Effects of ozone on DNA single-strand breaks and 8-oxoguanine formation in A549 cells

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Abstract

Animal studies have demonstrated that ozone exposure can induce lung tumors. Recent epidemiological studies have also shown that increased ozone exposure is associated with a greater risk of lung cancer. This study used single-cell gel electrophoresis (the Comet assay) and flow cytometry to investigate DNA damage in A549 cells exposed to ozone levels below the current ambient standard. Cells were exposed to ozone at levels of 0, 60, 80, and 120 ppb, and then DNA single-strand breaks and 8-oxoguanine levels were measured. Additionally, the formamidopyrimidine glycosylase (Fpg) repair enzyme was added to the Comet assay to enhance detection of oxidative damage. Vitamins C and E were also added to determine their inhibitory effects on ozone-induced 8-oxoguanine. Measurements of tail length, tail intensity, and tail moment of the Comet assay were shown to correlate with each other. However, tail moment appeared to be more sensitive than the other two indicators in detecting DNA single-strand breaks. Tail moments of cells exposed to 80 and 120 ppb of ozone were significantly higher than those exposed to 0 ppb ($P < 0.05$). These three indicators of DNA single-strand breaks with Fpg were shown to be increased and more sensitive than those without Fpg. After Fpg was introduced, the tail moments at ozone levels of 60, 80, and 120 ppb were significantly higher than those at 0 ppb ($P < 0.05$). Furthermore, 8-oxoguanine levels, determined by fluorescence intensity, at 80 and 120 ppb of ozone exposure were significantly higher than the level at 0 ppb. Pretreatment with vitamins C and E reduced the 8-oxoguanine levels caused by ozone. We conclude that ozone levels below current ambient standards may induce DNA breaks and oxidative DNA damage. Moreover, the Fpg repair enzyme in the Comet assay can increase the sensitivity of oxidative damage detection in vitro.

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1. Introduction

Increased ambient ozone levels are associated with pulmonary mortality and morbidity (Anderson et al., 1996; Burnett et al., 1997; Delfino et al., 1998; Hoek et al., 1997). Recent epidemiological studies have shown that increased ozone exposure is associated with a risk of lung cancer (Abbey et al., 1995; Lawrence et al., 1998). Animal studies have also demonstrated that ozone exposure can induce lung tumors (Hassett et al., 1985; Herbert et al., 1996; Last et al., 1987). However, the exact carcinogenic mechanisms of ozone remain less

clear, particularly at levels around the current ambient standard.

Ozone is a strong oxidant that can generate reactive oxygen species (ROS) in tissue, resulting in DNA damage (Pryor, 1994). Single-cell gel electrophoresis, or the Comet assay, has been used to assess the effects of ozone on cell lines, as ROS has been shown to induce DNA single-strand breaks (Frenkel, 1992). Increased DNA single-strand breaks have also been associated with ozone exposure in epidemiological and animal studies (Haney et al., 1999; Rojas et al., 2000).

Those studies were conducted at ozone levels much greater than 100 ppb for more than 1 h. The current ozone standard set by the US Environmental Protection Agency is 125 ppb in 1 h. Herein, we determined DNA single-strand breaks in A549 cells exposed to ozone levels much lower than the current ambient standard for

Abbreviations: Fpg, formamidopyrimidine glycosylase; ROS, reactive oxygen species.

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ozone in 1 h. Further, we also performed a Comet assay with formamidopyrimidine glycosylase (Fpg), a specific enzyme used to repair oxidative DNA damage, to enhance the sensitivity for detecting oxidative damage (Andrea et al., 1996; Leipold et al., 2000; Lavrukhin and Lloyd, 2000).

Studies have shown that 8-oxoguanine is one of the most prevalent DNA adducts caused by ROS (Loft and Poulsen, 1996; Lawrence, 2000). Production of 8-oxoguanine leads to G–A transversion in vitro and in animal studies; G–A transversion is a common mutation in the *p53* genes (Loft and Poulsen, 1996). In recent studies, 8-oxoguanine formation has been determined using flow cytometry (Futaki et al., 2002; Mei et al., 2002). Thus, in addition to DNA single-strand breaks, we used flow cytometry to further determine whether ozone levels below the current standard induce 8-oxoguanine, a specific type of DNA damage that may initiate carcinogenesis. At the same time, antioxidants have been shown to decrease the oxidative damage in previous studies (Heike et al., 1993; Qi et al., 2000). In this study, the antioxidant effects of vitamins C and E were also tested in ozone-treated A549 cells.

