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Involvement of haemoxygenase-1 in ozone-induced airway inflammation and hyperresponsiveness

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Abstract

Haemoxygenase catalyses the degradation of haem to bilirubin, and the inducible form of haemoxygenase, haemoxygenase-1, is highly induced in response to oxidative stress in vitro. The effect of haemoxygenase-1 in oxidant stress in vivo is not known. We determined the effect of exposure to ozone on haemoxygenase-1 expression, and the modulation of haemoxygenase-1 expression on ozone-induced lung neutrophilia and bronchial hyperresponsiveness in rats. Ozone caused a significant induction of lung haemoxygenase-1. Pretreatment of rats with haemoglobin, a potent inducer of haemoxygenase-1, resulted in a large induction of haemoxygenase-1 expression, and inhibited ozone-induced neutrophilia and bronchial hyperresponsiveness. Tin protoporphyrin, a competitive inhibitor of haemoxygenase, reduced the expression of haemoxygenase-1 induced by haemoglobin. It enhanced ozone-induced neutrophilia, but not the bronchial hyperresponsiveness, and reduced the protective effect of haemoglobin. Overall, there was an association between bronchial hyperresponsiveness and the neutrophilic response. These data indicate that haemoxygenase-1 plays an important role in modulating the effects of an oxidant, such as ozone in the lungs.

Keywords: Ozone; Haemoxygenase; Carbon monoxide (CO); Neutrophil; Bronchial hyperresponsiveness

1. Introduction

Haemoxygenase is an enzyme that catalyses the degradation of haem to form bilirubin and carbon monoxide. Two distinct iso-forms of haemoxygenase called haemoxygenase-1 and haemoxygenase-2 have been described and are the products of two distinct genes (Choi and Alam, 1996; Maines, 1988). Whereas, haemoxygenase-2 is constitutively expressed and is found abundantly in brain, testis and liver, haemoxygenase-1 is induced by haem, as well as non-haem agents, such as heavy metals and cytokines. Haemoxygenase-1 is induced by a variety of agents that lead to oxidant stress, including ultraviolet irradiation and hydrogen peroxide (Keyse et al., 1990; Lautier et al., 1992; Choi et al., 1996). Induction of haemoxygenase-1 is considered to be part of a generalised response to oxidative stress, manifested by glutathione depletion and oxidative degradation of protein (Ewing and Maines, 1993; Applegate et al., 1991). A protective role for haemoxygenase-1 has been postulated since the reactions of haemoxygenase lead to the degradation of a pro-oxidant (haem) and, ultimately, the formation of an antioxidant (bilirubin). This is supported by studies, in which transfection of the haemoxygenase gene led to protection against oxidative stress (Dennery et al., 1997; Lee et al., 1996).

One environmental source of oxidative stress is ozone, which is an important component of the photochemical oxidation product of air pollution, involving substrates emitted from automobile engines. Attention has been drawn to its potential adverse effects on respiratory health, because of its toxic effects related to its oxidative properties. Exposure of animals to ozone induces airway and alveolar epithelial necrosis, together with an inflammatory response, characterised by an influx of neutrophils, together with the increased expression of several pro-inflammatory cytokines and enzymes, such as interleukin-1β, tumour necrosis factor-α (TNF-α), cytokine-induced neutrophil chemoattractant (CINC), macrophage inflammatory protein

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(MIP-2) and inducible nitric oxide synthase (Pendino et al., 1994; Punjabi et al., 1994; Haddad et al., 1995a,b, 1996). In addition, exposure to ozone leads to an increase in bronchial responsiveness to bronchoconstrictor agents (Tsukagoshi et al., 1995), although the relationship of the inflammatory process to this phenomenon is unclear.

We have determined whether the oxidant stress of breathing ozone can induce the expression of haemoxygenase-1 in the lungs and whether this expression can modulate the various pro-inflammatory effects of ozone and bronchial hyperresponsiveness. We therefore examined the effect of the haemoglobin, an inducer of haemoxygenase (Pimstone et al., 1971), and of the Sn protoporphyrin, an inhibitor of haemoxygenase (Anderson et al., 1984; Kappas and Drummond, 1986), in rats exposed to ozone.

