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# Hydrogen peroxide cytotoxicity

## Low-temperature enhancement by ascorbate or reduced lipoate

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The principal mechanism of $H_2O_2$ toxicity is thought to involve the generation of hydroxyl (HO•) radicals through its interactions with $Fe^{2+}$ ions by the Fenton reaction. Of particular interest has been the demonstration by Ward, Blakely & Joner [(1985) Radiat. Res. **103**, 383–392] that the cytotoxicity of $H_2O_2$ is diminished at low temperature. We have now examined this phenomenon further with a mammalian epithelial cell line (CNCMI-221). Resistance of these cells to 100 $\mu$M-$H_2O_2$ added extracellularly exhibits a transition in the temperature range between 27 °C and 22 °C. We have found that the low-temperature resistance to cytotoxic concentrations of $H_2O_2$ is abolished by preincubation of cells with reductants such as ascorbate or reduced lipoic acid. This implies that the low-temperature resistance to $H_2O_2$ cytotoxicity may be due to inhibition of cellular reductive processes. The restoration of the cytotoxic action of $H_2O_2$ at 4 °C by ascorbate is prevented by pre-exposure of cells to desferrioxamine. This is evidence that transition-metal ions (such as iron ions) are involved in the cytotoxicity and is consistent with a mechanism of cell damage that depends on the Fenton reaction and a metal ion in the reduced state. Restoration of $H_2O_2$ cytotoxicity at low temperature by ascorbate is consistent with the artificial production of an intracellular reducing environment that at normal temperatures is sustained by cellular metabolism.

## INTRODUCTION

In recent years there has been a growing interest in $H_2O_2$ as a source of hydroxyl (HO•) radicals in studies of cytotoxic mechanisms. The toxicity of $H_2O_2$ has been studied in a variety of different cell lines (Ward *et al.*, 1983; Jones & Kennedy, 1983; Hoffmann *et al.*, 1984; Starke & Farber, 1985; Dallergri *et al.*, 1987; Link & Riley, 1988). Several studies have demonstrated that the cytotoxicity is dose-dependent (Hoffmann & Meneghini, 1979; Rubin & Farber, 1984; Spitz *et al.*, 1987).

The principal mechanism of cytotoxicity produced by $H_2O_2$ is thought to involve HO• radicals generated by the Fenton reaction in close proximity to the DNA strands. Several studies have provided support for this: (i) scavenger studies with chromatin *in vitro* show that the HO• radical is the principal species damaging DNA (Tullis, 1987); (ii) studies with bacterial spores demonstrate that HO• radicals are not damaging when generated extracellularly (Jacobs *et al.*, 1985); (iii) from studies with $\lambda$ and T bacteriophages it has been proposed that damage to DNA is caused by a site-specific Fenton reaction (Samuni *et al.*, 1983); (iv) in V79 cells (Larramendy *et al.*, 1987) or human fibroblasts (Mello-Filho & Meneghini, 1984) this could be prevented by pretreatment with *o*-1,10-phenanthroline.

Ward *et al.* (1985) have suggested that the cytotoxicity of $H_2O_2$ is associated with local multiply damaged sites in DNA. This suggestion arose as a result of failure to observe cell killing from singly damaged sites following exposure of Chinese-hamster ovary cells at 0 °C to doses of $H_2O_2$ that are toxic at 37 °C.

Our findings confirm that at low temperature (4 °C) $H_2O_2$ is non-toxic in a concentration range shown to produce a dose-dependent diminution of survival at 37 °C. Also, we demonstrate conditions in which $H_2O_2$ toxicity is restored at low temperature and provide further support for the involvement of reduced transition-metal ions in this effect.

## MATERIALS AND METHODS

### Materials

$H_2O_2$ [aq. 30% (w/v) solution, stabilizer-free] was obtained from Aldrich Chemical Co. Sodium dehydroascorbate was obtained from Koch–Light Laboratories. Ascorbic acid was obtained from Sigma Chemical Co. Reduced (sealed ampoules) and oxidized lipoic acid (DL-1,2-dithiolane-3-pentanoic acid) were obtained from Sigma Chemical Co. Desferrioxamine methanesulphonate was obtained from CIBA–GEIGY. [*Me*-$^3$H]-Thymidine (specific radioactivity 5 mCi/nmol) was obtained from Amersham International and diluted in phosphate-buffered saline (see below) to give a stock solution of 20 $\mu$Ci/ml. Eagle's medium and Earle's salts were obtained from Flow Laboratories.

