Ozone-induced oxygen radical release from bronchoalveolar lavage cells and airway hyper-responsiveness in dogs

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1. Ozone inhalation causes airway hyper-responsiveness and airway inflammation in dogs. The purpose of this study was to determine whether these effects are associated with increases in oxygen radical production from bronchoalveolar lavage (BAL) cells.

2. Twelve randomly selected dogs were studied twice, 4 weeks apart. On each study day, acetylcholine (ACh) airway responsiveness was measured before and 1 h after ozone (3 p.p.m., 30 min) or dry air inhalation, followed by BAL. The response to ACh was expressed as the concentration causing an increase in lung resistance of 5 $cmH_2O\ l^{-1}\ s^{-1}$ above baseline. Spontaneous and phorbol myristate acetate (PMA) (2·4 $\mu mol\ l^{-1}$)-stimulated oxygen radical release from washed BAL cells (4×10^6 cells ml^{-1}) was measured by luminol-enhanced chemiluminescence in a luminometer at 37 °C.

3. Ozone inhalation caused airway hyper-responsiveness. The concentration of ACh causing an increase in lung resistance of 5 $cmH_2O\ l^{-1}\ s^{-1}$ (the 'provocative' concentration) fell from 4·68 mg ml^{-1} (% S.E.M., 1·43) before, to 0·48 mg ml^{-1} (% S.E.M., 1·60) after ozone ($P < 0·0001$). Spontaneous chemiluminescence area under the curve (AUC) significantly increased after ozone from 4·08 mV (10 min) (% S.E.M., 1·28) after dry air to 8·25 mV (10 min; % S.E.M., 1·29) after ozone ($P = 0·007$). Ozone inhalation also increased PMA-stimulated chemiluminescence AUC from 18·97 mV (10 min; % S.E.M., 1·18) after dry air to 144·03 mV (10 min; % S.E.M., 1·45) after ozone ($P = 0·0001$). The increase in PMA-stimulated chemiluminescence was significantly correlated with ozone-induced ACh airway hyper-responsiveness ($r = 0·83$, $P < 0·001$).

4. These results indicate that inhaled ozone increases oxygen radical release from BAL cells and suggest that oxygen radicals are important in causing ozone-induced airway hyper-responsiveness.

Airway hyper-responsiveness is a characteristic feature of asthma. It can be increased in human subjects by inhalation of allergens, or ozone, and by viral respiratory infections. Each of these stimuli can also cause airway hyper-responsiveness in a number of animals (Holtzman *et al.* 1983*b*; Stevens, Jones, Manning, Lane & O'Byrne, 1989). In dogs, ozone inhalation results in the development of transient airway hyper-responsiveness, which is maximal 1 h after the end of the ozone inhalation, and which has resolved by 1 week (Holtzman *et al.* 1983*b*). The development of this airway hyper-responsiveness has been associated with an acute influx of inflammatory cells into the airways (Holtzman *et al.* 1983*a*).

A characteristic feature of activated phagocytes is the production of oxygen radicals (Klebanoff, 1988). When stimulated, these cells release superoxide ($O_2^{-}\cdot$) and hydrogen peroxide (H_2O_2) and their metabolites, including the extremely reactive and cytotoxic hydroxyl radical (OH·). Oxygen radicals can cause tissue injury and increase cellular arachidonic acid metabolism (Sporn, Peters, Golden & Simon, 1988), thereby leading to the generation of lipid mediators (Lansing *et al.* 1991).

In humans with chronic airflow obstruction (Postma, Renkema, Noordhoek, Faber, Sluiter & Kauffman, 1988) or asthma (Meltzer, Goldberg, Lad & Easton, 1989), the degree of airway hyper-responsiveness correlates with superoxide

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anion production by the polymorphonuclear leucocytes isolated from peripheral blood. Latex-stimulated luminol- and lucigenin-enhanced chemiluminescence from BAL cells in asthmatics are also significantly increased compared with controls (Kelly, Ward, Stenton, Bird, Hendrick & Walters, 1988). In cats, the production of oxygen radicals by the xanthine–xanthine oxidase reaction causes bronchoconstriction and airway hyper-responsiveness (Katsumata *et al.* 1990). Furthermore, Duddridge, Ward, Hendrick & Walters (1993) demonstrated that the improvement in airway responsiveness caused by 6 weeks of beclomethasone dipropionate inhalation was positively correlated with a reduction in unstimulated lucigenin-amplified chemiluminescence of BAL cells. Finally, work from our laboratory has shown that a combination of the antioxidants allopurinol and Desferal significantly inhibits the development of ozone-induced airway hyper-responsiveness (Matsui, Jones, Woolley, Lane, Gontovnick & O'Byrne, 1991). These results suggest that the production of oxygen radicals could be important in the pathogenesis of ozone-induced airway hyper-responsiveness. The purpose of this study was to determine whether inhaled ozone increases oxygen radical production from BAL cells and whether this production is correlated to the degree of ozone-induced airway hyper-responsiveness.

