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the State of California Air Resources Board

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Investigators: J.A. Nadel, M.D. (Principal Investigator)
H.A. Boushey, M.D.
D. Sheppard, M.D.
G.D. Leikauf, Ph.D.
J. Seltzer, M.D.
S.C. Lazarus, M.D.
R.A. Bethel, M.D.
Barbara Geffroy Bigby (Staff Research Associate)
Linda Scypinski (Staff Research Associate)

Cardiovascular Research Institute

University of California, San Francisco

San Francisco, CA 94143

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ABSTRACT

The purpose of these studies was to investigate various aspects of ozone effects in airways and to determine the mechanisms of these effects. The first group of studies in humans were designed to determine whether asthmatic subjects had a heightened sensitivity to ozone compared to normal, healthy individuals. These studies were carried out with a sequence of protocols to prevent excessive risks to the subjects. The results, taken together, do not suggest any striking difference between asthmatics and normal subjects in regard to airway responses to inhaled ozone. The second series of human studies were an extension of previous animal studies, designed to determine whether humans develop an inflammatory response to inhaled ozone similar to dogs. The studies confirm the animal studies and suggest mechanisms by which ozone and other oxidant pollutants may cause changes in lung function.

The third group of investigations dealt with the mechanisms by which ozone induces airway hyperresponsiveness. As in the past, we initiated the studies in animals, with the intention of applying the results to human investigations as soon as possible. The animal studies involved a series of experiments which demonstrated that airway epithelial damage (e.g., by oxidants) is capable of producing lipids that act as "mediators," initiating inflammatory responses. Among these responses is neutrophil mobilization into the airways. Subsequent activation of the neutrophils results in the production of more mediators that appear to be responsible for the final airway smooth muscle responses. Finally, we have discovered drugs that have the potential to prevent the effects of ozone in this system.

HUMAN STUDIES

Effect of Ozone on Airway Function in Asthmatic Subjects

The purpose of the first study proposed in our contract was to determine whether people with asthma develop a change in airway caliber or in bronchial reactivity on exposure to lower concentrations of ozone than do healthy nonasthmatic subjects. The rationale for this study derived from the fact that while genetic influences are clearly important in asthma, the bronchial hyperreactivity that is fundamental to the disease appears to be an acquired rather than an inborn phenomenon. We therefore hypothesized that the inducibility of bronchial hyperreactivity may be under genetic influence. If this is true, one might expect people with asthma to develop changes in airway reactivity after exposure to levels of ozone that have no effect on responsiveness in healthy people without a predisposition to asthma.

Our other reason for the study was simple. Asthma is associated with exaggerated sensitivity to a wide variety of agents that cause bronchoconstriction. Because ozone is such an agent, it seemed likely that it would cause greater bronchoconstriction in people with asthma, as we have shown to be the case for another common air pollutant, sulfur dioxide.

To date, we have studied 10 subjects under a variety of slightly different protocols, as detailed below. All exposures to ozone were conducted in an environmental chamber supplied with filtered air at ambient indoor air temperature and humidity.

Protocol #1: We proceeded cautiously at first, fearing that we might harm subjects if asthma was indeed associated with a striking increase in sensitivity to ozone. Knowing from our previous work that 0.4 ppm is just above the threshold concentration of ozone causing a change in airway reactivity and in lung mechanics in healthy people, we exposed our first subject to 0.25 ppm of ozone inhaled while performing moderate exercise (400 kpm/min or 67 watts) on a cycle ergometer for two 15-min periods during a 75-min exposure. As before, we assessed airway caliber by measuring forced expiratory volume in one second and forced vital capacity (FEV_1/FVC) and by measuring specific airway resistance (SRaw) by body plethysmography. We assessed airway reactivity by measuring the rise in SRaw provoked by inhalation of serially increasing concentrations of methacholine (0.016 to 1.0 mg/ml) from a DeVilbiss 646 nebulizer. We expressed reactivity in terms of the PC_{8u}, the concentration causing an 8-unit increase in SRaw over baseline. The values for PC_{8u} are reported in mg/ml.

Results

	Ozone (0.25 ppm)	
	before	after
FEV_1/FVC (L)	3.71/4.71	not done
SRaw (L x cm H_2O/L/s)	4.30	50.5
PC_{8u} (mg/ml)	0.37	not done

Conclusion: These data suggested that this subject was more sensitive than healthy people to ozone exposure, but that there might be a component of exercise-induced asthma that could not be distinguished from the response to ozone. Since the patient had a 10-fold increase in SRaw after ozone exposure, his reactivity to methacholine was not evaluated at that time. Twenty-four hours later, his SRaw was at a baseline level and his response to methacholine was the same as that before ozone exposure.

Protocol #2: Since the large change in SRaw after exposure to ozone in our first subject suggested heightened sensitivity to ozone, we used a lower ozone concentration for a shorter period of time in our next protocol. Three subjects were exposed to a low (0.25 ppm) level of ozone while performing a moderate workload (400 kpm) for 30 min.

Results

	Sham		Ozone	
	before	after	before	after
FEV_1/FVC				
Subject 2	2.3/3.5	2.4/4.2	2.3/4.7	2.5/3.5
Subject 3	3.3/4.1	3.3/3.9	3.4/3.8	3.7/4.0
Subject 4	4.3/4.4	4.2/4.4	4.1/4.6	4.2/4.7
SRaw				
Subject 2	20.0	24.3	15.8	23.5
Subject 3	9.0	10.4	5.4	5.1
Subject 4	4.7	8.1	4.3	5.6

PC_{8u}: Methacholine dose-response curves not done.

There were no significant differences in either $\Delta FEV_1/FVC$ or $\Delta SRaw$ between the sham and ozone exposures for these three subjects.

Conclusion: Subject 2 had a greater change in SRaw after ozone exposure, but for the other 2 subjects, the observed change in SRaw is similar after exercise with or without ozone exposure. Therefore, the change in bronchomotor tone may simply reflect exercise-induced asthma, rather than a response to ozone exposure.

