Antioxidant transport modulates peripheral airway reactivity and inflammation during ozone exposure

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Freed, Arthur N., Rafael Cueto, and William A. Pryor. Antioxidant transport modulates peripheral airway reactivity and inflammation during ozone exposure. J. Appl. Physiol. 87(5): 1595–1603, 1999.—We examined the effects of ozone (O3) and endogenous antioxidant transport on canine peripheral airway function, central airway function, epithelial integrity, and inflammation. Dogs were either untreated or pretreated with probenecid (an anion-transport inhibitor) and exposed for 6 h to 0.2 parts/million O3. Peripheral airway resistance (Rpa) and reactivity (ΔRpa) were monitored in three sublobar locations before and after exposure to either air or O3. Pulmonary resistance and transepithelial potential difference in trachea and bronchus were also recorded. Bronchoalveolar lavage fluid (BALF) was collected before, during, and after exposure. O3 increased Rpa and ΔRpa only in probenecid-treated dogs and in a location-dependent fashion. Pulmonary resistance and potential difference in bronchus increased after O3 exposure regardless of treatment. O3 markedly increased BALF neutrophils only in untreated dogs. With the exception of hexanal, O3 did not alter any BALF constituent examined. Probenecid reduced BALF ascorbate, BALF protein, and plasma urate. We conclude that 1) a 6-h exposure to 0.2 parts/million O3 represents a subthreshold stimulus in relation to its effects on peripheral airway function in dogs, 2) antioxidant transport contributes to the maintenance of normal airway tone and reactivity under conditions of oxidant stress, 3) O3-induced changes in Rpa and ΔRpa are dependent on location, and 4) peripheral airway hyperreactivity and inflammation reflect independent responses to O3 exposure. Finally, although anion transport mitigates the effect of O3 on peripheral airway function, it contributes to the development of airway inflammation and may represent a possible target for anti-inflammatory prevention or therapy.

airway hyperreactivity; anion transport; bronchoalveolar lavage; dog; lung; neutrophils; transepithelial potential difference; urate; vitamin C

EXPOSURE TO RELATIVELY LOW concentrations of ozone (O3) impairs pulmonary function and enhances nonspecific airway reactivity in animals (1, 33) and in normal (21) and asthmatic human subjects (27, 30). O3 exposure also causes airway mucosal damage throughout the tracheobronchial tree (8, 25), airway edema, and inflammation (8, 31, 52), and these O3-induced effects may contribute to the development of airway hyperreactivity. Potentially counterbalancing these destructive processes is the airway surface fluid, which is composed of plasma ultrafiltrate and locally secreted substances including α-tocopherol, albumin, ascorbate, ceruloplasmin, glutathione, lactoferrin, polyunsaturated fatty acids (PUFA), urate, and transferrin (11, 12, 20, 57). All of these substances are capable to some degree of scavenging oxygen-derived radicals and providing antioxidant protection to the epithelium (11).

O3-induced changes in small airway function are difficult to detect by using conventional pulmonary function tests. Thus most research has focused on O3-induced damage in the terminal airways and proximal alveoli (4, 7, 8) and has not addressed the relationship between peripheral airway injury and changes in peripheral airway resistance (Rpa) and airway reactivity (ΔRpa). O3-induced changes in Rpa and ΔRpa have been examined in dogs (19), but those experiments focused on local responses to high concentrations of O3 delivered directly into the peripheral lung via a bronchoscope. Weinman et al. (58, 59) reported significant O3-induced reductions in volume-adjusted forced expiratory flow rates measured at intermediate and low lung volumes (isoV FEF 25–75) in normal human subjects and interpreted this as impairment of small airway function. However, despite the development of an inflammatory response, Rpa was unaffected by O3 exposure (59). It is important to note that isoV FEF 25–75 and Rpa are unlikely to reflect peripheral airway function at the same location. In fact, Rpa is a measurement believed to be dominated by respiratory bronchioles and alveolar ducts (39), whereas isoV FEF 25–75 probably reflects “small” airways of unknown size. This difference may explain why isoV FEF 25–75 and Rpa are not similarly affected by O2 (58, 59). However, the relationship between O3-induced airway inflammation and impairment of peripheral airway function remains uncertain.