METHODS

Study design

Twelve randomly selected healthy adult mongrel dogs, weighing between 17 and 30 kg were housed and handled according to guidelines published by the Canadian Council on Animal Care (Olfert, Cross & McWilliam, 1993). They were studied on two randomly assigned experimental days separated by at least 2 weeks. On each study day, the dogs were anaesthetized with intravenous sodium pentobarbitone (30 mg kg^{-1}; Somnotol; MTC Pharmaceuticals, Mississauga, Ontario). The level of anaesthesia was checked by testing every 20 min for the absence of both a blinking reflex and jaw muscle tone. Additional anaesthetic was given to maintain the initial degree of anaesthesia (sufficient to allow successful artificial ventilation with no need for a muscle relaxant) throughout the experiment (13 mg, 2–4 times per experiment). Following induction, acetylcholine (ACh) airway responsiveness was measured before and 1 h after dry air or ozone inhalation. At the end of the second ACh dose–response determination, a bronchoalveolar lavage was performed.

Reagents

Sterile magnesium- and calcium-free phosphate-buffered saline (PBS) was prepared in our laboratory. Hanks' balanced salt solution without calcium chloride, magnesium sulphate and Phenol Red (HBSS), ACh, luminol and phorbol myristate acetate (PMA) were all purchased from Sigma.

Measurement of airway responses

An endotracheal tube (10 mm i.d.) was inserted and connected to a constant-volume ventilator (model 613; Harvard Apparatus, South Natick, MA, USA) set at a tidal volume of 10 ml kg^{-1} and a rate of 30 breaths min^{-1}. A balloon catheter was then inflated as previously described (Lemen, Benson & Jones, 1974) and placed in the oesophagus at the point of most negative end-expiratory pressure. The oesophageal catheter and a port at the proximal end of the endotracheal tube were both connected to a differential pressure transducer (model 267; Hewlett Packard, Palo Alto, CA, USA).

Transpulmonary pressure was measured as the difference between the mouth and the oesophageal pressures. Flow was measured using a pneumotachograph (no. 1; Fleisch, Switzerland), a differential pressure transducer (model 270; Hewlett Packard) and a pressure amplifier (model 8805C; Hewlett Packard). A continuous measurement of total lung resistance was computed from the flow and transpulmonary pressure measurements, obtained while the dogs were being ventilated, using a respiratory analyser (model 8816A; Hewlett Packard), utilizing the method described by Mead & Whittenberger (1953). Total lung resistance, transpulmonary pressure, flow and compliance were recorded on an eight-channel recorder (model 7758A; Hewlett Packard). The total lung resistance was corrected for the external resistance of the system.

Acetylcholine inhalation challenge

ACh was administered and inhaled by a method previously described (Jones, Lane, Manning & O'Byrne, 1987). Briefly, after measuring baseline total lung resistance, the dogs inhaled 0·15 M NaCl followed by doubling concentrations of ACh (0·07–80 mg ml^{-1}) while being ventilated. The ACh aerosol was generated from a nebulizer (Twinjet; Bennett, Maitland, Ontario, Canada), and delivered via the endotracheal tube. The nebulized output was 0·196 ml min^{-1} with particles of an aerodynamic mass median diameter of 2·5 μm (% S.D., 2·3). Before the administration of each concentration, the dogs' lungs were inflated to a transpulmonary pressure of 30 cmH_2O. Five inhalations of the aerosol, each of 3 s, were administered at each concentration until an increase in lung resistance of at least 5 $cmH_2O\ l^{-1}s^{-1}$ above the baseline was obtained. The increases in pulmonary resistance are not associated with increases in respiratory rate. The airway responsiveness was expressed as the concentration of ACh which increased total lung resistance by 5 $cmH_2O\ l^{-1}s^{-1}$, and was called the ACh provocative concentration.

Ozone generation and delivery

The ozone generation and delivery have been described previously (Jones *et al.* 1987; Matsui *et al.* 1991). Briefly, ozone was generated by passing oxygen through a high intensity electrical field. Ozone produced in this way was diluted with dry room air using a glass and Teflon mixing circuit. Ozone was administered via the endotracheal tube by allowing the dog to breath spontaneously from a flow-by system. Ozone concentration at the mouth was measured continuously using a calibrated photometric ozone analyser (model 8002; Bendix, Lewisburg, WV, USA). The dogs inhaled an ozone concentration of 3 p.p.m. for 30 min. As inhaled ozone at these concentrations can cause acute bronchoconstriction and increases in respiratory rates, the dogs were allowed to recover for 1 h, by which time the pulmonary resistance and respiratory rates had returned to baseline, before the second ACh inhalation challenge was performed. This ozone dose (time multiplied by concentration) has been previously shown to reliably cause airway hyper-responsiveness in approximately 95% of the dogs.

