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COMPARISON OF THE CYTOTOXICITY OF DIFFERENT HYDROPEROXIDES TO V79 CELLS

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We compared the cytotoxicty of hydrogen peroxide (H_2O_2), tertiary butyl hydroperoxide (t-BHP) and methyl linoleate hydroperoxide (MLHP) to V79 cells, using a colony formation assay. In all cases L-buthionine-(S,R)-sulphoximine enhanced the cytotoxicity, and Quin 2 inhibited it. Nordihydroguaiaretic acid (NDGA) and o-phenanthroline suppressed the cytotoxicity of H_2O_2 and t-BHP, but they had no effect on the cytotoxicity of MLHP. These results suggest that the biological effects of t-BHP are similar to those of H_2O_2 and not to those of lipid hydroperoxides. In the course of the experiments, we found that NDGA, an antioxidant and food additive, was a potent inhibitor of cytotoxicity of H_2O_2.

KEY WORDS: Hydroperoxide, hydrogen peroxide, t-butylhydroperoxide, lipid hydroperoxide, cytotoxicity.

INTRODUCTION

There are many reports describing the effects of hydroperoxides on mammalian cells or their components. Various parameters, e.g. cell proliferation, viability, DNA lesions, mutation or chromosomal aberrations have been measured in cells treated with hydrogen peroxide (H_2O_2), tertiary butyl hydroperoxide (t-BHP) or lipid hydroperoxide.[1–7] H_2O_2 and lipid hydroperoxide have been detected in cells and foods, but t-BHP does not exist in food or the human environment. The reasons why many researchers have used t-BHP in their studies might be its stability, ease of handling and, most important, no reported toxicity of its secondary degradation products. On the other hand, lipid hydroperoxides are difficult to keep for a long time and their secondary degradation products, e.g. alkenals, are more toxic to cells than the original lipid hydroperoxide.[7] It remains unclear what kinds of hydroperoxides existing in cells are simulated by t-BHP. Thus, we have compared the cytotoxicity of the three hydroperoxides by colony formation assay, one of the most reliable methods of assessing cytotoxic effects.[8]

MATERIALS AND METHODS

Reagents

Methyl linoleate and t-BHP (70% in water) were purchased from Nakarai Chemicals Ltd. (Kyoto, Japan). H_2O_2 was obtained from Mitsubishi Gas Chemical Co. Ltd.

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(Tokyo, Japan). L-Buthionine-(S,R)-sulphoximine (BSO), nordihydroguaiaretic acid (NDGA), o-phenanthroline and Quin-2-AM were purchased from Sigma Chemical Co. (St. Louis, U.S.A.). Methyl linoleate hydroperoxide (MLHP) was prepared by allowing 10 ml of methyl linoleate to stand in an incubator at 40°C for seven days. Crude MLHP was obtained by silica column chromatography in a mixture of diethyl ether and hexane. It was further purified by high performance liquid chromatography immediately before use to avoid formation of secondary degradation products.[9] Methyl linoleate hydroxide (MLH) was prepared by reduction of MLHP with sodium borohydride[10] and purified by thin layer chromatography; the sample was applied to a silica gel plate and chromatographed, using hexane/ether (1:1) as the solvent mixture. The structure of MLH was identified by mass spectrometry. MLHP and MLH, which consist of 4 stereoisomers,[9] were sonicated in 1% Tween 20 and filtered through a cellulose nitrate filter (pore size: 0.2 μm). Then their concentration in the sterilized solutions were determined by measurement of the absorbance at 233 nm. All other chemicals were reagent grade and were used without further purification.

Cell Culture

V79 cells from chinese hamster lung fibroblasts were grown in minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). The cells were cultured in a CO_2 incubator with 5% CO_2 in humidified air.

Standard Method of Colony Formation Assay

V79 cells were seeded in 200/60-mm petri dishes and incubated in MEM supplemented with 10% FBS. After 4 hours, the cells were washed with HEPES buffered saline (HBS) and incubated in HBS with or without hydroperoxide for 30 min at 37°C; one litre of HBS contained 8 g of NaCl, 0.4 g of KCl, 0.252 g $Na_2HPO_4 \cdot 12H_2O$, 1 g of glucose and 5.95 g of HEPES, its pH being adjusted to 7.3 with 0.1 N NaOH. After culture in MEM supplemented with 10% FBS for 5 days, the colonies were fixed with methanol and stained with Giemsa's solution. The number of colonies consisting of more than 50 cells was counted. The relative survival fraction was calculated by dividing the number of colonies on treated petri dishes by the number of colonies on untreated petri dishes. Results are expressed as the means and S.D. of four separately treated cultures unless specified otherwise. The data were analyzed statistically using the Student's t-test, comparing control vs. treatment groups.