Protocol #3: In our next experiment, we attempted to eliminate the confounding effect of exercise and increased the ozone exposure to 2 h, the duration necessary to cause a change in bronchial reactivity in healthy subjects. We first exposed a single subject was exposed to a low (0.25 ppm) level of ozone for 2 h, without exercise.

Results

	Sham		Ozone	
	before	after	before	after
FEV_1/FVC	2.7/4.4	2.6/4.6	2.1/3.4	2.3/3.5
SRaw	18.2	10.5	17.9	12.8
PC_{8u}	0.077	0.089	0.01	0.047

Conclusion: If anything, this subject was less reactive after methacholine with ozone exposure.

Protocol #4: Our next approach was to try and simulate conditions that caused a small response in healthy subjects, using an adequate duration of ozone exposure (2 h), and an increased ozone concentration (0.4 ppm), since 0.25 ppm had no effect in the previous experiment. We used a decreased workload in order to minimize the effects of exercise-induced asthma. Two subjects were exposed to 0.4 ppm ozone, with a moderate workload (400 kpm) for 2 h.

Results

	Sham		Ozone	
	before	after	before	after
FEV_1/FVC				
Subject 4	4.2/4.5	3.9/4.3	4.2/4.6	4.0/4.5
Subject 10	3.8/4.8	4.1/5.0	4.1/5.0	3.9/5.0
SRaw				
Subject 4	4.0	5.8	3.6	5.6
Subject 10	4.7	4.5	4.9	5.3
PC_{8u}				
Subject 4	0.25	0.2	0.063	0.125
Subject 10	0.2	0.5	0.09	0.27

Conclusion: There was no increase in airway reactivity to methacholine or bronchomotor response in these subjects.

Protocol #5: Since most healthy subjects respond to 0.6 ppm ozone, we asked in our next set of experiments whether asthmatic subjects had the same sensitivity to ozone as healthy subjects if exposed under the same conditions except for a decrease in workload. Three subjects were exposed to 0.6 ppm ozone while exercising at 400 kpm/min (67 watts) for 15 of each 30 min for 2 h.

Results

	Sham		Ozone	
	before	after	before	after
FEV_1/FVC				
Subject 3	3.2/3.3	3.2/3.4	3.1/3.3	1.8/2.0
Subject 5	3.1/3.7	3.2/3.6	3.0/3.5	1.8/2.6
Subject 6	3.0/3.8	2.9/3.7	3.0/3.9	2.7/3.5
SRaw				
Subject 3	5.0	5.1	5.8	18.3
Subject 5	5.1	5.1	6.0	20.7
Subject 6	9.1	5.2	5.7	10.9
PC_{8u}				
Subject 3	0.30	0.45	0.37	0.024
Subject 5	0.50	0.80	0.30	0.03
Subject 6	0.07	0.10	0.07	0.045

There were no significant differences in either $\Delta FEV_{1.0}/FVC$ or ΔPC_{8u} between the sham and ozone exposures. ΔSRaw was different after ozone exposure ($p < 0.02$) but the difference was not greater than we had observed in nonasthmatic subjects.

Conclusion: The changes in airway caliber and in bronchial reactivity observed in there asthmatic subjects were similar to what we previously have found in healthy subjects.

Protocol #6: In the last set of experiments, we chose a level of ozone just below that which causes a change in airway reactivity to methacholine in some healthy subjects. We reasoned that use of this level should magnify any potential difference in response to ozone between asthmatic and healthy subjects. A lower workload was used to minimize exercise-induced asthma. To permit us to identify worrisome changes in airway function, we had the subjects measure their peak flow throughout the exposure period. In this study, 4 subjects were exposed to 0.5 ppm of ozone at a moderate workload (300 kpm, or 50 watts) for 15 of every 30 min for 2 h.

Ozone-Induced Change in Bronchial Reactivity and Airway Inflammation in Human Subjects

The purpose of the second study proposed in our contract was to examine whether ozone-induced airway hyperreactivity is associated with airway inflammation in healthy people, as we have shown to be the case for dogs.

Methods

We studied 10 healthy volunteers, 3 women and 7 men (23-41 years old), who were informed of the risks of the experimental protocol and who signed consent forms approved by the UCSF Committee on Human Research. Eight subjects were studied after exposure to air (sham) and to ozone; the other 2 were studied only after exposure to ozone. The exposures consisted of 2 h of intermittent exercise in a chamber containing filtered air alone (sham) or filtered air with 0.4 or 0.6 ppm of ozone. Five subjects were exposed to 0.6 ppm ozone, the other 5 to 0.4 ppm. On the day prior to each exposure, baseline airway responsiveness to inhaled methacholine was measured. On the day of exposure, baseline specific airway resistance (SRaw) was determined shortly before within 10 min after exposure. One hour later, SRaw and airway responsiveness to methacholine were again measured. Bronchoalveolar lavage was performed 3 h after the end of the exposure.

Airway function and responsiveness

We measured the airway response to inhalation of serially increasing doses of methacholine aerosol. Five measurements of thoracic gas volume (Vtg) and airway resistance (Raw) by constant-volume whole-body plethysmography were made at 30-s intervals in the baseline state and 30 s after inhalation of 10 breaths of each dose of methacholine aerosol (0.25-32.0 mg/ml) delivered by a DeVilbiss no. 646 nebulizer equipped with a dose-metering device. SRaw was calculated (Raw x Vtg), and the mean SRaw was determined after each concentration of methacholine. Aerosol challenge was stopped when an increase in SRaw of at least 8 L x cm H_2O/L/s was achieved. The dose of methacholine that induced this change (PD_{8u}) was determined by interpolation from the plot of SRaw and the log methacholine concentration (Fig. A).