The purpose of this study was to examine the effects of O3 and endogenous antioxidant transport on canine Rpa and ΔRpa throughout the lung. We first examined the effect of a 6-h exposure to 0.2 parts/million (ppm) O3 on Rpa, ΔRpa, and several indexes of inflammation and injury in anesthetized dogs. We monitored bronchial transepithelial potential difference (PDtr) and concentrations of neutrophils, epithelial cells, total protein, peroxides, and aldehydes in bronchoalveolar lavage fluid (BALF) to determine whether any of these local markers were associated with peripheral airway dysfunction. We also documented BALF ascorbate, trolox equivalent antioxidant capacity (TEAC), and plasma urate to determine whether these potentially protective peripheral lung constituents were locally altered after acute O3 exposure. In addition, we recorded pulmonary resistance (RL) and tracheal transepithelial potential
difference \((PD_{tr})\) to determine whether changes in these central airway measurements occurred in association with or as paralleled changes in peripheral airway function.

We then examined the role of antioxidants in modulating oxidant stress in the lung periphery. Unlike other studies that used exogenous antioxidant supplementation to protect the lung from oxidant-induced injury (9, 49, 61), we interfered with endogenous antioxidant activity and evaluated its effect on O3-exposed peripheral airways. Specifically, we used probenecid (an anion-transport inhibitor) to inhibit antioxidant transport. Probenecid reduces urate levels in human peripheral airways. Specifically, we used probenecid to inhibit antioxidant transport with probenecid should amplify peripheral airway responses to O3.

**METHODS**

**Experimental Techniques**

Animal handling and preparation. Dogs were handled and maintained in accordance with the standards set forth in the Policy and Procedures Manual published by the Johns Hopkins University School of Hygiene and Public Health's Animal Care and Use Committee.

Peripheral airway preparation. Male mongrel dogs were anesthetized with pentobarbital sodium (18 mg/kg iv) and maintained on pentobarbital sodium (4 mg·kg\(^{-1}\)·h\(^{-1}\)·iv). Anesthetic depth was assessed by canthal reflex, heart rate, blood pressure, and the presence of spontaneous movement or breathing. After placement of an esophageal balloon, dogs were intubated with a stainless steel endotracheal tube and ventilated (17 ml/kg) on room air with a constant-volume ventilator (Harvard Apparatus, Holliston, MA). End-expiratory \(\text{CO}_2\) was monitored with a CO2 analyzer (LB-2, Beckman, Anaheim, CA) and maintained around 4.5% by adjusting ventilator frequency. Heart rate and blood pressure were monitored noninvasively throughout all experiments (Datascop ACCUTOR 1A; Datascop, Paramus, NJ). Rectal temperature was monitored with a thermistor (Yellow Springs Instrument, Yellow Spring, OH) and maintained with a warming pad.

Measurement of \(R_{l}\). Measurements of \(R_{l}\) were obtained by using a forced-oscillation method similar to that previously described (17). Briefly, with the ventilator momentarily stopped, airflow \((V)\) was recorded with a pneumotachograph attached to the tracheal tube and was oscillated sinusoidally at 4 Hz for 2 s by a loudspeaker while we simultaneously measured transpulmonary pressure \((P_{tp})\). \(P_{tp}\) was measured by using a differential transducer connecting the esophageal balloon to a catheter positioned 2 cm past the distal end of the endotracheal tube. \(R_{l}\) was calculated by relating the real portion \((Re)\) of the pressure differences along the airways to \(V\) at the trachea, i.e., \(R_{l} = Re(P_{tp})/V\).

Measurement of \(R_{pa}\). A bronchoscope (Olympus BF Type P10, OD = 5.5 mm, Olympus Corp. of America, New Hyde Park, NY) was visually guided into a sublobar segment until the tip occluded the bronchus. A map of the airway branching pattern was made and used at various times throughout the study to relocate the sublobar location. A polyethylene catheter (PE 190: 1.2 mm ID, 1.7 mm OD) attached to a pressure transducer was threaded through the port of the bronchoscope and was used to record pressure \((P_{b})\) at the tip of the scope. The wedged segment was ventilated with 200 ml/min of 5% \(\text{CO}_2\) in air delivered around the catheter and through the bronchoscope. \(R_{pa}\) was measured by stopping the ventilator at functional residual capacity. Under these conditions, \(P_{b}\) decays to a plateau pressure greater than the surrounding alveolar pressure \((\text{atmospheric})\). Thus \(R_{pa} = (cmH_2O·ml^{-1}·s^{-1}) = P_{b}/(200 ml/min)/1ml/60 s\) at 4°C and then centrifuged.

Measurement of \(\Delta R_{pa}\). Airway reactivity \((\Delta R_{pa} = R_{pa\text{max}} - R_{pa})\) was assessed 30 s after challenge \((R_{pa\text{max}})\) with a single dose of nebulized histamine (50 mg/ml for 30 s).