RESULTS AND DISCUSSION

Each hydroperoxide was added to HBS instead of MEM or MEM supplemented with 10% serum to avoid direct reaction of each hydroperoxide with components of the medium or the serum. Since incubation of the cells in HBS for more than 90 min had cytotoxic effects (the relative survival was 98% for 60 min incubation and 76% for 90 min incubation), the cells were incubated with relatively concentrated hydroperoxides for a short time. When the same amount of 1% Tween 20 (less than 25 μl) was added to HBS as a control for the experiments of MLHP or MLH, no cytotoxic effects were observed. Figure 1 shows that the toxicity of each hydroperoxide similarly increased with incubation time in the range of 45 min. This enable us to compare the

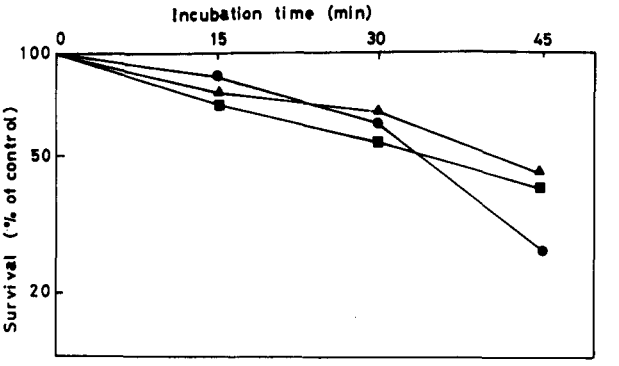

FIGURE 1 Time dependency of the survival of cells treated with different hydroperoxides. V79 cells were treated with hydroperoxides in HBS at 37°C. Each concentration was adjusted to give similar cytotoxic potency. The results are expressed as the mean of triplicate experiments. (●) 7 μM MLHP, (▲) 30 μM H_2O_2 (■) 300 μM t-BHP.

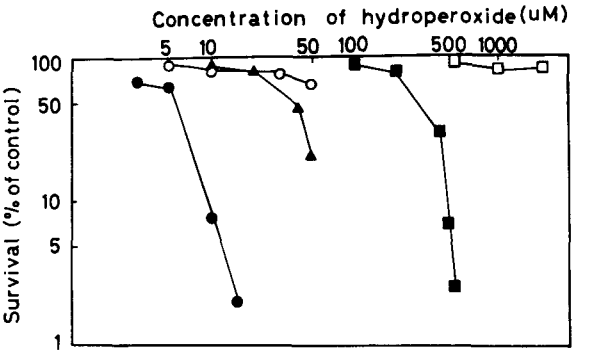

FIGURE 2 Typical dose effect curves of the relative survivals. V79 cells were treated with the hydroperoxides and the corresponding hydroxides in HBS for 30 min at 37°C. The number of colonies formed after 5 days was counted. The results are expressed as the mean of triplicate experiments. (●) MLHP, (○) MLH, (▲) H_2O_2, (■) t-BHP, (□) tertiary butyl alcohol.

cytotoxicity at the same incubation time. Figure 2 shows typical dose effect curves of the relative survivals. Although it was difficult to keep constant dose dependency in each experiment, MLHP was always most toxic, followed by H_2O_2. The hydroperoxide structure seems essential for cytotoxicity, since MLH and t-butyl alcohol were much less toxic than the corresponding hydroperoxides. Considering these results, we fixed the incubation time at 30 min and arranged the concentration of each hydroperoxide to keep the relative survivals at 20–40% in the following experiments; 2–10 μM of MLHP, 100–300 μM of t-BHP and 30–50 μM of H_2O_2 were added to HBS to adjust their toxic potency.

Effect of BSO, Quin-2-AM, o-phenanthroline and NDGA

BSO is an inhibitor of glutathione synthesis in cells and it is already known that BSO enhances cytotoxicity of t-BHP.[3] We added BSO to the medium after seeding and also