2. Methods and materials

2.1. A549 cell culture and exposure to ozone

The human A549 cell line was from the American Type Culture Collection and was cultured in a humidified 37°C environment in F12-K medium supplemented with 10% fetal calf serum and 1% penicillin and streptomycin. This cell line, derived from a patient with alveolar cell carcinoma of the lung, has been used as a model of human alveolar type II cells. The in vitro ozone exposure system was modified from a previous design, which included a stainless-steel exposure chamber, an ozone generator, and a humidifier (Tarkington et al., 1994). These three compartments were connected by Teflon tubes. Ozone generated by an ozonizer (Thermo Environmental Instrument Inc., Model 146, Franklin, MA, USA), was mixed with CO₂ and filtered air to provide predetermined ozone levels at 5% CO₂ for the experiments. The experimental air was first passed through a humidifier and then introduced from the top panel into the exposure chamber at approximately 3.5 L/min. A Teflon tube with multiple holes punched in it was used to distribute the humidified experimental air in order to ensure its even distribution inside the chamber. Ozone levels of 0, 60, 80, and 120 ppb at a constant humidity of 95 ± 5% were maintained in the 0.14-m³ exposure chamber throughout the experiment. An ozone analyzer (Thermo Environmental) was continuously used to monitor the ozone levels to ensure constant and even ozone concentrations inside the exposure

chamber. The human A549 cells were exposed to 0, 60, 80, and 120 ppb of ozone in the exposure chamber for 1 h. Three sets of experiments were conducted for each level of ozone concentration. The viability of A549 cells was determined by trypan blue exclusion assay.

2.2. Determination of DNA single-strand breaks by Comet assay

The alkaline Comet assay was used to determine DNA single-strand breaks. Quantities of 20 µL of A549 suspensions (10⁵ cells/mL) were mixed with 1 mL of 1.5% low-melting agarose and gelled to frosted slides pretreated with 0.1% normal-melting agarose on ice. After gelling, the slides were immersed in alkaline lysis buffer (2.5% SDS and 25 mM EDTA, pH = 13) for 10 min, then washed with PBS. They were then placed in buffer (30 mM NaOH and 1 mM EDTA) for electrophoresis at 21 V and 190 mA for 20 min. Subsequently, slides were rinsed and stained in sybr green. Individual cells were examined under a microscope with a photomicrographic system. All slides were coded and read blindly. The images were then recorded for further image analysis. We also used A549 cells that had been aliquoted previously as an internal standard to adjust for experimental variability (De Boeck et al., 2000).

In addition to the regular Comet assay, we also used Fpg, an endonuclease with specific activity toward oxidized purines, for the enhanced detection of oxidative damage. Briefly, slides and cells were prepared as described above and then immersed in lysis buffer for 10 min. After the slides were washed with enzyme reaction buffer, Fpg (Sigma) was added at a level of 10 µg/mL at 37°C for 30 min. Following electrophoresis, the slides were stained and the images were recorded.

Proprietary image processing software, which has been used in previous studies (Ma et al., 1996; Gwo et al., 2003), was applied to calculate the distribution of DNA on the agarose. Images of 100 randomly selected cells from each set of the experiment were analyzed under a fluorescence microscope adapted with excitation at 515–560 nm and a barrier filter of 590 nm. Parameters used in the analysis were tail length (%), tail intensity (%), and tail moment. Tail length (%) was defined as the proportion of tail length of the entire comet length; tail intensity (%) was defined as the proportion of tail intensity of the total Comet intensity; and tail moment was defined as the product of tail length and tail intensity. We used the percentage of tail length because of its ease in measurement, as in the measurement of tail intensity (Ma et al., 1996).

2.3. Determination of 8-oxoguanine levels by flow cytometry

For the detection of oxidative DNA damage, 8-oxoguanine, the OxyDNA assay kit was used (Biotrin,

