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Toxicity of Activated Oxygen: Lack of Dependence on Membrane Unsaturated Fatty Acid Composition

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SUMMARY: Membrane unsaturated fatty acid oxidation has been suggested as a mechanism of toxicity for a variety of activated oxygen species. We have tested this hypothesis by manipulating the fatty acid composition of an Escherichia coli mutant that is unable to synthesize unsaturated fatty acids. To provide a wide range of susceptibility to membrane oxidation we have replaced the naturally occurring monoenoic acyl chains with cyclopropanes to greatly reduce the unsaturation level and with linoleate to increase the membrane unsaturation. These cultures were treated with ozone, hydrogen peroxide, singlet oxygen and paraquat. In no case was there substantial protection from toxicity afforded by cyclopropanes nor was there enhancement of toxicity to cells with the polyunsaturated membranes. We suggest, therefore, that oxidation of membrane unsaturated fatty acids is not an essential component of the toxicity to E. coli of active oxygen species.

INTRODUCTION: The oxidation of membrane unsaturated fatty acids (UFA) and subsequent loss of membrane function has often been suggested as a mechanism of damage for a wide variety of toxicants, including oxygen and its activated forms (1-7). This hypothesis has been supported by several lines of reasoning. First, of the major cell components, UFA's are particularly reactive with active forms of oxygen. Second, lipid oxidation is often observed to be closely associated with cell damage or death attributed to active oxygen, and in several cases such damage and lipid oxidation are blocked by membrane antioxidants such as vitamin E (7-12). Third, oxygen and its active forms are likely to partition at severalfold higher concentrations in the hydrophobic lipid bilayer than in aqueous cell compartments. Fourth, the very short lifetime (and hence pathlength) of activated oxygen suggests that damage reactions are likely to occur close to the site of origin of the active species. Therefore, reactions at cell membranes

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would seem likely for activated species generated outside the cell or in organelle membranes.

Despite these considerations, the correlation between lipid oxidation and toxicity might merely reflect a coincidental association rather than a causal relationship between the two processes. Furthermore, activated oxygen species have been observed to react with such a wide variety of cell structures (e.g., proteins, DNA) that it is difficult to evaluate which site(s) of damage contribute to the primary toxic response.

In response to these problems, the following model is presented. E. coli mutants incapable of synthesizing UFA's will incorporate media-supplied fatty acids into their membranes. Cyclopropane fatty acids supplied in the medium will substitute for the naturally occurring membrane monoenoic acids and thereby cells can be grown that contain greatly reduced levels of UFA's in the membranes. Because cyclopropane fatty acids are resistant to oxidative degradation (13), such cells can provide a test of the relative importance of lipid oxidation in the toxicity mechanisms of a variety of agents. As a further test, we also have grown cells with polyunsaturated linoleic acid. The presence of two double bonds in the acyl chain should enhance reactivity at least twofold compared to a monoenoic acid (14). If lipid oxidation is indeed an important component of active oxygen toxicity, then cells in which UFA's have been replaced with cyclopropanes should demonstrate increased resistance to damage by these toxicants, and cells with linoleic acid should demonstrate increased sensitivity.

MATERIALS AND METHODS: E. coli strain 1060 B5 was generously provided by Dr. D. F. Silbert. This strain is defective in both synthesis of unsaturated fatty acid and β-oxidation of fatty acids (15). Cells were grown at 37°C in medium A (16) supplemented with 0.5% glucose, 0.5% caseamino acids and 0.0015% thiamine. Fatty acids were added from stock solutions (50 mM) in 90% ethanol to yield a final concentration of 50 μM (17). Growth of cultures was followed spectrophotometrically at 660 nm, and absorbance readings were converted into cell numbers as described previously (13). Viable cell counts were obtained by diluting cultures in 0.15 M NaCl and plating onto agar containing the above medium plus 0.1% Tween 40 and 0.025% oleic acid. That the apparent loss of viability following treatment of cells was not due to the formation of bacterial aggregates was confirmed by microscope examination of the cultures after treatment. Extraction of lipids and fatty acid analyses of cultures were performed as described previously (17).

Paraquat. Paraquat (methyl viologen) was added at concentrations of 0.1 and 0.9 mM to cultures growing in Medium A. In these experiments caseamino acids were omitted to increase the sensitivity of E. coli to paraquat toxicity (18).