Sodium dehydroascorbate, ascorbic acid and desferrioxamine methanesulphonate were dissolved in distilled water immediately before each experiment. $H_2O_2$ was diluted in distilled water immediately before addition to cells. Lipoic acid was dissolved in dilute ethanol. Phosphate-buffered saline was prepared by dissolving 8 g of NaCl, 0.2 g of KCl, 0.132 g of $CaCl_2,H_2O$, 0.1 g of $MgCl_2,6H_2O$, 1.5 g of $Na_2PO_4,2H_2O$ and 0.2 g of $KH_2PO_4$ in 1 litre of distilled water. All reagents were sterilized by filtration through a 0.22 $\mu$m-pore-size filter (Millipore).

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### Cell line

An established mammalian epithelial cell line (CNCM I-221) was used for all experiments. To minimize any variation due to repeated subculturing, the experiments were conducted with cells between passage numbers 21 and 30. Cells were grown in multi-well trays, each of diameter 1.5 cm (1.77 $cm^2$) (Falcon Plastics, Scientific Supplies). The culture medium consisted of Eagle's minimal essential medium with Earle's salts supplemented with 10% (w/v) foetal bovine serum (Imperial Labs), penicillin (10 $\mu$g/ml), streptomycin (10 $\mu$g/ml) and 20 mM-Hepes.

### Delayed-thymidine-incorporation assay for cell survival

The cytotoxic effect of $H_2O_2$ is not observable directly after treatment and takes several hours to become manifest. Changes in membrane permeability were found to be late in onset and unreliable indicators of the toxic action of $H_2O_2$. In the present studies we have used the delayed-[$^3$H]thymidine-incorporation assay as an index of toxicity, since this correlates very well with the plating efficiency assay, and is an index of reproductive viability (Jonas *et al.*, 1988). The method for determining cell survival as estimated by the delayed-thymidine-incorporation assay is as follows. After cells had been exposed to the agents to be tested (see Table 1 for protocol), the medium was removed and the cells were washed twice in fresh phosphate-buffered saline and re-incubated in serum-containing medium at 37 °C in an atmosphere of 2% $CO_2$ in air. After 18–24 h (which permits at least one cell division to occur) the cells were exposed to 1 $\mu$Ci of [$^3$H]thymidine/ml for 30 min at 37 °C. The cells were then washed in phosphate-buffered saline, fixed with 5% (w/v) trichloroacetic acid and washed twice with phosphate-buffered saline. The multi-wells were then dried and the acid-insoluble material was digested overnight in 250 $\mu$l of 1 M-NaOH per well. Samples (100 $\mu$l) of the digest were each mixed with 5 ml of scintillation fluid [consisting of 4 g of 2,5-bis-(5-t-butylbenzoxaz-2-oyl)thiophen/l of toluene/napthalene/2-methoxyethanol (55:8:37, by vol.)] and radioactivities were counted in an Intertechnique SL40 counter. The mean radioactivity count from four wells was expressed as a percentage of the radioactivity obtained from control cells that had not been exposed to the agents under test. These data are referred to as the survival index.

### Incubation with $H_2O_2$

The numbers of attached cells at the time of exposure were in the region of $5 \times 10^5$ cells/well for different experiments. Cells were incubated for 60 min at 4 °C or 37 °C in phosphate-buffered saline containing $H_2O_2$. The concentration of $H_2O_2$ used was between 5 $\mu$M and 80 $\mu$M, corresponding to a cell concentration of up to 800 fmol/cell.

In a separate experiment cells at a density of $1.5 \times 10^5$ cells/ml were incubated in phosphate-buffered saline containing 100 $\mu$M-$H_2O_2$ for 60 min at temperatures ranging from 4 °C to 37 °C.

