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INDUCTION AND REJOINING OF DNA SINGLE-STRAND BREAKS IN RELATION TO CELLULAR GROWTH IN HUMAN CELLS EXPOSED TO THREE HYDROPEROXIDES AT 0°C AND 37°C

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There was a 5-fold increase in cytotoxicity for cumene hydroperoxide, 10-fold for *tert*-butyl hydroperoxide and 25-fold for hydrogen peroxide, under metabolizing conditions (37°C) in comparison to nonmetabolizing conditions (0°C), when human P31 cells were exposed for 60 min. The induction of DNA single-strand breaks correlated poorly with cytotoxicity. Hydrogen peroxide was by far the most effective agent inducing single-strand breaks irrespective of temperature. Cumene hydroperoxide produced fewer strand breaks than *tert*-butyl hydroperoxide despite its greater cytotoxicity at either 37°C or at 0°C. The pattern of single-strand break induction did not change with temperature. The number of breaks, however, increased when the cells were exposed at 37°C. The pattern of rejoining was similar for hydrogen peroxide- and *tert*-butyl hydroperoxide-induced breaks at both temperatures whereas the rejoining of cumene hydroperoxide-induced breaks deviated somewhat from this pattern. The results indicate that there is no clear-cut relationship between induction of DNA single-strand breaks and cytotoxicity after hydroperoxide exposure.

KEY WORDS: Cytotoxicity, DNA damage, DNA repair, hydroperoxides, ionizing radiation, oxidative stress.

ABBREVIATIONS: IC_{50}, (IC_{30}) = Concentration inhibiting cell growth by 50% (30%).

INTRODUCTION

H_2O_2 is produced in mammalian tissues during normal metabolism partly as a consequence of O_2^- dismutation, either spontaneously or catalyzed by superoxide dismutases. H_2O_2 can also be produced by different oxidases or by ionizing radiation. Organic hydroperoxides arise, principally in membranes, as a result of oxygen addition to alkyl radicals. The peroxyl radicals formed in this process subsequently give rise to hydroperoxides by hydrogen atom abstraction. H_2O_2 passes readily across membranes and is well documented for its ability to induce DNA single-strand breaks.[1-16] We[10] and others[16-19] have recently shown that *tert*-butyl hydroperoxide similarly induces DNA single-strand breaks. The yield, however, is lower than with H_2O_2. The genotoxicity of peroxides has been proposed by some authors to be the result of a transition metal-driven Haber-Weiss reaction[11] (Fenton reaction)[12] leading to the generation of hydroxyl radical in the case of H_2O_2, and in the case of *tert*-butyl hydroperoxide and cumene hydroperoxide, to different alkoxyl radicals ($RO^{\cdot}$).[20-24]

However, peroxyl radicals ($ROO^{\cdot}$)[23–25] and other reactive intermediates[22,23] may also be formed from organic hydroperoxides in biological systems. Several mechanisms of hydroperoxide cytotoxicity have been suggested. Consensus is reached on the involvement of free radicals in the cytotoxicity. Proposed mechanisms include activation of poly(ADP-ribose)polymerase,[3] alteration in intracellular calcium homeostasis following glutahione and protein thiol depletion,[26] involvement of a calcium-dependent endonuclease,[13] production of DNA double-strand breaks[14] or locally multiply damaged sites in DNA,[9] onset of lipid peroxidation[27] and interference with cellular reductive processes.[28]

