

Short communication

The relationship between alveolar epithelial proliferation and airway obstruction after ozone exposure

Background: Proliferating cell nuclear antigen (PCNA) is a component of multiprotein complexes that are expressed during cell proliferation. Ozone induces cell necrosis and a consequent increase in cell proliferation.

Methods: To investigate the effects of acute ozone inhalation on cell proliferation and airway obstruction in BALB/C mice, we examined enhanced pause (Penh) as an index of airway obstruction and PCNA expression by immunohistochemical staining.

Results: Compared with controls that received filtered air, the ozone-exposed groups had increased PCNA expression in the alveolar epithelial cells. In rank order, the highest PCNA index was found following 2.0 p.p.m. ozone exposure. In the 2.0 p.p.m. ozone group, there was a PCNA index of $16.83 \pm 0.57\%$ (mean $\pm$ SEM; $P < 0.01$), compared with $4.25 \pm 0.5\%$ at 0.12 p.p.m., 6.83 ± 0.60 at 0.5 p.p.m. and $12.16 \pm 0.48\%$ at 1 p.p.m. Following ozone exposure, Penh was increased in a dose-dependent manner. There was a significant correlation between the PCNA index in alveoli and Penh ($r = 0.63$, $P < 0.01$).

Conclusions: These data suggest that ozone can induce alveolar epithelial cell proliferation in a dose-dependent manner, and that alveolar epithelial cell proliferation is correlated with airway obstruction.

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Ozone is the principal oxidant pollutant in photochemical smog. Along with substrates emitted from automobile engines, it is an important photochemical oxidation product of air pollution. It causes tissue injury not only in nasal airways (1, 2) but also in the pulmonary airways of animals (3). Ozone is known to decrease pulmonary function, increase airway hyper-responsiveness, and induce airway inflammation in humans (4, 5), guinea pigs (6), and dogs (7).

Ozone induces epithelial necrosis, which leads to increased epithelial cell proliferation. This effect, which is probably a reflection of the epithelial cell repair process, has been observed by 5-bromo-2-deoxyuridine (BrdU) incorporation and by tritiated thymidine labeling (8, 9). Proliferating cell nuclear antigen (PCNA) is a component of multiprotein complexes that are expressed during cell proliferation. The acute tracheobronchial epithelial injury and repair induced by ozone are thought to occur directly and indirectly through oxidative and inflammatory mechanisms involving neutrophils (3, 10).

Using barometric whole body plethysmography (WBP) and the increase in enhanced pause (Penh) as an index of airway obstruction, we measured responses to acute ozone inhalation in BALB/C mice (11).

The aim of this study was to assess cell proliferation in alveoli using PCNA expression. PCNA is an auxiliary protein of DNA polymerase delta and is located in the nuclear compartment of normal and transformed proliferating cells (12). We also examined the relationship between Penh and PCNA.

Material and methods

Animals and ozone exposures

Female BALB/c mice 5–6 weeks of age were obtained from DamulScience Laboratories (Daejeon, Korea). The mice were maintained on Ovalbumin-free diets. The mice were individually housed in rack-mounted stainless steel cages with free access to food and water. The mice housed in whole-body exposure chambers were exposed to normal ozone concentrations of 0.12 p.p.m., 0.5 p.p.m., 1 p.p.m., 2 p.p.m., and filtered room air for 3 h ($n = 6$ /group). Enhance pause (Penh) as an index of airway obstruction was measured immediately, 24 h, 48 h, and 72 h after ozone exposure. Ozone was generated with Sander model 50 ozonizers (Sander, Eltze, Germany). The concentration of ozone within the chambers was monitored throughout the exposure with ambient-air ozone motors (model 49C; Thermo Environmental Instruments Inc., Franklin, MA). The air-sampling probes were placed in the breathing zone of the mice. The mean chamber ozone concentrations ($\pm$ SEM) during the 3 h exposure period were 0.11 ± 0.02 p.p.m. (0.12 p.p.m.),

0.48 ± 0.05 p.p.m. (0.5 p.p.m.), 0.98 ± 0.03 p.p.m. (1 p.p.m.) and 1.95 ± 0.06 p.p.m. (2 p.p.m.). Temperature and humidity were maintained at a constant level within the chamber.