### Preincubation and incubation protocols

A series of experiments were carried out that involved the preincubation of cells with reducing agents at 4 °C or 37 °C in growth medium. This was followed by washing the cells twice with phosphate-buffered saline and replacing the medium by phosphate-buffered saline containing 50 $\mu$M-$H_2O_2$ at either 4 °C or 37 °C.

The sequence of treatment procedures is listed in Table 1. In experiments with reducing agents or their oxidized analogues, the agents were added immediately before preincubation III (stage 5). In experiments with desferrioxamine, the drug was added immediately before preincubation II (stage 3), when the cells had reached a density of approx. $5 \times 10^4$ cells/well.

**Table 1. Experimental protocol**

For full experimental details see the text. Abbreviations: SC MEM, culture medium consisting of Eagle's minimal essential medium with Earle's salts, 10% foetal bovine serum, penicillin (10 $\mu$g/ml), streptomycin (10 $\mu$g/ml) and 20 mM-Hepes; PBS, phosphate-buffered saline (see the text).

| Stage | Procedure | Incubation conditions | | |
|---|---|---|---|---|
| | | Medium | Temperature | Time |
| 1 | 1 ml of $2 \times 10^4$ cells inoculated into multi-wells | | | |
| 2 | Preincubation I | SC MEM | 37 °C | 24 h |
| 3 | Preincubation II (±desferrioxamine) | SC MEM | 37 °C | 24 h |
| 4 | Wash 2× with PBS | | | |
| 5 | Preincubation III (±reductants) | SC MEM | 4 °C/37 °C | 120 min |
| 6 | Wash 2× with PBS | | | |
| 7 | Add 50 $\mu$M-$H_2O_2$ | | | |
| 8 | Incubation in $H_2O_2$ | PBS | 4 °C/37 °C | 60 min |
| 9 | Wash 2× with PBS | | | |
| 10 | Post-incubation I | SC MEM | 37 °C | 24 h |
| 11 | Add [$^3$H]thymidine (1 $\mu$Ci/ml) | | | |
| 12 | Post-incubation II (labelling) | SC MEM | 37 °C | 30 min |
| 13 | Wash 5× with PBS | | | |
| 14 | Add trichloroacetic acid | | | |
| 15 | Wash 2× with PBS | | | |
| 16 | Digest and assay | | | |

**Table 2. Survival index of CNCM I-221 cells after exposure to $H_2O_2$ at different temperatures and cell concentrations**

For experimental details see the text. Survival indexes are given as means ± S.D. ($n = 4$).

| Temperature (°C) | Concn. of $H_2O_2$ ($\mu$M) | Cell density (cells/ml) | Concn. of $H_2O_2$ (fmol/cell) | Survival index (%) |
|---|---|---|---|---|
| 37 | 5 | $2 \times 10^5$ | 25 | 88 ± 4 |
| 37 | 10 | $2 \times 10^5$ | 50 | 75 ± 7 |
| 37 | 10 | $1 \times 10^5$ | 100 | 79 ± 13 |
| 37 | 30 | $1.5 \times 10^5$ | 200 | 54 ± 12 |
| 37 | 30 | $8 \times 10^4$ | 375 | 22 ± 4 |
| 37 | 40 | $1.5 \times 10^5$ | 268 | 28 ± 3 |
| 37 | 50 | $2 \times 10^5$ | 250 | 30 ± 13 |
| 37 | 50 | $1.5 \times 10^5$ | 500 | 18 ± 7 |
| 37 | 50 | $1.2 \times 10^5$ | 417 | 14 ± 3 |
| 37 | 80 | $1.5 \times 10^5$ | 400 | 21 ± 4 |
| 4 | 10 | $4 \times 10^5$ | 25 | 101 ± 22 |
| 4 | 30 | $4 \times 10^5$ | 75 | 94 ± 13 |
| 4 | 50 | $4 \times 10^5$ | 125 | 87 ± 13 |
| 4 | 50 | $1.5 \times 10^5$ | 334 | 94 ± 11 |
| 4 | 50 | $6 \times 10^4$ | 834 | 114 ± 4 |
| 4 | 50 | $1 \times 10^5$ | 500 | 104 ± 17 |
| 4 | 50 | $1 \times 10^5$ | 500 | 104 ± 16 |
| 4 | 50 | $6 \times 10^4$ | 834 | 103 ± 18 |
| 4 | 50 | $1.5 \times 10^5$ | 334 | 100 ± 6 |
| 4 | 50 | $1.5 \times 10^5$ | 334 | 104 ± 10 |
| 4 | 50 | $1.6 \times 10^5$ | 313 | 121 ± 16 |
| 4 | 80 | $4 \times 10^5$ | 200 | 88 ± 16 |