We have previously shown that selenium-supplemented P31 cells, with a 4-fold greater GSH peroxidase activity than control cells, show significantly fewer H_2O_2-induced single-strand breaks. The supplemented cells were also protected against *tert*-butyl hydroperoxide-induced breaks, but only at low concentrations of *tert*-butyl hydroperoxide (10–50 μM). The selenium-supplementation procedure did not change either H_2O_2- or *tert*-butyl hydroperoxide-induced cytotoxicity despite the decrease in single-strand breaks.[10] The present study was carried out to gain further insight into the mechanisms behind hydroperoxide-induced geno- and cytotoxicity. It concerns the effect of inhibiting or permitting metabolism during exposure to cumene hydroperoxide, H_2O_2 and *tert*-butyl hydroperoxide. The studied end-points were DNA single-strand break induction and rejoining, and cellular growth, following exposure at 0°C and 37°C. The involvement of a metabolic process in hydrogen peroxide-induced cytotoxicity is evident from the results of Ward and his colleagues.[6,9] They found that at 0° C, about a 50-fold higher dose of H_2O_2 was necessary to kill cells, than at 37°C.[6] I have not found reports of similar studies on the temperature dependence of *tert*-butyl hydroperoxide- and cumene hydroperoxide-induced cytotoxicity. Moreover, several reports exist on the rejoining of H_2O_2-induced DNA single-strand breaks.[1–8] However, only a few reports, often less detailed, are available for *tert*-butyl hydroperoxide,[17–19] whereas the rejoining of cumene hydroperoxide-induced breaks does not seem to have been studied before.

MATERIALS AND METHODS

Cell culture. P31 is a human cell line derived from a mesothelioma of probable asbestos-induced origin.[29] Stock cultures of P31 cells were maintained in Ham's F-10 medium (NordCell, Sweden) at 37°C in 5% CO_2 in humidified air. The medium was supplemented with 10% foetal calf serum, antibiotics, and L-glutamine (NordCell) as previously described.[30] Medium changes were three times a week and the cells were subcultured weekly to maintain exponentially growing cultures.

Hydroperoxides. Hydrogen peroxide was purchased from Merck, Germany, *tert*-butyl hydroperoxide and cumene hydroperoxide from Sigma, U.S.A.

Cytotoxicity assay. Six 10-ml tubes, each containing 4×10^5 recently trypsinized P31 cells, were filled with ice-cold or warm Hepes-buffered (20 mM, pH 7.4) unsupplemented Ham's F-10 medium and put in an ice bath or a water bath (37°C). Different amounts of hydroperoxide were added from a freshly prepared 100 mM stock solution and the cells were exposed for 60 min under repeated agitation at the relatively low cell density of 4×10^4 cells per ml.[15] After removal of the peroxide, the

cells were washed once (H_2O_2 and *tert*-butyl hydroperoxide) or twice (cumene hydroperoxide) with Hepes-buffered medium. Then cells from each tube were suspended in complete medium and spread onto two or three 60 mm-dishes (Nunc, Denmark) at about $1.2 \cdot \times 10^5$ cells per dish in 5 ml medium. Attached cells were trypsinized after 72 h of culture and counted in a Coulter Counter Model D (Coulter Electronics, U.K.). Cell growth was calculated as the number of cells at each dose point in comparison to the number of untreated control cells.

DNA radiolabelling for single-strand break assay. DNA single-strand breakage was detected with the DNA precipitation assay[31] modified by the use of double radiolabelling according to the principles outlined by Rydberg[32] and Sandström and Johanson[33] for the detection of DNA strand breaks with improved precision. Exponentially-growing cells were labelled with 3.7 kBq (0.1 μCi) [^{3}H]thymidine per ml; specific activity 74 GBq (2 Ci) per mmol (Amersham International, U.K.). In experiments requiring double-labelling (rejoining experiments), cells in an equal number of dishes were labelled with 0.74 kBq (0.02 μCi) [^{14}C]thymidine per ml; specific activity 2.2 GBq (59 mCi) per mmol. The labelling medium was removed after 42 h and replaced with non-radioactive medium for $\sim$ 1 h.

DNA Single-Strand Break Assay

Induction experiments. The cells were trypsinized and 2×10^6 cells were diluted, in ice-cold or warm (37°C) Hepes-buffered medium in 50-ml tubes, to 4×10^4 cells per ml. The exposure to the different hydroperoxides was carried out in an ice bath or a water bath (37°C) for 30 min at 37°C under repeated agitation. After centrifugation the medium was discarded and the cells were washed with ice-cold Hepes-buffered medium as in the cytotoxicity assay. Then individual samples of $3–4 \times 10^5$ cells in 100 μl medium were lysed and processed according to Olive.[31]