Bronchoalveolar lavage

A flexible fibre-optic bronchoscope (model BF-1T; Olympus Optical Co., Tokyo, Japan), with an o.d. of 6 mm, was positioned in a third generation bronchus of the middle lobe of the right lung.

Subsequently, five 20 ml aliquots of PBS without Ca^{2+} or Mg^{2+}, warmed to 37 °C, were injected via the bronchoscope. Immediately after each aliquot, the fluid was aspirated into a collection trap using a suction of 70–85 mmHg. The collected fluid, which varied between 50 and 87 % of the volume injected, was then centrifuged twice, each for a period of 10 min, at 170 *g*. A total cell count and cell viability were determined by the use of a haemocytometer and Trypan Blue exclusion dye. The cells were resuspended in PBS to a concentration of 4×10^6 cells ml^{-1}. For differential cell counts, an aliquot of BAL cells was resuspended at a concentration of 1×10^6 cells ml^{-1}, and six cytospin slides were prepared (Cytospin 3; Shandom Scientific Co., Sewichley, PA, USA), each with 60 μl of the cell suspension, and stained with a modified Wright–Giemsa (DiFF-Quick; American Scientific Products, McGaw Park, IL, USA). Differential cell counts were performed on 400 nucleated cells on two different slides per experimental day. Metachromatic cell counting was done on 10 000 toluidine-stained cells on each study day.

Chemiluminescence measurements

Measurements of oxygen radicals can be made by techniques which utilize chemiluminescence (Allen, Stjernholm & Steele, 1972). Luminol (cyclic hydrazide-5-amino-2,3-dihydro-1,4-phthalazinedione) serves as a substrate which reacts with oxygen radicals to produce nitrogen and an excited aminophthalate ion, which emits light as it relaxes to the ground state. Luminol is frequently used to increase the sensitivity of the chemiluminescence technique (Allen & Loose, 1976) and its light emission is dependent on both superoxide and myeloperoxidase-catalysed reactions (Allen & Loose, 1976). Spontaneous and phorbol myristate acetate (PMA, 2·4 μmol l^{-1})-stimulated oxygen radical release by 4×10^6 BAL cells was measured in duplicate by luminol-enhanced chemiluminescence in a luminometer (model 1250; LKB Wallac, Helsinki, Finland) at 37 °C and a pH of 7·1. The BAL cells were kept on ice and the luminol at room temperature (22–24 °C). The cells (4×10^6 cells ml^{-1}) in PBS and 1·6 ml luminol in HBSS ($3{\cdot}44 \times 10^{-5}$ M final concentration, pH 7·1) were placed in polystyrene 3 ml cuvets. The resulting light output was continuously recorded on a chart recorder (model SRLG; Sargent Welch) for 10 min (spontaneous chemiluminescence) and for a futher 10 min after addition of 10 μl PMA ($2{\cdot}4 \times 10^{-6}$ M final concentration; PMA-stimulated chemiluminescence; Fig. 1).

Analysis

The analysis of ACh provocative concentration measurements was performed using log-transformed data, and therefore summary statistics are expressed as the geometric mean and percentage standard error of the mean (% S.E.M.). Changes in airway responsiveness were measured by comparing the log difference of the pre- and post-ACh provocative concentrations after dry air and ozone using Student's two-tailed t test for paired observations, as were BAL cell differential counts, baseline total lung resistance and airway responsiveness.

To measure chemiluminescence, peak voltage (mV), peak time (min) and area under the curve (AUC) in 10 min (mV (10 min)) were calculated with a computer and a graphics tablet. The chemiluminescence results were analysed by a repeated ANOVA. The data were log transformed to normalize their distribution. The difference of the log difference in the ACh provocative concentrations between the dry air and ozone days was correlated with the logarithmic difference between dry air and ozone PMA-stimulated peak chemiluminescence, and with area under the curve chemiluminescence. $P \leqslant 0{\cdot}05$ was considered statistically significant.

