Toxic effects of ozone on murine L929 fibroblasts

Damaging action on transmembrane transport systems

Jolanda VAN DER ZEE, Tom M. A. R. DUBBELMAN and Johnny VAN STEVENINCK*

Sylvius Laboratories, Department of Medical Biochemistry, P.O. Box 9503, 2300 RA Leiden, The Netherlands

Exposure of murine L929 fibroblasts to ozone caused inactivation of the energy-coupled transport systems for 2-aminoisobutyric acid and Rb^+ . Ozone induced an increase of the apparent K_m for 2-aminoisobutyric acid transport, with a constant $V_{\max}$, indicating impairment of the carrier function, rather than a decrease in the number of transport sites. The uptake rate of 2-deoxy-D-glucose, on the other hand, increased after exposure of the cells to ozone. This was caused by an increased $V_{\max}$ of facilitated diffusion, without increased after ozone exposure. Together with the K^+ leakage described previously, this reflects ozone-induced impairment of the barrier function of the membrane.

INTRODUCTION

The reactivity of ozone towards various amino acids, unsaturated fatty acids, nucleic acid bases and sugar moieties has been shown previously in model experiments [1–3]. Therefore most cellular constituents are potential targets for reaction with ozone. In accordance, exposure of erythrocytes to ozone resulted in oxidation of membrane proteins and lipids, K^+ leakage, and inhibition of glyceraldehyde-3-phosphate dehydrogenase and the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ [4], whereas exposure of alveolar type II cells resulted in glutathione depletion [5]. Further, ozone-induced DNA degradation was observed in intact cells of *Escherichia coli* [6] and *Saccharomyces cerevisiae* [7].

Despite many studies on ozone-induced cellular damage, the ultimate cause of cell death after exposure to ozone is still unknown and, moreover, may be different for different cell types. In this context, we systematically studied the effects of ozone on murine L929 fibroblasts. In a previous paper it was shown that exposure of these cells to ozone resulted in inactivation of intracellular enzymes and depletion of GSH, whereas membrane damage was reflected by increased passive permeability for K^+ [8]. In further studies, ozone-induced damage of trans-membrane transport systems in these cells was investigated. In this context the effects of ozone on facilitated diffusion of 2-deoxyglucose, Na^+ -dependent 2-aminobutyric acid transport and energy-dependent Rb^+ uptake were measured. The results are presented in this paper.

MATERIALS AND METHODS

N.C.T.C. murine fibroblasts, clone L929 (A.T.C.C. number CCL1), tissue-culture products and newborn-bovine serum were obtained from Flow Laboratories. 2-Deoxy-D-[1- ^{14}C]glucose, 2-amino[1- ^{14}C]isobutyric acid, L-[1- ^{14}C]glucose and ^{86}Rb were purchased from Amersham International. All other chemicals were obtained from Sigma, Merck or Baker, and were of analytical grade.

L929 fibroblasts were grown as described previously

[9]. Exposure of the cells to ozone, at a flow rate of 0.4 ml of oxygen/min, containing 25 μmol of O_3/ml , was performed as described previously [8]. After exposure to ozone the cell layer was washed with phosphate-buffered iso-osmotic NaCl (PBS). Uptake of solutes was determined by incubating the cell layer for 5 min with 2 ml of 2.5 μM substrate in PBS at 20 °C on a shaker. Uptake was stopped by washing the cell layer with 3 $\times$ 4 ml of ice-cold PBS. The cells were subsequently scraped off into water, and a sample of this suspension was analysed for radioactivity. For kinetic analysis of 2-aminoisobutyric acid and 2-deoxyglucose transport, the same protocol was followed, but with different substrate concentrations. Passive Rb^+ influx was measured in the presence of 1 mM-ouabain, which completely inhibited active Rb^+ transport. For all measurements values were within the range of $\pm 6\%$, with four independent experiments for each datum point.

Protein was determined as described by Lowry *et al.* [10]. Lipid peroxidation was assayed by measuring thiobarbituric acid-reactive products at 532 nm [11].

RESULTS

In control experiments cells were exposed to pure oxygen for periods up to 2 h. This had no influence on uptake of Rb^+ , 2-deoxyglucose or 2-aminoisobutyric acid.

Both before and after exposure to ozone, active Rb^+ uptake [reflecting the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ activity] and passive Rb^+ uptake were linear with time up to 60 min. Upon exposure of the cells to ozone, active Rb^+ uptake decreased, whereas passive Rb^+ uptake increased (Fig. 1).

2-Aminoisobutyric acid influx is linear with time up to 40 min, and this substrate is accumulated in L929 fibroblasts, indicating that the transport system is energy-dependent [9]. Preincubation of the cells with 1 mM-ouabain for 5 min completely inhibited the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ activity, but did not affect 2-aminoisobutyric acid transport (results not shown). As shown in Fig. 2, 2-aminoisobutyric acid influx was

* To whom reprint requests should be addressed.

