed as outlined by Gilliland and coworkers (Gilliland et al., 1990a,b), except that known amounts of the IS rcRNA were reverse-transcribed into complementary DNA (cDNA) in a volume of 20 μl containing PCR buffer plus 5 mM MgCl₂, 1 mM each dNTP, 10 units rRNasin, 125 ng oligo(dT)₁₂₋₁₈ (Becton Dickinson, Bedford, MD), 100 ng total RNA from maxilloturbulantes, and 40 units of MMLV reverse transcriptase (Promega). For each RNA sample from individual animals, a known concentration of IS rcRNA molecules was added that was similar in concentration to the RNA samples. This was determined in a preliminary range-finding experiment using pooled samples of each experimental group to be between 10⁻⁹ and 10⁻¹⁰ molecules per sample. A standard curve was also prepared by adding tenfold serial dilutions of the IS (10⁻⁹ to 10⁻¹⁰ molecules per tube) to a constant amount of RNA (pooled from all samples). All RNA samples were then incubated at 42°C for 15 min, followed by an incubation at 95°C for 4 min. A PCR master-mix consisting of PCR buffer, 4 mM MgCl₂, 6 pmol each of rMuc-5AC forward (5'–CATCATTCTTGGTTAGGCTGAGG-3') and reverse (5’–GGTACCAGGTCTACACTCCTGG-3’) primers, and 1.25 units Taq DNA polymerase were added to the cDNA samples, for a final volume of 30 μl (Taq polymerase was added to the PCR master-mix after it had been heated to 85°C for 5 min). Samples were then immediately heated to 95°C for 4 min and then cycled 36 times at 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s, after which an additional final extension step at 72°C for 10 min was included.
PCR products (10 μl) were electrophoresed on a 3% agarose gel (NuSieve 3:1; FMC Bioproducts, Rockland, ME) and visualized by ethidium bromide staining. Densitometry was carried out using a Bio-Rad ChemiDoc image acquisition system and Quantity One (v4.0) quantitation software (Bio-Rad, Hercules, CA), running on a Dell OptiPlex GX1 computer. The density ratio of the rMuc-5AC PCR product band to the corresponding IS PCR product band present in each sample was determined as described by Gilliland and colleagues (Gilliland et al., 1990a,b). A standard curve was constructed by plotting the log of the density ratio (i.e., rMuc-5AC PCR product band/IS PCR product band) versus the log of IS serial dilution concentrations added to the standards (i.e., 10⁴–10⁹ molecules/tube). Linear regression was performed on the standard curve to determine slope and y-intercept, which yielded the amount of rMuc-5AC mRNA (molecules) present in the pooled RNA standard sample when mRNA/IS = 1.

The rMuc-5AC mRNA value was then divided by each IS serial dilution concentration to arrive at an “actual” ratio. A transformed standard curve was then calculated by plotting the original density ratio versus the actual ratio, and linear regression was performed. The transformed standard curve was used to calculate the single point measurements of the experimental samples, which were obtained with the following equation:

\[
\text{actual mRNA (molecules)} = \frac{\text{actual IS} \times 10^{[\log(\text{density ratio})-y \text{-intercept/slope}]}}{\text{slope}}
\]

Data are expressed as the number of rMuc-5AC mRNA molecules per ng of total sample RNA that was added to the RT-PCR reaction.

Statistical analysis. Data are expressed as mean ± standard error of the mean (SEM). Data were analyzed using a completely randomized analysis of variance. Multiple comparisons were made by Student-Newman-Keuls post hoc test. Criterion for significance was taken to be \( p \leq 0.05 \).

RESULTS

Histopathology

Rats instilled with endotoxin and exposed to filtered air. Intranasal instillations of endotoxin induced focal areas of mild reddening and consolidation of the pulmonary parenchyma principally surrounding the main axial airways of the right and left lung lobes. These gross lesions were usually most severe near the hilar regions, but similar, but less severe, lesions were often found in the mid and distal aspects of the lobes. Approximately 25–50% of the parenchyma were affected in the animals receiving 20 μg of endotoxin. Slightly less severe gross and microscopic pulmonary lesions were observed in rats instilled with 2 μg of endotoxin.

Microscopically, the affected areas of lung consisted of a mild-moderate acute bronchopneumonia. The principal features in the alveolar parenchyma included mild congestion of alveolar capillaries, interstitial edema within alveolar septa, type II cell hyperplasia and a mixed inflammatory cell infiltrate of mononuclear cells (monocytes/macrophages and lymphocytes) and neutrophils. Aggregates of large vacuolated alveolar macrophages containing phagocytized cellular debris were widely scattered throughout the affected areas of the alveolar parenchyma along with modest amounts of eosinophilic proteineous material in the alveolar lumens (pulmonary edema). In addition, the preterminal and terminal bronchioles in the areas of pneumonia often had hypertrophic surface epithelium. Intersitial edema and accumulation of mononuclear cells, neutrophils, and some eosinophils also surrounded these distal airways and adjacent pulmonary arteries.