Exposures

Subjects were exposed for 2 h in a chamber (6 x 9 x 8 ft) while wearing noseclips and exercising on a cycle ergometer for 15 min of each 30 min. Workloads were 83 watts for women and 100 watts for men. Ozone was generated by passing 100% oxygen through an ozonator (Wellsbach no. T-408) and concentrations were maintained at 0.60 ± 0.01 ppm (mean ± SD) or 0.40 ± 0.01 ppm as measured by an ultraviolet ozone analyzer (Dasibi no. 1003 AH). Air

Results

	Sham		Ozone	
	before	after	before	after
PEF*				
Subject 2	490	475	460	430
Subject 7	565	570	545	500
Subject 8	580	560	540	510
Subject 9	585	580	610	600
SRaw				
Subject 2	8.4	12.1	14.5	25.5
Subject 7	3.8	4.2	5.0	6.6
Subject 8	3.7	3.2	4.8	7.9
Subject 9	4.4	5.1	3.8	7.6
PC_{8u}				
Subject 2	0.16	0.12	0.05	0.009
Subject 7	0.4	0.42	0.55	0.37
Subject 8	0.26	0.40	0.35	0.145
Subject 9	0.31	0.29	0.31	0.21

*Peak expiratory flow in L/min.

By paired t-test, the baseline values before sham and ozone exposures did not differ. Sham exposure had little effect on any parameter. The increase in reactivity caused by 0.50 ppm of ozone was significant (PC_{8u} fell from 0.315 to 0.183% methacholine; $p = 0.03$); the rise in SRaw (from 7.03 to 11.9 L x cm H_2O/L/s) was short of significant in this small sample.

Summary of Findings: Only recently have tools been developed to diagnose asthma clearly from other pulmonary disorders. In this series of experiments, different protocols were used to locate the threshold of response to ozone in a group of well-characterized exercising asthmatic subjects. Initially, low concentrations of ozone and/or short periods of exposure were used for fear of provoking severe bronchospasm in a group suspected of having heightened sensitivity to ozone. The protocols that were used are shown in Table 1.

Conclusion: A level of ozone (0.5 ppm) that we would expect, on the basis of our prior experience, to have at most a modest but significant effect on airway caliber and airway reactivity in healthy subjects had a similar effect in subjects with mild asthma. Taken together, our results in these 10 subjects do not exclude the possibility that asthma is associated with heightened airway sensitivity to ozone, but they suggest that any such heightening is modest, unlike the striking increase in sensitivity to the airway effects of sulfur dioxide.

was filtered (type 91184, Mine Safety Appliances), and chamber temperature and relative humidity were 71.5 ± 7.5°F and 55 ± 15%, respectively.

For sham exposures, an initial small concentration of ozone was added to the chamber prior to the start of exposure to produce the characteristic odor of ozone. The concentration of ozone thereafter was less than 0.005 ppm.

Bronchoalveolar lavage

Prior to bronchoscopy, all subjects received 0.8-1.0 mg atropine sulfate IM, and 5 received codeine sulfate (30 mg IM) or morphine sulfate (10 mg IM). Oxygen was given by nasal cannula at 4-5 L/min throughout the procedure. The upper airway was anesthetized with topical application of 4% lidocaine; 1% lidocaine was applied to the lower airways through the bronchoscope as necessary to inhibit cough. Lavage was performed through a flexible fiberoptic bronchoscope (Pentax FB-19D, O.D. 6.3 mm) wedged into a subsegmental airway in the anterobasal segment of the right lower lobe with the subject supine. Hanks' balanced salt solution, without calcium, magnesium or phenol red, warmed to 37°C (pH 7.36-7.44), was instilled in five 20- to 25-ml boluses and withdrawn immediately after each instillation by suction. The bronchoscope was not moved from the wedged position until 120 ml were instilled and suctioning was completed. The fluid was immediately placed on ice, filtered through gauze to remove mucus, and centrifuged for 5 min at 200 g at 4°C. The supernatant was removed and frozen at -70°C for later analysis of arachidonic acid metabolites. The cell pellet was resuspended for the total cell count and differential analysis.

Cell counts

Cells were resuspended in Hanks' solution and counted with a hemocytometer. Samples of resuspended cells were cytocentrifuged at 800 rpm for 5 min (model 7 cytospin; Shandon Scientific Co., Sewickley, PA) and stained by the May-Grunwald-Giemsa stain for differential cell counts. Epithelial cells, macrophages (and monocytes), lymphocytes, neutrophils, eosinophils, and metachromatically staining cells were identified. A total of 500-1000 cells were counted. Cells of undefined type (smudged or without definite morphology) were also counted. A blinded review of one slide chosen at random for each lavage yielded counts that did not differ from the original observations.

Quantification of arachidonic acid metabolites

Prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), 6-keto-prostaglandin ($PGF_{1\alpha}$) and thromboxane B_2 (TxB_2) were measured by radioimmunoassay without extraction (NEK-020, NEK-024 and NEK-025, New England Nuclear, Boston, MA;

CA-503, Clinical Assays, Cambridge, MA).

Assays for leukotrienes were performed on supernatant after acidification, application to Sep-Pak columns (Waters Associates, Milford, MA) and elution with methanol. C6-peptide leukotrienes were assayed using specific rabbit antiserum for LTC_4, LTD_4 and LTE_4 [1], with [^{3}H]LTD_4 (36 Ci/mmol; New England Nuclear, Inc., Boston, MA) and synthetic LTD_4 as the competing ligand (Merck Frost Laboratories, Pointe Claire Dorval, Quebec, Canada). Leukotriene B_4 was assayed using specific rabbit antiserum for LTB_4 [2], with [^{3}H]LTB_4 (180-221 Ci/mmol; Amersham Corp., Arlington Heights, IL) and synthetic LTB_4 as the competing ligand (Merck Frost Laboratories, Pointe Claire Dorval, Quebec, Canada). Each value was corrected for the recovery of radiolabeled compound.

Data analysis

Means of PD_{8u} are expressed as the geometric mean. The change in PD_{8u} from before to after exposure were compared using Student's t-test for paired data. Changes in SRaw from before to after exposure were compared using Student's 2-tailed t-test for unpaired data.

Differences between mean total cell counts for sham and ozone exposures were compared by Student's t-test. Differences between differential cell counts were compared by the paired t-test.

