

# Proliferation of Airway Epithelium After Ozone Exposure

## Effect of Apocynin and Dexamethasone

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Ozone is an environmental pollutant with potent oxidizing properties. We investigated whether exposure to ozone-induced cell proliferation in the lungs of rats, and determined the effect of an antioxidant and of a glucocorticosteroid in Brown-Norway (BN) rats. Following single ozone exposure (0.5, 1.0, or 3.0 ppm for 6 h), proliferating cell nuclear antigen (PCNA) expression, as determined with immunohistochemistry, was significantly increased in the bronchial epithelium and alveolar epithelium as compared with controls exposed to filtered air with a maximal effect at 24 to 48 h ( $p < 0.001$ ). Apocynin (5 mg/kg, orally), a reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase inhibitor, reduced the PCNA index in bronchial epithelium induced by ozone (3 ppm, 6 h) from  $11.5 \pm 1.3\%$  (percent of nuclear cells expressing PCNA) to  $4.4 \pm 1.3\%$  (mean  $\pm$  SEM;  $p < 0.05$ ). Dexamethasone (3 mg/kg, intraperitoneally) also reduced the PCNA index in bronchial epithelium, from  $19.2 \pm 2.3\%$  to  $10.9 \pm 2.6\%$  ( $p < 0.05$ ). Dexamethasone but not apocynin inhibited ozone-induced neutrophil influx. Rats exposed repeatedly to ozone (3.0 ppm, 3 h, on three occasions 48 h apart) expressed a lower PCNA index in bronchial epithelium than did rats exposed only once at  $1.9 \pm 0.7\%$  versus  $6.0 \pm 0.9\%$ , respectively ( $p < 0.05$ ). The proliferative epithelial response following a single exposure to ozone is modulated through oxidative and inflammatory mechanisms probably involving neutrophils. Salmon M, Koto H, Lynch OT, Haddad E-B, Lamb NJ, Quinlan GJ, Barnes PJ, Chung KF. Proliferation of airway epithelium after ozone exposure: effect of apocynin and dexamethasone.

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Ozone is an important component of the photochemical oxidation product of air pollution involving substrates emitted from automobile engines, and attention has been drawn to its potential adverse effects on respiratory health because of its potential toxic effects related to its oxidative properties (1). Exposure of animals to ozone induces airway and alveolar epithelial necrosis, together with an inflammatory cellular response characterized by an influx of neutrophils (2-4) and increased expression of several proinflammatory cytokines and enzymes, such as interleukin-1 $\beta$  (IL-1 $\beta$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), cytokine-induced neutrophil chemoattractant (CINC), macrophage inflammatory protein (MIP-2), and inducible nitric oxide synthase (iNOS) (4-7). Ozone induces epithelial necrosis with a consequent increase in epithelial-cell proliferation that has been observed either through incorporation of 5-bromo-2-deoxyuridine (BrdU) or tritiated thymidine labeling, which is probably a reflection of the epithelial-cell repair process (3, 8, 9). The relationship between neutrophil influx and epithelial-cell necrosis and repair is unclear. Direct

oxidative injury induced by ozone may occur immediately after exposure to ozone, but further damage to the epithelium may occur with the migration of neutrophils into the airway epithelium, with the release of oxidants and proteolytic enzymes in the vicinity of the epithelium. Recent studies with monkeys have provided evidence for the contribution of granulocytes to injury and repair of the tracheobronchial epithelium after short-term exposure to ozone (2). However, other studies, with rats, have indicated that the acute epithelial injury induced by ozone is likely to be a direct effect of ozone and not an indirect effect of neutrophils (10).

In order to understand further the direct role of ozone as an oxidant, or of inflammatory factors in the proliferation of the epithelium induced by ozone, we studied the effects of the antioxidant inhibitor of superoxide-generating reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, apocynin (11), and of the antiinflammatory corticosteroid dexamethasone. To assess cell proliferation, we examined the expression of the proliferation marker proliferating cell nuclear antigen (PCNA) which is an auxiliary protein of DNA polymerase- $\delta$  located in the nuclear compartment of both normal and transformed proliferating cells (12, 13).

## METHODS

### Ozone Exposure

Pathogen-free, male Brown-Norway (BN) rats weighing 220 to 300 g (Harlan, Bicester, UK) were housed at all times in a caging system

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(Maximiser; Thorens Caging Systems Inc., Hazleton, PA). Rats were exposed to ozone in a 30,000-cm<sup>3</sup> Perspex box, with ozone being generated by passing laboratory air through a Sander Ozoniser (Model 500; Erwin Sander GmbH, Uetze, Germany) at a rate of 0.5 L/min and mixed with compressed air at a rate of 8 L/min. The ozone concentration was measured at regular time points, using specific sampling tubes (Dräger Ltd, Blyth, UK) introduced via an outlet port to the chamber.

#### Time Course and Dose Response of PCNA Expression

The time course of PCNA expression in the lungs following single exposure to ozone was investigated by exposing rats ( $n = 2$  for each time point) to 3.0 ppm ozone for 6 h and then collecting lung tissue at 4, 12, 24, 48, 96, and 168 h after exposure. The effect of increasing concentrations of ozone was investigated by exposing rats ( $n = 3$  for each dose) to 0.5, 1.0, or 3.0 ppm ozone for 6 h; control rats received filtered air only. Tissues were collected at the time of maximal response to ozone as determined by the time-course experiments.