Acute whole lung exposure to \(\text{O}_3\). Room air was pumped through a ventilator with a valve that directed air into a stainless steel flow-through chamber containing a low-pressure Hg lamp \((\text{O}_3\) generator) and then into a 4-liter glass mixing chamber. The valve allowed the bulk of the ventilator output to bypass the \(\text{O}_3\) generator, which was then mixed with the \(\text{O}_3\) in the glass chamber before entering the lungs via a Teflon tube. The inhaled \(\text{O}_3\) concentration was sampled every 30 s at the level of the stainless steel endotracheal tube with an \(\text{O}_3\) monitor (model 1003-AH, Daisi Environmental, Glendale, CA) and fed back to a computer that adjusted airflow through the valve leading to the \(\text{O}_3\) generator. Temperature and relative humidity were maintained at 21–23°C and ~55%, respectively.

Measurement of \(PD_{a}\) and \(PD_{tr}\). \(PD_{a}\) was measured via two 3 M KCl/3.5% agar-filled polyethylene-tube bridges (PE 190: 1.2 mm ID, 1.7 mm OD) connected to a pair of calomel half cells and a high-input impedance voltmeter. The recording bridge rested on the inner surface of the midtrachea, and the reference bridge was inserted in the subcutaneous tissue of the neck via a needle. In a similar fashion, two agar-filled bridges were used to monitor \(PD_{tr}\) in a small airway located in the lower lobe.

Analysis of BALF. TOTAL AND DIFFERENTIAL CELL COUNTS. Bronchoalveolar lavage (BAL) was done by using three 20-ml aliquots of warm \((38°C)\) isotonc Hanks’ balanced salt solution. BALF was delivered via a PE 190 catheter that was threaded through the suction port of the bronchoscope. The 20-ml syringes and PE 190 catheter were used to gently suction the BALF from the wedged sublobar segment. BALF samples were temporarily stored at 4°C and then centrifuged at 4°C for 15 min at 1,350 rpm. The cell pellet from a 5-ml sample was resuspended in 1 ml of supernatant, and a 10-µl sample was placed on a hemocytometer to determine total cell number. Macrophages, lymphocytes, neutrophils, eosinophils, and epithelial cells were counted after being stained with Diff-Quik. Trypan blue exclusion was used to document cell viability.

MEASUREMENT OF ASCORBATE AND TEAC IN BALF. For the ascorbate assay, 10 ml of BALF were treated at the time of recovery with 3% perchloric acid and stored at ~70°C. From this sample, 0.5 ml of supernatant was mixed with 0.1 ml of 2,4-dinitrophenylhydrazine-thiourea-copper solution and incubated for 3 h at 37°C. The sample was then mixed with 0.75 ml of ice-cold 65% \(\text{H}_2\text{SO}_4\) and allowed to stand at room temperature for an additional 30 min. Absorbance at 520 nm was then measured (45). Ascorbic acid standards were measured daily to ensure optimal calibration. The standards were prepared in 3% perchloric acid to match the samples as closely as possible. These standards yielded linear calibration curves in the range of 0.2–2 µg/ml.

A spectrophotometric technique was used to quantify the TEAC of BALF. This technique measures the relative ability of antioxidants to scavenge 2,2’-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) radical cation in comparison with the antioxidant efficacy of standard quantities of a water-soluble...
vitamin E analog (Trolox). The TEAC of BALF recovered from air and O₃-exposed lungs was measured at 734 nm in Trolox equivalents (mmol/l) (38).

**MEASUREMENT OF PEROXIDES, TOTAL PROTEIN, AND ALDEHYDES IN BALF.** A commercial quantitative peroxide assay (PeroxOQuant, Pierce, Rockford, IL) was used to quantify hydrogen peroxide and organic hydroperoxides in BALF, where the peroxides are expressed as equivalents of H₂O₂ µmol/l. In this assay, H₂O₂ reacts with sorbitol to form peroxyl radical, which then oxidizes ferrous to ferric ion in the presence of xylenol orange to yield a purple product with a maximum absorbance at 560 nm.

A commercially available Coomassie, dye-binding, colorimetric assay (Pierce) was used to quantify total BALF protein (µg/ml). Samples were read spectrophotometrically at 595 nm and evaluated by using a bovine serum albumin standard curve.