Analyses of radioimmunoassay results were compared by Student's t-test, using the Welch approximation of degrees of freedom [3]. A probability of less than 5% was considered significant. Mean values for cell counts, specific airway resistance, and arachidonate metabolites are reported as mean ± SD unless otherwise indicated.

Results

Airway responsiveness

Sham exposure did not cause a change in airway responsiveness (Table 2). The mean PD_{8u} before exposure was 2.95; after exposure it was 2.59 mg/ml ($p > 0.50$). It changed by less than 2-fold in 6 of 8 subjects and changed by 2.6-fold in both remaining subjects (subjects 4 and 10, Table 2). Exposure to 0.4 ppm ozone caused a slight increase in mean airway responsiveness. The mean PD_{8u} before exposure was 2.44; after exposure it was 1.30 mg/ml ($p < 0.025$). PD_{8u} decreased by 2-fold or more in 3 of 5 subjects, but the changes were small, with the largest change being 2.7-fold (subject 3, Table 2). Exposure to 0.6 ppm ozone caused a greater increase in airway responsiveness (decreases in PD_{8u} ranged from 3- to 16-fold). The mean PD_{8u} before exposure was 4.44; after exposure it was 0.75 mg/ml ($p < 0.01$). Three of these subjects

responded to inhalation of concentrations of methacholine in the range found in asthmatic subjects in our laboratory (less than 1.0 mg/ml). In summary, sham exposure did not change airway responsiveness, but ozone exposure increased it significantly.

Sham exposure did not affect specific airway resistance (immediately post-exposure, SRaw = 4.24 ± 1.23 L·cm H_2O/L/s; baseline SRaw = 3.84 ± 0.91; $p > 0.40$). Exposure to both concentrations of ozone caused increases in specific airway resistance (Table 2), but mean SRaw 1 h after exposure, when airway responsiveness was measured, did not differ from the value on the previous day when the baseline responsiveness to methacholine was determined (4.59 ± 0.73 vs. 4.11 ± 0.82 L·cm H_2O/L/s respectively; $p > 0.10$).

Bronchoalveolar lavage (BAL)

Analysis of the fluid obtained by BAL showed differences between the effects of sham and ozone exposure in the types of cells present, but not in the volume recovered nor in the total number of cells obtained (Tables 3a and 3b). Likewise, the cell concentrations did not differ (1.925 ± 0.848 vs 1.920 ± 0.397 x 10^5 cells/ml). Differential cell counts of BAL fluid showed that ozone caused a striking increase in the mean percentage of neutrophils (Tables 3a and 3b). The mean percentages of epithelial cells, lymphocytes, and eosinophils were not significantly altered by ozone exposure. The mean percentage of macrophages/monocytes was significant after ozone, but the absolute cell number was not. The number of unidentifiable cells in lavage from ozone-exposed subjects also did not differ from sham-exposed lavages. Metachromatically staining cells (possibly mast cells or basophils) were seen rarely and were not quantitated.

After sham exposure, the mean neutrophil count was 2.3 ± 2.6%. In 7 of 8 subjects, less than 3% were counted; in one subject, the neutrophil count was 9.0%. After exposure to ozone, neutrophil counts were increased to 18.5 ± 11.7%. In the 3 subjects who had less than 2-fold decreases in PD_{8u} (subjects 1, 4, and 10) increases in the percentage of neutrophils were not different from the increases caused by sham exposure ($p > 0.05$) and were smaller than the increases noted in the subjects with greater than 3-fold decreases in PD_{8u} (8.0 ± 4.8% vs 30.8 ± 4.0%, $p < 0.01$).

Analysis of cyclooxygenase and lipoxygenase metabolites of arachidonic acid in BAL fluid demonstrated significant changes for some cyclooxygenase products (Table 4). $PGF_{2\alpha}$, TxB_2, and PGE_2 concentrations increased significantly, whereas concentrations of C6-peptide leukotrienes, 6-keto-$PGF_{1\alpha}$, and LTB_4 did not change significantly after ozone exposure. There were no correlations between changes in the concentration of cyclooxygenase products of arachidonic acid and either changes in methacholine reactivity or neutrophil concentrations after ozone. The mean concentrations of C6-peptide leukotrienes were 1690 ± 2041 pg/ml after sham exposure and 635 ± 976 pg/ml

after ozone exposure ($p > 0.10$). The mean concentrations of LTB_4 were 72 ± 67 pg/ml after sham exposure and 113 ± 121 pg/ml after ozone exposure ($p > 0.20$).

Discussion

Our data show that ozone-induced increases in bronchial responsiveness are associated with an increase in the number of neutrophils in BAL fluid in humans, as has been previously shown in dogs [4]. In addition, we have shown that ozone exposure causes an increase in the levels of PGE_2, $PGF_{2\alpha}$, and TxB_2 in BAL fluid.

The increase in neutrophils in BAL fluid was most marked in those subjects who developed greater than 3-fold increases in airway responsiveness, although the relationship was variable. For example, the subject with the greatest change in responsiveness did not have the highest neutrophil count.

Our findings differ from those made in dogs [4] in that we did not find an increase in recovered airway epithelial cells after ozone exposure. This difference may be due to the much higher concentrations of ozone used in the dog exposures (3 ppm) or to differences in lavage technique.

Our demonstration of an association between increases in the numbers of neutrophils in BAL fluid and increases in airway responsiveness does not prove a causal relationship; they may represent separate, unrelated responses to an earlier event or sequence of events. Studies of animals provide conflicting circumstantial evidence on the importance of the neutrophil in the induction of airway hyperresponsiveness caused by ozone. In dogs, the time course of the increase in airway hyperresponsiveness is similar to the time course of the appearance and disappearance of neutrophils in airway lavage fluid [5]. In another study of dogs, neutrophil depletion by treatment with cytotoxic drugs prevented the increase in airway responsiveness and the influx of neutrophils produced by ozone [6], although the cytotoxic agents required to induce neutropenia may have had effects on other cells.

