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I. R. Komanapalli · B. H. S. Lau

Ozone-induced damage of *Escherichia coli K-12*

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Abstract *Escherichia coli* K-12 transformed with pACYC184 plasmid DNA was exposed to ozone (O_3) in aqueous solution. The damage to the membrane, protein, plasmid DNA, and cell survival were investigated. Cell viability was unaffected by short-term O_3 exposure (1–5 min) but membrane permeability was compromised as indicated by protein and nucleic acid leakage and lipid oxidation. The intracellular components, protein and DNA, remained intact. With longer durations of O_3 exposure (up to 30 min) cell viability decreased with a more significant increase in lipid oxidation and protein and nucleic acid leakage. The proteins leaking out were further oxidized by O_3. The total intracellular proteins run on sodium dodecyl sulfate/polyacrylamide gel electrophoresis, and plasmid DNA run on agarose gel, showed progressive degradation corresponding to the decrease in cell viability. The data indicate that membrane components are the primary targets of O_3 damage with subsequent reactions involving the intracellular components, protein and DNA.

Introduction

Ozone (O_3) behaves like a double-edged sword with the potential for beneficial and harmful effects. It is a potent microbicidal agent in sewage-disposal plants (Menzel 1984), water disinfection (Glaze 1987), and food preservation (Rice et al. 1982). It is also widely used to sterilize biological safety cabinets (Akey 1987), decontaminate bioclean rooms (Masaoka et al. 1982) and for destruction of toxic industrial impurities (Borek and Mehlman 1983). Being a powerful oxidant, it is one of the most important components of air pollutants affecting plants, animals and humans (Lipmann 1991). The primary target of long-term or chronic ozone exposure in humans is the respiratory system with lesions in the bronchioles (Huang et al. 1989). Its toxic effects have been studied extensively in plant and animal cells (Heath 1980; Menzel 1984) and, to a lesser extent, in bacteria (Hamelin and Chung 1974; Hamelin et al. 1977); however, no consensus exists as to how it exerts its toxicity. There is controversy about whether O_3 induces toxic effects via oxidation of lipids or proteins in the biomembranes (Goldstein and McDonagh 1975; Mudd et al. 1969; Ohlrogge and Kernan 1983). Because of these discrepancies further investigations are needed to clarify the effects of O_3. Bacteria offer a feasible system for such a study.

To elucidate the mechanism by which O_3 causes cell damage and eventually cell death, we conducted a comprehensive study using *Escherichia coli* K-12. This bacterium was chosen because of its ease of handling and high degree of reproducibility, thus avoiding the biological variations inherent in animal and plant cells. In water- and sewage-treatment plants, *E. coli* is used as an indicator organism for fecal contamination. In this study, we determined the effects of short- and long-interval ozone exposure on membrane permeability, total proteins, and plasmid DNA. These effects were correlated with cell viability.

I. R. Komanapalli · B. H. S. Lau (✉)
Department of Microbiology and Molecular Genetics,
Loma Linda University, Loma Linda,
California 92350, USA.
Fax: (909) 824 4035
e-mail: bLau@ccmail.llu.edu

Materials and methods

Growth conditions and ozone treatment

E. coli K-12 (CSH50, Cold Spring Harbor Laboratory) transformant harboring pACYC184 plasmid (Cm, Tc) was grown in Luria-Bertani (LB) medium supplemented with chloramphenicol at a final concentration of 40 μg/ml. Overnight cultures were diluted 1:10 in fresh LB medium and incubated until the absorbance at 520 nm reached 0.600 (early log phase). Cells were then centrifuged, washed once with sterile distilled water, and resuspended in pH 7.4 phosphate buffer (10 mmol/l; Sigma Chemical Co., St. Louis, Mo.). O_3 was generated from pure oxygen using an ozonizer (Ultraviolet

Products Inc., San Gabriel, Calif.). The concentration of O_3 output was determined spectrophotometrically using the potassium iodide method (Shechter 1973). The test suspensions (5 ml with 10^8 cells/ml) were exposed to 10^7 nmol O_3/min (600 ppm) at a constant flow rate of 20 ml/min for 1–30 min by allowing the gas to bubble directly into the cell suspensions at room temperature (22 °C). Control experiments were performed under identical conditions using oxygen gas.

Determination of cell viability

Aliquots of untreated and treated samples were removed and appropriate dilutions plated on LB medium solidified with 1.5% agar (Difco, Detroit, Mich.). The plates were incubated overnight at 37 °C and the colonies counted.

