Toxic effects of ozone on murine L929 fibroblasts

Damage to DNA

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Damage to DNA caused by exposure of L929 fibroblasts to ozone was reflected by the generation of strand breaks, DNA inter-strand cross-links and DNA–protein cross-links. Addition of propan-2-ol, a hydroxyl-radical scavenger, did not affect the formation of strand breaks. In model experiments it appeared that both purines and pyrimidines were involved in DNA inter-strand and DNA–protein cross-links.

INTRODUCTION

DNA is generally considered to be a critical cellular target for lethal, carcinogenic and mutagenic effects of drugs, radiation and environmental chemicals [1–3]. DNA-damaging species can induce a variety of DNA damage, namely strand breaks, base modifications and cross-links. In model experiments the reactivity of ozone, an aggressive air pollutant and a strong oxidizing agent, towards nucleic acids and its derivatives, has been shown. All base and sugar moieties are degraded by ozone. Strand breaks are induced in calf thymus DNA, possibly mediated by attack of hydroxyl radicals on the deoxyribose ring [4]. Furthermore, ozone is able to relax, linearize and then degrade native plasmid DNA molecules in solution [5,6].

However, little is known about the effects of ozone on DNA in intact cells. Ozone-induced DNA degradation was observed in *Escherichia coli* [7] and *Saccharomyces cerevisiae* [8] but, in general, the putative effects of ozone on DNA in intact cells are controversial [3]. Previously it was shown that exposure of murine L929 fibroblasts to ozone resulted in damage to intracellular components. GSH was depleted and several enzymes were inactivated [9]. Thus it is not unlikely that exposure to ozone may cause damage to the cellular DNA as well.

As will be shown in the present paper, ozone, at relatively high concentrations, caused extensive DNA damage, as reflected by strand breaks, DNA inter-strand cross-links and DNA–protein cross-links. Thus, despite its high reactivity, ozone, or a reactive secondary product, could apparently reach the nucleus of intact cells and react with cellular DNA.

MATERIALS AND METHODS

N.C.T.C. murine fibroblasts (clone L929, A.T.C.C. no. CCL1), tissue-culture products and newborn-bovine serum were obtained from Flow Laboratories. [U-¹⁴C]-Adenosine (500 mCi/mmol), [U-¹⁴C]cytidine (463 mCi/mmol), [2-¹⁴C]thymidine (52 mCi/mmol) and [U-¹⁴C]-guanosine (500 mCi/mmol) were purchased from Amersham International. Filters (Metricel; 0.45 µm pore size) were obtained from Gelman, proteinase K from

Boehringer and DNA (type I, from calf thymus) from Sigma. All other chemicals were from Sigma or Merck and were of analytical grade.

Murine L929 fibroblasts were grown as described previously [10]. Uniform labelling of DNA was obtained with [2-¹⁴C]thymidine (0.1 µCi/dish). Confluent cell layers (7 × 10⁴ cells/cm²) were washed twice with PBS (phosphate-buffered iso-osmotic NaCl solution, pH 7.4). Exposure to ozone, at a flow rate of 0.4 ml of oxygen (containing 25 µmol of O₃/ml)/min, was performed as described previously [9]. Before exposure to X-rays, the cells were put on ice. Subsequently, the cells were irradiated by using a Muller RT-100 X-ray apparatus operating at 100 kV and 8 mA. Irradiation was carried out at a dose rate of 15 rad/s. After exposure to ozone or to X-rays the cells were kept on ice and carefully scraped off into 1 ml of PBS.

DNA strand breaks were determined with the alkaline elution technique described by Kohn *et al.* [11]. Briefly, 10⁶ cells were filtered on Metricel filters (0.45 µm pore size) and lysed with a buffer containing 0.2% Sarkosyl. In the experiments with X-rays only and ozone in combination with X-rays, cellular proteins were digested with proteinase K (0.5 mg/ml) before alkaline elution. DNA was eluted with 0.1 M-tetrapropylammonium hydroxide buffer, pH 12.2, at a flow rate of 0.66 ml/h for 20 h.