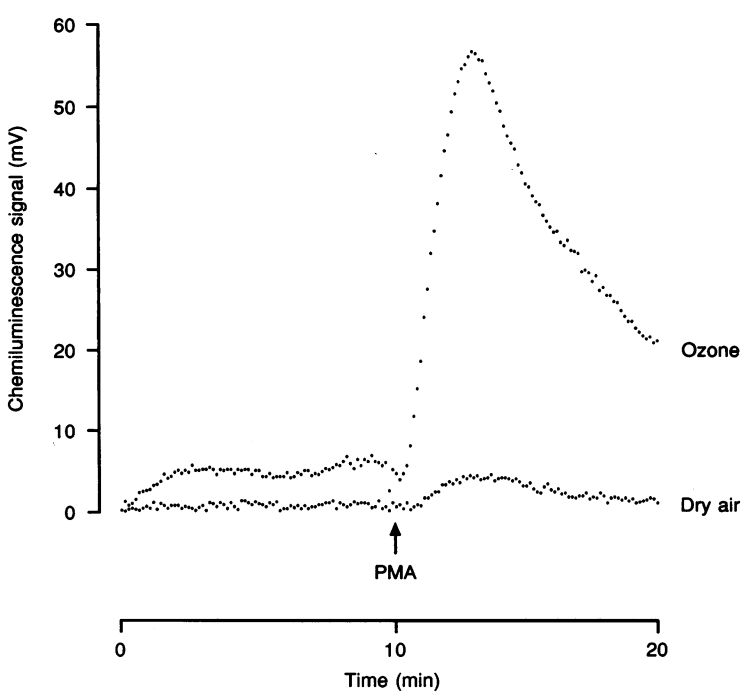

Figure 1. Change in airway responsiveness

Two typical traces of spontaneous and PMA-stimulated chemiluminescence from one dog after ozone and dry air inhalation.

Table 1. Cell counts in bronchoalveolar lavage fluid of dogs after dry air or ozone (3 p.p.m., 30 min) inhalation

	Dry air	Ozone	*P* value
Total BAL fluid recovered (BALF) (ml)	71·17 ± 3·44	67·75 ± 2·5	60·19
Total cells ml^{-1} BALF (10^4 cells ml^{-1})	37·72 ± 4·18	45·45 ± 4·44	0·07
Absolute BAL cell count (10^4 cells ml^{-1})			
Macrophages	30·21 ± 3·68	24·01 ± 2·17	0·18
Neutrophils	1·85 ± 0·36	11·74 ± 3·01	0·00
Lymphocytes	3·37 ± 0·49	4·11 ± 0·68	0·32
Eosinophils	2·25 ± 1·05	3·72 ± 1·17	0·06
Epithelial cells	0·05 ± 0·02	1·86 ± 0·68	0·02
Metachromatic cells	0·14 ± 0·05	0·12 ± 0·02	0·69
BAL cell differential count (%)			
Macrophages	79·57 ± 3·00	57·81 ± 6·19	0·01
Neutrophils	4·77 ± 0·75	22·63 ± 5·31	0·00
Lymphocytes	9·32 ± 1·33	9·23 ± 1·11	0·95
Eosinophils	6·21 ± 2·28	6·95 ± 1·81	0·67
Epithelial cells	0·14 ± 0·07	3·37 ± 1·20	0·02
Metachromatic cells	0·39 ± 0·13	0·28 ± 0·05	0·40

P values were calculated by comparing dry air and ozone treatments by a paired *t* test. Values are means ± S.E.M.

RESULTS

Ozone inhalation caused airway hyper-responsiveness in all dogs. The mean ACh provocative concentration before ozone inhalation was 4·68 mg ml^{-1} (% S.E.M., 1·43) and decreased to 0·48 mg ml^{-1} (% S.E.M., 1·60) after ozone inhalation ($P < 0·0001$; Fig. 2). The mean ACh provocative concentration was 5·04 mg ml^{-1} (% S.E.M., 1·50) before, and 4·68 mg ml^{-1} (% S.E.M., 1·43) after dry air inhalation ($P = 0·61$; Fig. 2). The mean log difference of the pre- minus post-ACh provocative concentration was 1·04 (S.E.M., 0·13) after ozone and 0·03 (S.E.M., 0·06) after dry air ($P < 0·0001$). The baseline pulmonary resistances before