Aldehydes in the BALF supernatant were analyzed as oximes of pentafluorobenzylhydroxylamine by gas chromatography, using electron-capture detection as previously described (13, 14). Briefly, 2 ml of a solution (1–20 µg/ml) containing the aldehydes hexanal, heptanal, or nonanal or 2 ml of BALF were allowed to react with 0.5 ml of a pentafluorobenzylhydroxylamine solution (1.0 µg/ml) for 2 h. Three drops of 18 N H₂SO₄ were then added, and the oximes were precipitated from the reaction mixture using 1 ml of hexane containing decafluorobiphenyl as the internal standard. The hexane layer was washed with 5 ml of 0.1 N H₂SO₄ and dried over anhydrous sodium sulfate. A gas chromatograph (Hewlett Packard model 5890, series II) with a 63Ni electron-capture detector and an autosampler (Hewlett Packard 7361A), connected to a cool on-column injector with electronic pressure control, was used for the analysis. An HP-5 25 m × 0.2 mm × 0.33 µm column with a 5 m × 0.53-mm retention gap was used for the separation. Helium (2.9 ml/min) was used as a carrier and argon-methane as a makeup gas. The chromatographic conditions were as follows: detector temperature, 280°C; temperature programming, 50°C for 1 min; temperature ramp, 5°C/min; final temperature, 220°C. Two micrograms of sample were injected.

Measurement of urate in plasma. Plasma urate concentrations were assayed by using a uricase reaction, and the decrease in absorbance of urate was measured spectrophotometrically at 320 nm.

**Experimental Protocols**

**Series 1: Untreated control dogs.** Rₐ and POTENTIAL DIFFERENCE DURING A 6-H EXPOSURE TO 0.2 PPM O₃. Dogs (mean weight = 19.2 ± 1.4 (SE) kg, n = 6) were anesthetized and exposed for 6 h to room-temperature humidified air. The same dogs were exposed 1 wk later for 6 h to 0.2 ppm O₃ in humidified room air. Rₐ, PDₜᵣ, and PDₜᵣ were recorded every 30 min throughout the exposure. Dogs were allowed to recover between the 6- and 24-h measurements.

Rₐ and ΔRₐ during a 6-h exposure to 0.2 ppm O₃. Dogs (mean weight = 20.5 ± 1.5 kg, n = 6) were anesthetized and exposed for 6 h to room-temperature humidified air. The same dogs were exposed 1 wk later for 6 h to 0.2 ppm O₃ in humidified room air. Rₐ and ΔRₐ were recorded in the right upper lobe (RUL), left middle lobe (LML), and the right lower lobe (RLL) before O₃ exposure (0 h), after 3 and 6 h of exposure, and at 24 h. We selected 0.2 ppm O₃ for our 6-h exposure protocol based on estimates made by Morgan et al. (40), who suggested that breathing at rest 0.2 ppm of O₃ through a tracheal tube was roughly equivalent to breathing 0.2 ppm of O₃ through the mouth while exercising. Thus the anesthetized dogs used in this study were exposed to O₃ concentrations similar to those used in many human studies.

**BALF ANALYSES.** BAL was done in the left upper lobe (LUL) at 0 h, the right middle lobe (RML) at 3 h, the left lower lobe (LLL) at 6 h, and the cardiac lobe at 24 h, respectively.

**Series 2: Probenecid treatment.** Rₐ, POTENTIAL DIFFERENCE, Rₐ, and ΔRₐ DURING A 6-H EXPOSURE TO 0.2 PPM O₃. Dogs (mean weight = 18.6 ± 0.8 kg, n = 6) were given probenecid (37 mg·kg⁻¹·day⁻¹) orally three times a day for 3 days before each exposure. Dogs were treated with probenecid on the fourth day, which was the first day of the exposure. Dogs were anesthetized and exposed for 6 h to room-temperature humidified air. One week later, the same dogs were pretreated again with probenecid and exposed for 6 h to 0.2 ppm O₃ in humidified room air. Rₐ, PDₜᵣ, and PDₜᵣ were recorded every 30 min throughout the experiment. Rₐ and ΔRₐ were recorded in the RUL, LML, and RLL before O₃ exposure (0 h), after 6 h of exposure, and at 18 h postexposure (24 h), respectively.