The principal lesion in the main intrapulmonary axial airways, and several of the other large diameter, preterminal bronchioles branching off of the axial airways, was a conspicuously thickened, columnar surface epithelium with numerous mucous (goblet) cells containing copious amounts of AB/PAS-stained mucosubstances (mucous cell metaplasia; Fig. 2). This endotoxin-induced mucous cell metaplasia occasionally extended into the surface epithelium lining more distal preterminal and terminal airways.
Rats instilled with saline and exposed to ozone. Repeated ozone exposures induced site-specific lesions that were restricted to cen triacini throughout the lung lobes. The centriacinar lesions were characterized by a mild neutrophilic inflammation (aveolitis/bronchiolitis), increased numbers of alveolar macrophages, and regenerative hyperplasia centered predominantly in terminal bronchioles and proximal alveolar ducts. Mucous cell metaplasia, a principal feature in the endotoxin-instilled rats, was not present in the airway epithelium lining the large- and small-diameter conducting airways (Fig. 2).

Rats instilled with endotoxin and exposed to ozone. The lungs of animals that were instilled with endotoxin and exposed to ozone had gross and microscopic lung lesions that were characteristic of both toxicants, described above. However, the mucous cell metaplasia in the axial airways of these coexposed rats was more severe (see morphometric analyses below) than that observed in the rats instilled with endotoxin but exposed only to filtered air (0 ppm ozone).

Rats instilled with saline and exposed to filtered air. No exposure-related histologic lesions were present in the lungs of control rats exposed only to filtered air and intranasally instilled with saline.

Bronchoalveolar Lavage

Cellularity. Endotoxin instillation caused a dose-dependent increase of neutrophils and lymphocytes recovered in BALF from rats (Figs. 3 and 4). By comparison, ozone exposure alone caused significant accumulations of BALF macrophages but not of neutrophils or lymphocytes. Exposure of rats to ozone enhanced by twofold the numbers of BALF neutrophils elicited by instillation with 20 μg of endotoxin, and of BALF lymphocytes elicited by 2 μg endotoxin.

Secreted mucosubstances. Instillation of rats with 20 μg, but not 2 μg, endotoxin caused a significant increase (16% increase) in Muc5AC recovered in BALF (Fig. 5). Ozone exposure alone did not alter the amount of Muc5AC in BALF. However, ozone exposure caused the potentiation of BALF Muc5AC in rats instilled with 2 μg endotoxin (100% increase), and enhanced Muc5AC in rats instilled with 20 μg endotoxin (33%).

FIG. 3. Effects of endotoxin instillation and ozone exposure on neutrophil accumulation in bronchoalveolar lavage fluid (BALF). Animals were instilled with endotoxin and exposed to 0 or 1 ppm ozone for two consecutive days. Seventy-two h later animals were sacrificed and neutrophils were enumerated in BALF as described in Materials and Methods. Data is expressed as mean ± SEM; n = 6; a = significantly different from respective control instilled with saline (0 μg endotoxin), b = significantly different from respective control exposed to air, c = significantly different from respective control instilled with 2 μg endotoxin.

FIG. 4. Effects of endotoxin instillation and ozone exposure on lymphocyte and macrophage accumulation in bronchoalveolar lavage fluid (BALF). Animals were instilled with endotoxin and exposed to 0 or 1 ppm ozone for two consecutive days. Seventy-two h later animals were sacrificed and lymphocytes and macrophages were enumerated in BALF as described in Materials and Methods. Data is expressed as mean ± SEM; n = 6; a = significantly different from respective control instilled with saline (0 μg endotoxin), b = significantly different from respective control exposed to air, c = significantly different from respective control instilled with 2 μg endotoxin.
Lavage elastase. Ozone exposure alone had no effect on elastase concentration in BALF (Fig. 6). Instillation with endotoxin caused a dose-dependent increase in BALF elastase (33% increase after 2 μg endotoxin; 75% increase after 20 μg).

Ozone exposure enhanced endotoxin-induced increases in elastase after both 2 μg (22% increase) and 20 μg endotoxin (15% increase).

Morphometry

Intraepithelial mucosubstances. Instillation of rats with 2 or 20 μg of endotoxin caused mucous cell metaplasia in the respiratory epithelium lining proximal and distal airways as indicated by significant increases in the volume density of intraepithelial mucosubstances (Fig. 7). Endotoxin-induced mucous cell metaplasia was dose-dependent in distal airways. Exposure of rats to ozone alone did not alter amounts of

FIG. 5. Effects of endotoxin instillation and ozone exposure on mucin secretion detected in bronchoalveolar lavage fluid (BALF). Animals were instilled with endotoxin and exposed to 0 or 1 ppm ozone for two consecutive days. Seventy-two h later animals were sacrificed and concentrations of mucin glycoprotein 5AC in BALF was determined as described in Materials and Methods. Data is expressed as mean ± SEM; n = 6; a = significantly different from respective control instilled with saline (0 μg endotoxin), b = significantly different from respective control exposed to air, c = significantly different from respective control instilled with 2 μg endotoxin.