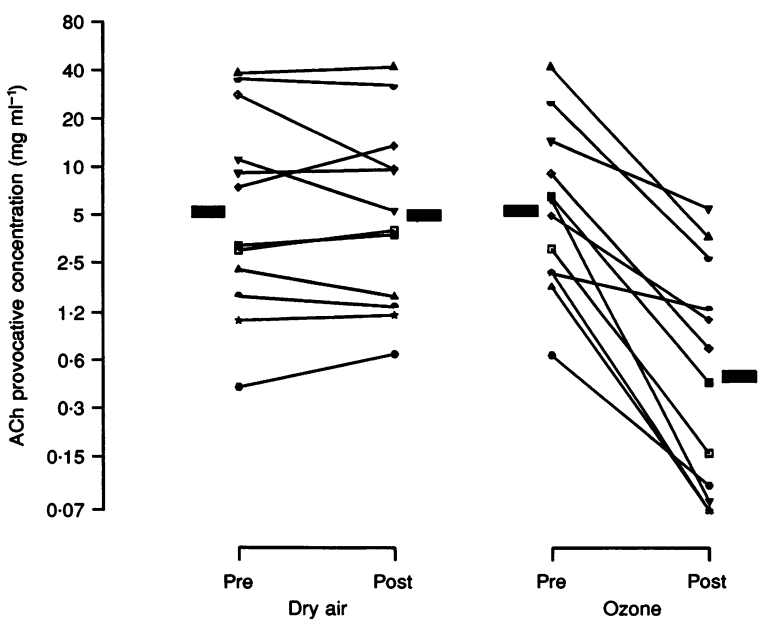

Figure 2. Chemiluminescence measurements from one dog

The ACh provocative concentration before and after inhaled dry air and ozone (3 p.p.m., 30 min) ($n=12$). The bars represent mean values. The mean log difference of the pre- minus post-ACh provocative concentration is significantly greater for ozone treatment.

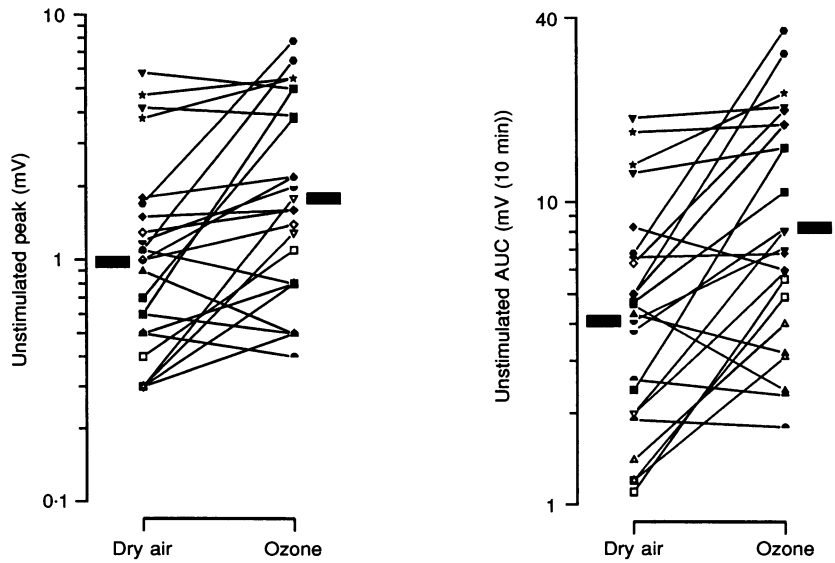

Figure 3. Spontaneous chemiluminescence

Ozone inhalation (3 p.p.m., 30 min) significantly enhanced spontaneous peak and area under the curve (AUC) chemiluminescence. Bars represent mean values.

the ACh inhalation challenges on the dry air study day were 0·68 $cmH_2O\ l^{-1}\ s^{-1}$ (S.E.M., 0·08) and 0·70 $cmH_2O\ l^{-1}\ s^{-1}$ (S.E.M., 0·05), and on the ozone study day were 0·52 $cmH_2O\ l^{-1}\ s^{-1}$ (S.E.M., 0·09) and 1·28 $cmH_2O\ l^{-1}\ s^{-1}$ (S.E.M., 0·22). Neither the baseline pulmonary resistances nor baseline airway responsiveness were significantly different between the two study days.

There were no differences in the amount of BAL fluid recovered, total cell counts, or total number of cells per millilitre on the two study days. Ozone inhalation increased neutrophil ($P < 0·009$) and epithelial ($P < 0·02$) cell counts in BAL, either expressed as absolute number of cells per millilitre of recovered lavage fluid or as a percentage of total cells (Table 1). Ozone significantly decreased the percentage of macrophages in BAL ($P = 0·01$), but there were no significant differences in the other cell types.