Hydrogen peroxide. Cells were treated with H_2O_2 while in 0.15 M NaCl to avoid reactions of H_2O_2 with the culture medium. Cells suspended in 0.15 M NaCl were added to tubes containing various concentrations of H_2O_2. After 5 min, catalase was added (1350 units); after an additional 10 min, one-tenth volume of a tenfold concentrated solution of medium was added. Preliminary experiments showed that adding catalase as above was sufficient to remove essentially any residual H_2O_2 from the NaCl cell suspension. Growth of the cultures at 37°C was then followed spectrophotometrically.

Ozone. Ozone gas produced by a Welsbach generator was bubbled through a 0.15 M NaCl solution for 3 hr at 25°C to produce a saturated solution (0.8 mM). Aliquots (0.1 or 0.4 ml) of this solution were added to E. coli cultures suspended in 8 ml of 0.15 M NaCl. Ozone was allowed to react with the cells for 6 min at room temperature, after which tenfold concentrated medium was added and the growth of the culture was continued at 37°C.

Singlet oxygen. Rose Bengal (0.5 g) and silica gel G (4 g) were slurried in acetone and used to apply a thin coating of dye to the inside of a 30 x 2.5 cm Vigreaux distillation column. This column was illuminated by four 15-watt fluorescent bulbs in parallel, and oxygen was passed through the column at a rate of 150 ml/min. Gas flowing from the column was bubbled through teflon tubing into the suspensions of E. coli at 20-30 ml/min. Control cultures were treated simultaneously with oxygen. Aliquots from the cell suspensions were removed for viable cell counts at various times.

RESULTS AND DISCUSSION

The fatty acid composition of logarithmic stage cells supplemented with cyclopropane, oleic or linoleic acids is given in Table 1. Cultures that have grown for many generations on cyclopropane or linoleic acid still contain low levels of 16:1 and 18:1, presumably due to "leakiness" of the mutation in UFA

TABLE 1. Esterified fatty acid composition (mole % ± SE) of logarithmic E. coli cultures supplemented with cyclopropane, oleic and linoleic acids

	FATTY ACID SUPPLEMENT		
	Cy17:0	18:1	18:2
14:0	2.9 ± 0.6	3.1 ± 0.5	1.6 ± 0.1
16:0	51.8 ± 3.0	42.6 ± 0.9	50.6 ± 2.3
16:1	3.1 ± 0.4	3.2 ± 0.2	3.2 ± 0.6
Cy17:0	37.8 ± 4.2	1.9 ± 0.2	1.8 ± 0.1
18:1	4.0 ± 0.4	47.9 ± 0.6	4.6 ± 0.3
Cy19:0	n.d.	1.7 ± 0.3	n.d.
18:2	n.d.	n.d.	37.6 ± 1.2
Total % UFA	7.1	51.1	45.4

Abbreviations: n.d.: not detected. Cy17:0: cis-9,10-methylene hexadecanoic acid. Cy19:0: cis-9,10-methylene octadecanoic acid. UFA: unsaturated fatty acid.

synthesis. Nevertheless, the three cultures represented in Table 1 differ at least sevenfold in the % unsaturation in their membranes and at least tenfold in the number of acyl chain double bonds per cell.

Paraquat. Hassan and Fridovich (18) have suggested that paraquat toxicity to E. coli is dependent upon intracellular superoxide production. In Fig. 1 the sensitivity to paraquat of E. coli cultures containing cyclopropane, oleic and linoleic acids is shown. With all fatty acid supplements, growth was completely inhibited by 0.9 mM paraquat. Lower concentrations of paraquat gave correspondingly less inhibition, but the extent of inhibition was not substantially dependent upon the cell's membrane fatty acid composition. Thus for both high and low levels of toxicity, we could detect neither protection of these cells by removal of unsaturated fatty acid nor enhancement of toxicity when oleic was replaced by linoleic acid.

Hydrogen peroxide. Fig. 2 shows growth of cultures after treatment with four concentrations of H_2O_2. H_2O_2 (1.0 mM) was sufficient to kill essentially all cells in the cultures (as confirmed by viable cell counts). Lower concentrations of H_2O_2 appeared to kill or damage proportionately fewer cells, so that the growth of the culture continued after a lag time that increased with higher H_2O_2 levels. Replacement of UFA in the cell membranes, however, afforded essentially no protection to the cells from either the high or low doses of H_2O_2, nor did increased UFA cause increased sensitivity.