2. Methods

2.1. Protocol

Virus-free inbred male Brown–Norway (BN) rats weighing 250–300 g (Harlan Olac, Bicester, Oxon, UK) were kept in a special caging system with its own air circulation (Maximizer; Thorens Caging System, Hazelton, PA). We studied the effect of hemoglobin, an inducer of HO-1, and tin protoporphyrin, an HO-1 inhibitor, in rats exposed to 3 ppm ozone for 6 h. Rats were anesthetized with an i.p. injection of 2 mg/kg midazolam (Roche Products, Welwyn Garden City, UK) and an s.c. injection of 0.4 mg/kg Hypnorm (Janssen Pharmaceuticals, Wantage, UK), which contains 0.315 mg/ml of fentanyl citrate and 10 mg/ml of fluanisone. After adequate anaesthesia was achieved, rats were administered 300 mg/kg rat hemoglobin (Sigma, St. Louis, MO) intravenously, 16 h before exposure of filtered laboratory air or 3 ppm ozone for 6 h. For Sn protoporphyrin studies, Sn protoporphyrin (Porphyrin Products, Logan, UT) was prepared in 0.1 M NaOH, to which an equal volume of saline was added and the pH was adjusted to 7.4, and administered to rats (50 μmol/kg s.c.) 21 h before exposure to air or ozone.

2.2. Ozone exposure

Ozone was generated by passing laboratory air (1 l/min) through a Sander ozoniser (Model IV, Sander, Vetze, Germany). The output was diluted with compressed air (10 l/min) controlled by a gas flowmeter (Platon Flow Control, Basingstoke, UK) and fed into a 32-l box made of Perspex. The concentration of ozone was determined by using specific gas sampling tubes (Dragerwerk, Lubeck, Germany) and was maintained at 3 ppm by regular measurement at the output port of the box. Conscious rats were exposed to ozone for 6 h, and control rats breathed filtered air only.

2.3. Measurement of airway responsiveness to acetylcholine

Airway responsiveness was measured 18–24 h after a 6 h exposure to 3 ppm of ozone. Rats were anaesthetized as above. A tracheal cannula (1.02-mm OD) was inserted into the cervical trachea through a tracheostomy and tied snugly with suture material. A polyethylene catheter was inserted into the left carotid artery to monitor blood pressure and heart rate with a pressure transducer. The right external jugular vein was cannulated for administration of intravenous drugs and fluids. The animals were then connected to a small-animal respirator (Harvard Apparatus, Edenbridge, Kent, UK) and ventilated with 10 ml/kg of air at a rate of 90 strokes/min. Transpulmonary pressure was measured with a pressure transducer (model FCO 40 ± 1000 mm H_2O, Furness Controls, Bexhill, Sussex, UK), with one side attached to an air-filled catheter inserted into the right pleural cavity, and the other side attached to a catheter connected to a side port of the intratracheal cannula. The ventilatory circuit had a total volume of 20 ml.

Airflow was measured with a pneumotachograph (model F1L, Mercury Electronics, Glasgow, Scotland) connected to a transducer (model FCO 40 ± 20 mm H_2O, Furness Controls). The signals from the transducers were digitized with a 12-bit analog-to-digital board (NB-MIO-16, National Instruments, Austin, TX) connected to a MacIntosh II computer (Apple Computer, Cupertino, CA) and analyzed with a software (Lab VIEW 2, National Instruments, Austin, TX). Lung resistance (R_L) was calculated according to the method of Neergard and Wirz (1927). Arterial blood pressure was also monitored throughout the experiments.

Aerosols were generated with an ultrasonic nebulizer (model 2511, PulmoSonic, DeVilbiss, Hazeltown, PA) and were administered to the airways through a separate ventilator system that bypassed the pneumotachograph. The volume of this circuit was 50 ml. The mean mass diameter of the aerosol was 3.8 μm, with a geometric standard deviation of 1.3, measured with a laser droplet and particle analyzer (model 2600C, Malvern Instruments, Derbyshire, UK).

The animals were initially injected with propranolol (1 mg/kg i.v.) to inhibit adrenergic effects and suxamethonium (1.5 mg/kg) to stop spontaneous breathing. A dose of inhaled saline was administered for 45 breaths, and the subsequent R_L value was used as the baseline value for the calculation of acetylcholine responsiveness. Starting 3 min after saline exposure, increasing half-$\log_{10}$ concentration of acetylcholine were administered by inhalation (45 breaths), with the initial concentration set at 10^{-4} M. Increasing half-log concentration were administered at 5–7-min intervals with one hyperinflation of twice the tidal volume applied between each acetylcholine concentration, performed by manually blocking the outflow of the ventilator. Animals were allowed to recover to baseline R_L.