Determination of lipid oxidation

Cell suspensions were centrifuged at 2 000 rpm for 10 min at 22 °C and supernatants collected for malondialdehyde determination by thiobarbituric acid fluorometric assay (Yagi 1976). Fluorometric measurements were made at 553 nm with 515 nm excitation, using a LS-3 fluorescence spectrophotometer (Perkin-Elmer, Norwalk, Conn.). The value of the fluorescence was calculated using standards prepared from tetraethoxypropane (Sigma Chemical Co., St. Louis, Mo.).

Protein determination and analysis

Supernatants of cell suspensions were assayed for total protein content using the Coomassie protein assay reagent (Pierce, Rockford, Ill.). The absorbance of the protein/dye mixture was measured spectrophotometrically at 595 nm. The amount of protein was calculated by comparing it with bovine serum albumin standards. The supernatants were concentrated using a Centriprep concentrator-3 (Amocon Inc., Beverly, Mass.) with the molecular-mass cut-off of 3 kDa. For protein analysis, the cell suspension was centrifuged at 2 000 rpm for 10 min at room temperature, and the pellet was either frozen at –80 °C or used immediately. Extraction of proteins from cell pellets and sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) of the resultant cell extracts were carried out according to the method of Laemmli (1970), using 12.5% polyacrylamide gels and staining with 0.25% Coomassie blue. The concentrated supernatants were likewise analyzed.

Measurement of tryptophan oxidation

The amount of tryptophan present in the supernatants was measured by fluorescence reading at 336 nm and excitation at 280 nm using an F-2 000 fluorescence spectrophotometer (Hitachi, Irvindale, Ill.). The increase in fluorescence indicates increased amounts of unoxidized tryptophan and a decrease in fluorescence correlates with the amounts of tryptophan residues being oxidized (Burstein et al. 1973).

Determination of nucleic acid leakage

The UV absorbance of nucleic acids, nucleotides and nucleosides in the supernatants was measured at 260 nm in a Beckman model DU spectrophotometer (Beckman, Fullerton, Calif.).

Extraction and analysis of plasmid DNA

Plasmid DNA was extracted from cell pellets using the Wizard Mini-Prep DNA purification kit (Promega, Madison, Wis.). One half of the DNA sample extracted by this procedure was subjected to restriction-enzyme digestion by *Eco*RI (Promega, Madison, Wis.), while the other half was left undigested. Both samples were run on 0.8% agarose gels.

Statistical analyses

Data were analyzed with one-way analysis of variance followed by Tukey's multiple-range test for honestly significant difference. The results were expressed as means ± SE. A P value less than 0.05 was considered significant. All statistical procedures were performed with Statgraphics software version 5.0 (STSC Inc., Rockville, Md.).

Results

Repeated experiments involving treatments with oxygen gas showed no effects on all the parameters tested. Data with oxygen treatments are thus not presented for comparison. The number of viable *E. coli* K-12 cells remained constant during the short interval (1–5 min) of O_3 exposure (Fig. 1a). There was a significant increase of malondialdehyde, a product of lipid oxidation, at 3–5 min (Fig. 1b). A statistically significant increase of the protein content in the supernatant was observed from 2 min to 5 min (Fig. 1c). The changes of tryptophan were not statistically significant (Fig. 1d). A time-dependent leakage of nucleic acids, as indicated by the increase of UV absorbance, was observed (Fig. 1e).

The effect of a long interval (up to 30 min) of O_3 exposure, reflecting higher ozone concentrations, is shown in Fig. 2. The cell count dropped by a factor of 10 at 10 min and continued to drop thereafter (Fig. 2a). The numbers of viable cells at 20 min and 30 min were not significantly different, indicating that the effect had reached a plateau. There was a significant time-dependent increase of malondialdehyde at 5–30 min of O_3 exposure (Fig. 2b). Figure 2c shows a significant increase of extracellular protein at 5 min of O_3 exposure, peaking at 15 min. There was a time-dependent increase of oxidation of tryptophan residues from 5 min to 30 min, as reflected by a decrease of fluorescence (Fig. 2d). The measurement of UV-absorbing substances indicates a linear increase of nucleic acid components from 5 min to 30 min (Fig. 2e).