**BALF ANALYSES.** BAL was done in the LUL at 0 h, the RML at 6 h, and the LLL and cardiac lobe at 24 h, respectively.

**Statistical Methods**

The Friedman two-way analysis of variance by ranks was used for within-series analyses of Rₐ, PDₜᵣ, PDₜᵣ, Rₐ, ΔRₐ, and BALF cell/ml data. Nonparametric multiple comparisons were done by using a Student-Newman-Keuls test for between-treatment comparisons. The Kruskal-Wallis one-way analysis of variance was used for between-series comparisons of BALF data that were pooled for analysis regardless of location or time. Either the Student-Newman-Keuls or Dunn’s test applied to ranks was used to compare individual treatment means. All values are means ± SE. Statistical significance was judged at P < 0.05.

**RESULTS**

Rₐ and Potential Differences During a 6-h Exposure to 0.2 ppm O₃

Rₐ recorded before air exposure in untreated dogs was 0.80 ± 0.06 cmH₂O·l⁻¹·s⁻¹ (n = 6) and was almost identical to the baseline value (0.83 ± 0.05 cmH₂O·l⁻¹·s⁻¹) recorded before O₃ exposure 1 wk later (Fig 1). After 6 h, a small but significant difference (P < 0.05) in Rₐ was detected between air (1.01 ±
0.11 cmH$_2$O·l$^{-1}$·s$^{-1}$ and O$_3$ exposure (1.33 ± 0.21 cmH$_2$O·l$^{-1}$·s$^{-1}$). No significant difference existed between the two treatments by 24 h. Responses in probenecid-treated dogs were similar. RL was 0.99 ± 0.04 cmH$_2$O·l$^{-1}$·s$^{-1}$ (n = 6) before air exposure and was unchanged (0.99 ± 0.08 cmH$_2$O·l$^{-1}$·s$^{-1}$) 1 wk later, before O$_3$ exposure. O$_3$ increased (P < 0.05) RL at 6 h but was not significantly different from baseline 18 h after the exposure (Fig. 1).

Baseline PD$_T$ before air exposure in control dogs was greater (P < 0.05) than that recorded before O$_3$ exposure (Fig. 2). PD$_T$ increased during the air exposure, resulting in values recorded at 6 and 24 h that were similar to those recorded during O$_3$ exposure, which did not change significantly during the O$_3$ exposure. Responses in probenecid-treated dogs were similar.

PD$_T$ in untreated dogs decreased (P < 0.05) after 6 h of exposure to either air or O$_3$ when compared with 0-h measurements. However, PD$_T$ was reduced even further 18 h after O$_3$ exposure (P < 0.050), whereas it increased 18 h after air exposure. Similar changes in PD$_T$ occurred in response to O$_3$ in probenecid-treated dogs, whereas no significant changes in PD$_T$ were observed after exposure to air (Fig. 2).

Rpa and ΔRpa During a 6-h Exposure to 0.2 ppm O$_3$

Regardless of lobar location, Rpa in O$_3$-exposed, untreated dogs was not significantly different (P = 0.797) from that recorded during air exposure (Fig. 3). Similarly, peripheral airway reactivity, ΔRpa, to histamine was not affected (P = 0.197) by O$_3$. O$_3$ increased (P < 0.0001) Rpa and ΔRpa in probenecid-treated dogs (Fig. 3).

Interlobar variation in Rpa and ΔRpa was not statistically significant in untreated control dogs (Fig. 4). In addition, although O$_3$ tended to increase baseline Rpa (P = 0.053), it did not significantly alter ΔRpa (P = 0.562). Significant interlobar variation was detected in probenecid-treated dogs in contrast to untreated dogs.

Fig. 2. Bronchial (A; PD$_{br}$) and tracheal (B; PD$_{tr}$) transepithelial potential difference recorded in untreated control and probenecid-treated dogs before (0 h), immediately after (6 h), and 18 h (24 h) after exposure to either room-temperature humidified air (○) or 0.2 ppm humidified O$_3$ (●). *P < 0.05 compared with baseline at 0 h. †P < 0.05 comparing air with O$_3$. n = 6 dogs.

Fig. 3. Baseline peripheral airway resistance (Rpa; ○, ○) before and maximum Rpa (Rpa$_{max}$; □, ■) after histamine aerosol challenge. Peripheral airway reactivity (ΔRpa = Rpa$_{max}$ − Rpa) was recorded in untreated control and probenecid-treated dogs before (0 h), immediately after (6 h), and 18 h (24 h) after exposure to either air (A; open symbols) or O$_3$ (B; closed symbols). *P < 0.05 compared with baseline at 0 h. †P < 0.05 comparing air with O$_3$. n = 6 dogs.