Rejoining experiments. The cells were trypsinized and both ^{3}H-labelled and ^{14}C-labelled cells were exposed to H_2O_2 for 10 min or to *tert*-butyl or cumene hydroperoxide for 30 min exactly as above. Meanwhile ^{3}H-labelled and ^{14}C-labelled control cells were held at the same temperature as the exposed cells, but in 10-ml tubes. After washing in ice-cold medium the ^{3}H-labelled control cells were suspended in 1 ml ice-cold Hepes-buffered medium and mixed with ^{14}C-labelled exposed cells and vice versa. Initial strand breakage was determined from 100 μl aliquots of this mixture. The remainder was mixed with 4 ml 37°C Hepes-buffered medium to give 5 ml cell suspension containing 8×10^5 ml cells per ml. The cell suspension was then kept at 37°C. Two 500-μl aliquots were withdrawn from each tube after 5, 10, 15, 30 and 60 min and instantly chilled in tubes containing 4 ml ice-cold medium to prevent further rejoining. After centrifugation, the medium was discarded and the cell pellets were lysed and processed as previously. The mean percentage precipitated DNA was calculated using an equal number of mixtures of ^{3}H-labelled exposed cells and ^{14}C-labelled control cells and mixtures of ^{14}C-labelled exposed cells and ^{3}H-labelled control cells.

Calculation of number of DNA strand breaks. The logarithm of the fraction of precipitated DNA was a linear function of the radiation dose up to at least 20 Gy for P31 cells. Ionizing radiation induces 1000 single-strand breaks per cell per Gy.[34] By

using this number a constant can be found that will satisfy the equation,

$$\text{number of breaks} = -\text{constant} \cdot \log(\text{precipitated DNA})$$

The constant that satisfied the equation in the dose interval 0–20 Gy was 5×10^4 for P31 cells.

Irradiation. The cells, to be studied in experiments on the rejoining of ionizing radiation-induced breaks, were irradiated in a Gammarad 900 (Scanditronix, Sweden) irradiation chamber at 0°C. The radiation source was ^{137}Cs (γ-rays) and the dose rate was 0.7 Gy per min as determined by thermoluminescence dosimetry. Initial strand breakage and rejoining were determined as with hydroperoxide-exposed cells.

RESULTS

The cytotoxicity of a 60-min exposure to cumene hydroperoxide, hydrogen peroxide and *tert*-butyl hydroperoxide was compared at 0°C and 37°C. The time of exposure (60 min) was chosen to facilitate comparisons with our earlier work[10] and also with studies by other groups.[5,8,16,18,27,28] All hydroperoxides caused significantly less growth inhibition at 0°C in comparison to exposure at 37°C. Cumene hydroperoxide was the most cytotoxic agent at concentrations above 1 mM at 0°C and showed less temperature dependence than both *tert*-butyl hydroperoxide and H_2O_2 (Figure 1). A 5-fold increase in cumene hydroperoxide concentration gave the same growth inhibition (IC_{50}) at 0°C compared to 37°C, in comparison to a 10-fold increase for *tert*-butyl hydroperoxide (IC_{30}) and a 25-fold increase for H_2O_2 (IC_{50}). H_2O_2 was the most noxious of the hydroperoxides up to 1 mM at 0°C and at all tested