Fig. 1. Active (●) and passive (○) Rb^+ transport in L929 fibroblasts as a function of ozone exposure time

Rb^+ concentration in the medium was $2.5 \mu\text{M}$. The uptake velocity is given as a percentage of the initial uptake velocities (2.0 for active and $0.41 \text{ pmol/min per mg}$ of protein for passive Rb^+ uptake).

Fig. 2. 2-Aminoisobutyric acid transport in L929 fibroblasts as a function of ozone exposure time

2-Aminoisobutyric acid concentration in the medium was $2.5 \mu\text{M}$. Initial uptake velocity was $24 \text{ pmol/min per mg}$ of protein. Inset shows Lineweaver-Burk plot of 2-aminoisobutyric acid transport: ○, control cells; ●, cells exposed to ozone for 30 min.

inhibited by ozone. Kinetic analysis revealed that the inhibition is characterized by an increase in the apparent K_m value, whereas the apparent V_{max} remained constant at $10 \text{ nmol/min per mg}$ of protein (Fig. 2). The increase in the apparent K_m occurred gradually during exposure to ozone, values of 1.0, 1.4, 1.9, 2.1 and 2.5 mM being

Fig. 3. Influence of ozone on the uptake of 2-deoxyglucose and L-glucose, at substrate concentrations of $2.5 \mu\text{M}$ in the medium

The uptake velocity is given as a percentage of the initial uptake velocity of 2-deoxyglucose ($10.6 \text{ pmol/min per mg}$ of protein): ○, 2-deoxyglucose; ●, 2-deoxyglucose in the presence of 20 mM -glucose; △, L-glucose uptake.

Fig. 4. Lineweaver-Burk plot of 2-deoxyglucose transport before (○) and after (●) exposure to ozone for 30 min

found after exposure times of 0, 10, 20, 30 and 45 min respectively.

2-Deoxyglucose uptake is linear with time up to 60 min. Even after prolonged incubation periods, the intracellular free sugar concentration remained negligible, as over 98% of the intracellular sugar appeared to be phosphorylated (results not shown). As shown in Fig. 3, exposure of the cells to ozone caused an increase in 2-deoxyglucose uptake. Kinetic analysis demonstrated that the V_{max} of transport increased from 7.9 ± 0.4 (s.e.m.) before, to $10.1 \pm 0.3 \text{ nmol/min per mg}$ of protein after, 30 min exposure to ozone, whereas the K_m remained constant at $4.3 \pm 0.2 \text{ mM}$ in six independent experiments (Fig. 4). Addition of 20 mM -glucose inhibited 2-deoxyglucose uptake by about 80%, both

before and after exposure to ozone (Fig. 3). The uptake of L-glucose was not affected by ozone (Fig. 3).

In further experiments, ozone exposure was discontinued after 30 min and the cells were subsequently incubated in medium for 4 h. During this post-incubation period, transport velocities remained constant, indicating that ozone-induced damage was both non-progressive and irreversible after cessation of ozone exposure.

Even after 45 min exposure to ozone no thiobarbituric acid-reactive products could be measured. In control experiments the cells were exposed to 30 mM-H₂O₂. After 10 min lipid peroxidation was reflected by the accumulation of thiobarbituric acid-reactive products (0.315 A_{532} unit/mg of protein).

DISCUSSION

Passive ouabain-insensitive Rb⁺ transport increased upon exposure of the cells to ozone (Fig. 1), indicating increased passive permeability of the membrane for cations. Apparently this increased permeability is not caused by major structural damage to the plasma membrane, as the permeability for a larger molecule such as L-glucose was not increased (Fig. 3).

Active ouabain-sensitive Rb⁺ transport, reflecting the activity of the (Na⁺+K⁺)-ATPase, decreased upon exposure of the cells to ozone (Fig. 1). This cannot be attributed to ATP depletion, as ozone does not affect the cellular ATP concentration [8]. Ozone-induced inactivation of the (Na⁺+K⁺)-ATPase in erythrocyte membranes is caused primarily by destruction of phospholipids, necessary for the enzyme integrity. Ozone-induced destruction of phospholipids was reflected by a fast increase in thiobarbituric acid-reactive products [12]. In the present study no generation of thiobarbituric acid-reactive products was found during exposure to ozone. This cannot be attributed to fast secondary reactions of these products, as a pronounced generation of thiobarbituric acid-reactive products was observed when the cells were incubated with H₂O₂. This virtually excludes a phospholipid-mediated inactivation of the (Na⁺+K⁺)-ATPase, and thus other mechanisms of enzyme inhibition should be considered. For instance, photodynamic inactivation of the (Na⁺+K⁺)-ATPase in erythrocyte membranes is presumably mediated by photo-oxidation of an essential thiol group of the enzyme molecule [13]. A similar mechanism may be involved in ozone-induced inactivation of the (Na⁺+K⁺)-ATPase in fibroblasts. In this context it is interesting that the sensitivity of the (Na⁺+K⁺)-ATPase of erythrocytes to ozone [4] is much higher than the sensitivity of the enzyme in L929 fibroblasts, as described in the present paper. Probably this reflects different mechanisms of inactivation.