## RESULTS

Table 2 gives the data from several experiments that show a dose-dependence of survival at 37 °C but not at 4 °C. The Table also demonstrates the dependency of survival on cell density, which accounts for the large variation in survival index at particular concentrations of $H_2O_2$. The concentration of $H_2O_2$ to which the cells were exposed is expressed in terms of fmol/cell to eliminate these effects of cell density, to which the system was found to be very sensitive, as also noted by others (Ziegler-Sylakakis & Andrae, 1987; Spitz *et al.*, 1987). The data show clearly that lowering of the incubation temperature to 4 °C abolishes the cytotoxic effect of $H_2O_2$ exhibited at 37 °C.

In a further study a range of temperatures between 0 °C and 37 °C was investigated. Cells at a density of $1.5 \times 10^5$ cells/ml were exposed to 100 $\mu$M-$H_2O_2$ for 60 min. The data show that there is a slight fall in survival index to 80% as the temperature is raised from 0 °C to 22 °C, as illustrated in Fig. 1. A steep gradient between 22 °C and 27 °C was observed, suggesting a transition temperature at about 25 °C.

### Preincubation with ascorbate

Cytotoxicity of $H_2O_2$ in the concentration range that is effective at 37 °C was observed at 4 °C in cells that had been exposed during preincubation III to 1 mM-ascorbate (Table 3). A 2 h preincubation period was required. Periods of exposure to 1 mM-ascorbate for less than 2 h had little or no effect. Analogous experiments with dehydroascorbate demonstrated that the effect was dependent on the temperature of the preincubation (Table 3). Preincubation with dehydroascorbate at 4 °C exhibited a toxic action independent of $H_2O_2$, but at 37 °C preincubation with dehydroascorbate had the effect of restoring the toxic action of $H_2O_2$ at 4 °C in a manner similar to that observed with ascorbate.

### Preincubation with lipoic acid

Cells were exposed during preincubation III to 100 $\mu$M- or 500 $\mu$M-lipoic acid (oxidized or reduced) before treatment with $H_2O_2$ at 4 °C. Preincubation with

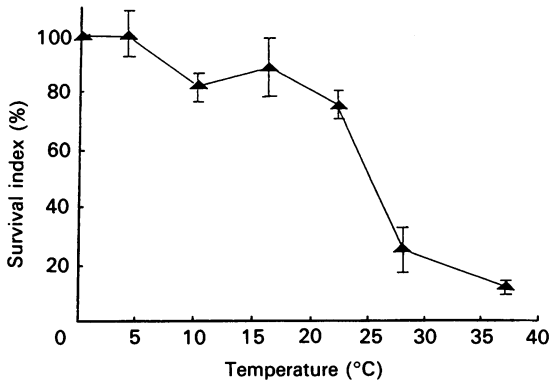


**Fig. 1. Survival data for cells exposed to 100 $\mu$M-$H_2O_2$ at temperatures between 0 °C and 37 °C**

Cells at a density of $1.5 \times 10^5$ cells/ml were exposed for 30 min to 100 $\mu$M-$H_2O_2$ in phosphate-buffered saline at pH 7.4 at the temperatures indicated. The plot shows the variation of survival index with temperature. Means ± S.D. are shown ($n = 4$).