DNA strand breaks were also assayed by the technique of fluorimetric analysis of DNA unwinding as described by Birnboim & Jevcak [12]. This technique is based on the fact that unwinding of double-stranded DNA in alkaline solutions is increased considerably when strand breaks are present. Remaining double-stranded DNA, after a fixed unwinding period, is measured with ethidium bromide, which shows fluorescent enhancement with double-stranded, but not with single-stranded, DNA. After exposure to ozone the cells were carefully scraped off into PBS. To aliquots (0.2 ml) of this cell suspension (2 × 10⁶ cells/ml), 0.2 ml of a solution containing 9 M-urea, 10 mM-NaOH, 2.5 mM-cyclohexanediamine-tetra-acetate and 0.1% SDS was added. Further procedures were performed as described by Birnboim & Jevcak [12]. Fluorescence was determined in an Aminco SPF-500 spectrophotofluorimeter (excitation 520 nm;

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emission 590 nm). All determinations were done in triplicate.

DNA-protein cross-links were assayed by a modification of the technique described by Tsutsui *et al.* [13]. Cells (2×10^6) were scraped off into PBS (1 ml) and lysed with a same volume of 20 mM-Tris/HCl, pH 7.4, containing 20 mM- Na_2EDTA and 2% SDS. To 0.5 ml of the lysate, 64 μl of 2 M-KCl was added and the mixture was incubated on ice for 10 min after vortex-mixing. The precipitate was pelleted by centrifugation at 2400 g for 4 min (4°C) and washed three times with 1 ml of ice-cold 10 mM-Tris/HCl (pH 7.4)/1 mM-EDTA/0.1 M-KCl by vortex-mixing for 30 s vigorously and centrifuging each time. The washed precipitate was dissolved in 1 M-HCl and neutralized with the same volume of 1 M-NaOH. Precipitated protein-bound DNA was measured by liquid-scintillation counting.

Binding of labelled reagents to DNA was determined as follows. DNA (0.75 mg/ml in 50 mM-sodium phosphate, pH 7.4) was exposed to ozone for 15 min in the presence of a radiolabelled nucleoside (0.08 μmol ; 10000 d.p.m./nmol) in a final volume of 8 ml. After exposure, sodium acetate was added to 1 ml samples to a final concentration of 0.3 M, and the DNA was precipitated with ethanol at -70°C . After centrifugation (48000 g) the pellet was washed five times with 80% ethanol to remove excess label. For measuring the binding to protein, bovine serum albumin (2 mg/ml in 50 mM-sodium phosphate, pH 7.4) was exposed to ozone for 15 min in the presence of 0.10 μmol of nucleoside (final vol. 10 ml). After exposure to ozone the protein was precipitated with ice-cold trichloroacetic acid (final concn 1 M) and centrifuged (3000 g). To remove unbound nucleosides the pellet was washed five times with ice-cold trichloroacetic acid.

RESULTS

In control experiments L929 fibroblasts were exposed to pure oxygen for 2 h. No DNA damage could be measured after this treatment, indicating that the effects described below should be attributed to ozone.

Induction of strand breaks by exposure of L929 cells to ozone was studied with two different techniques, namely alkaline elution and fluorimetric analysis of DNA unwinding. Exposure to ozone for up to 120 min did not cause an increase in the alkaline elution rate. Addition of proteinase K to eliminate the effect of possible DNA-protein cross-links did not influence the elution rate. Exposure of fibroblasts to a dose of X-rays of 300 or 500 rad resulted in an increased rate of elution (Fig. 1), showing that strand breaks were formed. When the cells were exposed to ozone for 60 min before the dose of X-rays, the elution velocity also increased, but much less than after exposure to X-rays alone (Fig. 1). This indicates that exposure to ozone had induced the generation of DNA inter-strand cross-links.

With the fluorimetric-analysis-of-DNA-unwinding technique, the ethidium bromide fluorescence decreased upon treatment with ozone, indicating that strand breaks were induced (Fig. 2). Addition of 100 mM-propan-2-ol had no effect on the formation of strand breaks. With this technique the effects of ozone and X-rays were additive (Fig. 3).

Formation of ozone-induced DNA-protein cross-

Fig. 1. Alkaline-elution kinetics of DNA from L929 fibroblasts

a, Control cells and cells exposed to ozone for 60 min; b, cells exposed to 300 rad of X-rays; c, cells exposed to 500 rad of X-rays; d, cells exposed to ozone for 60 min and then to 300 rad of X-rays; e, cells exposed to ozone for 60 min and subsequently to 500 rad of X-rays.

links is shown in Fig. 4. Measurable cross-linking started after 30 min exposure of the cells to ozone.