#### Drug Pretreatments

Drug pretreatments were done on rats exposed once to ozone. For experiments with apocynin, rats were lightly anesthetized (0.3 ml/kg Hypnorm, consisting of 0.315 mg/ml fentanyl citrate and 10 mg/ml fluanisone, given intramuscularly), and were then given a 5 mg/kg oral dose of apocynin (4-hydroxy-3-methoxyacetophenone) dissolved in 1 ml of 5% ethanol. Control rats were anesthetized in the same way, but were given vehicle alone. Dexamethasone (3 mg/kg) was administered intraperitoneally, with controls receiving saline only. All drug pretreatments were given 1 h before ozone exposure commenced. Control rats breathing filtered air and treated with vehicle were also studied.

#### Triple Ozone Exposure

We examined the effect of three consecutive ozone exposures (3 ppm, 3 h each) given every 48 h. Lung tissues were collected 48 h after the final exposure. Lung tissues from rats receiving a single ozone exposure were also collected 48 h after exposure. Rats exposed to filtered air only were used as controls ( $n = 6$  in all groups).

#### Tissue Processing

Rats were killed with an overdose of sodium pentobarbitone (500 mg/kg intraperitoneally). The trachea and lungs were rapidly removed and insufflated via the trachea with O.C.T. Tissue Tek mounting medium (Raymond A. Lamb, London, UK) diluted 1:1 with phosphate-buffered saline (PBS). Regions of trachea, main bronchus, and left and right lung lobes were mounted on cork blocks, snap-frozen in isopentane, and stored at  $-20^{\circ}\text{C}$ .

#### PCNA Immunohistochemistry

Cryostat sections (5  $\mu\text{m}$ ) were cut horizontally and perpendicular to the plane of the airways, and were then thaw-mounted onto glass microscope slides pretreated with poly-L-lysine (Sigma Chemicals, Poole, UK). Tissue sections were fixed in acetone at  $4^{\circ}\text{C}$  for 5 min and rinsed in PBS for a further 10 min. Endogenous peroxidase was blocked by immersing sections in methanol containing 0.3% hydrogen peroxide for 30 min and then washing them in PBS. Primary anti-PCNA (PC10 clone) monoclonal antiserum (Dako, High Wycombe, UK) was then applied at a concentration of 1:150 in PBS containing 0.05% bovine albumin and normal horse serum, and the sections were incubated for 1 h at room temperature. A rat adsorbed, biotinylated, secondary antimouse monoclonal antiserum (Vector Laboratories, Peterborough, UK) was then applied for 30 min, followed by a 45-min incubation with an avidin-biotin complex reagent (ABC-Elite kit, Vector Laboratories). Negative control sections were performed with normal mouse IgG or with the incubation medium without primary antibody. PCNA-positive cells were visualized with permanent peroxidase stain, using a 3,3-diaminobenzidine tetrahydrochloride solution (Sigma Chemicals) with glucose oxidase/nickel color enhancement, as described in the method of Shu and colleagues (14). Nuclei were counterstained with a light hematoxylin stain, were dehydrated, and were mounted under glass coverslips with DPX mounting medium (Raymond A. Lamb).

#### Neutrophil Recruitment into the Lungs

Lung tissue sections (5  $\mu\text{m}$ ) were stained with an antirat granulocyte antibody to assess neutrophil influx. Briefly, sections were fixed in acetone for 5 min at  $4^{\circ}\text{C}$  and rinsed in PBS buffer, and endogenous peroxidase was then blocked with 0.3% hydrogen peroxide in methanol for 20 min. A mouse antirat granulocyte antibody (clone MOM/3F12/F2; Serotec Ltd, Oxford, UK) was applied to sections for 1 h at room temperature. A secondary biotinylated antiserum to mouse IgG (Vector Laboratories) was applied for a further 30 min. Granulocytes were visualized with Sigma Fast red TR/Naphthol AS-MX for alkaline phosphatase (Sigma Chemicals) with all nonstaining cells counterstained with hematoxylin. Sections were mounted under glass coverslips, and granulocytes counted in tissue sections, using a light microscope. Evaluation of neutrophil influx was done in lung sections from regions of airway and parenchyma. Neutrophil counts around airways included epithelium, submucosal, and smooth-muscle regions, and were expressed as the number of positively stained granulocytes per millimeter of basement-membrane length. Counts in the parenchyma were assessed as the number of neutrophils in 12 random fields at  $\times 200$  magnification.

#### Quantification of PCNA-positive Cells

Quantification of PCNA-positive cells was performed on sections of trachea and main bronchus, and on two sections of lung, one from the upper lung, incorporating larger bronchi and conducting airways, and one from the hilar region for medium to small airways. The PCNA index was calculated for all individual airways ranging from 100  $\mu\text{m}$  to 800  $\mu\text{m}$  in diameter in each tissue section, using manual eye-counts under a light microscope at  $\times 1,000$  magnification. The number of airways counted ranged from 10 to 32. The proliferative index was calculated as the number of positive PCNA-stained epithelial cells divided by the total number of epithelial cells lining the perimeter of the airway. Counts to a maximum of 500 cells were made by one observer (M.S.). The slides were blinded to this observer. The PCNA index in alveolar epithelium was calculated from a total of 10 fields selected at random (magnification:  $\times 400$ ). The diameter of all airways was determined with a graticule placed in the eyepiece of the microscope, and was taken as the maximum width perpendicular to the long axis of the airway measured from the basement membrane. The PCNA indexes for tracheae, main bronchi, airways  $< 800$   $\mu\text{m}$  in diameter, and alveolar epithelium were analyzed separately.