**Table 3. Effect of preincubation of cells with reducing agents (120 min) on the cytotoxicity of $H_2O_2$ (50 μM for 60 min)**

Reducing agents were present during preincubation III (stage 5 of the experimental protocol). $H_2O_2$ incubation was in phosphate-buffered saline in all cases (stage 8 of the experimental protocol). Cell density was $1.5 \times 10^5$ cells/ml. Survival indexes are given as means ± S.D. ($n = 4$).

| Preincubation additions... | Incubation temperatures | | Survival index (%) | | | | |
|---|---|---|---|---|---|---|---|
| | Preincubation III | Incubation | Control | Ascorbate (1 mM) | Dehydro-ascorbate (1 mM) | Reduced lipoate (100 μM at 37 °C) (500 μM at 4 °C) | Oxidized lipoate (100 μM at 37 °C) (500 μM at 4 °C) |
| Control | 37 °C | 37 °C | 100 ± 14 | 107 ± 20 | – | – | – |
| + $H_2O_2$ | 37 °C | 37 °C | 46 ± 6 | 39 ± 8 | – | – | – |
| Control | 37 °C | 4 °C | 100 ± 7 | 99 ± 14 | 90 ± 11 | 113 ± 19 | 100 ± 10 |
| + $H_2O_2$ | 37 °C | 4 °C | 97 ± 16 | 43 ± 4 | 46 ± 12 | 71 ± 4 | 98 ± 12 |
| Control | 4 °C | 37 °C | 100 ± 18 | – | – | – | – |
| + $H_2O_2$ | 4 °C | 37 °C | 42 ± 16 | – | – | – | – |
| Control | 4 °C | 4 °C | 100 ± 18 | 108 ± 17 | 55 ± 13 | 91 ± 10 | 83 ± 9 |
| + $H_2O_2$ | 4 °C | 4 °C | 90 ± 15 | 89 ± 21 | 69 ± 20 | 103 ± 8 | 76 ± 13 |

reduced lipoic acid re-established the $H_2O_2$-induced cytotoxicity at 4 °C (Table 3), but to a lesser extent than ascorbate. The ethanol solvent of lipoic acid limited the maximum concentration that could be used, and 500 μM-lipoic acid applied at 37 °C was found to be toxic by itself (results not shown). Despite the greater lipophilicity of lipoic acid, preincubation at 4 °C with reduced lipoic acid did not restore the cytotoxic action of $H_2O_2$ even at the higher concentrations used.

### Protection by desferrioxamine

Pre-exposure to desferrioxamine for 24 h at 37 °C (preincubation II) before treatment with ascorbate (preincubation III) protected the cells from the toxic effect of $H_2O_2$ incubation at 4 °C observed with ascorbate preincubation alone (Table 4). This desferrioxamine-induced protection increased in a dose-dependent manner and was complete at a concentration of 200 μM-desferrioxamine. No toxic effect was evident in cells treated with desferrioxamine alone.

**Table 4. Effect of desferrioxamine on the restoration of $H_2O_2$-induced cytotoxicity at 4 °C by ascorbate**

Desferrioxamine was present during preincubation II (stage 3 of the experimental protocol), followed by ascorbate during preincubation III (stage 5), followed by incubation with $H_2O_2$ (stage 7). Survival indexes are given as means ± S.D. ($n = 4$).

| Pre-exposure to desferrioxamine (200 μM) for 24 h at 37 °C | Preincubation with ascorbate (1 mM) for 120 min at 37 °C | Exposure to $H_2O_2$ (50 μM) for 60 min at 4 °C | Survival index (%) | |
|---|---|---|---|---|
| 0 | 0 | 0 | 100 ± 11 | |
| 0 | 0 | + | 116 ± 16 | |
| 0 | + | 0 | 109 ± 20 | * |
| 0 | + | + | 46 ± 8 | * |
| + | 0 | 0 | 100 ± 15 | |
| + | 0 | + | 80 ± 14 | |
| + | + | 0 | 83 ± 18 | |
| + | + | + | 112 ± 22 | |

* $P < 0.005$.

## DISCUSSION

The addition of ascorbate to cells that were subsequently incubated at 4 °C with $H_2O_2$ was shown to restore the cytotoxicity to the same degree as was observed with $H_2O_2$ treatment alone at 37 °C. The requirement for an incubation period of 2 h and the fact that dehydroascorbate at concentrations of 1 mM also restores the cytotoxic effect of $H_2O_2$ at 4 °C if preincubation takes place at 37 °C suggest that ascorbate autoxidizes extracellularly and is taken up by the cells as dehydroascorbate. The half-life of ascorbate oxidation is less than 2 h (Lewin, 1976). Intracellularly it then becomes metabolically re-reduced to ascorbate. This process has been proposed by Bridges & Hoffmann (1986), who measured the rate of ascorbate uptake by K562 cells. Cells incubated with $^{14}C$-labelled ascorbate for 2 h exhibited a linear uptake rate of 0.029 nmol/min per $10^7$ cells. Cells were also found to have a high content of ascorbate as measured by h.p.l.c. following exposure to dehydroascorbate. This would explain why dehydroascorbate was equally effective when applied to cells at 37 °C and why it did not restore the cytotoxicity when cells were preincubated with dehydroascorbate at 4 °C.