Regardless of the type of exposure, baseline Rpa in the RLL was greater than Rpa in the LML, and Rpa in the LML was greater than that recorded in the RUL (Fig. 5). O$_3$ increased (P < 0.05) Rpa in the RUL and LML of probenecid-treated dogs, compared with either baseline (0 h) or air control. Although the increase in RLL Rpa was not statistically significant, it was the only location to show an O$_3$-induced increase in airway reactivity. ΔRpa after exposure to O$_3$ in the RLL was significantly greater than that recorded in the RUL or the LML, and this difference was seen only in probenecid-treated dogs (Fig. 5).

BALF Analyses

Total and differential cell counts. An average of 40 ± 2 and 40 ± 3 ml (n = 24) of BALF was recovered from sublobar segments in control dogs exposed to air and
O₃, respectively. Cell viability exceeded 97% in all cases. Except for the 6-h time point where more cells were recovered after O₃ exposure than after air exposure (P < 0.05), total number of cells per milliliter of BALF recovered on air and O₃ days was not significantly different. Compared with air exposure (2 ± 1%), neutrophils tended to increase after a 6-h exposure to O₃ (9 ± 4%) and were significantly (P < 0.05) increased at 24 h (39 ± 11%). No differences were evident between macrophages, lymphocytes, eosinophils, and epithelial cells recovered before, during, or after either exposure (Fig. 6).

An average of 44 ± 1.7 and 40 ± 2 ml (n = 24) of BALF was recovered from sublobar segments in probenecid-treated dogs exposed to air and O₃, respectively. Cell viability exceeded 99% in all cases. Total number of cells per milliliter of BALF recovered on air and O₃ days did not differ significantly. No significant differences were evident among macrophages, lymphocytes, eosinophils, and epithelial cells recovered before or after exposure to either air or O₃ (Fig. 7). Although neutrophils in the cardiac lobe and LLL tended to increase 24 h after each exposure regimen, a statistically significant change (P < 0.05) was seen only in the air-exposed cardiac lobe (6.1 ± 1.6%) when compared with its baseline value (0 h: 2.2 ± 0.8%, n = 6).

Measurement of ascorbate and TEAC in BALF. Exposure to O₃ did not significantly alter the concentration of ascorbic acid or the total antioxidants of BALF recovered from either control or probenecid-treated dogs at any time during an experiment (Fig. 8). However, the concentration of ascorbic acid (0.62 ± 0.04 µg/ml) recovered in BALF from probenecid-treated dogs pooled regardless of time or location was significantly reduced when compared with untreated control samples (1.02 ± 0.06 µg/ml, n = 48, P < 0.0001). Drug treatment did not affect BALF TEAC (Fig. 8).

Measurement of peroxide, total protein, and aldehydes in BALF. Exposure to O₃ did not significantly alter the concentrations of peroxide or total protein recovered in BALF from either control or probenecid-treated dogs at any time during an experiment (Fig. 9). However, the concentrations of peroxide (0.67 ± 0.03 µmol/l) and total protein (247 ± 15.1 µg/ml) recovered in BALF from probenecid-treated dogs pooled regardless of time or location were significantly reduced when compared with untreated control samples (1.02 ± 0.06 µg/ml, n = 48, P < 0.001; and 203 ± 24.1 µg/ml, n = 48, P < 0.0001, respectively). Finally, although O₃ did not significantly alter the concentrations of hexanal (P > 0.107), heptanal (P > 0.457), or nonanal (P > 0.572) in a time-specific fashion, significantly more (P < 0.05) hexanal was recovered from untreated dogs when pooled samples from the O₃ and air experiments were compared (Fig. 10). Treatment with probenecid significantly reduced heptanal recovery in air-exposed bronchi when compared with its untreated counterpart (Fig. 10). No other significant effects were seen after treatment with probenecid.

**DISCUSSION**

The present study shows that O₃ increased peripheral airway resistance and reactivity only in dogs treated with the antioxidant transport inhibitor probenecid, and these effects were heterogeneously distributed throughout the canine lung (Figs. 3 and 5). These observations support the hypothesis that endogenous antioxidant activity moderates the effect of O₃ on airway function in the lung periphery.
Although O₃ transiently decreased central airway function (Fig. 1), this decrement was not accompanied by any detectable change in PD<sub>TR</sub>. This suggests that O₃ exposure did not grossly injure the tracheal mucosa. However, transepithelial potential difference tended to improve with time in the air-exposed group. Although this may reflect poor initial contact between the record-

Fig. 7. Differential cell counts expressed as percentage of total cells in BALF recovered from probenecid-treated dogs before (0 h), immediately after (6 h), and 18 h (24 h) after a 6-h exposure to either humidified room air (open bars) or O₃ (hatched bars). asterisk (*) indicates P < 0.05 compared with baseline at 0 h; n = 6 dogs.