#### Antioxidant Effect of Apocynin

In order to determine the antioxidant effect of apocynin, we conducted two studies. First, we determined the effect of apocynin on NADPH activation in neutrophils *in vitro* from three rats. Rat whole blood (5 to 6 ml) was obtained by cardiac puncture and added to ethylenediamine tetraacetic acid (EDTA). Erythrocytes were removed through dextran sedimentation, and the leukocyte-rich plasma was centrifuged with Ficoll-Paque PLUS (Pharmacia Biotech, Milton Keynes, UK). The remaining erythrocytes were lysed in hypotonic saline solution, and the neutrophil fraction was resuspended in 4-(2-hydroxyethyl)-1-piperazine-*N*-2-ethanesulfonic acid (HEPES) buffer containing 145 mM NaCl, 5 mM KCl, 1 mM CaCl<sub>2</sub>, 0.7 mM MgCl<sub>2</sub>, 10 mM HEPES (pH 7.4), 5 mM glucose, and 1 mg/ml bovine serum albumin (BSA). Neutrophil superoxide generation was determined through chemiluminescence (15) with a plate-shaking luminometer (Labtech International, Uckfield, UK). The reaction mixture (200  $\mu\text{l}$ ) contained 25  $\mu\text{M}$  lucigenin in HEPES buffer, apocynin (range: 1 mM to 3  $\mu\text{M}$ ) or vehicle, and  $1 \times 10^5$  neutrophils. Following preincubation at  $37^{\circ}\text{C}$  for 10 min, the reaction was initiated by the addition of phorbol myristate acetate (PMA) to give a final concentration of 100 nM. Superoxide anion formation was measured as the peak of superoxide-dismutase-inhibitable light output during the initial 30 min of the reaction.

Second, we determined the effect of apocynin on the levels of reduced glutathione (GSH) and oxidized glutathione (GSSG) in bronchoalveolar lavage fluid (BALF) of rats exposed to ozone. Rats were pretreated orally with either apocynin (5 mg/kg) or vehicle alone at 1 h before exposure to ozone (3 ppm for 6 h) or air ( $n = 2$  for each group). Twenty-four hours after exposure, rats were killed with an overdose of sodium pentobarbitone (500 mg/kg intraperitoneally) and the lungs were lavaged with 10 ml of 0.9% NaCl solution. BALF was

centrifuged and the cell pellet was reconstituted. Total cell counts were done and cytopins were made on which a differential cell count was obtained, so that the number of neutrophils could be derived. GSH and GSSG were measured with high-performance liquid chromatography (HPLC) with an electrochemical detector, based on the method of Smith and colleagues (16). Briefly, BALF samples stored at  $-80^{\circ}\text{C}$  were thawed and deproteinized by the addition of methanol, and were then concentrated by freeze-drying for 18 h. After re-suspending the concentrates in  $200\ \mu\text{l}$  of running buffer,  $20\ \mu\text{l}$  of sample was injected into a Hypersil ODS column (Jones chromatography, Mid-Glamorgan, UK). With an applied electrode potential of 1.3V, peaks were quantified from standard curves for GSH and GSSG, and the redox ratio was determined by dividing the GSSG fraction by the total glutathione.

#### Data Analysis

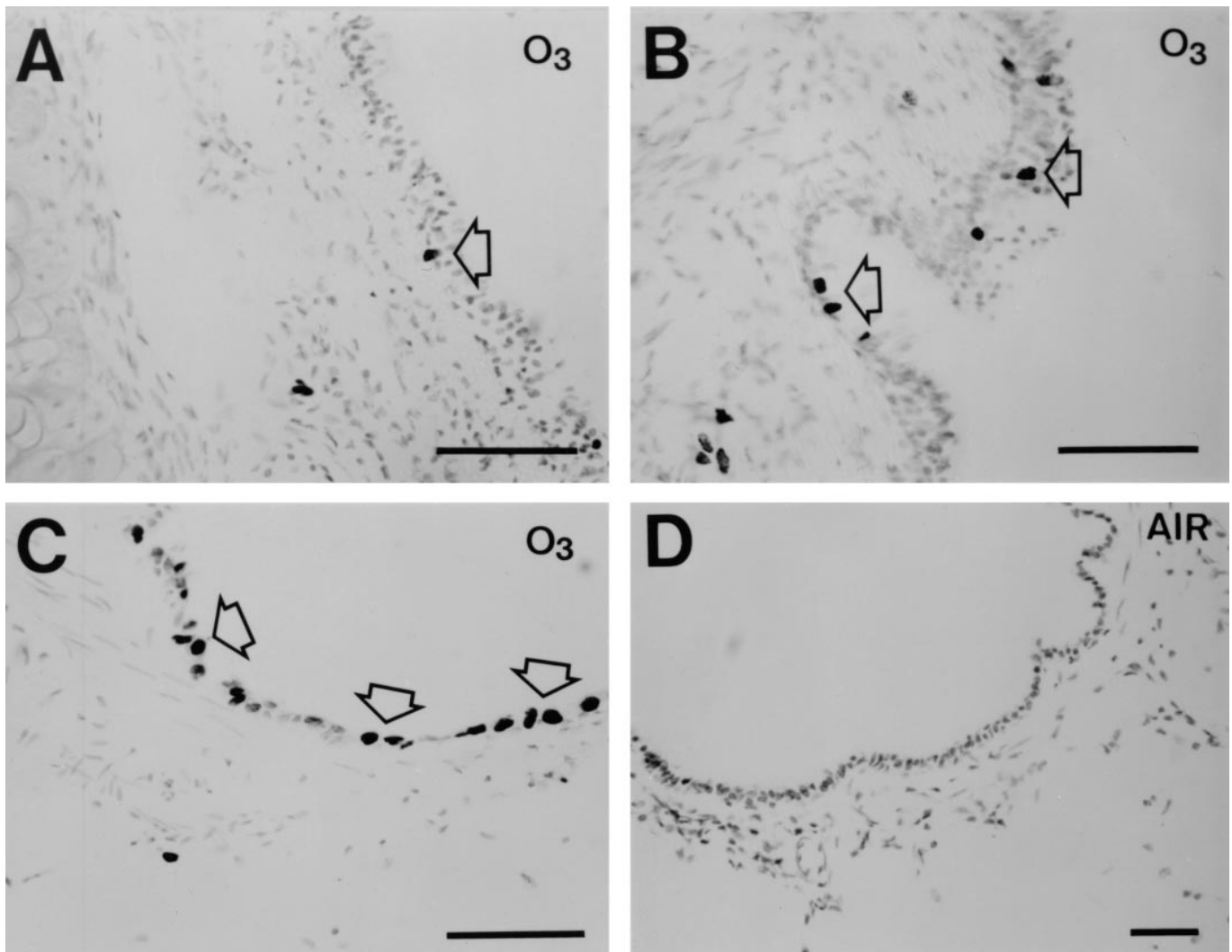
Data for PCNA indexes are expressed as arithmetic means and SE of the pooled indices from each airway category counted in all animals. Statistical analysis was done through Kruskal-Wallis nonparametric analysis of variance (ANOVA), with Dunn's multiple comparison tests used to evaluate significant differences between groups. The relationship between airway diameter and PCNA index was determined through linear regression analysis. Values of  $p < 0.05$  were considered significant.