Ozone. Various investigators (25, 26) have suggested that O_3 toxicity is due to reactions with membrane UFA, although evidence against this mechanism has also been presented (27). In our experiments, cells containing different levels of membrane unsaturation were treated for 6 min with either 0.01 or 0.04 mM O_3. The toxic effects in the ozone-treated cultures were reflected by either a decrease in culture OD, presumably due to lysis of cells, or to a lag before growth resumed. As shown in Fig. 3, cells containing cyclopropane rather than unsaturated fatty acids were not protected from O_3 damage and, in fact, appeared more susceptible. Cells containing linoleic acid were slightly more sensitive

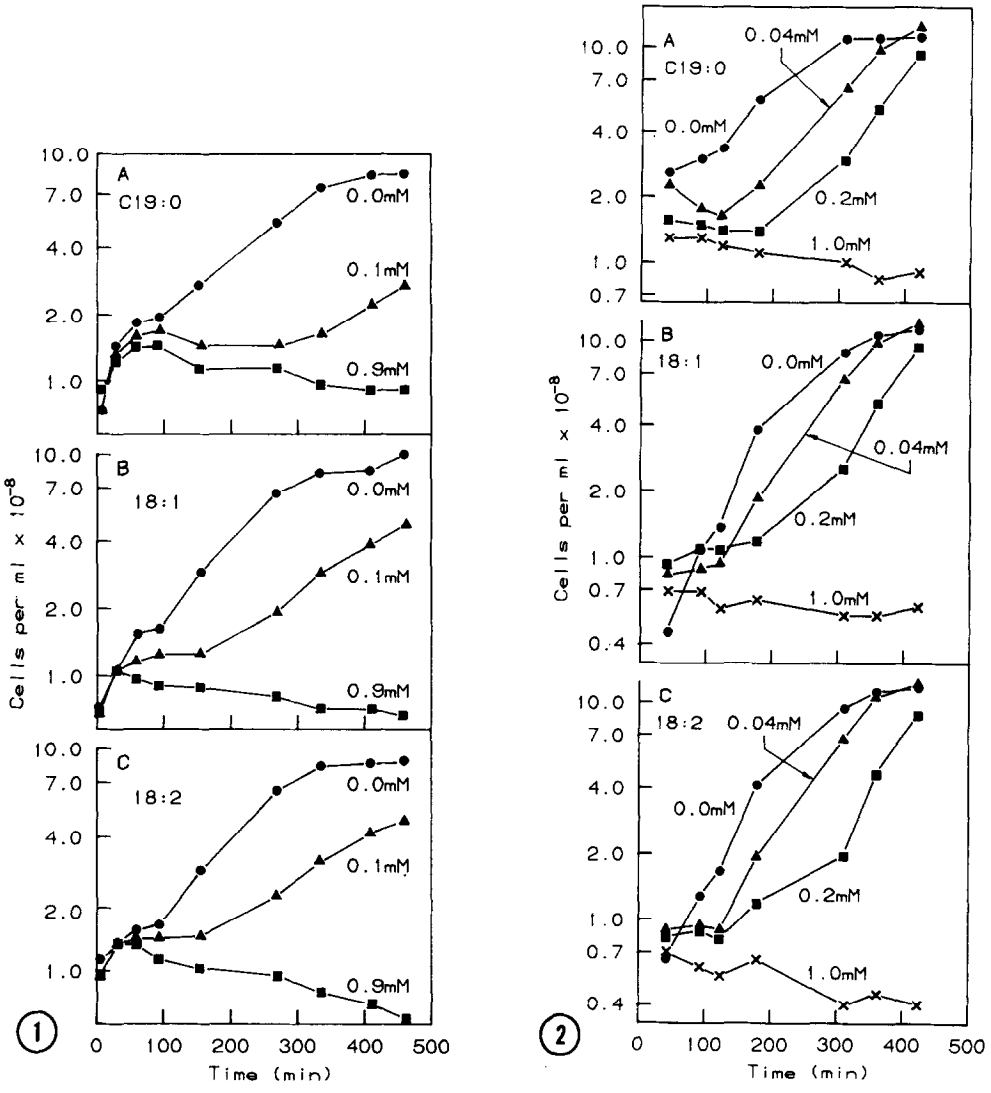

Fig. 1. Effect of paraquat on growth of Escherichia coli cells supplemented with (A.) 50 μM cis-9,10-methyleneoctadecanoic acid, (B.) 50 μM oleic acid, (C.) 50 μM linoleic acid.
●—●, 0 mM paraquat. ▲—▲ 0.1 mM paraquat. ■—■ 0.9 mM paraquat.