Determination of airway responsiveness

Airway responsiveness was measured by barometric plethysmography using whole body plethysmography (Buxco, Troy, NY) immediately after ozone exposure while the animals were awake and breathing spontaneously as a modification of the method described by Hamelmann et al. (11). Before taking readings, the box was calibrated with a rapid injection of 150 μ L air into the main chamber. Measured were pressure differences between the main chamber of the WBP containing animal, and a reference chamber (box pressure signal). This box pressure signal is caused by volume and resultant pressure changes in the main chamber during the respiratory cycle of the animal. A pneumotachograph with defined resistance in the wall of the main chamber acts as a low pass filter and allows thermal compensation. The time constant of the box was determined to be approximately 0.02 s. Mice were placed in the main chamber, and baseline reading were taken and averaged for 3 min.

Bronchoalveolar lavage (BAL) fluid preparation and analysis

Bronchoalveolar lavage was performed immediately after the last measurement of airway responsiveness. The mice were deeply anesthetized intraperitoneally with 50 mg/kg of pentobarbital sodium and were killed by exsanguination from the abdominal aorta. The trachea was cannulated with a polyethylene tube through which the lungs were lavaged three times with 1.0 ml of physiologic saline (4.0 mL total). The BAL fluid was filtered through wet 4 $\times$ 4 gauze. Trypan blue exclusion for viability and total cell count was performed. The BAL fluid was centrifuged at 150 g for 10 min. The obtained pellet was immediately suspended in 4 mL of physiologic saline, and total cell numbers in the BAL fluid were counted in duplicate with hemocytometer (improved Neubauer counting chamber). Then, a 100- μ L aliquot was centrifuged in a cytocentrifuge (Model 2 Cytospin; Shandon Scientific Co., Pittsburgh, PA). Differential cell counts were made from centrifuged preparations stained with Diff-quick, counting 500 or more cells in each animal at a magnification $\times$ 1000 (oil immersion).

Immunohistochemistry for PCNA

Immunohistochemistry was performed on tissue mounted on Probe-On Plus slides (Fisher Scientific, Pittsburgh, PA). All steps in the staining procedure were performed at 50°C with a Microprobe system that took advantage of the capillary gap action (13) produced by two opposed Probe-On Plus slides. The slides were incubated in anti-PCNA antibody for 15 min (Pharmingen, USA), and then for 10 min in goat antimouse IgG (Sigma, St. Louis, MO). To block alkaline phosphatase activity the slides were incubated for 2 min in redusol (Biomedex, Foster City, CA), and antigen/antibody complexes were detected by a 7-min incubation with avidine alkaline phosphatase (Dako) (14). The chromogen 3-amino-9-ethylcarbazole (Research Genetics, Huntsville, AL) was applied for 10 min, followed by a 30-s application of hematoxylin. PCNA antibodies were diluted in primary antibody diluent (Research Genetics, Huntsville, AL) at a final concentration of 5 μ g/mL. Samples of human spleen known to be PCNA-positive were used as positive controls and were included with each staining reaction. For negative controls, the primary antibody incubation was omitted. All slides were examined by two observers using standard light microscopy.

Quantification of PCNA-positive cells

Lung sections were used to quantify PCNA-positive cells. PCNA-positive cells in the alveoli were counted by eye under a light

microscope at 400 $\times$ magnification. The proliferative index was calculated as the number of PCNA-positive alveolar epithelial cells divided by the total number of alveolar epithelial cells lining the perimeter of the airway. The PCNA index was calculated from a total of 10 fields selected at random (magnification: 400 $\times$).

Statistical analysis

All data were analyzed using the SPSS version 7.5 for Windows. Data are expressed as mean $\pm$ SEM. Inter-group comparisons were assessed by a nonparametric method using Mann-Whitney *U*-test. The correlation between variables was examined by Spearman rank correlation coefficient. A *P*-value of less than 5% was regarded as statistically significant.