## RESULTS

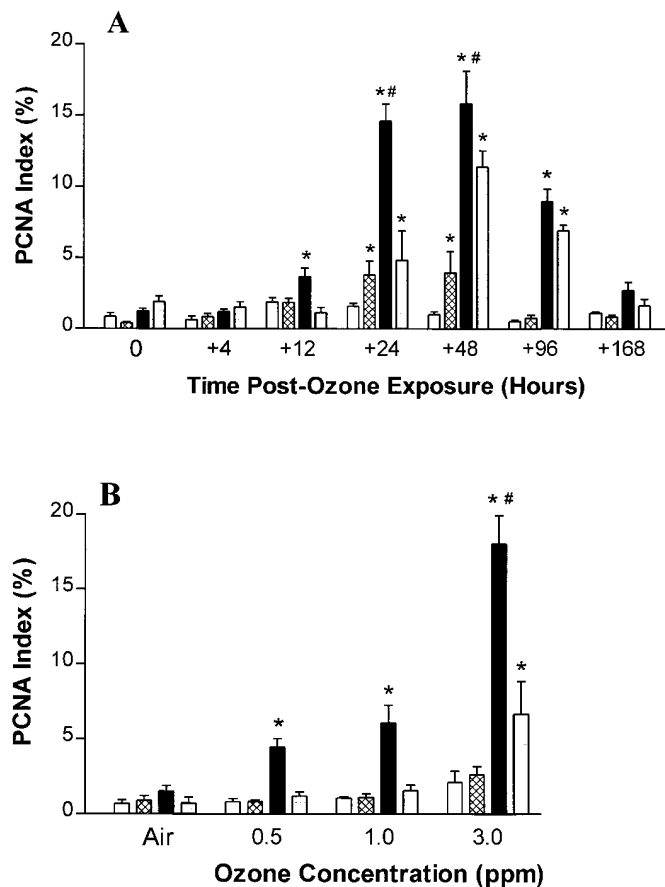
### Single Ozone Exposure

Following ozone exposure, PCNA expression was increased in the epithelial lining of the airways and alveoli of all rats as compared with controls receiving filtered air. There was no increase in PCNA expression in other cell types apart from epithelial cells (Figure 1). There was a significant increase in PCNA index in bronchial epithelium, from  $1.1 \pm 0.3\%$ , in rats exposed to filtered air, as compared with  $19.2 \pm 2.3\%$  in rats exposed to 3.0 ppm ozone for 6 h (Figure 2;  $p < 0.001$ ). In rank order, the PCNA index in the lung following ozone exposure was highest in bronchi, followed by alveolar epithelium and then main bronchus; the lowest index was found in trachea. The highest indices were generally found in airways  $< 300\ \mu\text{m}$  in diameter (Figure 3). There was a significant correlation between the PCNA index and the airway size for counts obtained for ozone-exposed rats ( $r = -0.21$ ;  $p < 0.05$ ). In the air-exposed control rats, PCNA index was low, being on the order of 1%, with no variation between airway sizes.

The time-course of PCNA expression in the intrapulmonary airways following exposure of rats to 3.0 ppm ozone for



**Figure 1.** Representative photomicrographs of airways, demonstrating an increase in PCNA index with use of a human anti-PCNA antibody (PC10) following ozone exposure (3 ppm, 6 h). (A–C) Positively staining cells in airway epithelium of trachea and airways with diameters of  $700\ \mu\text{m}$  and  $150\ \mu\text{m}$ , respectively. (D) Photomicrograph from a rat exposed to filtered air, showing an intrapulmonary airway. Arrows point to positively staining cells; horizontal bar =  $20\ \mu\text{m}$ .