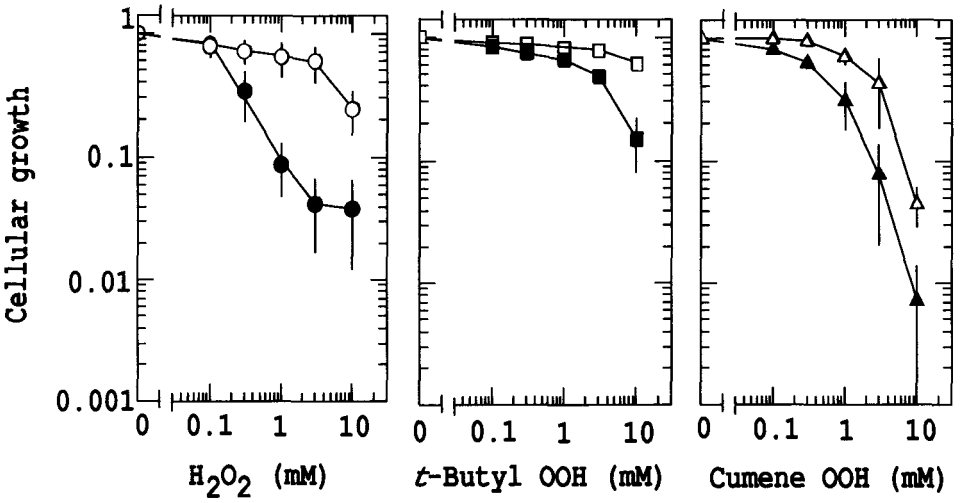

FIGURE 1 P31 cells, at 4×10^4 cells per ml, were exposed to different concentrations of H_2O_2 (*a*), *tert*-butyl hydroperoxide (*b*) and cumene hydroperoxide (*c*) at 0°C (open symbols) or 37°C (filled symbols) for 60 min. Cell growth in relation to growth of unexposed control cells was determined 72 h later by counting the number of attached cells. Results are the mean ± S.D. of 3–6 experiments.

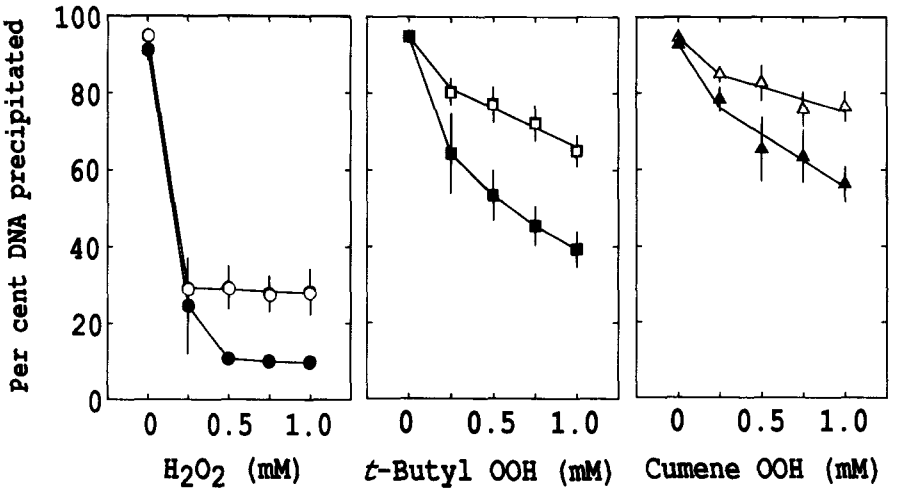

FIGURE 2 P31 cells, at 4 × 10^4 cells per ml, were exposed to different concentrations of H_2O_2 (*a*), *tert*-butyl hydroperoxide (*b*) and cumene hydroperoxide (*c*) at 0°C (open symbols) or 37°C (filled symbols). The induction of DNA single-strand breaks was measured after 30 min. Results are the mean ± S.D. of triplicate determinations in two experiments.

concentrations at 37°C, while the cytotoxicity of *tert*-butyl hydroperoxide, despite a 10-fold increase compared to 0°C, still was comparatively low at 37°C.

Our previous report[10] showed that the maximum number of DNA single-strand breaks in P31 cells exposed to 100 μM H_2O_2 at 37°C was detected after 10 min. The number of DNA strand breaks decreased after this reaching almost control levels at 60 min. This was probably a reflection of the fact that H_2O_2 is rapidly decomposed in cell-containing medium, not only intracellularly by the H_2O_2-degrading enzymes glutathione peroxidase and catalase, but also to some degree extracellularly by transition metal ions since Ham's F-10 medium contains both Fe^{2+}- and Cu^{2+}- ions. Furthermore, Hepes buffer has been shown to stimulate the generation of free radicals by copper ions and H_2O_2[35] adding to this effect. The induction of *tert*-butyl hydroperoxide-induced breaks did not show a similar change with time as H_2O_2-induced breaks and reached its maximum after 60 min with only a minor increase from 30 min.[10] Considering the time-dependent variation of both H_2O_2- and *tert*-butyl hydroperoxide-induced breaks I chose 30 min as the time of exposure in the current experiments. H_2O_2 was once again shown to be an extremely effective DNA single-strand breaking agent in these experiments and for cells exposed at 37°C the assay was saturated already at 500 μM (Figure 2). *tert*-Butyl hydroperoxide and cumene hydroperoxide both caused DNA single-strand breakage to much less extent than H_2O_2. The pattern of strand break induction was the same irrespective of metabolic conditions. That is H_2O_2 was more effective than *tert*-butyl hydroperoxide than cumene by hydroperoxide in producing single-strand breaks. The measured yields of breaks after a 30-min exposure increased when the cells were exposed at 37°C, despite being allowed the effect of full rejoining.