FIG. 6. Effects of endotoxin instillation and ozone exposure on elastase detected in bronchoalveolar lavage fluid (BALF). Animals were instilled with endotoxin and exposed to 0 or 1 ppm ozone for two consecutive days. Seventy-two h later animals were sacrificed and concentrations of elastase in BALF was determined as described in Materials and Methods. Data is expressed as mean ± SEM; n = 6; a = significantly different from respective control instilled with saline (0 μg endotoxin), b = significantly different from respective control exposed to air, c = significantly different from respective control instilled with 2 μg endotoxin.

FIG. 7. Effects of endotoxin instillation and ozone exposure on volume density of intraepithelial mucosubstances in proximal and distal axial pulmonary airways. Animals were instilled with endotoxin and exposed to 0 or 1 ppm ozone for two consecutive days. Seventy-two h later animals were sacrificed and tissues collected and processed as described in Materials and Methods. Data is expressed as mean ± SEM; n = 6; a = significantly different from respective control instilled with saline (0 μg endotoxin), b = significantly different from respective control exposed to air, c = significantly different from respective control instilled with 2 μg endotoxin.
intraepithelial mucosubstances when compared to air-exposed animals. However, ozone exposure significantly enhanced endotoxin-induced mucous cell metaplasia in both proximal and distal airways.

**Epithelial cell density.** Endotoxin instillation did not alter the numeric cell density in the respiratory epithelium of proximal and distal airways (Fig. 8). In addition, changes in epithelial cell density were not significant in rats exposed to ozone alone, or coexposed to ozone and endotoxin.

**Mucin-Specific Gene Expression**

**Mucin (rMuc-5AC) mRNA.** Significant increases in expression of rMuc 5AC mRNA in axial conducting airways of rats was induced by instillation with 2 and 20 μg endotoxin (Fig. 9). Exposure to ozone alone caused a significant decrease in steady state levels of mucin mRNA. By comparison, increased expression of rMuc-5AC by 20 μg endotoxin was enhanced when rats were coexposed to ozone.

**DISCUSSION**

Exposure of rat airways to bacterial endotoxin elicits the mobilization of soluble and cellular inflammatory mediators that precede epithelial alterations and the hyperproduction and secretion of mucin glycoproteins. Inhalation of ozone is well documented to cause airway inflammatory and epithelial responses in rodents, but in contrast to endotoxin exposure, the mucus apparatus lining pulmonary airways is relatively unaffected. In the present study, exposure of rats to ozone potentiated airway alterations induced by endotoxin. Specifically, ozone enhanced the endotoxin-induced increases in airway neutrophil influx, lavage elastase, mucus secretion, storage of intraepithelial mucosubstances, and mucin gene expression.

The mechanism by which endotoxin promotes mucus production in airway epithelium is unknown. In cultured airway cells and nasal explants, endotoxin administered in vitro directly induces mucin gene expression, apparently in the absence of secondary mediator(s) (Hotchkiss et al., 1998; Li et al., 1997). In animal studies, endotoxin elicits production in airways of several mediators known to induce mucin gene expression and mucous cell metaplasia, including TNF-α, IL-1, platelet activating factor, and neutrophil-derived elastase. Thus, in addition to direct effects of endotoxin on airway
epithelium, many soluble mediators may contribute to endotoxin-induced mucous cell metaplasia in vivo.

We have demonstrated in F344 rats that endotoxin-induced mucous cell metaplasia is partially dependent on neutrophils. In endotoxin instilled rats depletion of circulating neutrophils blocks neutrophilic inflammation completely, and significantly inhibits mucous cell metaplasia in both nasal and pulmonary airways by approximately 60% (Hotchkiss and Harkema, 1994; Wagner et al., 2001a). In the present study, ozone enhanced both airway neutrophil accumulation and increases in mucus storage and secretion that was elicited by the instillation of 20 μg endotoxin. These data suggest that ozone’s effects may be mediated by neutrophils or neutrophil-derived products. Because endotoxin-induced mucous cell metaplasia is driven by neutrophilic inflammation, enhancement by ozone may be due simply to increased numbers or the activation of neutrophils. Data was collected at a single timepoint, 72 h after the last endotoxin instillation and ozone exposure. Thus, is it unknown if ozone caused a higher peak in numbers of neutrophils elicited early after endotoxin instillation, or if it caused their persistence in airways beyond that invoked by endotoxin alone.