In the present experiments prolonged preincubation with desferrioxamine abolished the action of ascorbate. The requirement for a 24 h preincubation period suggests a low rate of desferrioxamine uptake, or alternatively a slow release of iron from the cells. Low rates of uptake of desferrioxamine by cells have been reported previously (Halliwell & Gutteridge, 1985). Desferrioxamine speci-

fically binds $Fe^{3+}$ rather than $Fe^{2+}$ and is known to inhibit superoxide-driven reduction of $Fe^{3+}$ (Halliwell & Gutteridge, 1985). It is probable that desferrioxamine also prevents reduction of $Fe^{3+}$ by ascorbate.

Ward *et al.* (1987) have demonstrated that different extents of DNA damage result from exposure to $H_2O_2$ at 0 °C and 37 °C. Numerous double-strand breaks are observed at 37 °C, whereas only single-strand breaks are found at 0 °C. This could result either from differences in the extent of damage inflicted or differences in the activity of repair processes, or a combination of these factors. Our data on cell survival suggest that less damage is inflicted at low temperature.

The restoration of the full toxic effect of $H_2O_2$ at 4 °C by reducing agents and its abolition by pretreatment with desferrioxamine are consistent with the proposal that the toxic damage is produced by HO· radicals generated through the agency of the Fenton reaction. The difference in DNA damage produced by $H_2O_2$ at 37 °C and at 4 °C is a reflection of the amount and degree of reduction of iron ions (or similar transition-metal ions) associated with chromatin. Studies by Imaly *et al.* (1988) indicate that DNA damage in *Escherichia coli* following exposure to low concentrations of $H_2O_2$ is dependent on cell metabolism and suggest that NADH may be involved.

A similar argument could also be applied to other potential cellular targets in which the density of HO· radicals generated in their vicinity determines the degree and distribution of damage sustained. This may be of relevance in connection with reperfusion injury (Bulkley, 1987; Garlick *et al.*, 1987; Halliwell *et al.*, 1985).

The above interpretation depends on the assumption that intracellular iron tends to be in the oxidized state in cells kept at low temperatures. This might be due to the lack of reducing equivalents normally generated by cellular metabolism. In their absence, the redox potential of the iron–oxygen couple would rapidly convert most of the iron into Fe(III), which is unreactive towards $H_2O_2$. It cannot be ruled out that superoxide may be a potential reductant that becomes less available at low temperature (Kyle *et al.*, 1988). In the case of damage to the genome some of the most vulnerable sites would be those in the locality of 'zinc finger' domains of DNA-binding proteins (Sunderman & Barber, 1988). Although zinc does not undergo redox reactions, if the zinc were replaced by redox-reactive metal ions then damaging free-radical reactions may result (Willson, 1977, 1988; Williams, 1984). Under normal circumstances the concentration of reduced metal ions in chromatin would be expected to be low. Consequently the production of a high local density of lesions in DNA by HO· radicals derived from the Fenton reaction will depend on a large proportion of the potentially reactive metal ions being in the reduced state or capable of being reduced by an intrinsic mechanism or by reductants introduced from outside the cell.

Although our data on cytotoxicity are consistent with this argument, the mechanism cannot be regarded as simple. The existence of a transition temperature at about 25 °C suggests that membrane-related functions may be involved. Any influence of membrane fluidity on the penetration of $H_2O_2$ into the cell can be excluded by the re-establishment of $H_2O_2$ toxicity at low temperature by pre-exposure to the reducing agents. The low-temperature data suggest that any membrane involvement is indirect, since the effect of the lipid-soluble reductant (lipoic acid) was similar to that of ascorbate.

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