The challenge was stopped when an increase in R_L exceeding 200% over the initial baseline value was obtained. The $-\log_{10}$ transformation of the provocative concentration of acetylcholine, producing a 200% increase in R_L ($-\log PC_{200}$), was calculated by a log-linear interpolation of concentration–response curves.

2.4. Bronchoalveolar lavage (BAL) and cell counting

After measurement of lung function parameters, the rats were administered an overdose of pentobarbital sodium (200 mg/kg i.v.), and the lungs were lavaged with 2 ml aliquots of saline solution 10 times through a polyethylene tube introduced through the tracheostomy. Lavage fluid was centrifuged ($500 \times g$ for 10 min at 4°C), and the cell counts were determined from cytospin preparations, which were made by centrifuging at 300 rpm for 5 min and stained with May–Grünwald stain. Cells were identified as macrophages, neutrophils, eosinophils, lymphocytes, basophils and epithelial cells by standard morphology. Five hundred cells were counted under ×400 magnification, and the percentage and absolute number of each cell type were calculated.

2.5. Western blot analysis

Following BAL, lungs were removed rapidly and snap-frozen in liquid nitrogen at −80°C until used. Frozen tissues were ground in liquid nitrogen and homogenized in 50 mM Tris/HCl pH 7.4 containing 10 mM $MgCl_2$, 0.5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml benzamidine. Protein concentrations of lysates were determined by Bradford assay. The proteins were solubized by boiling in Laemmli buffer (0.0625 mM Tris/HCl pH 6.8 containing 10% (v/v) glycerol, 1% (w/v) sodium dodecyl sulphate (SDS), 1% (w/v) β-mercaptoethanol and 0.01% w/v bromophenol blue). The proteins (50 μg/lane) were resolved by electrophoresis in 10% (w/v) SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The nitrocellulose was blocked in 10% dried milk protein in PBS containing 0.05% v/v Tween-20. The blots were washed and incubated for 1.5 h in the presence of a polyclonal rabbit anti-rat haemoxygenase-1 antibody (StressGen, Victoria, Canada) at 1:1000 dilution. The blots were washed and then incubated for 1 h with goat anti-rabbit immunoglobumin G conjugated to horse-radish peroxidase (DAKO, Glostrup, Denmark) at 1:4000 dilution. The blots were washed again and the bands were visualized using ECL kit (Amersham, Amersham Place, UK). Rat testis microsomal lysates (Affiniti Prod., Exeter, UK) were used as a positive control for haemoxygenase-1. The level of haemoxygenase-1 protein was quantified using laser densitometry.

2.6. Data analysis

All values are expressed as mean ± S.E.M. PC_{200} acetylcholine data were $\log_{10}$-transformed and reported as geometric mean. Non-parametric analysis of variance (Kruskal–Wallis methods) was used to determine significant variance among the groups. We used Mann–Whitney U test to analyze for significant difference between individual groups, and a P value < 0.05 was considered significant.

3. Results

3.1. Induction of lung haemoxygenase-1 after ozone exposure

A representative haemoxygenase-1 protein expression measured by Western blot analysis is shown in Fig. 1a. Injected haemoglobin or ozone exposure (3 ppm, for 6 h) induced haemoxygenase-1 protein in the lung tissue from air-exposed animals (1.8-fold, $P < 0.05$ and 4.1-fold, $P < 0.05$, respectively), 18–24 h following exposure compared to air-exposed animals. Haemoglobin-injected, together