An important role for airway epithelial cells in mediating the hyperresponsiveness induced by ozone is suggested by other studies. Injury to airway epithelium is a well-known effect of ozone exposure in animals [7-9], and there is some evidence implicating the epithelial cell in the change in responsiveness in dogs. In this species, ozone exposure caused an increase in epithelial cells as well as in neutrophils in lavage fluid [4]. When ozone-induced hyperresponsiveness was prevented by pretreatment with BW755C, a cycloxygenase and lipoxygenase inhibitor, the increase in the number of epithelial cells in lavage fluid was also prevented [10]. We are unable with certainty to exclude the epithelial cell from a role in ozone induced airway hyperresponsiveness in our human subjects, as our sampling technique (BAL) may not have adequately assessed changes in airway epithelial cell populations or cellular products.

The measured levels of arachidonic acid metabolites varied widely among ozone- and sham-exposed individuals. Despite the wide variability of these measurements, we found statistically significant increases in PGE_2, $PGF_{2\alpha}$, and TxB_2. Giri and Hollinger [11] have reported increased levels of $PGF_{2\alpha}$ and PGE_2 in pooled BAL and pleural fluids in ozone-exposed rats, supporting our findings.

Our demonstration that increased concentrations of the products of arachidonic acid are present in BAL fluid after ozone exposure suggests that such inflammatory mediators may play an important role in hyperresponsiveness. This is underscored by the finding in dogs that ozone-induced hyperresponsiveness is blocked by pretreatment with cyclooxygenase and lipoxygenase inhibitors [10,12]. Both indirect and direct studies of the airway effects of inflammatory mediators have shown that they may alter smooth muscle responsiveness to other stimuli, but the effects of particular mediators or families of mediators seem to differ greatly with species, the source of the muscle, and the dose and route of administration of the agent.

Conclusion: Ozone-induced increases in airway reactivity are associated with increased concentrations of neutrophils in bronchoalveolar lavage fluid and with increased concentrations of PGE_2, $PGF_{2\alpha}$, and TxB_2 in human subjects. The patterns of interaction among cells and inflammatory mediators await elucidation.

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Table 1. Protocols Used to Examine Airway Response to Ozone in Asthmatic Subjects

Exp. #	Ozone conc.	Workload	Time	
			Exercise	Exposure (total)
1	0.25	none		2 h
2	0.25	moderate	2 15-min periods	75 min
3	0.25	moderate	30 min	30 min
4	0.40	moderate	2 h	2 h
5	0.50	moderate	4 15-min periods	2 h
6	0.60	moderate	4 15-min periods	2 h

Findings: A significant but modest effect on airway caliber and reactivity was found in asthmatic subjects only after exposure to levels of ozone of 0.50 ppm and above (experiments #5 and #6).

Table 2. Specific Airway Resistance and Airway Responsiveness to Methacholine

Subject*	Day before Sham		Day of Sham Exposure				Day before Ozone		Day of Ozone Exposure			
	SRaw** baseline	PD_{8u}*** mg/ml	SRaw baseline	SRaw after Sham 5 min	1 h	PD_{8u} mg/ml	SRaw baseline	PD_{8u} mg/ml	SRaw baseline	SRaw after Ozone 5 min	1 h	PD_{8u} mg/ml
1	4.53	2.0	4.64	5.13	4.72	3.8	4.21	2.9	4.52	5.43	4.30	2.4
2	5.21	2.1	4.87	5.87	4.65	2.4	4.55	2.6	5.58	7.65	5.90	1.1
3	4.46	5.1	3.71	3.48	3.09	8.5	4.98	4.6	3.73	4.92	3.97	1.7
4	5.44	3.4	5.12	5.69	5.83	1.3	5.59	0.8	5.09	5.68	5.25	0.7
5	3.97	5.4	4.18	4.35	4.14	5.8	4.08	3.1	4.64	9.04	4.83	1.2
6	--	--	--	--	--	--	3.40	5.8	2.83	4.44	4.02	1.5
7	--	--	--	--	--	--	4.71	5.7	4.12	6.43	4.41	0.7
8	2.87	1.3	2.19	2.86	2.91	0.8	2.82	1.6	3.30	5.30	4.19	0.2
9	3.78	2.4	2.83	2.72	3.10	2.4	3.43	6.5	2.36	6.13	3.48	0.4
10†	3.85	4.7	3.57	3.85	3.91	1.8	3.34	5.0	3.78	6.74	5.54	3.2

*Subjects 1-5 were exposed to 0.4 ppm ozone, subjects 6-10 to 0.6 ppm.

**L·cm H_2O/L/s.

***Provocative methacholine dose at which baseline SRaw increased 8 L·cm H_2O/L/s.

†This subject's SRaw failed to reach 8 L·cm H_2O/L/s over baseline on some occasions. The provocative concentrations listed are those causing a doubling of SRaw.

Table 3a. Differential Cell Counts (Percentage) in BAL Fluid

Subject*	Total Cell Number x 10^5	BAL Volume Returned (ml)	Sham Exposure				
			Epithelial Cells	Neutrophils	Macrophages/ Monocytes	Lymphocytes	Eosinophils
1	69	48	5.3	0.6	91.0	3.0	0.1
2	88	70	5.9	2.1	80.8	11.0	0.2
3	70	71	2.2	0.2	94.4	3.3	0.0
4	86	54	2.7	0.4	79.2	17.6	0.1
5	200	57	0.1	2.1	95.5	1.5	0.8
6	--	--	--	--	--	--	--
7	--	--	--	--	--	--	--
8	71	57	9.0	9.0	74.1	7.8	0.1
9	109	57	2.6	2.7	88.6	6.1	0.0
10	199	66	2.2	1.65	87.8	7.4	0.9
mean	112	60	3.8	2.3	86.4	7.2	0.27
± SD	56	8	2.8	2.8	7.6	5.2	0.36

*Subjects 1-5 were exposed to 0.4 ppm ozone, subjects 6-10 to 0.6 ppm (see Table 2b).