The SDS-PAGE analysis (Fig. 3a) of the supernatant shows increasing amounts of proteins at 5–15 min of O_3 exposure. Thereafter, the 20- and 30-min-treated samples had decreased amounts of protein. This observation correlates well with the leakage of protein content measured by Coomassie reagent assay. The patterns of intracellular proteins of *E. coli* exposed longer to O_3 are also observed on SDS-PAGE (Fig. 3b). The intensity of the bands on the gel decreased with time, indicating a gradual decrease in the amount of total cellular proteins, noticeable at 15–30 min.

Figure 4a depicts the effect of prolonged O_3 exposure on plasmid DNA. DNA degradation began at 10 min and increased through 15 min and 20 min of O_3 exposure, with complete degradation observed at 30 min. Figure 4b shows the plasmid DNA digested with the restriction enzyme *Eco*RI after O_3 exposure. This di-

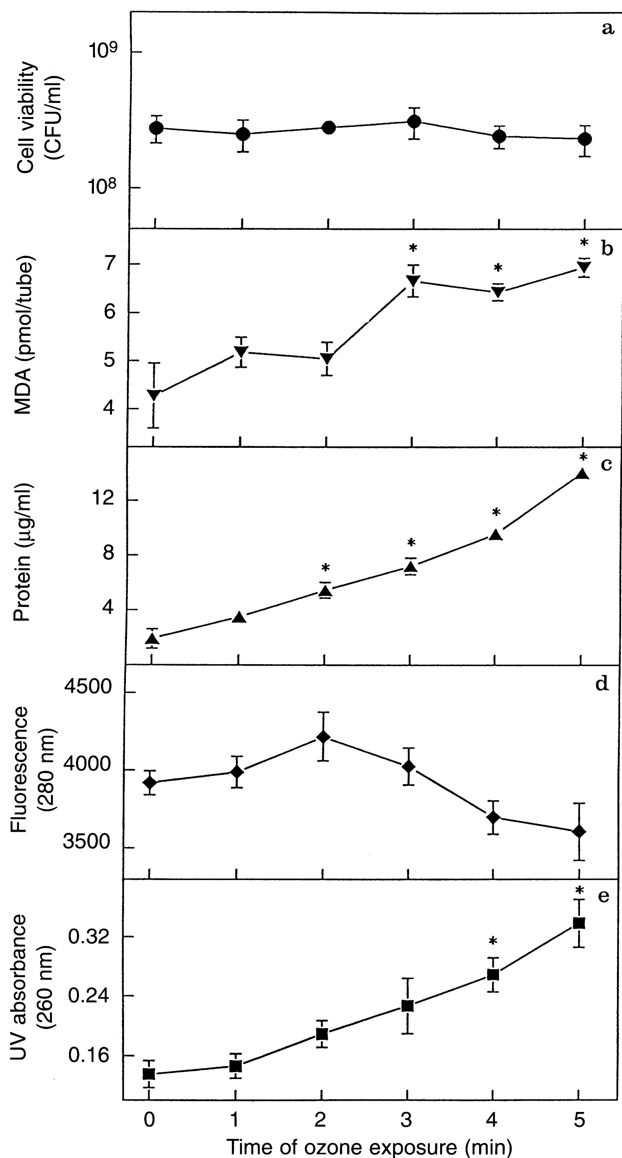

Fig. 1a–e Effect of short-interval ozone exposure on cell viability (**a**), lipid oxidation (**b**), protein leakage (**c**), tryptophan oxidation (**d**), and nucleic acid leakage (**e**). Bacteria were exposed to 107 nmol O3/min for 1–5 min. Each point is the mean of three experiments, and *vertical bars* standard errors. * Significant difference (P<0.05) from the respective control at 0 point