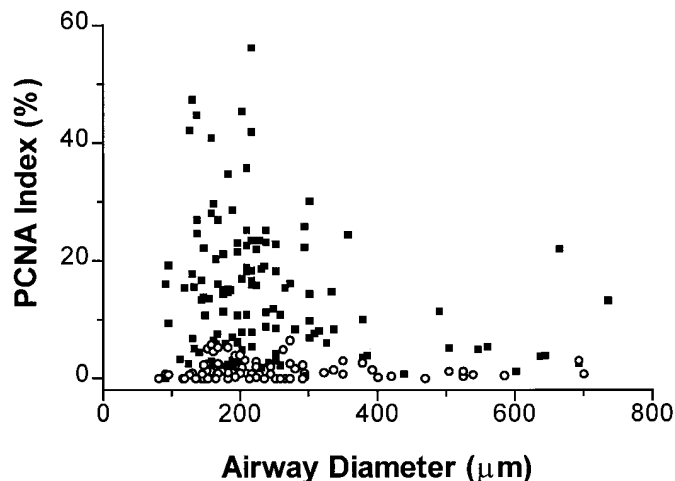
**Figure 2.** (A) Time-course of PCNA expression in epithelial cells, expressed as PCNA index in trachea (*open bar*), main bronchus (*hatched bar*), intrapulmonary airways < 800  $\mu\text{m}$  (*solid bar*) and alveoli (*stippled bar*) following ozone exposure (3.0 ppm, 6 h). Each bar represents mean PCNA index ( $\pm$  SEM) of all the airways sampled from two rats. Mean PCNA index was highest between 24 and 48 h after ozone exposure; \* $p < 0.05$  compared with time 0; # $p < 0.001$  compared with other time points. (B) Epithelial PCNA expression in trachea, main bronchus, intrapulmonary airways < 800  $\mu\text{m}$ , and alveoli after ozone exposure at 0.5, 1.0, and 3.0 ppm for 6 h. Each bar represents the mean PCNA index ( $\pm$  SEM) of all the airways sampled from three rats; \* $p < 0.05$  compared with air-exposure; # $p < 0.001$  compared with other concentrations of ozone.

6 h was significantly increased from 12 to 96 h after ozone exposure as compared with air-exposed controls ( $p < 0.05$ ), and was maximal between 24 and 48 h after exposure ( $p < 0.001$ ). PCNA index returned to baseline values 7 d after ozone exposure (Figure 2A). There was a dose-related response at all ozone concentrations used, as compared with the response in filtered-air controls ( $p < 0.05$ ). At 3.0 ppm, there was a greater PCNA index of  $18.1 \pm 1.9\%$  ( $p < 0.001$ ), as compared with  $4.5 \pm 0.6\%$  at 0.5 ppm and  $6.1 \pm 1.2\%$  at 1.0 ppm (Figure 3B).

There was little epithelial damage in tracheae and main bronchi, but more frequent evidence of epithelial damage in the intrapulmonary airways. Epithelial damage appeared mainly as disruption of the apical surface membrane, and was associated on consecutive sections with a high concentration of PCNA-positive cells.

#### Triple Ozone Exposure

Exposure of rats to ozone on three occasions 48 h apart led to a reduced mean PCNA index of  $1.9 \pm 0.7\%$  in the intrapulmo-

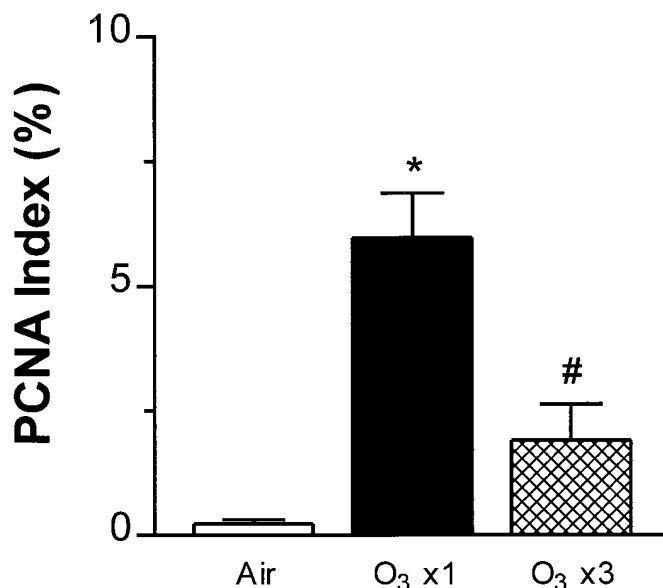


**Figure 3.** Plot of PCNA index against airway diameter measured in all airways less than 800  $\mu\text{m}$  in diameter from rats exposed to either filtered air (*open circles*) or ozone (*solid squares*) (3.0 ppm, 6 h). Airways less than 300  $\mu\text{m}$  in diameter were more susceptible to ozone exposure. Regression analysis showed a negative correlation between airway size and proliferation index following ozone ( $p < 0.05$ ).

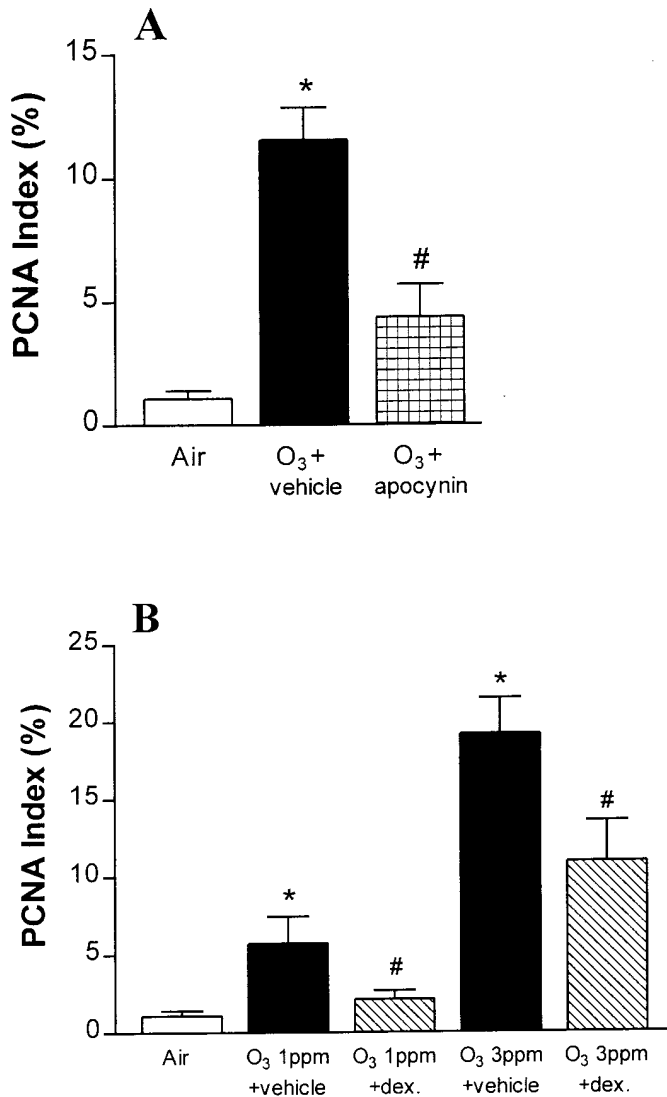
nary airways as compared with that in rats exposed to ozone on a single occasion, which had an index of  $6.0 \pm 0.9\%$  ( $p < 0.05$ ; Figure 4).