Fig. 8. Concentrations of ascorbate (A) and trolox equivalent antioxidant capacity (TEAC; B) in BALF recovered from untreated control and probenecid-treated dogs before (0 h), during (3 h), immediately after (6 h), or 18 h (24 h) after a 6-h exposure to either humidified room air (open bars) or O₃ (hatched bars). §P < 0.05 when comparing control with probenecid ascorbate, regardless of time or location; n = 6 dogs.

Fig. 9. Concentrations of peroxide (A) and total protein (B) in BALF recovered from untreated control and probenecid-treated dogs before (0 h), during (3 h), immediately after (6 h), or 18 h (24 h) after a 6-h exposure to either humidified room air (open bars) or O₃ (hatched bars). §P < 0.05 when comparing either control peroxides with probenecid peroxides or control protein with probenecid protein, regardless of time or location; n = 6 dogs.

Fig. 10. Concentrations of hexanal (A), heptanal (B), and nonanal (C) in BALF recovered from untreated control and probenecid-treated dogs before (0 h), during (3 h), immediately after (6 h), or 18 h (24 h) after a 6-h exposure to either humidified room air (open bars) or O₃ (hatched bars). §P < 0.05 when comparing air to O₃ regardless of time or location. §P < 0.05 when comparing control heptanal to probenecid heptanal, regardless of time or location; n = 6 dogs.
ing bridge and the mucosal surface, it is unclear why this would occur only during exposure to air. If this were the case, then we might be missing an O₃-induced effect on PDₑₑ. However, we also would be underestimating the significant decrease in PDₑₑ seen in Fig. 2. This decrease in potential difference is believed to reflect O₃-induced disruption of the mucosal barrier and is consistent with the enhanced mucosal permeability previously reported in O₃-exposed rats and humans (4, 28). Treatment with probenecid did not affect any O₂-induced changes in potential difference, although it markedly reduced its variance. This may result from the inhibition of intrapulmonary transport of lipid mediators (5, 6, 60), many of which can modulate ion channel function in airway epithelial cells (2, 34, 55), or a direct effect of probenecid on chloride channels (10).

The marked neutrophilic inflammation observed in untreated control airways (Fig. 6) indicates that O₃ penetrates deep into the lung periphery; apparently, at concentrations that are too low to significantly affect normal peripheral airway function (Figs. 3 and 5). The fact that treatment with probenecid inhibits O₃-induced inflammation (Fig. 7) while enhancing the effects of O₃ on peripheral airway function raises questions concerning the relationship between airway inflammation and airway reactivity. Some studies report good correlations between airway inflammation and airway hyperresponsiveness in dogs (16, 24). However, other studies in rats (15), guinea pigs (41), dogs (35), and humans (51) suggest that neutrophil infiltration does not contribute to the development of airway hyperreactivity caused by acute exposure to high concentrations of O₃. Our data (Figs. 3, 4, and 6) are consistent with those obtained from normal human (59) and asthmatic (3) subjects that reveal a dissociation between inflammation and airway function and suggest that a low-dose O₃-induced inflammatory response is itself insufficient to significantly alter airway reactivity.

Our data suggest that O₃-induced airway hyperreactivity and inflammation are independent phenomena and that O₂-induced inflammatory cell influx is dependent on a probenecid-sensitive transport process. Inhibition of leukotrienes in general (53), and leukotriene B₄ in particular (54), reduces O₂-induced neutrophil infiltration in dogs. Whether probenecid inhibits leukotriene B₄ transport is unknown, but our data are consistent with this effect. Other mediators, such as interleukin-1 (IL-1) (42) and IL-8 (26), also modulate neutrophil recruitment into the lung, and IL-8 and growth-related oncoprotein-α have been implicated in the development of O₂-induced inflammation in human peripheral airways (31). Although the effect of probenecid on cytokine transport has not been investigated, tenidap (another potent anion-transport inhibitor) (37) does interfere with the posttranslational release and maturation of IL-1 (32). Thus it is possible that the anti-inflammatory activity of probenecid results from either a direct or indirect effect on either leukotriene or cytokine transport.