Table 3b. Differential Cell Counts (Percentage) in BAL Fluid

Subject*	Total Cell Number x 10^5	BAL Volume Returned (ml)	Ozone Exposure: Epithelial Cells	Neutrophils	Macrophages/ Monocytes	Lymphocytes	Eosinophils
1	127	58	0.3	2.8	93.5	3.4	0.0
2	95	56	0.7	23.2	60.2	14.3	1.6
3	190	76	0.8	1.6	95.6	2.0	0.0
4	93	51	3.5	8.7	65.4	22.0	0.4
5	97	55	1.9	13.5	78.6	5.7	0.3
6	120	50	0.8	32.1	58.3	8.7	0.1
7	135	67	2.2	35.2	57.7	3.8	1.1
8	77	60	4.4	30.2	62.2	3.0	0.2
9	83	64	1.4	25.7	64.0	8.9	0.0
10	156	70	0.6	12.4	71.6	12.3	3.1
mean	117	61	1.7	18.5**	70.7**	8.4	0.68
± SD	36	8	1.8	11.7	14.1	6.3	0.87

*Subjects 1-5 were exposed to 0.4 ppm ozone, subjects 6-10 to 0.6 ppm.

**Paired t-test, $p < 0.05$.

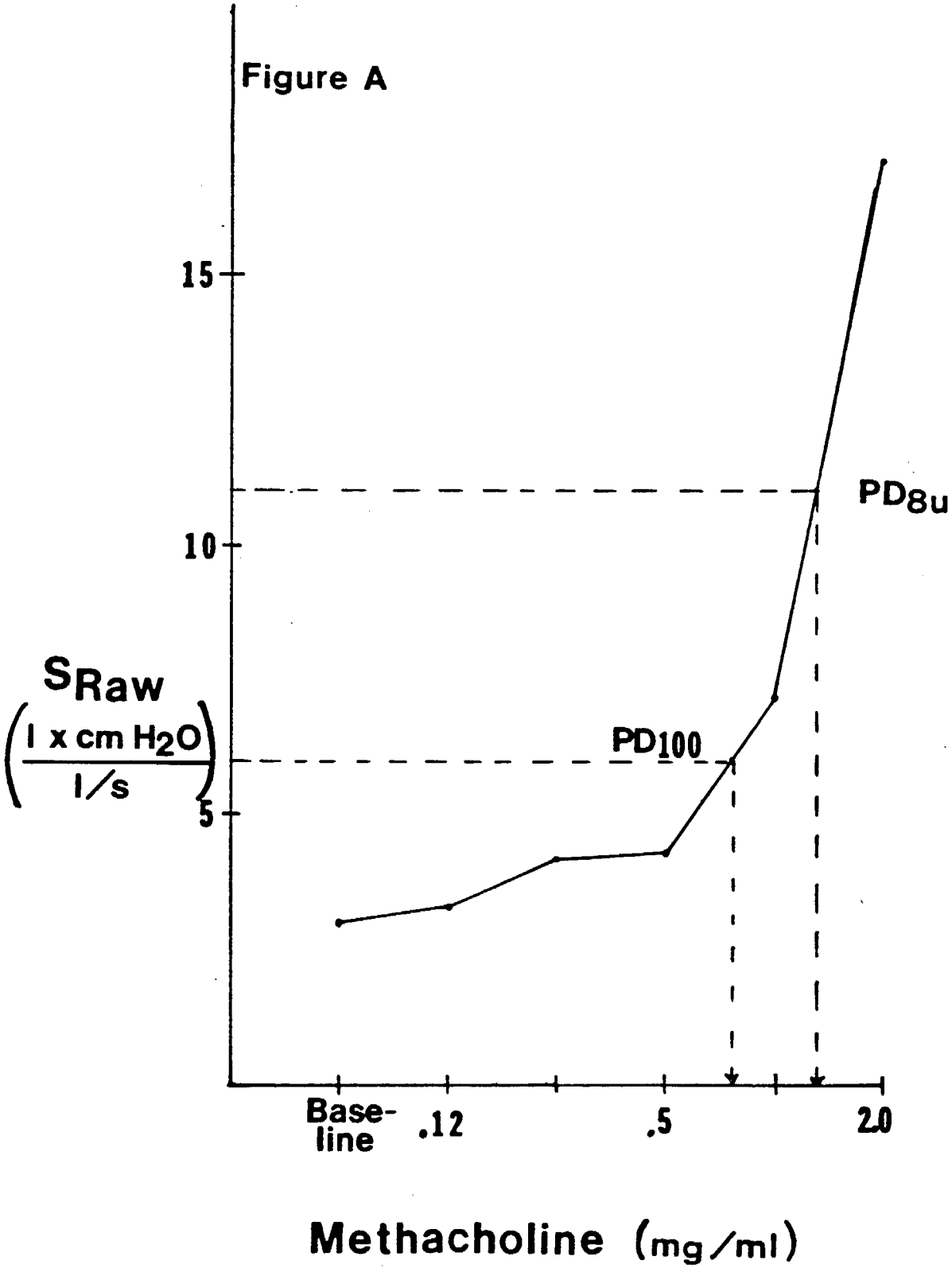
Table 4. Concentrations of Arachidonic Acid Products (in pg/ml) in Bronchoalveolar Lavage Fluid

	Sham				Ozone			
Subject*	PGE_2	$PGF_{2\alpha}$	TxB_2	6-keto-$PGF_{1\alpha}$	PGE_2	$PGF_{2\alpha}$	TxB_2	6-keto-$PGF_{1\alpha}$
1	3.3	25	200	31	46.0	202	170	22
2	2.9	30	160	20	71.0	101	253	32
3	7.0	28	117	20	61.0	156	348	25
4	8.8	48	170	12	72.0	56	305	25
5	17.0	21	196	9	165.0	116	416	24
6	----	----	----	----	18.0	1680	108	15
7	----	----	----	----	15.8	64	28	24
8	3.2	38	170	110	12.5	84	310	100
9	3.7	22	92	5	27.5	70	125	33
10	2.5	56	132	30	28.0	56	170	20
mean ± SD (n = 8**)	6.1 ± 4.7	34 ± 13	155 ± 38	30 ± 34	60.4† ± 47.5	105† ± 52	262† ± 101	35.0 ± 27
mean ± SD (n = 10)	---- ± ----	---- ± ----	---- ± ----	---- ± ----	51.7† ± 43.4	259† ± 502	223† ± 122	32 ± 25

*Subjects 1-5 were given 0.4 ppm ozone; subjects 6-10 were given 0.6 ppm.