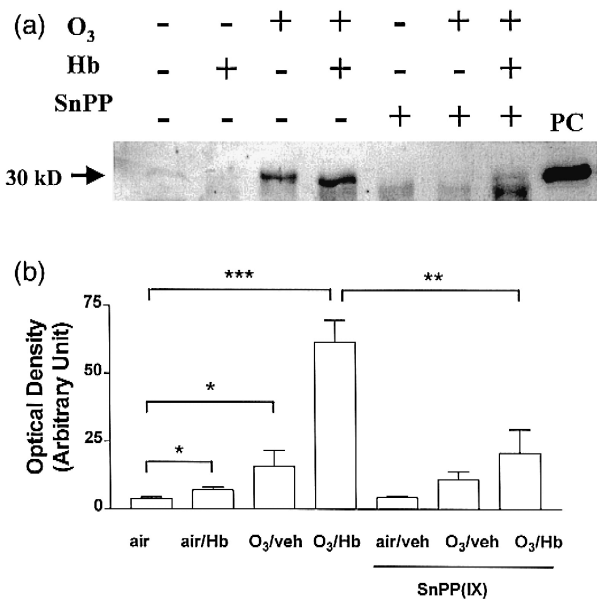

Fig. 1. Western blot analysis of haemoxygenase-1 expression from lungs of rats exposed to ozone (3 ppm, for 6 h), and the effect of the haemoxygenase-1 inducer, haemoglobin, and of the haemoxygenase-1 inhibitor, Sn protoporphyrin, 18–24 h following exposure. Rat lung protein was extracted and subjected to Western blot analysis. Panel a shows representative Western blot analyses of haemoxygenase-1 protein expression. Rat testis microsomal lysates were used as positive control (lane PC) with a specific band 32-kDa protein. Panel b shows the mean optical density (±S.E.M.) of the specific 32-kDa bands expressed in the lungs. Haemoxygenase-1 was expressed at low levels in air-exposed animals, or in air-exposed animals treated with haemoglobin or Sn protoporphyrin. However, ozone exposure induced haemoxygenase-1 expression and haemoglobin pretreatment increased this induction further. On the other hand, Sn protoporphyrin decreased haemoxygenase-1 induction by haemoglobin and ozone. $n = 5–7$ in each group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. PC: positive control, Hb: haemoglobin, SnPP: tin protoporphyrin.

with ozone-exposed rats, caused a marked increase in haemoxygenase-1 protein expression (16.2-fold, $P < 0.001$). By contrast, the haemoxygenase-1 inhibitor, Sn protoporphyrin, significantly inhibited the expression of HO-1 protein (Fig. 1b).

3.2. Cell counts in BAL fluid

The recovery rates of BAL fluid were similar in all groups (approximately 90% of instilled fluid; data not shown). There were no significant differences in the number of macrophages, lymphocytes and eosinophils recovered in BAL fluid between the groups. Compared to naive animals, the number of neutrophils recovered in BAL fluid was increased after 3 ppm ozone exposure for 6 h (air: $0.15 \pm 0.06 \times 10^6$ cells/ml vs. ozone/vehicle: $2.6 \pm 0.2 \times 10^6$ cells/ml, $P < 0.01$). Haemoglobin injection itself did not have any effect on BALF neutrophil counts. However, haemoglobin reduced the neutrophilia induced by ozone exposure ($1.4 \pm 0.2 \times 10^6$ cells/ml, $P < 0.01$). Sn protoporphyrin enhanced ozone-induced neutrophil influx into BAL fluid ($4.9 \pm 0.5 \times 10^6$ cells/ml, $P < 0.01$ compared to Sn protoporphyrin (−)/ozone/vehicle rats), which was reduced by haemoglobin administration ($2.0 \pm 0.4 \times 10^6$ cells/ml, $P < 0.01$). Sn protoporphyrin alone did not have any effect on neutrophil influx into BAL fluid in air-exposed animals (Fig. 2).

3.3. Bronchial responsiveness to acetylcholine

Exposure to 3 ppm ozone for 6 h caused significant bronchial hyperresponsiveness to acetylcholine in the groups of animals without Sn protoporphyrin treatment. Hb pretreatment prior to ozone exposure reduced this hyperresponsiveness to acetylcholine ($-\log PC_{200}$: ozone/vehicle: 2.7 ± 0.1 vs. ozone/haemoglobin: 2.2 ± 0.1, $P < 0.01$). Animals, pretreated with Sn protoporphyrin prior to ozone exposure, had significant bronchial hyperresponsiveness, which was greater than that found in Sn protoporphyrin (−)/ozone/vehicle rats, but not significantly different compared to Sn protoporphyrin (−)/ozone/vehicle rats. Bronchial hyperresponsiveness induced by ozone was reduced by haemoglobin administration ($-\log PC_{200}$: 2.4 ± 0.1, $P < 0.05$; Fig. 3).