#### Effect of Apocynin and Dexamethasone

Apocynin reduced the PCNA index in bronchial epithelium in vehicle-treated rats from  $11.6 \pm 1.3\%$  to  $4.4 \pm 1.3\%$  ( $p < 0.05$ ; Figure 4).

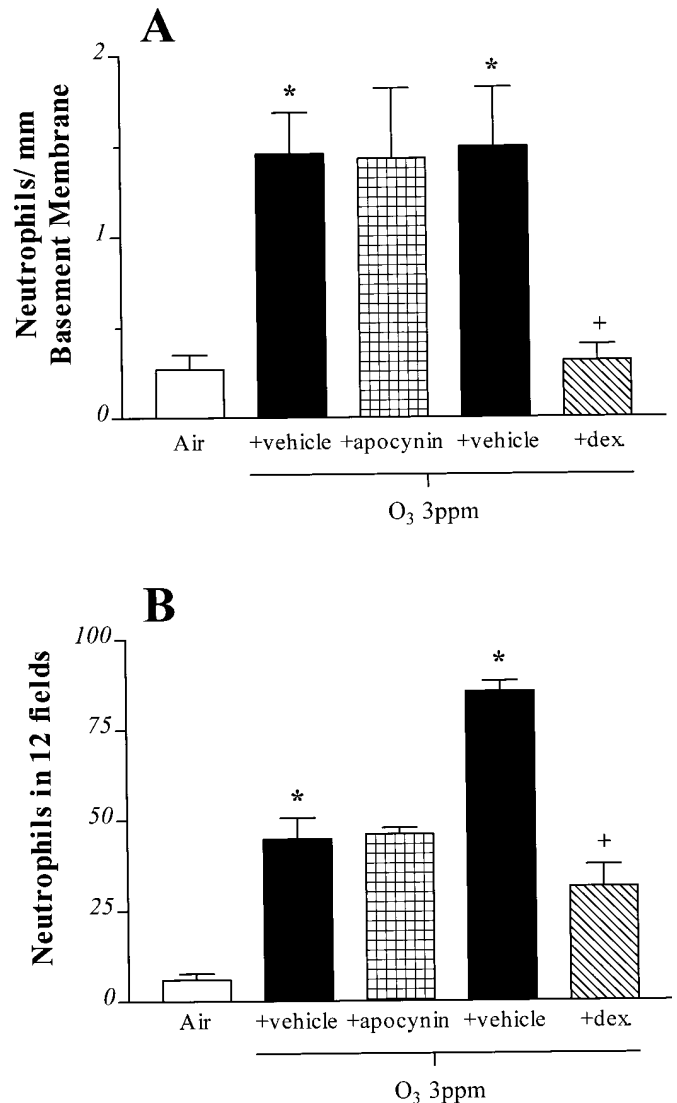


**Figure 4.** Effect of a single (ozone  $\times 1$ ) compared with a triple ozone exposure (ozone  $\times 3$ ; 3.0 ppm, 3 h) on PCNA index in intrapulmonary airways < 800  $\mu\text{m}$ . Each bar represents the mean PCNA index ( $\pm$  SEM) of all airways sampled from six rats. PCNA index was significantly increased in rats exposed to a single dose of ozone as compared with air-exposed controls. Rats exposed to a triple dose of ozone had a significantly reduced bronchial PCNA index; # $p < 0.05$  compared with single exposure; \* $p < 0.001$  compared with air-exposure.



**Figure 5.** (A) Effect of apocynin pretreatment (5.0 mg/kg) on bronchiolar epithelial PCNA index following ozone exposure (3.0 ppm, 6 h). Each bar represents the mean PCNA index ( $\pm$  SEM) of all intrapulmonary airways  $<$  600  $\mu$ m sampled for six rats. PCNA index was significantly increased in the ozone-exposed group as compared with air-exposed controls; apocynin significantly reduced PCNA index; \* $p$   $<$  0.001 compared with air-exposure; # $p$   $<$  0.05 compared with ozone and vehicle treatment. (B) Effect of dexamethasone pretreatment (dex; 3.0 mg/kg) on bronchiolar epithelial PCNA index following ozone exposure (3.0 ppm, 6 h). Each bar represents the mean PCNA index ( $\pm$  SEM) of all intrapulmonary airways  $<$  800  $\mu$ m sampled from six rats. PCNA index was significantly increased in rats exposed to 1.0 ppm and 3.0 ppm ozone as compared with air-exposed controls. Dexamethasone significantly reduced the PCNA index at both 1.0 ppm and 3.0 ppm ozone; \* $p$   $<$  0.001 compared with air-exposure; # $p$   $<$  0.05 compared with corresponding ozone level plus vehicle.