Ozone increased both spontaneous and PMA-stimulated chemiluminescence (Fig. 1). The geometric mean

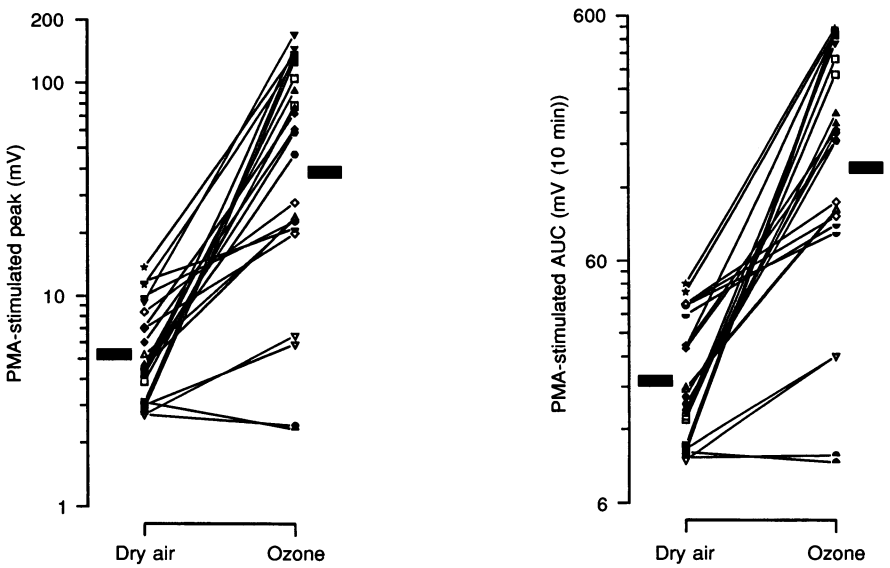

Figure 4. PMA-stimulated chemiluminescence

Ozone inhalation (3 p.p.m., 30 min) significantly enhanced PMA-stimulated peak and AUC chemiluminescence. Bars represent mean values.

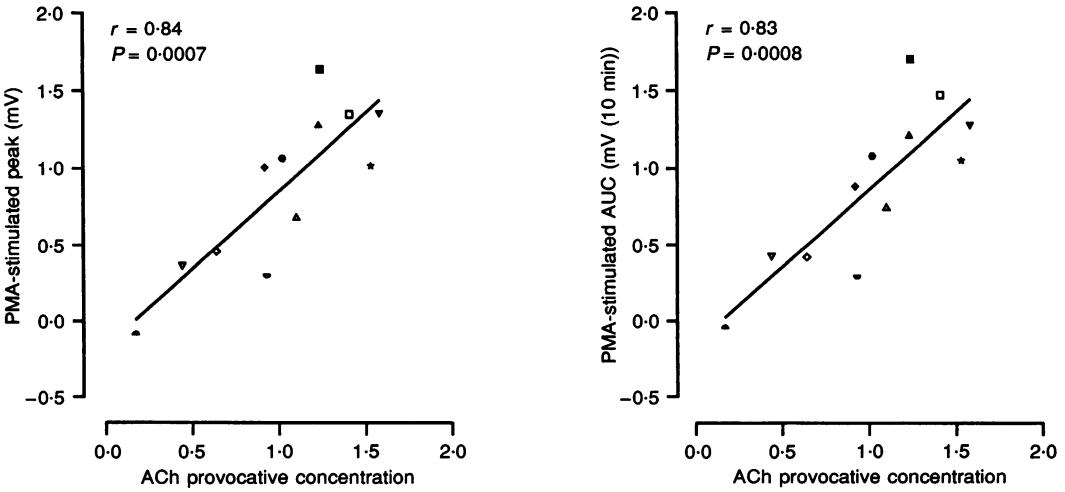

Figure 5. Airway hyper-responsiveness correlated with chemiluminescence signals

Both the ozone-induced increase in PMA-stimulated peak and AUC correlates significantly with the ozone-induced increase in ACh airway hyper-responsiveness. A higher numerical value means increased ozone-induced airway hyper-responsiveness (x-axis, log difference ozone minus log difference dry air) or increased ozone-induced oxygen radical production (y-axis, log ozone minus log dry air).

spontaneous peak was 0·98 mV (% S.E.M., 1·30) after dry air and 1·79 mV (% S.E.M., 1·30) after ozone ($P = 0·02$). The geometric mean spontaneous area under the curve was 4·08 mV (10 min) (% S.E.M., 1·28) after dry air and 8·25 mV (10 min) (% S.E.M., 1·29) after ozone ($P = 0·007$) (Fig. 3). The PMA-stimulated peak increased from 5·24 mV (% S.E.M., 1·16) after dry air to 39·15 mV (% S.E.M., 1·46) after ozone ($P < 0·0001$). The PMA-stimulated AUC increased from 18·97 mV (10 min) (% S.E.M., 1·18) after dry air, to 144·03 mV (10 min) (% S.E.M., 1·45) after ozone ($P < 0·0001$) (Fig. 4). The mean time to the PMA-stimulated peak chemiluminescence was not different between ozone and dry air inhalation days ($P = 0·37$).