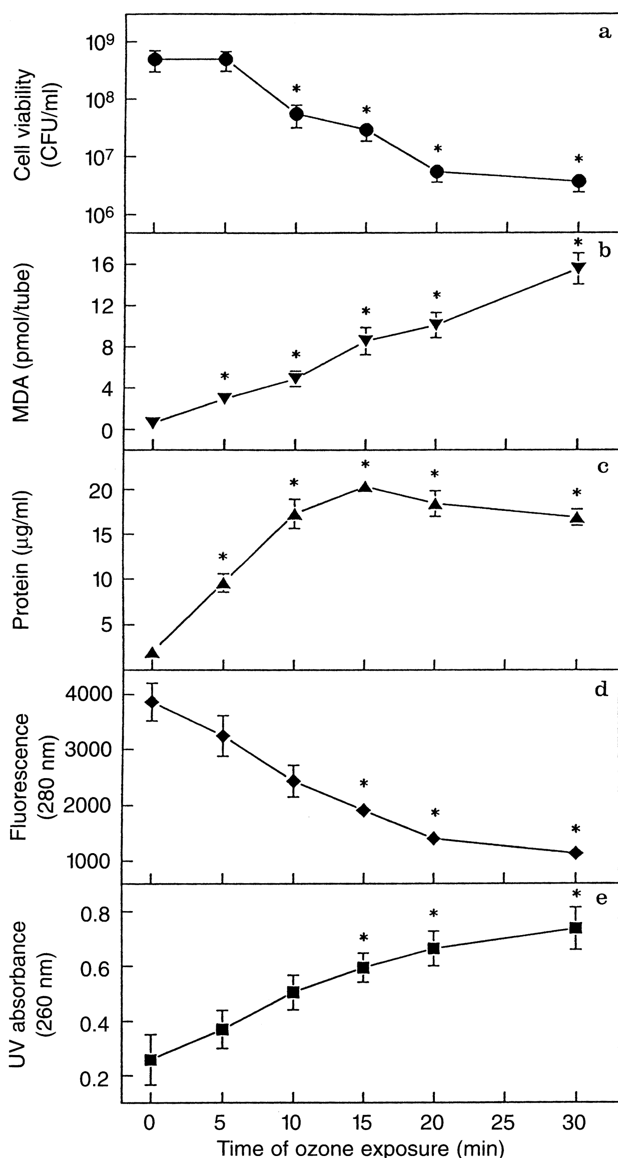

Fig. 2a–e Long-interval exposure of *Escherichia coli* K-12 suspension to 107 nmol O_3/min for 5–30 min. The effect on cell viability (**a**), lipid oxidation (**b**), protein leakage (**c**), tryptophan oxidation (**d**), and nucleic acid leakage (**e**) was observed. *Bars* standard errors of triplicate determinations. * significant difference (P<0.05) from the respective control at 0 point

gestion resulted in the linearization of plasmid DNA. There was no difference in the amount of linear DNA in the control (lane 2) and 5-min-exposed (lane 3) samples. There was a decrease in the amount of linear DNA from 10 min to 30 min with increased degradation, as indicated by increasing smearing.

Discussion

Exposure to O_3 for a short time had no effect on cell viability and tryptophan oxidation. Conversely, a significant leakage of protein was observed, followed by malondialdehyde and nucleic acid components. The oxidation of phospholipids and proteins, both of which have structural and functional roles in biomembranes (Cronan and Vagelos 1972), could alter the ability to regulate permeability. The presence of malondialdehyde byproducts and protein and nucleic acid components in the supernatant after O_3 exposure indicates membrane damage, without apparent effect on cell viability.

With longer O_3 exposure, cell viability decreased from 10 min onwards. The small difference in viability observed between 20 min and 30 min indicates that increasing periods of O_3 exposure did not kill more bacteria. A possible explanation is that cellular debris from