Transport of 2-aminoisobutyric acid proceeds via a Na⁺-dependent active-transport system [14]. Although Na⁺-dependent transport systems and the membrane (Na⁺+K⁺)-ATPase are interdependent [15], the inactivation of the amino acid-transport system cannot be attributed to inhibition of the ATPase. This is indicated by the observation that preincubation with ouabain (completely abolishing the ATPase activity) did not affect 2-aminoisobutyric acid transport. Ozone-induced inhibition of 2-aminoisobutyric acid transport was characterized by an increased apparent K_m and a constant apparent V_{max} (Fig. 2). The increased apparent K_m

cannot simply be interpreted as a decrease of affinity of the carrier for the amino acid substrate, as the apparent K_m of co-transport systems is a composite constant containing, besides the affinity constant for the substrate, several other terms [16–18]. The constant apparent V_{max} suggests that ozone does not completely inactivate carrier molecules, as this would most probably have been reflected by a decrease in apparent V_{max} [16].

The uptake velocity of 2-deoxyglucose increased upon exposure of the cells to ozone. The increase is not caused by increased passive diffusion, as indicated by the following observations. For simultaneously occurring carrier-mediated transport and passive diffusion, Lineweaver–Burk plots are biphasic [19]. This was not found experimentally (Fig. 4). Further, the percentage of inhibition of 2-deoxyglucose transport by glucose was the same in control cells and in cells exposed to ozone (Fig. 3), indicating that all 2-deoxyglucose transport remained carrier-mediated. Finally, L-glucose uptake occurs via passive diffusion and is unaffected by ozone. This makes it very unlikely that passive diffusion of 2-deoxyglucose would be increased by ozone. Probably ozone causes an increase in the velocity of the translocation step of 2-deoxyglucose transport, by an as yet unknown mechanism.

Comparison of the present results with earlier studies [8] reveals that, on a time-scale basis, membrane damage and inactivation of several intracellular enzymes proceed with about the same velocity during exposure to ozone. Therefore both membrane deterioration and intracellular damage may contribute to ozone-induced cell inactivation.

This work was supported by a grant of the Netherlands Organization for the Advancement of Pure Scientific Research. Z.W.O. (grant no. 98-61).

REFERENCES

1. Mudd, J. B., Leavitt, R., Ongun, A. & McManus, T. T. (1969) *Atmos. Environ.* **3**, 669–682
2. Menzel, D. B. (1976) in *Free Radicals in Biology* (Pryor, W. A., ed.), vol. 2, pp. 181–201, Academic Press, New York
3. Shinriki, N., Ishizaki, K., Sato, S., Miura, K., Sawadaishi, K. & Ueda, T. (1984) *Chem. Pharm. Bull.* **32**, 3636–3640
4. Van der Zee, J., Tijssen-Christianse, K., Dubbelman, T. M. A. R. & Van Steveninck, J. (1987) *Biochim. Biophys. Acta* **924**, 111–118
5. Rietjens, I. M. C. M., Alink, G. M. & Vos, R. M. E. (1985) *Toxicology* **35**, 207–217
6. Hamelin, C. & Chung, Y. S. (1981) *Mol. Gen. Genet.* **185**, 560–561
7. Dubeau, H. & Chung, Y. S. (1984) *Mol. Gen. Genet.* **197**, 120–124
8. Van der Zee, J., Dubbelman, T. M. A. R., Raap, T. K. & Van Steveninck, J. (1987) *Biochem. J.* **242**, 707–712
9. Dubbelman, T. M. A. R. & Van Steveninck, J. (1984) *Biochim. Biophys. Acta* **771**, 201–207
10. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
11. Stocks, J. & Dormandy, T. L. (1971) *Br. J. Haematol.* **20**, 95–111
12. Chan, P. C., Kindya, R. J. & Kesner, L. (1977) *J. Biol. Chem.* **252**, 8537–8541
13. Girotti, A. W. (1976) *Photochem. Photobiol.* **24**, 525–532

14. Taub, M. & Englesberg, E. (1978) *J. Cell. Physiol.* **97**, 477–485
15. Zibirre, R., Poronnik, P. & Koch, G. (1986) *J. Cell. Physiol.* **129**, 85–93
16. Van den Broek, P. J. A. & Van Steveninck, J. (1982) *Biochim. Biophys. Acta* **693**, 213–220
17. Heinz, E., Geck, P. & Wilbrandt, W. (1972) *Biochim. Biophys. Acta* **255**, 442–461
18. Ghazi, A. & Shechter, E. (1981) *Biochim. Biophys. Acta* **644**, 305–315
19. Van den Broek, P. J. A. & Van Steveninck, J. (1980) *Biochim. Biophys. Acta* **602**, 419–432

Received 10 December 1986/22 April 1987; accepted 30 April 1987