Ionizing radiation induces 1000 single-strand breaks per cell per Gy[34] in a linear relationship. Thus, by calibrating the DNA precipitation assay with the strand-breaking effect of ionizing radiation, it was possible to recalculate the raw data (percentage

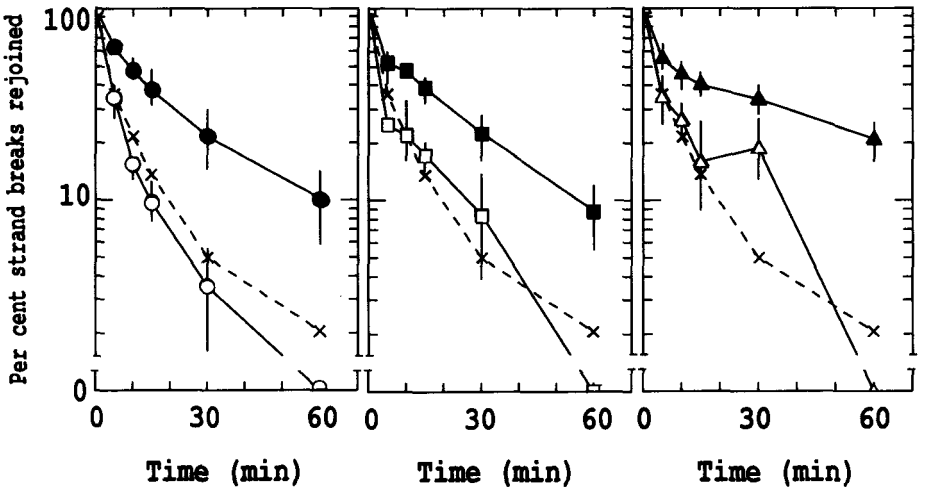

FIGURE 3 P31 cells, at 4 × 10^4 cells per ml, were exposed to 300 μM H_2O_2 (*a*) for 10 min, 1 mM *tert*-butyl hydroperoxide (*b*) for 30 min or 1 mM cumene hydroperoxide (*c*) for 30 min at 0°C (open symbols) or 37°C (filled symbols). Then the rejoining of the induced single-strand breaks was followed at 37°C after washing at 0°C. The dotted line (-x-) shows, for comparison, the rejoining of γ-ray induced breaks. Results are the mean and maximal variation of double determinations in duplicate from two experiments per agent. Error bars are omitted in the curve showing rejoining of γ-ray induced breaks to help keep the figure clear.

precipitated DNA) to number of strand breaks. The rejoining of strand breaks induced at 0°C returned to control levels after 60 min. These facts made it possible to compare the rejoining between different hydroperoxides and different experiments at the two temperatures. The calculated number of initial strand breaks from hydroperoxide exposure was recalculated to 100% in all experiments independent of temperature. "Zero"% was the calculated number of strand breaks of cells exposed at 0°C after 60 min rejoining. For unknown reasons this most often resulted in a small negative value, which means that the assay at this time detected fewer breaks in exposed cells than in control cells. The mean numbers were 100 for H_2O_2, 400 for *tert*-butyl hydroperoxide and 500 fewer breaks per cell for cumene hydroperoxide. The rejoining curve for breaks induced by ionizing radiation was determined in comparison and did not produce negative numbers at 60 min. The recalculations of the rejoining experiments resulted in Figure 3.