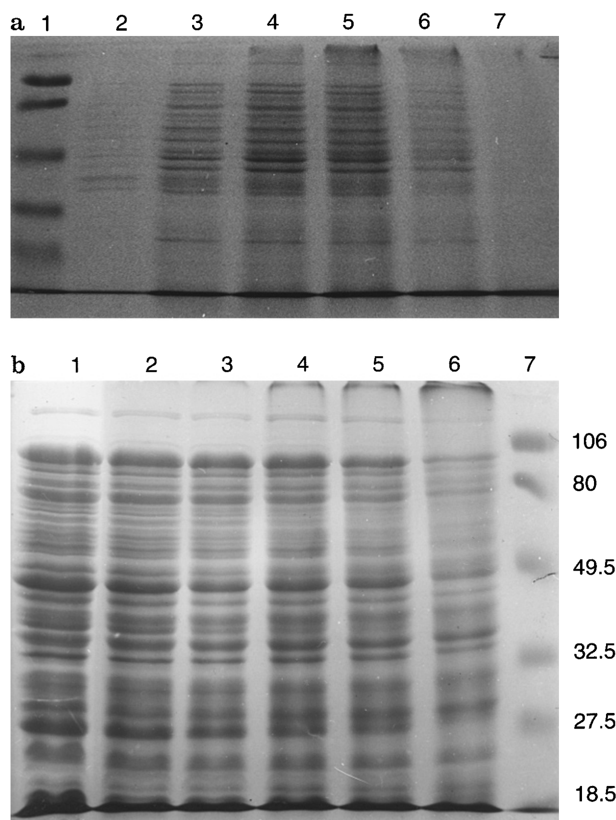

Fig. 3 a Sodium dodecyl sulfate/polyacrylamide gel electrophoresis analysis of concentrated proteins in supernatants of *E. coli* K-12 exposed to ozone (107 nmol O_3/min) for 0–30 min. *Lane 1* BioRad low-molecular-mass standards, *lane 2* 0 min (unexposed cells), *lane 3* 5 min, *lane 4* 10 min, *lane 5* 15 min, *lane 6* 20 min, *lane 7* 30 min. **b** Cellular proteins extracted from *E.coli* K-12 exposed to ozone (107 nmol/min) for 0–30 min. *Lanes 1–6* 0, 5, 10, 15, 20 and 30 min, respectively, of ozone treatment. *Lane 7* standards

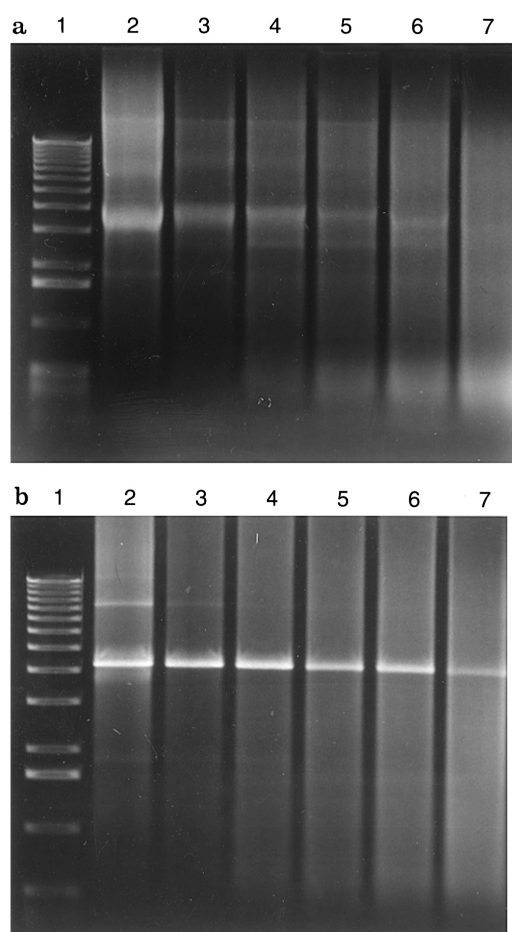

Fig. 4 a Electrophoresis of plasmid pACYC184 DNA extracted from *E.coli* K-12 exposed to 107 nmol O_3/min. *Lane 1* 1 kb DNA standard; *lane 2* 0 min (without ozone), *lane 3* 5 min, *lane 4* 10 min, *lane 5* 15 min, *lane 6* 20 min, *lane 7* 30 min of ozone exposure. **b** Agarose gel electrophoresis of *Eco*RI-restriction-enzyme-digested plasmid pACYC184 extracted from *E. coli* K-12. *Lane 1* standard, *lanes 2–7* samples with 0 min (no ozone), 5 min, 10 min, 15 min, 20 min, and 30 min ozone exposure

the disabled organisms may shield the surviving *E. coli* from the effects of O_3. Finch et al. (1988) reported that even slight cell lysis significantly increases the ozone demand and thus decreases its efficiency, which may account for the plateau of cell viability observed in this study (Fig. 2a). There was further increased malondialdehyde production and increased protein and nucleic acid leakage, as well as tryptophan oxidation. There was also further degradation of proteins that leaked into the supernatant, as indicated by the increased oxidation of tryptophan residues.

Our results demonstrate that the initial target of O_3 toxicity on *E. coli* K-12 is the membrane, affecting both lipid and protein components. In spite of the membrane permeability being compromised at short intervals of O_3 exposure, the bacterial cell is still capable of surviving as there is no apparent damage of intracellular components and no change in viability. This correlates with the study of Pryor (1992), suggesting that a substantial fraction or all of the O_3 reacts within a cell's membrane. Only with prolonged O_3 exposure are intracellular proteins and DNA affected, and there is a decrease in cell viability. Hamelin (1985) has suggested ozonation as a method for generating fragments for DNA sequence analysis.

This study, directed at the differences between short and long exposures, permits a determination of the earliest events in O_3 toxicity. Elucidation of the sequential events taking place in a cell when exposed to O_3 would lead to a better understanding of its impact on higher life forms.

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