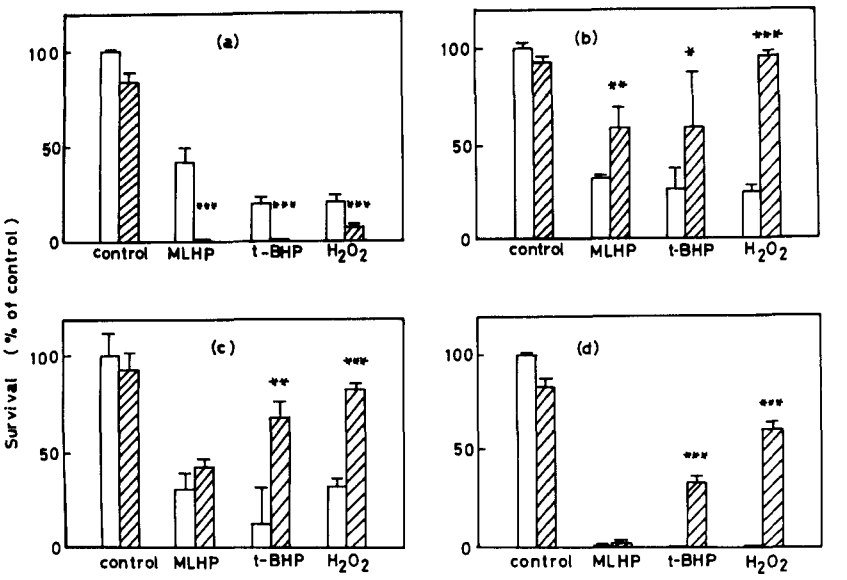

FIGURE 3 Effects of BSO, Quin-2, o-phenanthroline and NDGA on the cytotoxicity of hydroperoxides. V79 cells were exposed by hydroperoxide without (□) or with (■) each reagents for 30 min at 37°C: (a) 50 μM of BSO, (b) 10 nM of Quin-2-AM, (c) 25 μM of o-phenanthroline. In experiment (d), V79 cells pretreated with (■) or wthout (□) 5 μM of NDGA were exposed to hydroperoxide for 30 min at 37°C. The results are expressed as the mean ± S.D. of four separately treated cultures. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

to HBS at the time of treatment with hydroperoxide. After treatment with hydroperoxide, the cells were incubated in the medium without BSO. Figure 3(a) shows that BSO enhanced the cytotoxic effects of all types of hydroperoxides used in our experiment. This suggests that intracellular glutathione reduced these hydroperoxides in the presence of glutathione peroxidase, consistent with the view that the hydroperoxide structure is essential to the cytotoxicity.

When the lipophilic Quin-2-AM is added to cells, it diffuses across the plasma membrane and is hydrolyzed to Quin-2 by cytosolic esterases. Then, Quin-2 tightly binds Ca^{2+} in the cytosol.[11] It is reported that Quin 2 reduces cellular toxicity caused by H_2O_2.[12] We added 25 μl of Quin-2-AM dimethylsulphoxide solution to HBS (final 10 nM) 5 min before treatment with hydroperoxide. The effect of Quin-2-AM on the relative survival fraction were quite similar (Figure 3(b)). On the other hand, 25 μl of dimethyl sulphoxide had no effect. Since Quin-2-AM reduced the toxic effect of all three hydroperoxides, Ca^{2+} seems an important factor in cytotoxicity induced by any hydroperoxides.

We added o-phenanthroline (final 25 μM), a potent iron chelator rendering the metal incapable of generating hydroxyl radicals ($\cdot OH$)[2], to HBS and incubated for 10 min before treatment with hydroperoxides. Figure 3 (c) shows that o-phenanthroline inhibited and cytotoxicity caused by H_2O_2 and t-BHP, confirming previous findings.[3,13] On the other hand, no inhibitory effect of o-phenanthroline on the cytotoxicity by MLHP was observed, suggesting that iron in the cells is not necessary for the toxic mechanism of MLHP.

NDGA, a lipid soluble antioxidant, is known to trap free radicals and inhibit lipoxygenases from a wide variety of sources.[14] In our experiments we avoided direct reaction of NDGA with each hydroperoxide added to HBS and the lipid fraction of serum. We seeded 200 cells and incubated them in MEM supplemented with 10% FBS for 2 hours. After changing the medium to MEM free of FBS, we added 25 μl of 1 mM NDGA ethanol soluton (final 5 μM) and incubated the cells for 4 hours. After washing the cells with HBS, the cells were treated with each hydroperoxide. Under these conditions, 25 μl of ethanol had neither enhancing effects nor inhibitory effects on cytotoxicity of any hydroperoxides. Since NDGA had potent inhibitory effects on the cytotoxicity caused by t-BHP and H_2O_2, we changed the concentration of these hydroperoxides in order that the survival of the cell without treatment with NDGA become nearly 0%. Even in this concentration, NDGA also showed potent inhibitory effects (Figure 3 (d)). On the other hand, the cytotoxicity of MLHP was not reduced. When the survival fraction of the cells treated with MLHP was arranged to 30–40%, no inhibitory effect of NDGA was observed (data not shown).

Considering the effects of the chelators and the inhibitors, the cytotoxic mechanism of t-BHP seems similar to that of H_2O_2. On the other hand, the absence of an effect of NDGA or of o-phenanthroline on the cytotoxicity of MLHP suggests that the cytotoxic effects of lipid hydroperoxides are different from those of t-BHP.

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