Fig. 2. Effect of hydrogen peroxide on growth of E. coli cells supplemented with (A.) 50 μM cis-9,10-methyleneoctadecanoic acid, (B.) 50 μM oleic acid, (C.) 50 μM linoleic acid. ●—● 0 mM H_2O_2, ▲—▲ 0.04 mM H_2O_2, ■—■ 0.2 mM H_2O_2, X—X 1.0 mM H_2O_2. Cells suspended in 0.15 M NaCl were treated with H_2O_2 for 5 min, after which catalase and concentrated medium were added.

to O_3 than those containing oleic, but still were less sensitive than the cyclopropane cultures.

Singlet oxygen. Singlet oxygen, generated upon the absorption of light by photosensitizing dyes in the presence of oxygen, is believed responsible for the photodynamic toxicity of a variety of drugs and diseases (28, 29). In our

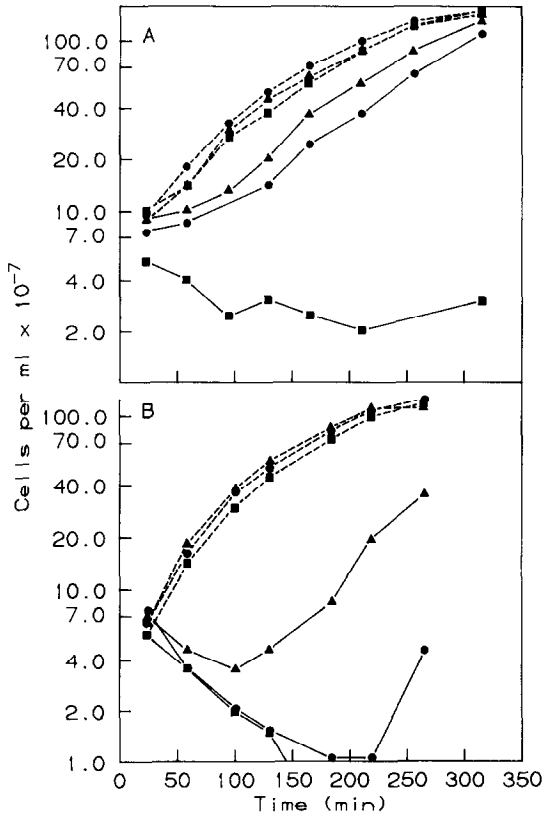

Fig. 3. Effect of ozone treatment on growth of E. coli cultures containing various membrane fatty acid compositions. 0.1 ml (A) or 0.4 ml (B) of a solution of 0.15 M NaCl saturated with ozone (~0.8 mM) was added to cells suspended in 0.15 M NaCl. After 6 min, concentrated medium was added and the growth of the cultures was monitored at 660 nm.
■ Cultures supplemented with 50 μM cis-9,10-methylenehexadecanoic acid.
▲ Cultures supplemented with 50 μM oleic acid.
● Cultures supplemented with 50 μM linoleic acid.
Dotted lines are control cultures.

experiments, singlet oxygen was produced in the gas phase by light action on the dye Rose Bengal (30). In Fig. 4 the number of surviving viable cells is plotted versus the time of exposure to singlet oxygen. We again observed no large differences in the susceptibility of the various cultures to killing by singlet oxygen.

Harley et al. using a similar mutant observed that cyclopropane fatty acids protect E. coli against hyperbaric oxygen toxicity (25). Therefore the active species in our study likely damage cells by mechanisms different from hyperbaric O_2.