Ozone-induced ACh airway hyper-responsiveness was significantly correlated to ozone-induced increases in PMA-stimulated peak chemiluminescence ($r = 0·84$, $P = 0·0007$) and area under the curve ($r = 0·83$, $P = 0·0008$) (Fig. 5). Also, a significant positive correlation was found between number of BAL neutrophils and PMA-stimulated peak ($r = 0·70$ $P = 0·011$) and AUC ($r = 0·72$, $P = 0·008$) after ozone inhalation. A negative correlation was found between the numbers of BAL macrophages and the PMA-stimulated peak ($r = -0·76$, $P = 0·004$) and AUC ($r = -0·77$, $P = 0·004$) after ozone inhalation.

DISCUSSION

This study has demonstrated, for the first time, that inhaled ozone increases the spontaneous production and the capacity to produce oxygen radicals from BAL cells, and that these increases are significantly correlated with the magnitude of ozone-induced ACh airway hyper-responsiveness.

The positive correlation between the magnitude of ozone-induced ACh airway hyper-responsiveness and the PMA-stimulated chemiluminescence is supported by previous reported findings. Cluzel *et al.* (1987) demonstrated that the severity of asthma, established by a clinical score which combines symptoms, bronchial obstruction and treatment requirements, was positively correlated with luminol-enhanced chemiluminescence from cultured alveolar macrophages stimulated with opsonized zymozan (Cluzel *et al.* 1987). It has also been shown that the degree of airway hyper-responsiveness from subjects with chronic airflow obstruction (Postma *et al.* 1988) and asthma (Meltzer *et al.* 1989) is positively correlated to the PMA- (Postma *et al.* 1988) or FMLP-(Meltzer *et al.* 1989) stimulated $O_2^{-}\cdot$ generation of peripheral blood polymorphonuclear leucocytes. By contrast, Kelly *et al.* (1988) found no correlation between methacholine airway responsiveness and luminol-enhanced chemiluminescence from mixed cell BAL samples of asthmatics.

Ozone inhalation is a potent stimulus for the induction of neutrophil migration and airway hyper-responsiveness in several species, including dogs (Fabbri *et al.* 1984) and humans (Matsui *et al.* 1991). It has been suggested that neutrophil infiltration into the airways may be necessary for the development of airway hyper-responsiveness (O'Byrne *et al.* 1984*b*). Matsui *et al.* (1991) have demonstrated that ozone-induced airway hyper-responsiveness in dogs, but not ozone-induced neutrophil influx into the airways, was abolished by pretreatment with the combination of allopurinol (which inhibits xanthine oxidase activity) and Desferal (which inhibits the generation of iron-dependent hydroxyl radicals). These

results suggested that oxygen radicals, perhaps released from activated neutrophils, are important in causing ozone-induced airway hyper-responsiveness. However, the release of oxygen radicals was not measured in that study. The $O_2^-\cdot$–H_2O_2-generating NADPH oxidase of neutrophils and macrophages plays an important role in the killing of many pathogenic bacteria and fungi during infections (Babior, 1984). These reactive oxidants generated during the respiratory burst (or oxidants derived from them such as HOCl and OH·) are also implicated in the host tissue damage which may occur during inflammation or in inflammatory diseases such as rheumatoid arthritis (Brown, 1988).

Phorbol esters mimic the elaboration of endogenous diacylglycerol in neutrophils, which is required for the activation of the protein kinase C receptor. The latter is a calcium phospholipid-dependent kinase and probably bypasses cyclic AMP- and cyclic GMP-modulated pathways (Nishizuka, 1984). In a pilot study, we established that the PMA concentration used in this study (2·4 μM) was the optimum concentration, yielding highest chemiluminescence signals when compared with 0·24 or 12 μM. Furthermore, we could also inhibit PMA-stimulated luminol-enhanced chemiluminescence of BAL cells after ozone by 87%, by adding superoxide dismutase (SOD; 0·8 mg ml^{-1}; 3020 U (mg protein)$^{-1}$) to the reaction medium. It is known that SOD blocks the effects of $O_2^-\cdot$ radicals by preventing their reaction with H_2O_2 to generate the highly reactive hydroxyl radicals (Halliwell & Gutteridge, 1984) and preventing the release of iron from ferritin (Gutteridge, Richmonds & Halliwell, 1979). This suggests that PMA-stimulated luminol-enhanced chemiluminescence of BAL cells after ozone is measuring oxygen radicals derived from $O_2^-\cdot$.