We began ozone exposures 6 h after instillation with endotoxin, at a time when airway neutrophil recruitment induced by endotoxin is near maximal. Significant numbers of airway neutrophils were therefore exposed directly to ozone. Neutrophil response to ozone exposure has not been adequately characterized in vitro or in vivo, but ozone exposure of monocyctic and epithelial cells in culture systems in vitro elicits their production of inflammatory mediators (Jaspers et al., 1997; Samet et al., 1992). Similarly, ozone may directly stimulate neutrophils to undergo degranulation or oxidative burst within airways. In the present study, ozone significantly increased airway elastase concentrations induced by instillation with 2 μg endotoxin without increasing neutrophil numbers. These data suggest that airway neutrophils in endotoxin-instilled, ozone-data-exposed rats were more activated to produce inflammatory mediators than airway neutrophils in endotoxin-instilled rats breathing filtered air. Using a specific elastase inhibitor, we have recently demonstrated that endotoxin-induced mucous cell metaplasia in F344 rats is elastase dependent (Wagner et al., 2002b). Elastase has been implicated in ozone-induced airway reactivity and mucus hypersecretion (Matsumoto et al., 1999; Nogami et al., 2000). Thus, although elastase was not detected in BALF from saline-instilled and ozone-exposed rats, ozone may be enhancing endotoxin-induced mucous cell metaplasia by the same, elastase-dependent mechanism.

It is notable that ozone’s effects are not additive with those induced by endotoxin. Indeed, ozone exposure alone did not engender significant responses of neutrophilic inflammation, BALF elastase accumulation, mucus secretion and storage, or gene expression. Ozone served only to enhance or act synergistically with endotoxin. Endotoxins can initiate cellular responses by binding to the CD14 receptor on inflammatory cells, or to the soluble form of CD14 to interact with epithelial and endothelial cells (Heumann and Roger, 2002). A significant number of recent studies show that endotoxin also binds and activates Toll-like receptors (TLR), a previously described class of cell surface receptors that are linked intercellularly to NF-κB pathways and are important in innate and adaptive immune responses (Modlin, 2002). TLRs are pattern recognition receptors that are activated by lipids and lipid moieties on lipopolysaccharides, lipoproteins, and peptidoglycans, and thereby recognize a variety of bacterial, viral, and fungal products. However, at least one animal model suggests that TLRs are also important in ozone-induced lung injury. Using mice that are deficient in TLR-4, a specific Toll-like receptor, Kleeberger and coworkers (2000, 2001) showed that TLR-4 is necessary to fully develop ozone-induced lung permeability. It was also demonstrated that induction of pulmonary Tlr4 gene expression by ozone was required for injury in this model. In these studies, mice were exposed to 0.3 ppm ozone, whereas in the present study we exposed rats to 1 ppm. In our model, we hypothesize that ozone’s ability to upregulate TLR4, and thereby provide more available receptors to transduce cellular responses to endotoxin, may serve to prolong and magnify the effects of endotoxin on epithelial and inflammatory cells. Other studies suggest that upregulation of Tlr genes in both inflammatory and epithelial cells is important for prolonging the inflammatory processes (Modlin, 2002). Extending our exposure regimen to employ more and lower doses of ozone, and assessing gene induction (i.e., Tlr4) at earlier timepoints may support the hypothesis that TLR-4 mediates the ozone-induced enhancement we observe in this animal model.

Our results illustrate a unique interaction between two airborne toxicants to alter airway epithelium that would not have been predicted from the known toxicological profile of either pollutant given alone. Ozone enhanced the toxicity of endotoxin, a ubiquitous biogenic substance, at ozone exposure concentrations that alone are nontoxic to rat airways. Because safety standards of air pollutants are primarily based on the toxicological effects of a single pollutant (e.g., ozone), it is possible that the health risk of breathing a mixture of pollutants is underestimated. Our demonstration of ozone enhancement of endotoxin-induced mucous cell metaplasia supports this premise. It is notable that the doses of endotoxin and ozone used in this study are reasonable models for human exposures. Humans living in highly polluted areas and that have high domestic or occupational exposure to endotoxin may be at an increased risk to adverse health effects. Conversely, individuals with a polymorphism in TLR4 and have altered response to inhaled endotoxin may exhibit less sensitivity to coexposures (Schwartz, 2001). Controlled, human exposures are required to test this hypothesis. Furthermore, our results with ozone and endotoxin might be extended to predict the potential airway responses to exposures to other oxidant gases and biogenic substances. Indeed we have recently demonstrated ozone enhancement of airway lesions induced in allergic airways (Wag-
ner et al., 2002a) and by instillation with vanadium (Wagner et al., 2001b). Further research is needed to elucidate the mechanism of these toxic interactions and the role played by inflammatory cells and their soluble mediators.

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REFERENCES