The pattern of rejoining of breaks induced at 37°C was quite similar for all three hydroperoxides and seemed to consist of two processes. Successive linear regression analysis yielded a fast process with a $t_{1/2}$ of 1–3 min and a slower process with a $t_{1/2}$ in the 20–25 min range for H_2O_2 and *tert*-butyl hydroperoxide, but substantially longer for cumene hydroperoxide (about 45 min). The pattern of rejoining of hydroperoxide-induced breaks showed larger inter-variation at 0°C than at 37°C (Figure 3). The data obtained at 0°C could also be interpreted as consisting of two processes for both H_2O_2 and *tert*-butyl hydroperoxide. An initial fast process had a $t_{1/2}$ in the 2–4 min range and a slower process a $t_{1/2}$ of 10–15 min. The rejoining of breaks induced by cumene hydroperoxide under non-metabolizing conditions was again different. The cells exposed to cumene hydroperoxide initially showed rejoining

similar to that of cells exposed to either γ-rays, H_2O_2 or *tert*-butyl hydroperoxide, with a fast initial rejoining process. The difference in the rejoining of these breaks as compared to H_2O_2-, *tert*-butyl hydroperoxide- and γ-ray-induced breaks was noted between 15 and 30 min when the rejoining levelled off in cells exposed to cumene hydroperoxide. This result suggests the existence of a process interfering with the sealing of the breaks. Despite this levelling off no remaining breaks could be detected at 60 min in the cumene hydroperoxide-exposed cells.

DISCUSSION

The induction of DNA single-strand breakage seemed to be associated with the hydrophilicity of the three hydroperoxides. Thus, H_2O_2 was more effective than *tert*-butyl hydroperoxide, which was in turn more effective than cumene hydroperoxide in causing DNA breakage. Permitting metabolism during the exposure slightly increased the measured yield of strand breaks after 30 min (Figure 2). However, taking into account the rapid rejoining of breaks at 37°C (cf. Figure 3) the number of induced breaks at 37°C, in comparison to 0°C, must have increased several-fold. It is therefore logical that the *remaining* breaks at 37°C, in comparison to all breaks induced at 0°C, rejoin relatively more slowly. From the data two rejoining processes are generally apparent. The initial process is fast ($t_{1/2} \sim$ 2–3 min) and appears to be the same independent of temperature and hydroperoxide. The rejoining of ionizing radiation-induced single-strand breaks consists of two apparent components with half-times of about 2 and 15–20 min.[36,37] From the data obtained in this study (Figure 3), half-times of 2.8 and 21 min were calculated for ionizing radiation. The rather large relative difference for the first component in comparison to the previously obtained data may depend on a difference in the set-up of the experiments. The current experiments did not instantly give the desired temperature of 37°C. The similarity of the rejoining curves suggests that single-strand breaks caused by ionizing radiation resembles breaks induced by H_2O_2 and *tert*-butyl hydroperoxide. The second rejoining process is found to be slower in cells exposed at 37°C ($t_{1/2}$ 20–25 min) than in cells exposed at 0°C ($t_{1/2}$ 10–15 min). Since the longer half-time is about the same as that obtained with ionizing radiation (21 min) this result does not agree with the finding that breaks induced by H_2O_2, from glucose oxidase, are repaired more slowly than breaks caused by ionizing radiation in human leukocytes.[4]

Five out of six rejoining experiments, with cells exposed to hydroperoxides at 0°C, resulted in less breaks in exposed cells than in control cells after 60 min of DNA rejoining. This was not the case with ionizing radiation as the damaging agent and might indicate that DNA-protein or DNA-DNA crosslinks are formed by hydroperoxides. Crosslinks would give this effect in the DNA precipitation assay as they would tend to decrease the amount of DNA released during alkali treatment at 65°C. In a previous communication[10] we reported that we could not detect crosslinks after exposure to H_2O_2 and *tert*-butyl hydroperoxide. However, the possibility exists that the crosslink assay might not have been sensitive enough to detect hydroperoxide-induced crosslinks. The presence of a thymine-tyrosine crosslink in mammalian chromatin after H_2O_2 exposure was recently detected by gas chromatography/mass spectrometry analysis.[38] This finding lends support to a previous study indicating the occurrence of DNA-protein, as well as DNA inter-strand, crosslinks in isolated chromatin after H_2O_2-exposure.[39]