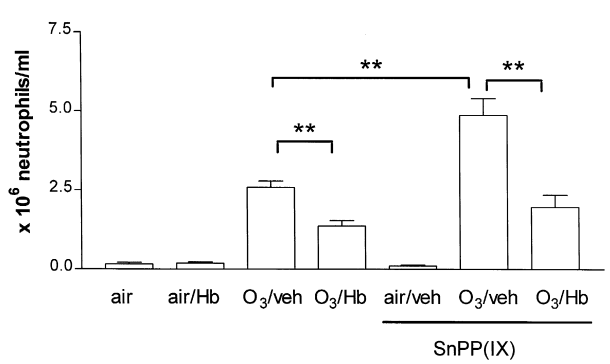

Fig. 2. Mean neutrophil counts (mean ± S.E.M.) in BAL fluid. Ozone (3 ppm, for 6 h) increased neutrophil counts and haemoglobin significantly reduced this effect. Sn protoporphyrin administration augmented ozone-induced neutrophilia. ** $P < 0.01$. $n = 6–8$ in each group.

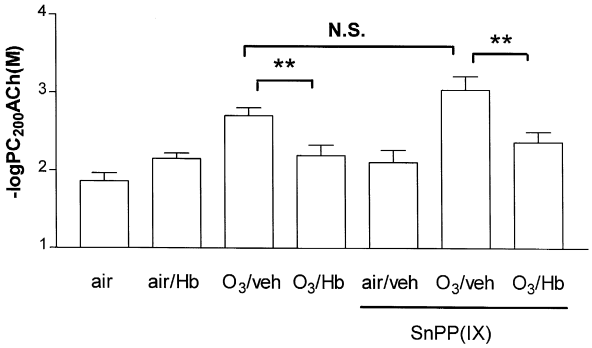

Fig. 3. Bronchial responsiveness measured as $-\log PC_{200}$ ACh (negative log of the concentration of acetylcholine needed to increase baseline resistance by 200%; mean ± S.E.M) following ozone exposure and the effects of haemoglobin and Sn protoporphyrin. Ozone increased bronchial responsiveness and haemoglobin reduced this. Sn protoporphyrin did not significantly enhance ozone-induced bronchial hyperresponsiveness. * $P < 0.05$, ** $P < 0.01$; $n = 6–8$ in each group.

4. Discussion

Because recent studies have shown that oxidative stress can augment haemoxygenase-1 induction (Keyse and Tyrrell, 1989; Keyse et al., 1990; Applegate et al., 1991), we studied the effect of ozone inhalation, which induced a marked increase in haemoxygenase-1 expression as measured by Western blot analysis. We also examined the functional role of haemoxygenase-1 in vivo by measuring the neutrophilia and the bronchial hyperresponsiveness induced by ozone exposure and by examining two known modulators of haemoxygenase-1, namely an inducer of haemoxygenase-1, haemoglobin, and an inhibitor, Sn protoporphyrin. We found that haemoglobin was able to partly reduce the neutrophilia and bronchial hyperresponsiveness induced by ozone, while tin protoporphyrin augmented ozone-induced neutrophilia, but had no effect on bronchial hyperresponsiveness. Thus, modulation of haemoxygenase-1 expression can modify the inflammatory response, and to some extent, the bronchial hyperresponsiveness to ozone.

The concentrations of haemoglobin that we used to induce haemoxygenase-1 were similar to those described by Otterbein et al. (1995), who showed that doses above 15 mg/kg, up to 300 mg/kg, protected against lethal endotoxaemia in rats. In this study, haemoxygenase activity was increased approximately threefold in the lungs, and HO-1 mRNA expression was also increased in the lungs, effects that were observed at haemoglobin doses above 10 mg/kg. These doses of haemoglobin are also needed to saturate haptoglobin binding capacity (Hershko, 1975).