Luminol-enhanced chemiluminescence is a well established, albeit indirect, method of measuring the release of oxygen radicals from activated alveolar macrophages from rabbit, sheep and guinea-pig (Allen & Loose, 1976). Recently, luminol-enhanced chemiluminescence has been used to measure the respiratory burst of BAL macrophages from asthmatics and normal controls (Cluzel *et al.* 1987), and from patients with idiopathic pulmonary fibrosis (Strausz, Muller-Quernheim, Steppling & Ferlinz, 1990). Strausz *et al.* (1990) have suggested that alveolar macrophages are the most important source of oxygen radicals in BAL. However, Williams & Cole (1981) have concluded that human alveolar macrophages produce little, if any, luminol-dependent chemiluminescence. Also, Ward *et al.* (1990) suggested that luminol-dependent chemiluminescence measures oxygen radicals only from polymorphonuclear leukocytes. In the present study, we found a negative correlation between the numbers of macrophages in BAL and PMA-stimulated chemiluminescence, but a positive correlation between numbers of neutrophils in BAL and PMA-stimulated chemiluminescence. This indirect evidence suggests that neutrophils rather than macrophages are an important source of PMA-stimulated oxygen radical release from canine BAL cells. While epithelial cells were also significantly increased in BAL after ozone, we were unable to find any correlation between the increase in these cells and the increase in the oxygen radical production. However, these conclusions are dependent on associations only, and do not exclude the possibility that activated alveolar macrophages, epithelial cells, or other airway cells may be the cell(s) of origin of the oxygen radical release.

The production of oxygen radicals by inhaled aerosolized xanthine and xanthine oxidase can cause a dose-dependent increase in bronchoconstriction and airway hyper-responsiveness in cats (Katsumata *et al.* 1990). These induced increases in lung resistance were significantly inhibited by pretreatment with the inhaled antioxydative enzymes, polyethylene glycol-SOD and/or polyethylene glycol-catalase. This was a not a non-specific protein-scavenging effect, as heat-inactivated polyethylene glycol-SOD had no inhibiting effects (Katsumata *et al.* 1990).

Ozone inhalation increases ACh airway responsiveness. Exogenous inflammatory mediators, like prostaglandin (PG) D_2 and $F_{2\alpha}$, leukotriene (LT) B_4 and thromboxane (TX) A_2 invoke airway smooth muscle hyper-responsiveness, and drugs that antagonize the synthesis or effects of these mediators can prevent airway hyper-responsiveness (O'Byrne, Walters, Aizawa, Fabbri, Holtzman & Nadel, 1984*a*; O'Byrne *et al.* 1985). In humans, inhalation of ozone can lead to increases in PGE_2, $PGF_{2\alpha}$, TXB_2, neutrophil elastase, and protein levels in bronchoalveolar lavage fluid (Devlin *et al.* 1991). H_2O_2 has been shown to induce eicosanoid release in rat (Sporn *et al.* 1988) and guinea-pig (Cohen, Palmer, Welsh & Sheppard, 1991) alveolar macrophages, and human (Leikauf, Zhao, Zhou & Santrock, 1993) and bovine (Leikauf, Driscoll & Wey, 1988) tracheal epithelial cells. Based on *in vitro* experiments, it has been suggested that inhaled ozone can react with unsaturated fatty acids in membrane phospholipids (Roehm, Hadley & Menzel, 1971). If ozone inhalation *in vivo* increases the formation of H_2O_2, this could lead to an increase in lipid peroxidases and eicosanoid production. Ozone inhalation could also oxidize amino acids, which could lead to altered proteins with altered functions (Mehlman & Borek, 1985). Both processes can lead to an increase in airway responsiveness. However, oxygen radicals may alter airway function through other mechanisms, which include β-adrenergic receptor dysfunction (Engels, Oosting, Hendricks & Nijkamp, 1985).

Allergen inhalation has been demonstrated to increase BAL cell oxygen radical production (Calhoun & Bush, 1990), and we have demonstrated that this correlates with allergen-induced airway hyper-responsiveness in dogs (Stevens, Woolley, Wattie & O'Byrne, 1994). Thus, not only oxidative stimuli, such as ozone, which cause airway hyper-responsiveness, but also non-oxidative stimuli, such as

allergen inhalation, cause an increase in the capacity of BAL cells to produce oxygen radicals.

In conclusion, this study has demonstrated that ozone inhalation increases oxygen radical release from BAL cells. The increase in the capacity to produce oxygen radicals is correlated with the ozone-induced increase in the airway hyper-responsiveness. These results, taken together with our previous evidence that the antioxidants allopurinol and Desferal prevent ozone-induced airway hyper-responsiveness (Matsui *et al.* 1991), support the hypothesis that oxygen radicals from activated inflammatory cells (probably neutrophils) are involved in causing ozone-induced airway hyper-responsiveness in dogs.

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Acknowledgements

The authors thank Cathy Lane and Jennifer Wattie for their technical assistance.

Received 12 April 1994; accepted 21 December 1994.