Dublin, Ireland). This reagent has affinity for the imidazilidone group, which is part of 8-oxoguanine. The manufacturer's protocols were followed. Briefly, 1×10^5 – 5×10^5 cells were harvested and transferred to polystyrene flow cytometry tubes, washed with PBS, pelleted, and resuspended in 0.5 mL of PBS. After 0.5 mL of 2% paraformaldehyde was added, the solution was placed on ice for 15 min. The cells were washed twice, resuspended in 1 mL of ice-cold ethanol, and stored at -20°C until analysis. After washing, the nonspecific sites were blocked by incubation with blocking solution at 37°C for 1 h. After washing, 100 μL of FITC-conjugated protein specific for 8-oxoguanine was incubated for 1 h in the dark at room temperature. After washing, the cells were rinsed, resuspended, and analyzed with a fluorescence-activated cell sorter (FACSCalibur system, Becton Dickinson). Fluorescence readings were done for 10^4 cells for each set of experiments and analyzed with CELLQuest software. Mean fluorescence intensity was used to represent the 8-oxoguanine level. Cells were also treated with vitamins C and E, respectively, at concentrations of 0, 50, 100, and 200 μM for 1 h before exposure to ozone. After 1 h of ozone exposure at 120 ppb, 8-oxoguanine levels were determined using flow cytometry.

2.4. Statistical analysis

Student's *t* test was used to compare tail length (%), tail intensity (%), and tail moment between the controls and different ozone exposure groups. Similarly, mean fluorescence intensity for 8-oxoguanine levels was also compared between the controls and different ozone exposure groups. Last, the effect of vitamins C and E on mean fluorescence intensity was also compared between the controls and treated groups. A *P* value of less than 5% was considered significant.

3. Results

After ozone treatment, cell viability decreased with the concentration of ozone (Fig. 1). Cell viabilities were 95% at 0 ppb, 88% at 60 ppb, 83% at 80 ppb, and 75% at 120 ppb of ozone.

Tail length (%), tail intensity (%), and tail moment of DNA single-strand breaks increased with increased ozone levels (Table 1). Tail length increased from 58.1% to 65.1% when the ozone level increased from 0 to 120 ppb. Over the same range of ozone levels, tail intensity increased from 58.2% to 70.4%, and the tail moment increased from 102.2 to 116.1. At 80 and 120 ppb of ozone, tail moments were significantly higher than that of the control ($P < 0.05$). However, the difference was not significant for either tail length or tail intensity. Subsequently, Fpg was added to the

Fig. 1. Cell viability after exposure to ozone. Data are plotted as mean $\pm$ SE ($n = 3$).

Table 1

DNA single-strand breaks induced by ozone using Comet assay with and without Fpg enzymes

Concentrations of ozone (ppb)	% tail length ($\pm$ SE)	% tail intensity ($\pm$ SE)	Tail moment ($\pm$ SE)
Without Fpg			
0	58.1 $\pm$ 4.9 ^a	58.2 $\pm$ 7.8	102.2 $\pm$ 1.3
60	58.5 $\pm$ 5.2	63.9 $\pm$ 7.6	104.0 $\pm$ 5.3
80	63.0 $\pm$ 5.5	68.4 $\pm$ 7.6	114.3 $\pm$ 1.0*
120	65.1 $\pm$ 5.2	70.4 $\pm$ 6.6	116.1 $\pm$ 2.8*
With Fpg			
0	74.5 $\pm$ 5.5	78.4 $\pm$ 5.0	110.9 $\pm$ 3.0
60	76.2 $\pm$ 4.8	86.2 $\pm$ 4.6	162.0 $\pm$ 1.5*
80	83.2 $\pm$ 4.6	93.0 $\pm$ 3.5*	190.1 $\pm$ 1.5*
120	85.9 $\pm$ 4.7*	93.5 $\pm$ 3.2*	199.1 $\pm$ 1.1*

* $P < 0.05$ compared to the experiment at 0 ppb.

^aMean $\pm$ SE ($n = 3$), 100 cells were scored for each experiment.

Comet assay. Tail length, tail intensity, and tail moment with Fpg treatment were greater than those without Fpg. Tail lengths increased with ozone levels from 74.5% to 85.9%, and measurements at 120 ppb were significantly greater than those at 0 ppb. Tail intensity also increased with ozone levels from 78.4% to 93.5%. Tail intensities at 80 and 120 ppb were significantly greater than those at 0 ppb. Furthermore, tail moment increased from 110.9 to 199.1. Tail moments at ozone levels of 60, 80, and 120 ppb were greater than those at 0 ppb ($P < 0.05$).

Similarly, 8-oxoguanine levels determined by mean fluorescence levels increased with ozone levels (Fig. 2). At 0 ppb of ozone, 8-oxoguanine was 10.7; at 60 ppb, it was 67.6; at 80 ppb, it was 328.3 ($P < 0.05$); and at 120 ppb, it was 290.1 ($P < 0.05$).