**Excluding subjects 6 and 7.

†$p < 0.05$.



Method used for calculating PD_{8u}, the concentration of methacholine causing an increase in specific airway resistance of 8 units over the initial value.

ANIMAL STUDIES

Introduction

The purpose of our studies was to describe effects of ozone on airways and to understand how they come about. When possible, studies were performed in humans. In the present studies, we discovered that damage to the airway epithelium was associated with airway smooth muscle hyperresponsiveness, and we needed animal studies (physiologic, pharmacologic, cellular, and biochemical) to understand the mechanisms. The physiologic studies showed the importance of neutrophils to the responses. The pharmacologic studies suggested the importance of arachidonic acid metabolism, particularly thromboxane A_2. They further showed that specific drugs prevented the responses and suggested possible new forms of therapeutic interventions that may be important to patients with asthma, bronchitis, and other diseases of the airways. The cellular studies revealed products of arachidonate metabolism that may explain the series of events leading to airway hyperresponsiveness.

Effect of Ozone on Airways in Dogs

Protocol #1

Problem: Neutrophils appear to play an important role in the changes that occur after ozone. Furthermore, we have shown that neutrophil chemotactic factors (e.g., leukotriene B_4) are released from epithelial cells. It seems reasonable that these released chemotactic factors could be involved in the cascade leading to airway hyperresponsiveness of exposure to ozone.

Methods: We studied the effect of leukotriene B_4 aerosols on airway responsiveness to inhaled acetylcholine aerosols and on the cellular components and cyclooxygenase metabolites in bronchoalveolar lavage fluid in dogs.

Results: Inhalation of leukotriene B_4 aerosols had no effect on resting total pulmonary resistance but increased airway responsiveness, an effect that was maximum in 3 h and that returned to control levels within a week. Three hours after leukotriene B_4, the number of neutrophils and the concentration of thromboxane B_2 recovered in lavage fluid increased markedly. Pretreatment with the thromboxane synthetase inhibitor OKY-046 prevented the increases in airway responsiveness and in thromboxane B_2 but did not alter neutrophil chemotaxis.

Conclusions: These findings suggest the following sequence of events after exposure to ozone: ozone activates airway epithelial cells to release arachidonate metabolites (particularly leukotriene B_4); the metabolites stimulate neutrophil migration into the airways; neutrophils then release thromboxane in the airways, a material that increases airway responsiveness.

Protocol #2

Problem: Thus far, our studies suggested that ozone damages airway epithelium, causing the release of neutrophil chemotactic factors, leading to neutrophils moving into the airways. The next question was how neutrophils could produce airway hyperresponsiveness. We knew that neutrophils release thromboxane A_2. To determine whether thromboxane A_2 may be involved in ozone-induced airway hyperresponsiveness.

Methods: We studied the effect of a thromboxane synthetase inhibitor (OKY-046, 100 ug/kg/min, i.v.) in 5 dogs exposed to ozone. Airway responsiveness was assessed by determining the provocative concentration of acetylcholine aerosol that increased total pulmonary resistance by 5 cm $H_2O \cdot L^{-1} \cdot s$.

Results: Ozone (3 ppm) increased airway responsiveness as demonstrated by a decrease in acetylcholine provocative concentration from 2.42 mg/ml (GSEM, 1.56) to 0.14 mg/ml (GSEM, 0.75). OKY-046 significantly inhibited this effect without altering pre-ozone responsiveness or the ozone-induced increase in neutrophils and airway epithelial cells in bronchoalveolar lavage fluid. To further examine the role of thromboxane A_2, we studied the effect of a thromboxane A_2 mimetic, U-46619, on airway responsiveness in 5 additional dogs. U-46619 in subthreshold doses (i.e., insufficient to increase baseline pulmonary resistance) caused a 4-fold increase in airway responsiveness to acetylcholine. Subthreshold doses of histamine had no effect.

Conclusions: These studies provide important information suggesting that thromboxane A_2, a cyclooxygenase product of arachidonic acid, may be an important mediator of ozone-induced airway hyperresponsiveness.

Protocol #3

Problem: It may not always be possible to prevent exposures to air pollutants such as ozone. Therefore, we have begun to look for drugs that can prevent the effects of inhaled ozone.

Methods: Because the experimental drug BW755C is known to inhibit the formation of oxygenation products of arachidonic acid, we investigated the effect of BW755C on ozone-induced hyperresponsiveness in dogs. Airway

responsiveness was assessed with dose-response curves of acetylcholine aerosol versus pulmonary resistance in two sets of experiments: in one set (placebo treatment), five dogs were given only saline treatment and were studied before treatment or ozone exposure and then after treatment both before and after ozone (3.0 ppm, 2 h); in another set (BW755C treatment), the same dogs were studied before BW755C treatment or ozone and then after treatment (10 mg/kg intravenously) both before and after ozone.

Results: When the dogs were given no BW755C treatment, ozone caused a marked increase in airway responsiveness to acetylcholine. When the dogs were given BW755C, responsiveness was no different during treatment than before treatment, but more importantly, responsiveness did not increase significantly after ozone.

Conclusions: We conclude that BW755C markedly inhibits ozone-induced airway hyperresponsiveness in dogs, probably by inhibiting the formation of oxygenation products of arachidonic acid. Thus, this experimental drug was able to prevent ozone-induced responses, suggesting a possible strategy for therapy of diseases associated with airway obstruction.