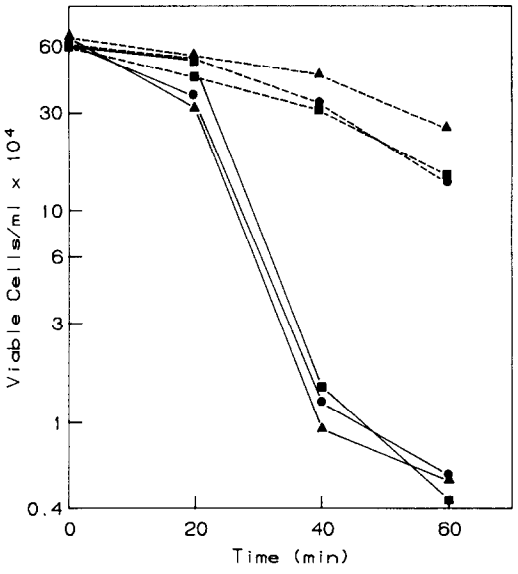

Fig. 4. Effect of singlet oxygen treatment on growth of E. coli cultures containing various membrane fatty acid compositions. Oxygen (dotted lines) or singlet oxygen (solid lines) was bubbled at 20-30 ml/min into cells suspended in 0.15 M NaCl. At the times indicated, dilutions onto solid media were made for viable cell counts.
■ Cultures supplemented with 50 μM cis-9,10-methylenehexadecanoic acid.
▲ Cultures supplemented with 50 μM oleic acid.
● Cultures supplemented with 50 μM linoleic acid.

The results in Figs. 1-4 argue against the hypothesis that oxidation of membrane UFA is an important component of the toxicity of ozone, H_2O_2, singlet oxygen or paraquat toward E. coli. We suggest, therefore, that many previously observed correlations between cell damage and lipid oxidation may reflect secondary or coincidental associations and do not represent a primary mechanism of toxicity.

REFERENCES

1. Borg, D. C., Schaich, K. M., Elmore, J. J., Jr. and Bell, J. A. (1978) Photochem. Photobiol. 28, 887-907.
2. Freeman, B. A., Topolosky, M. K. and Crapo, J. D. (1982) Arch. Biochem. Biophys. 216, 477-484.
3. Fridovich, I. (1978) Science 201, 875-880.
4. Goldstein, B. D. and Balchum, O. J. (1967) Proc. Soc. Exp. Biol. Med. 126, 356-358.
5. Mead, J. F. (1976) Free Radicals in Biology. (Pryor, W. A., ed.) Academic Press, New York, pp. 51-68.
6. Shimazaki, K., Sakak, T., Kondo, N. and Sugahara, K. (1980) Plant Cell Physiol. 21, 1193-1204.
7. Tsen, C. C. and Collier, H. B. (1960) Can. J. Biochem. Physiol. 38, 957-964.
8. Goldstein, B. D., Buckley, R. D., Cardenas, R. and Balchum, O. J. (1970) Science 169, 605.
9. Hafeman, D. G. and Hoekstra, W. G. (1977) J. Nutr. 107, 656-665.
10. Leung, H. W., Vang, M. J. and Mavis, R. D. (1981) Biochim. Biophys. Acta 664, 266-272.

11. Sevanian, A., Hacker, A. D. and Elsayed, N. (1982) Lipids 17, 269-277.
12. Tappel, A. L. and Dillard, C. J. (1981) Fed. Proc. 40, 174-178.
13. Ohlrogge, J. B., Gunstone, F. D., Ismail, I. A. and Lands, W. E. M. (1976) Biochim. Biophys. Acta 431, 257-267.
14. Gunstone, F. D. and Hilditch, T. P. (1945) J. Chem. Soc. 1945, 836-841.
15. Silbert, D. F., Ladenson, R. C. and Honegger, J. L. (1973) Biochim. Biophys. Acta 311, 349-361.
16. Davis, B. D. and Mingoli, E. S. (1950) J. Bacteriol. 60, 17-28.
17. Ohlrogge, J. B., Barber, E. D., Lands, W. E. M., Gunstone, F. D. and Ismail, I. A. (1976) Can. J. Biochem. 54, 736-745.
18. Hassan, H. M. and Fridovich, I. (1979) J. Biol. Chem. 254, 10846-10852.
19. Pauls, K. P. and Thompson, J. E. (1981) Physiol. Plant 53, 255-262.
20. Scott, D. B. M. and Lesher, E. C. (1963) J. Bacteriol. 85, 567-576.
21. Swanson, E. S., Thomson, W. W., and Mudd, J. B. (1973) Can. J. Bot. 51, 1213-1219.
22. Foote, C. S. (1976) Free Radicals in Biology. (Pryor, W. A., ed.) Academic Press, New York, pp. 85-133.
23. Ito, T. (1978) Photochem. Photobiol. 28, 493-508.
24. Bezman, S. A., Burtis, P. A., Izod, T. P. and Thayer, M. A. (1978) Photochem. Photobiol. 28, 325-329.
25. Harley, J. B., Santangelo, G. M., Rasmussen, H. and Goldfine, H. (1978) J. Bacteriol. 134, 808-820.