When cells were treated with vitamin C at the levels of 0, 50, 100, and 200 μM before ozone exposure, mean fluorescence intensity of 8-oxoguanine significantly

Fig. 2. 8-oxoguanine levels detected by fluorescence in A549 cells after ozone exposure. Data are plotted as mean $\pm$ SE ($n = 3$), 10,000 cells were scored for each experiment. * $P < 0.05$, compared to the experiment at 0 ppb.

decreased from 261.3 to 46.1, 44.7, and 25.4 (inhibited by 72–86%) (Fig. 3). Similar results were also found with vitamin E treatment. The mean fluorescence intensity of 8-oxoguanine significantly decreased from 204.8 to 56.5, 35.4, and 28.0 after vitamin E treatment (inhibited by 82–90%) (Fig. 3).

4. Discussion

The results showed that ozone exposure at 80 ppb could induce increased oxidative DNA damage, including 8-oxoguanine and DNA single-strand breaks. Adding Fpg further increased the sensitivity in detecting oxidative damage caused by ozone.

The Comet assay has been used to determine oxidative DNA damage. In a previous epidemiological study, subjects with higher exposure to ozone had higher levels of DNA single-strand breaks (Rojas et al., 2000). The average ozone levels in the high-exposure area ranged from 160 to 170 ppb. In rats exposed to 250 and 500 ppb for 3 h, cells from bronchoalveolar lavage had higher levels of DNA single-strand breaks (Haney et al., 1999). Similar findings were observed in in vitro studies. Human bronchial cell lines also had higher levels of DNA single-strand breaks after exposure to 100 and 200 ppb of ozone for 2 h (Lee et al., 1996). When normal human tracheobronchioepithelial cells were exposed to 400 ppb of ozone for 40 min, DNA single-strand breaks were significantly increased (Lee et al., 1996). Thus, increased oxidative damage may be detected when exposed to an ozone level of 100 ppm for more than 1 h. In this study, we used a regular Comet assay and observed a significant increase in DNA single-strand breaks with an ozone level of 80 ppb for 1 h. The mechanisms through which ozone induces DNA single-

Fig. 3. Inhibitory effects of vitamins C and E on ozone-induced 8-oxoguanine levels detected by fluorescence in A549 cells. Data are plotted as mean $\pm$ SE ($n = 3$), 10,000 cells were scored for each experiment. * $P < 0.05$, compared to the experiment at 0 ppb.

strand breaks are less clear. A recent study suggested that DNA single-strand breaks induced by ozone may be mediated through H_2O_2 production (Diaz-Llera et al., 2002). In the same study, formation of DNA single-strand breaks was inhibited by pretreatment using catalase.

Using Fpg to enhance the sensitivity of the Comet assay we were able to detect increased oxidative damage at an ozone level of 60 ppb. The Fpg enzyme can remove 8-oxoguanine and other oxidative damage. Studies have revealed that 30–50% of oxidative damage removed by Fpg is due to 8-oxoguanine (Boiteux et al., 1992; Karakaya et al., 1997). The Comet assay with Fpg has been demonstrated to be more sensitive in previous studies (Andrea et al., 1996; Hodges et al., 2001). Our study also showed similar findings. It appears that the Comet assay with Fpg enzymes is a useful methodology for detecting oxidative DNA damage.

Although the Comet assay has been used to assess oxidative DNA damage caused by ozone, it is not clear whether ozone can cause specific DNA damage. Studies

have shown that 8-oxoguanine is a sensitive marker of oxidative DNA damage and is implicated in carcinogenesis (Park and Floyd, 1992; Loft and Poulsen, 1996). However, 8-oxoguanine has not been used in previous studies to detect the effects of ozone. In this study, we used flow cytometry to determine 8-oxoguanine levels in A549 cells caused by ozone. Interestingly, increased 8-oxoguanine levels were observed in A549 cells exposed to ozone levels as low as 80 ppb. Our results support previous epidemiological and animal studies that ozone may play a role in carcinogenesis. A previous study also showed a decrease in 8-oxoguanine levels in chromium- and H_2O_2 -treated cells after administering vitamins C and E (Qi et al., 2000). Our study also produced similar finding. This suggests that ozone-induced ROS may be scavenged by antioxidants.

Our results indicate that ozone exposure at 60–80 ppb for 1 h can induce DNA single-strand breaks and specific 8-oxoguanine formation in A549 cells. We conclude that possible carcinogenesis due to chronic ozone exposure warrants further study.

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