Although haemoglobin induced haemoxygenase activity, it may also have other effects of relevance in this study. Thus, haemoglobin can also stimulate ferritin, independent of haemoxygenase activity (Taylor et al., 1998). Ferritin, by its ability to sequester free iron into ferritin complexes, may inhibit hydroxyl radical formation and, therefore, acts as an antioxidant (Maines, 1988). Induction of HO-1 enzyme by ozone was accompanied by the presence of neutrophilia and bronchial hyperresponsiveness, while a much greater induction with haemoglobin of haemoxygenase-1 expression, led to significant reduction in ozone-induced lung neutrophilia and bronchial hyperresponsiveness. Thus, the level of induction of haemoxygenase-1 achieved by ozone was not sufficient to modulate ozone-induced functional effects.

Haemoxygenase-1 induction protected against ozone-induced neutrophilia and bronchial hyperresponsiveness. We therefore hypothesized that suppression of haemoxygenase-1 activity would enhance these effects. We used synthetic metalloporphyrins, such as Sn protoporphyrin, which have been shown to be potent competitive inhibitors of haemoxygenase (Anderson et al., 1984; Kappas et al., 1984; Kappas and Drummond, 1986). Sn protoporphyrin has been demonstrated to be the most potent inhibitor of haemoxygenase activity in various tissues, including rat lung at the doses used (Drummond and Kappas, 1981, 1982; Yoshinaga et al., 1982) and was well-tolerated. Although we showed that the expression of haemoxygenase-1 was not affected by Sn protoporphyrin in control rats, other studies, using the same dose of Sn protoporphyrin, caused complete loss of haemoxygenase enzymatic activity (Otterbein et al., 1995). We showed that haemoxygenase-1 expression induced by ozone was inhibited by Sn protoporphyrin. However, the greater induction observed with ozone exposure in haemoglobin-treated rats was markedly reduced. In parallel, Sn protoporphyrin enhanced the neutrophilia induced by ozone. The lack of a concomitant significant effect on bronchial hyperresponsiveness may be related to a maximal response already achieved. When Sn protoporphyrin and haemoglobin were administered together prior to ozone exposure, the neutrophilic response and the bronchial hyperresponsiveness were similar to ozone-exposed rats not receiving any pretreatment. Our results complement the observations obtained with haemoglobin, and indicate that haemoxygenase, particularly haemoxygenase-1, activity can modulate ozone-induced effects. The studies using Sn protoporphyrin do not allow us to distinguish whether haemoxygenase-1 or haemoxygenase-2 were inhibited since the metalloprotoporphyrins inhibit both activities (Maines and Trakshel, 1992).

Ozone-induced neutrophilia is likely to be secondary to the release of neutrophil chemoattractants, in particular CINC, since an anti-CINC antibody blocked ozone-induced neutrophilia (Koto et al., 1997). Ozone also induces the expression of other cytokines and enzymes such as TNF-α, interleukin-16, interleukin-1β and iNOS, which may also be involved in the recruitment of inflammatory cells, such as neutrophils. It is not known whether haemoxygenase-1 is necessary for the induction of these cytokines. Binding of the transcription factor, activating protein-1 (AP-1), to DNA has been shown to be necessary for the transcriptional activation of haemoxygenase-1 by lipopolysaccharide (Camhi et al., 1995). It is more likely for haemoxygenase-1 and these cytokines to be expressed concomitantly. In a rat model with haemoxygenase-1 overexpression in the airway epithelium, death resulting from hyperoxia was reduced, together with the accompanying neutrophilia (Otterbein et al., 1999).

In the current study, the modulation of ozone-induced bronchial hyperresponsiveness, induced by altering haemoxygenase-1 activation, paralleled that of ozone-induced neutrophilia, supporting a link between the presence of neutrophils in the airways and bronchial hyperresponsiveness. In previous studies, we have observed that antioxidants, such as allopurinol and desferoxamine can inhibit ozone-induced bronchial hyperresponsiveness, but not the neutrophilia (Tsukagoshi et al., 1995). In addition, inhibition of the neutrophilia by an antibody to the neutrophil chemoattractant, CINC, did not inhibit BHR (Koto et al., 1997). Overall, taken together, the relationship between neutrophilia and bronchial hyperresponsiveness is complex and may not entirely be a direct cause-and-effect relationship. We have also observed that apocynin, another antioxidant, inhibited ozone-induced airway epithelial proliferation, but not the neutrophilia (Salmon et al., 1998), which would indicate that ozone-induced epithelial proliferation may be involved in the increase in bronchial hyperresponsiveness after ozone.

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