Figure 5A). Dexamethasone also significantly reduced the PCNA index in airway epithelium as compared with that in vehicle-treated controls. At an ozone concentration of 1.0 ppm, the index was reduced from  $5.7 \pm 1.2\%$  to  $2.1 \pm 0.6\%$  ( $p$   $<$  0.05), and at 3.0 ppm from  $19.2 \pm 2.3\%$  to  $10.9 \pm 2.6\%$  ( $p$   $<$  0.05; Figure 5B). In addition, apocynin had no effect on the ozone-induced increase in neutrophil counts in airway wall

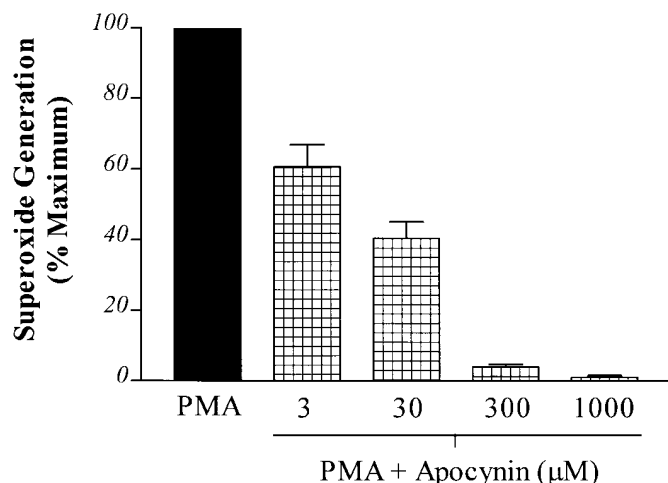


**Figure 6.** Effect of apocynin and dexamethasone on ozone-induced increases in neutrophil counts in airway walls per millimeter of basement-membrane length (A) and in 12 fields of lung parenchyma at  $\times 200$  magnification (B). Rats exposed to ozone following vehicle treatment showed significant increases in neutrophil counts in airways and parenchyma. Apocynin did not inhibit the neutrophil influx, whereas dexamethasone significantly reduced the number of neutrophils; \* $p$   $<$  0.01 compared with corresponding vehicle-treated animals; \* $p$   $<$  0.001 compared with air-exposed rats.

and lung parenchyma, whereas dexamethasone significantly inhibited neutrophil influx in both regions (Figure 6A and B).

#### Antioxidant Effect of Apocynin

**In vitro effect on neutrophils.** Apocynin competitively inhibited, in a dose-dependent manner, NADPH oxidase superoxide generation induced by PMA. The basal and PMA-stimulated superoxide values were  $0.06 \pm 0.03$  and  $5.51 \pm 0.45$  relative light units, respectively. The mean concentration of apocynin needed to inhibit oxidase activity by 50% ( $IC_{50}$ ) was  $9.6 \mu$ M (95% CI: 5.3 to 17.3). At the maximal apocynin concentration studied (1 mM), superoxide generation by NADPH oxidase was inhibited by 99% (Figure 7).



**Figure 7.** Effect of increasing apocynin concentration on NADPH oxidase activity induced by phorbol myristate acetate (PMA, 100 nM) in purified rat neutrophils ( $n = 3$ ). Ninety-nine percent of NADPH oxidase activity was inhibited at the highest concentration of apocynin used (1 mM).

**Glutathione levels in BALF.** Air-exposed rats showed a mean redox ratio of 15.4%, compared with a value of 15.5% for air-exposed rats with apocynin pretreatment. Following ozone exposure, the mean redox value increased to 32.0%, indicating oxidation of glutathione, whereas apocynin pretreatment of ozone-exposed rats reduced this value to 18.3%, indicating an antioxidant effect of apocynin. The absolute values of GSH and GSSG measured in the BALF samples are shown in Table 1. The air-exposed and the apocynin pretreated, air-exposed rats had few neutrophils recovered in BALF (means:  $8.60 \times 10^3$  and  $5.60 \times 10^3$ , respectively). Both ozone-exposed and apocynin pretreated, ozone-exposed rats showed a much greater mean number of neutrophils in BALF ( $11.89 \times 10^5$  and  $9.71 \times 10^5$ , respectively).

## DISCUSSION

In this study, we have demonstrated that a single exposure to ozone induces a significant increase in PCNA index in the bronchial epithelium of the BN rat, indicative of increased cell proliferation. PCNA expression was maximal between 24 and 48 h after ozone exposure, and was dose-dependent. Rats ex-

posed to ozone on three consecutive occasions exhibited a significantly lower PCNA index in bronchial epithelium than did animals given only a single exposure. PCNA index was significantly reduced after ozone exposure by pretreatment with both apocynin, a compound with antioxidant properties, and by the glucocorticosteroid dexamethasone.

We have used specific antibodies to PCNA to study the proliferation of cells in the airways following ozone exposure. PCNA immunoreactivity has been shown to correlate with other markers of the S phase of the cell cycle, such as findings in flow cytometry, the tritiated thymidine labeling index, BrdU incorporation, and Ki67 immunoreactivity (17–19). Blockade of PCNA production has been found to inhibit cell division, indicating that PCNA plays an important role in the process of cell proliferation (20). PCNA may be involved in the process of DNA repair in addition to *de novo* synthesis of DNA (21). This may be relevant to ozone exposure, since ozone causes DNA damage (22). However, PCNA production may be stimulated by certain growth factors, such as platelet-derived growth factor (PDGF), without inducing DNA synthesis (23). Thus, the degree of proliferation may be overestimated by the use of the PCNA index if these factors are operative with ozone exposure.