The involvement of the various purines and pyrimidines in the observed DNA inter-strand and DNA-protein cross-links was studied in model experiments by exposing a solution of calf thymus DNA or albumin

Fig. 2. Generation of DNA strand breaks during exposure of L929 fibroblasts to ozone, as reflected by a decrease of relative fluorescence with the fluorimetric-analysis-of-DNA-unwinding technique

Fig. 3. Generation of DNA strand breaks by X-rays in L929 fibroblasts, as revealed by the fluorimetric-analysis-of-DNA-unwinding technique

○, Cells exposed to various doses of X-rays; ●, cells exposed to ozone for 60 min and subsequently to various doses of X-rays.

together with nucleosides to ozone. The results are presented in Table 1. In control experiments with [14 C]-ribose it appeared that this sugar was not bound to DNA or albumin during exposure to ozone. Therefore nucleoside binding reflects binding of the base moiety.

Fig. 4. Generation of DNA-protein cross-links during exposure of L929 fibroblasts to ozone

Table 1. Binding of nucleosides to calf thymus DNA and bovine serum albumin after exposure to ozone

For details, see the Materials and methods section. In the absence of ozone no binding was observed.

Nucleoside	DNA (pmol/mg)	Albumin (μ mol/mol)
Adenosine	124	2.6
Guanosine	52	1.0
Thymidine	40	2.1
Cytidine	228	1.0

DISCUSSION

The results presented indicate that exposure of L929 fibroblasts to ozone causes damage to the DNA, namely strand breaks, DNA inter-strand cross-links and DNA-protein cross-links. The strand breaks could be detected with the fluorimetric analysis of DNA unwinding (Fig. 2), but not with the alkaline elution technique. This discrepancy is based on the fact that the former technique measures the rate of unwinding of DNA molecules in alkaline, which is greatly increased by strand breaks and reflected by a decrease of ethidium bromide fluorescence [12]. The alkaline elution technique also measures strand unwinding, but requires, in addition, physical separation of fast-to-be-eluted single-stranded and slow-to-be-eluted double-stranded molecules. DNA inter-strand and DNA-protein cross-links interfere with this separation, obscuring the results when measuring strand breaks [11]. The present results indicate that fluorimetric analysis of DNA unwinding is very useful in detecting strand breaks in the presence of simultaneously occurring DNA inter-strand cross-links. With the alkaline elution method the interfering effect of DNA-protein cross-links (but not of DNA inter-strand cross-links) can be abolished by digestion of the protein by proteinase K. With proteinase K pretreatment the alkaline elution technique can be used to detect DNA inter-strand cross-links, as these cross-links will decrease the effect of a given frequency of strand breaks (induced, e.g., by a fixed dose of X-rays) on the elution rate [11]. As shown in Fig. 1, exposure of L929 fibroblasts to ozone decreased the effect of subsequent exposure to X-rays considerably, thus demonstrating the generation of DNA inter-strand cross-links by ozone.

Because of these DNA-DNA cross-links, the alkaline elution technique could not be used to determine DNA-protein cross-linking. We therefore utilized the K^+ /SDS method. This procedure involves the formation of SDS-protein complexes, followed by precipitation of the complexes with KCl. Labelled DNA covalently attached to protein is also precipitated, whereas protein-free DNA stays in solution. As shown in Fig. 4, ozone induced not only DNA inter-strand cross-links, but also, after a lag time of about 30 min, DNA-protein cross-links. As shown in Table 1, both purines and pyrimidines are covalently coupled to DNA and albumin during exposure to ozone. This indicates that all DNA bases can be involved in the observed DNA inter-strand and DNA-protein cross-links.

Hoigné & Bader [14] demonstrated that oxidation by ozone may occur either by a direct reaction between

target molecules and ozone or via hydroxyl radicals as oxidizing intermediates. Although the hydroxyl-radical scavenger propan-2-ol did not inhibit the formation of DNA strand breaks in L929 cells, this does not exclude a role of hydroxyl radicals in this process. Hydroxyl radicals are very reactive, often giving rise to so-called site-specific reactions. Usually hydroxyl-radical scavengers cannot effectively interfere with such site-specific reactions [15,16].

Exposure of L929 fibroblasts to ozone caused inhibition of intracellular enzymes [9], glutathione depletion [9], damage to the cell membrane [17] and, as shown here, DNA damage. These various forms of damage appear to develop simultaneously. Therefore it is impossible, as yet, to ascribe ozone-induced cell death to a particular type of cellular damage. Membrane deterioration, cytoplasmic damage and DNA modification may all contribute to the cell inactivation caused by ozone.

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