Results

The proportion of neutrophils in bronchoalveolar lavage (BAL) fluid was significantly higher in the group exposed to 2 p.p.m. ozone than in the filtered air and 0.12 p.p.m. ozone groups ($11 \pm 1.5\%$ vs. $3 \pm 0.7\%$ vs. $0.9 \pm 0.15\%$; *P* < 0.01, respectively). Following ozone exposure, PCNA expression (Fig. 1) was increased in the cell lining of alveoli compared with controls that received filtered air. In rank order, the highest PCNA index was found following 2 p.p.m. ozone exposure. Compared with the response in filtered-air controls, there was a dose-related response at all ozone concentrations (*P* < 0.01). At 2.0 p.p.m., there was a greater PCNA index of $16.83 \pm 0.57\%$ (mean $\pm$ SEM; *P* < 0.01, Fig. 2), compared with $4.25 \pm 0.5\%$ at 0.12 p.p.m., $6.83 \pm 0.60\%$ at 0.5 p.p.m. and $12.16 \pm 0.48\%$ at 1 p.p.m. In rank order, the Penh was highest after 2 p.p.m. ozone exposure, followed by 1 p.p.m. exposure. The lowest Penh was found at 0.12 p.p.m. At 2.0 p.p.m., there was a greater Penh of 1.573 ± 0.201 (*P* < 0.01), compared with 1.044 ± 0.035 at 0.12 p.p.m., 1.176 ± 0.198 at 0.5 p.p.m. and 1.191 ± 0.099 at 1 p.p.m. (Fig. 3). There was a significant correlation between the alveolar PCNA index and Penh (Fig. 4; *r* = 0.635, *P* < 0.01).

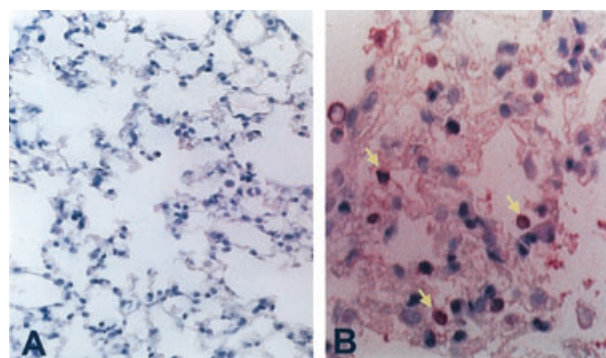


Figure 1. Photomicrographs of airways from BALB/C mice showing (A) PCNA-negative (filtered air) and (B) PCNA-positive cells (ozone exposure, 2 p.p.m., 3 h) in the alveoli. PCNA was detected with human anti-PCNA antibody. Arrow indicates positive-staining cell. Original magnification, 100 $\times$, 200 $\times$.

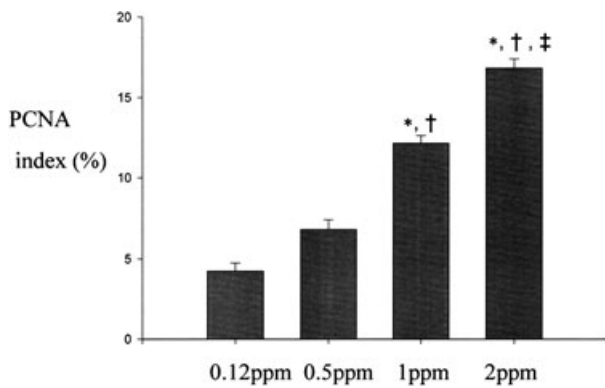


Figure 2. PCNA index in the intrapulmonary airways of BALB/C mice following ozone exposure. * $P < 0.01$ compared with control group. † $P < 0.05$ compared with 0.12 p.p.m. group. ‡ $P < 0.001$ compared with 0.2, 0.5 and 1 p.p.m. groups.

Discussion

In this study, we observed that a single exposure to ozone induced a significant increase in the alveolar PCNA index in BALB/C mice. This finding is indicative of increased cell proliferation. In addition, PCNA expression was correlated with airway obstruction.