Probenecid also interferes with membrane transport of ascorbate (36, 62), glutathione (22, 56), and urate (47), thus altering antioxidant levels in the lung. We documented a 50–60% decrease in plasma urate in probenecid-treated dogs, and this may reflect reduced local concentrations of uric acid in the peripheral lung. Although the concentration of ascorbic acid and the TEAC of BALF suggest that O₃ had little effect on airway antioxidant levels, probenecid reduced ascorbate levels in BALF (Fig. 8). Although O₂ exposure did not result in significant changes in BALF protein, which can also function as an antioxidant (23, 46), protein concentrations were reduced by probenecid (Fig. 9). Theoretically, reductions in ascorbate, urate, and total protein could account for the increased airway obstruction and peripheral airway reactivity seen in probenecid-treated animals (Figs. 3 and 5). Probenecid can also inhibit the transport of prostaglandins (5), leukotrienes (43, 60), and cAMP and cGMP (18). However, cyclooxygenase-derived metabolites contribute to O₂-induced changes in baseline airway function and airway reactivity in dogs (29, 44) and normal humans (50, 52). Thus the inhibition of membrane transport of prostaglandins by probenecid would be expected to decrease, not increase, peripheral airway reactivity (Figs. 3 and 5). Lipoxigenase-derived metabolites also contribute to O₂-induced airway hyperresponsiveness in dogs (54); thus interference with leukotriene membrane transport should also inhibit O₂-induced peripheral airway hyperreactivity. Finally, the inhibition of either cAMP or cGMP transport could theoretically increase peripheral airway reactivity. However, the average histamine-induced ΔRpa in air-exposed untreated dogs (Fig. 3: ΔRpa = 0.57 ± 0.06 cmH₂O·ml⁻¹·s⁻¹, n = 53) was not significantly different from that seen in dogs treated with probenecid (Fig. 3: ΔRpa = 0.61 ± 0.07 cmH₂O·ml⁻¹·s⁻¹, n = 54, Mann-Whitney U-test: P = 0.891). This suggests that inhibition of cyclic nucleotide transport did not affect peripheral airway reactivity in this study. Thus it appears that the enhanced peripheral airway reactivity seen in probenecid-treated dogs probably results from interference with antioxidant transport.

We measured concentrations of the three aldehydes that are produced by ozonation of the most prevalent unsaturated fatty acids (UFA) in lung lipids. It is important to note that UFA undergo ozonation, but only (n-6) PUFA can undergo autoxidation. Thus all three aldehydes reflect the amount of direct ozonation that has occurred. Heptanal is produced from ozonation of palmitoleic acid, an n-7 UFA that is present in the lung, and nonanal from oleic acid, an n-9 UFA that is very prevalent. However, hexanal can be produced either by the ozonation of any n-6 UFA or by the O₃-initiated autoxidation of any n-6 PUFA in the lung (13, 14, 48) and is formed in greater amounts than are the other two aldehydes.

The fact that the overall concentration of hexanal was significantly increased in O₂-exposed airways (Fig. 10) suggests that the exposure regimen used in this study increased oxidative stress in the lung periphery...
O₃ did not alter either heptanal or nonanal above background levels. Increased concentrations of all three aldehydes were previously reported for BALF samples recovered from rats exposed to 0.5–10 ppm O₃ (13, 48). Thus our inability to detect changes in either heptanal or nonanal may simply reflect the relatively low concentration of O₃ used in this study.

In summary, O₃ did not alter peripheral airway function in normal dogs but did increase Rpa and ΔRpa in probenecid-treated dogs in a location-dependent fashion. Bronchial but not tracheal mucosal permeability was increased immediately after and 18 h after O₃ exposure. O₃ markedly increased BALF neutrophils, and treatment with probenecid inhibited this O₃-induced inflammation. With the exception of hexanal, dogs;

Probenecid significantly reduced BALF ascorbate, BALF total protein, and plasma urate. On the basis of these activity was increased immediately after and 18 h after O₃ exposure. O₃ markedly increased BALF neutrophils, and treatment with probenecid inhibited this O₃-induced inflammation. With the exception of hexanal, dogs;

Probenecid significantly reduced BALF ascorbate, BALF total protein, and plasma urate. On the basis of these observations, we conclude that 1) a 6-h exposure to 0.2 ppm O₃ represents a subthreshold stimulus in relation to its effects on peripheral airway resistance and reactivity in normal dogs; 2) impairment of antioxidant transport contributes to the development of O₂⁻induced peripheral airway hyperreactivity in probenecid-treated dogs; 3) peripheral airway hyperreactivity and inflammation reflect independent responses to O₃ exposure; and 4) O₃-induced inflammation is dependent on probenecid-sensitive organic anion transporters, which may represent novel targets for anti-inflammatory drugs.

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