Studies of Arachidonate Metabolism

Protocol 4

Problem: It is now clear that inflammation of the airways is produced by exposure to inhaled ozone, and that this inflammatory process is associated with hyperresponsive airway smooth muscle similar to asthma. Inhaled ozone causes damage to the airway epithelial cells, and we suspected that the damaged epithelium produced meidators that subsequently initiated the inflammatory process. For these reasons, in a previous contract with the California Air Resources Board, we studied the potential role of airway epithelial cells in causing inflammation. The major cellular metabolic pathway that results in production of mediators is the arachidonate system. We showed that the incubation of suspensions of canine tracheal epithelial cells of greater than 95% purity with arachidonic acid (25-100 µg/ml) for 60-120 min resulted in the generation of a maximum of 36.2 ± 9.1 picomoles of leukotriene $B_4/10^6$ cells, less than 2.0 picomoles of leukotrienes C_4, D_4, and $E_4/10^6$ cells, and 1030 ± 463, 767 ± 500, and 324 ± 100 picomoles/10^6 cells of 15-, 12-, and 5-hydroxy-eicosatetraenoic acids, respectively (mean ± SEM, n = 8). The identity of leukotriene B_4 was established by chromatographic and spectral properties, by reactivity with mono-specific anti-plasma, and by the chemotactic activity for neutrophils. Thus, the epithelium may be an important source of mediators of inflammation and hypersensitivity of pulmonary airways.

Because the animal studies described important, new mechanisms of airway inflammation, and because human arachidonate metabolism has been shown to

differ from animals in some cell systems, in the present studies it was key to determine how human airway epithelial cells differ from the findings in dogs. Fortunately, in other studies we have recently discovered how to maintain the viability of human airway epithelial cells, and we are now able to culture these cells.

Methods: Epithelial cells of 99% purity and 92% viability were isolated from human tracheas obtained post-mortem and the cellular pathways for lipoxygenation of arachidonic acid were examined in vitro. The lipoxygenase metabolites were identified by comparison with synthetic standards during reverse-phase and straight-phase high-pressure liquid chromatography, UV spectroscopy, and gas chromatography-mass spectrometry.

Results: Epithelial cells incubated without arachidonic acid failed to generate detectable quantities of metabolites, while cells incubated with 1-50 µg/ml of arachidonic acid for 1-30 min invariably generated predominantly 15-lipoxygenase products, including 15-hydroxyeicosatetraenoic acid (15-HETE), four isomers of 8,15-dihydroxyeicosatetraenoic acid (two 8,15-diHETEs and tw 8,15-leukotrienes), at least one isomer of 14,15-dihydroxyeicosatetraenoic acid, and smaller amounts of 8-HETE and 12-HETE, but little or no detectable 5-HETE or 5,12-diHETEs.

Conclusions: The capacity of epithelial cells from human pulmonary airway to selectively generate 15-lipoxygenase metabolites of arachidonic acid suggests a potential role for the products as mediators of airway epithelial function. These new findings suggest mechanisms by which airway epithelial cells affect airway function. They also suggest new avenues of approach to therapy in obstructive airway diseases such as asthma.

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SUMMARY AND CONCLUSIONS

(1) People with mild asthma do not appear to have heightened sensitivity to the effects of ozone on airway caliber or on bronchomotor responsiveness to methacholine.

(2) In human subjects, the increase in airway responsiveness caused by ozone is associated with airway mucosal inflammation, as inferred from an increase in the number of polymorphonuclear cells and in the

concentrations of Prostaglandins E_2 and $F_{2\alpha}$ and of thromboxane B_2 in fluid obtained by bronchial lavage.

(3) Human tracheal epithelial cells selectively generate 15-lipoxygenase products of arachidonic acid. The generation of these potent chemical mediators of inflammation suggests that the epithelial cell is an important intermediary in the response to inhaled pollutants.

(4) In dogs, the inhalation of leukotriene B_4, a chemotactic factor released by canine tracheal epithelial cells, produces an increase in airway responsiveness, and increases in the number of neutrophils and quantity of thromboxane B_2 recovered in bronchial lavage fluid.

(5) In dogs, the effects of inhalation of leukotriene B_4 are prevented by pretreatment with a thromboxane synthetase inhibitor. This suggests that the release of thromboxane A_2 may be responsible for the increase in airway responsiveness.

(6) In dogs, pretreatment with a thromboxane-synthetase inhibitor blocks the effect of ozone on airway responsiveness without blocking its effect on the number of polymorphonuclear cells in bronchial lavage fluid. This suggests that thromboxane A_2 may be an important mediator of ozone-induced airway hyperresponsiveness.

(7) In dogs, pretreatment with an inhibitor of the formation of oxygenation products of arachidonic acid inhibits the effects of ozone on airway responsiveness. This suggests that oxygenation products of arachidonic acid may mediate the effects of ozone on airway responsiveness.

RECOMMENDATIONS

On the basis of the results obtained in this and in previous contract periods, we recommend that the following studies be done:

(1) Examination of the mechanisms of tolerance to repeated exposures to ozone: It is known that repeated exposure to ozone causing diminishing effects on airway caliber and airway responsiveness. It is not known whether this "tolerance" is associated with diminished airway mucosal inflammation. Because the effects of chronic inflammation may be severe, we recommend that the effects of repeated exposure to ozone on the number of polymorphonuclear cells and levels of prostaglandin and thromboxane be examined in people or in dogs.

(2) Because asthma appears to be associated with a striking increase in sensitivity to sulfur dioxide, but not to ozone, we recommend that the sensitivity of asthmatic subjects to nitrogen oxides and acid particulate matter be compared to that of healthy subjects.

(3) Because inflammation is a fundamental response to injury, we recommend that bronchial lavage fluid be examined in human subjects after exposure to other common pollutants, such as nitrogen oxides and acid particulate matter.

DISCLAIMER

The statements and conclusions in this report are those of the contractor and not necessarily those of the California Air Resources Board. The mention of commercial products, their source or their use in connection with material reported herein is not to be construed as either an actual or implied endorsement of such products.

LIST OF PUBLICATIONS RESULTING FROM WORK DONE UNDER THIS CONTRACT

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