PCNA may be involved in both DNA repair and *de novo* DNA synthesis (15). Flow cytometry, tritiated thymidine labeling, BrdU incorporation and Ki67 immunoreactivity have all shown that PCNA immunoreactivity correlates with other markers of the S phase of the cell cycle (16, 17).

Ozone induces epithelial necrosis with a consequent increase in epithelial cell proliferation. This effect has been observed by BrdU incorporation and by tritiated thymidine labeling, and is probably a reflection of the epithelial cell repair process (18). Short-term exposure to 0.2 p.p.m. ozone induces epithelial shedding and neutrophil recruitment. It also leads to an increase in the CXC chemokines growth-related oncogene- α and IL-8 in BAL fluid, and to a decrease in T-cell numbers, activation markers and the CD4⁺/CD8⁺ ratio in BAL fluid (19).

The proliferating epithelial response following a single exposure to ozone is probably modulated through oxidative and inflammatory mechanisms involving neutrophils (3). Exposure of animals to ozone induces airway and alveolar epithelial necrosis, together with an inflammatory cellular response characterized by an influx of neutrophils (20). The link between the neutrophil and epithelial proliferative responses after ozone exposure is supported by the apparently similar time courses of both reactions. Maximal epithelial proliferation and neutrophil influx occur by 24 h (21). In this study the proportion of neutrophils was increased in the BAL fluid of mice exposed to 2 p.p.m. ozone indicating a potential role for neutrophils in the epithelial proliferative response after ozone exposure. Increased expression of several proin-

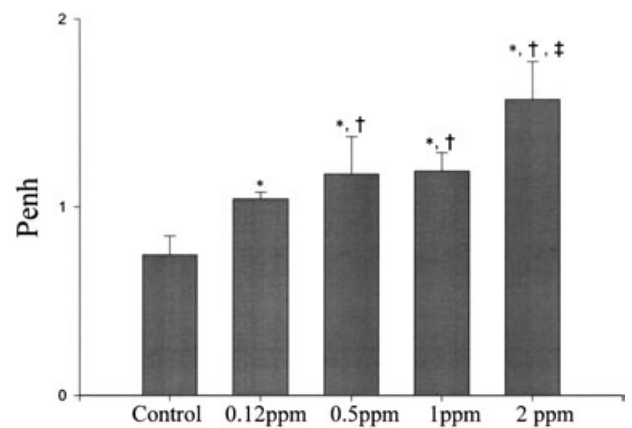


Figure 3. Enhanced pause (Penh) according to ozone concentration. * $P < 0.01$ compared with 0.12 p.p.m. group. † $P < 0.05$ compared with 0.5 p.p.m. group. ‡ $P < 0.01$ compared with 0.2, 0.5 and 1 p.p.m. groups.

flammatory cytokines and enzymes, such as interleukin-1, tumor necrosis factor- α , cytokine-induced neutrophil chemoattractant, macrophage inflammatory protein, and inducible nitric oxide synthase, have been reported (21–23). Blockade of PCNA production inhibits cell division, indicating that PCNA plays an important role in the process of cell proliferation (24). This may be relevant to ozone exposure, since ozone damages DNA (25). In this study, immunohistochemistry revealed that PCNA expression was dose-dependent on ozone, suggesting that ozone induces epithelial injury in alveoli, which results in a dose-dependent increase in alveolar epithelial cell proliferation.

We observed that Penh, an index of airway obstruction, was increased in a dose-dependent manner in mice exposed to ozone. Penh was also correlated with PCNA expression, indicating that acute ozone exposure in mice induces airway obstruction, which may be mediated by non-IgE mechanism (26) and alveolar epithelial cell proliferation. This is the first report on the relationship between airway obstruction and epithelial cell proliferation in mice exposed to ozone.

In summary, using PCNA as a marker of cell proliferation, we have shown that ozone exposure induces the proliferation of alveolar epithelial cells, and that PCNA is related to Penh. These findings suggest that ozone exposure induces alveolar epithelial cell proliferation and airway obstruction in a dose-dependent manner.

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