The proliferative response induced by ozone in the present study was exclusively localized to epithelial cells, and the greatest degree of proliferation occurred in the distal airways, particularly in those with a diameter  $< 300 \mu\text{m}$ , and in alveolar epithelium. Ozone has a very high reactivity and could penetrate into an air-tissue boundary. Pryor has calculated that ozone cannot cross the lung-lining-fluid layer without reacting where this layer is thicker than about  $0.1 \mu\text{m}$  (24). Because the lung-lining-fluid layer varies from 20 to  $0.1 \mu\text{m}$  in thickness from the trachea to the bronchioles (25), the more distal airways would be more susceptible to interaction with ozone. The reaction of ozone with biochemical substrates is extremely fast, with the result that ozone can be decomposed close to the gas-mucus interface in the lower conducting airways (26). The lung-lining-fluid layer is the most likely site of initial ozone interaction with absorption targets (27). Lung antioxidants, such as reduced glutathione, ascorbic acid, uric acid, and vitamin E, represent another line of defense against ozone injury, and are present within the epithelial lining fluid (28). However, it is not known whether the concentrations of these antioxidants are lower in the lining fluid of more distal airways. Lung antioxidant defenses as measured by increased activity of superoxide dismutase, catalase, and glutathione peroxidase can be augmented by a single acute exposure to ozone (29, 30), which probably underlies the reduced proliferation of the airway epithelium following three successive exposures to ozone.

In order to examine the contribution of superoxide anions in ozone-induced proliferation of airway epithelial cells, we studied the effect of apocynin, which is a potent selective inhibitor of the superoxide anion  $\text{O}_2^-$ -generating NADPH oxidase that acts by inhibiting the assembly of this enzyme in neutrophil membranes (11, 31). Stimulated neutrophils metabolically activate apocynin by releasing myeloperoxidase and reactive oxygen species, leading to the production of metabolites that prevent the formation of a functional  $\text{O}_2^-$ -generating NADPH oxidase in cell membranes of neutrophils (32). Thus, cells that lack peroxidase are insensitive to apocynin, with the result that apocynin activation is limited to cells such as macrophages and neutrophils, which have the capacity to release peroxidase. We have shown that apocynin is capable of inhibiting superoxide generation by rat neutrophils *in vitro*, and the finding of an effect of apocynin *in vivo* in preventing oxidation

TABLE 1

REDUCED (GSH) AND OXIDIZED (GSSG) GLUTATHIONE LEVELS IN BRONCHOALVEOLAR LAVAGE FLUID OF RATS EXPOSED TO AIR OR OZONE AND THE EFFECT OF APOCYNIN

Rat*	GSH ( $\mu\text{mol}$ )	GSSG ( $\mu\text{mol}$ )	Redox Ratio†
Air-exposed 1	38.2	5.9	13.4
Air-exposed 2	69.3	14.5	17.3
Air + apocynin 1	67.7	12.2	15.3
Air + apocynin 2	72.7	13.4	15.6
$\text{O}_3$ exposed 1	21.6	13.0	37.5
$\text{O}_3$ exposed 2	34.4	12.3	26.4
$\text{O}_3$ + apocynin 1	65.5	11.9	15.3
$\text{O}_3$ + apocynin 2	47.0	12.7	21.2

Definition of abbreviations: GSH = reduced glutathione; GSSG = oxidized glutathione.

\* Each value represents the result from one rat.

† Redox ratio =  $(\text{GSSG} \div \text{total glutathione}) \times 100$ .

of GSH in BALF indicates that at the dose used, apocynin exerted an antioxidant effect. Thus, our data with apocynin support a contribution of inflammatory cells, and particularly neutrophils, to the release of superoxide anions, which may in turn stimulate airway epithelial-cell proliferation. Apocynin did not inhibit neutrophil influx induced by ozone, as we have previously demonstrated (33), and therefore apocynin acts on neutrophils that have already been chemoattracted to the airway mucosa. Apocynin also has a minor effect in inhibiting mitogen-induced proliferation *in vitro* (34).

Corticosteroids are potent antiinflammatory agents that inhibit a wide range of inflammatory mechanisms of possible relevance to the mechanisms induced by ozone exposure (35). We have previously shown that ozone exposure of BN rats increased neutrophil influx in the lungs, together with an increased expression of neutrophil chemotactic cytokines CINC and MIP-2 (4, 5). CINC may be involved in the neutrophil influx because an antibody to CINC inhibited ozone-induced neutrophil influx (36). Corticosteroids at doses similar to those used in the present study inhibited both CINC and MIP-2 expression together with neutrophil influx, further indicating the relationship between neutrophil chemotactic cytokines and the observed neutrophil influx. It is possible that the inhibitory effects of corticosteroids on epithelial proliferative responses are in part secondary to neutrophil recruitment in the lungs. This link between the neutrophil and epithelial proliferative responses after ozone exposure is supported by the apparently similar time-courses of both the proliferative epithelial response and neutrophil influx after ozone exposure, with maximal responses by 24 h (4). One of the effects of oxidative stress is activation of the transcription factor nuclear factor- $\kappa$ B (NF- $\kappa$ B), leading to its binding to specific sites on the 5'-promoter sequences of several genes, including those for cytokines such as CINC and MIP-2 (37, 38). Hydrogen peroxide and other oxidants have been shown to rapidly activate NF- $\kappa$ B in certain cell lines *in vitro* (39, 40). Corticosteroids inhibit ozone-induced activation of NF- $\kappa$ B in the lungs (4). However, steroids also have other relevant effects in relation to ozone-induced inflammation, such as inhibition of release of inflammatory mediators and growth factors.

In summary, we have shown, using PCNA as a marker of cellular proliferation, that ozone exposure induces the proliferation of airway and alveolar epithelial cells, and that this response is inhibited by apocynin and dexamethasone. Our observations support a potential role for the neutrophil in this proliferative response.

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