The pattern of rejoining of cumene hydroperoxide-induced breaks deviated from the rejoining of H_2O_2- and *tert*-butyl hydroperoxide-induced breaks (Figure 3). At 37°C I found a larger fraction of unrejoined breaks after 60 min and at 0°C I found that the rejoining levelled off between 15 and 30 min. It is possible that the relatively low number of induced breaks in comparison to the other two hydroperoxides may have affected the result. The variation in the data points, however, was not larger in cumene hydroperoxide-exposed cells than in the cells exposed to either H_2O_2 or *tert*-butyl hydroperoxide (Figure 3). Some studies on the rejoining of ionizing radiation-induced DNA strand breaks (summarized in Ref. 40) have found a similar levelling off, and at the same time as I found with the DNA rejoining of cumene hydroperoxide-exposed cells. These authors explained their results by incisions made in the DNA to remove base-damaged sites by endonucleases, which would transiently increase the number of openings in the DNA, and would be detected as breaks. This is also a likely explanation of the current results. For example, several base modifications, needing excision repair to be corrected, have been identified after H_2O_2-exposure.[41]

The principal finding of comparing the cytotoxicity, of a 60-min exposure to cumene hydroperoxide, hydrogen peroxide and *tert*-butyl hydroperoxide, at 0°C and 37°C was that the increase in cytotoxicity under metabolizing conditions was correlated with the hydrophilicity of the three agents. Under the same conditions the pattern of DNA single-strand breakage did not change between the hydroperoxides. This implies, assuming that other types of DNA damage are being formed by the same mechanism that creates DNA single-strand breaks, that there exists at least one mechanism of hydroperoxide-induced cytotoxicity separate from DNA damage. This finding agrees with a recent report by Farber and co-workers.[16] They found that two antioxidants, *N*,*N*′-diphenyl-*p*-phenylenediamine and butylated hydroxytoluene, as well as acidification of the culture medium, prevented cell killing, but not DNA strand breakage, induced by *tert*-butyl hydroperoxide. Thus, cell killing could be separated from induction of DNA strand breaks.

The lipophilic nature of cumene hydroperoxide suggests that lipid peroxidation may be involved in what seems to be the main mechanism of cumene hydroperoxide-induced cell killing at doses above 1 mM. Cumene hydroperoxide is likely to be almost equally trapped in the cellular membranes independent of temperature and be much more difficult to wash away than both H_2O_2 and *tert*-butyl hydroperoxide. Thus, exposure at 0°C might give nearly the same cumene hydroperoxide dose as exposure at 37°C and just delay the onset of cytotoxicity somewhat. The low number of single-strand breaks induced by cumene hydroperoxide supports this conclusion. Cumene hydroperoxide trapped in membranes, in most cases far from the nucleus, would find it difficult to cause DNA strand breaks by a site-specific mechanism. The less lipophilic *tert*-butyl hydroperoxide and H_2O_2 are likely to move about in the cell more freely and thus more frequently find the targets in DNA.

The alteration in Ca^{2+} homeostasis that occurs following peroxidative injury in rat hepatocytes[26] was recently shown[42] to be blocked by the Ca^{2+}-channel blockers verapamil and nifedipine without interfering with lipid peroxidation. Antioxidants preventing lipid peroxidation also prevented accumulation of Ca^{2+}, which suggests that peroxidative injury might interfere with the flow of Ca^{2+} ions and precede the observed alteration in Ca^{2+} homeostasis. Prevention of lipid peroxidation has been shown by Farber and his colleagues[16,27] to prevent killing of rat hepatocytes after exposure to *tert*-butyl hydroperoxide. It is of interest to note that the same group in

an earlier communication concluded that the mechanism by which *tert*-butyl hydroperoxide lethally injured hepatocytes would seem to be distinct from that of H_2O_2. Yet they found, based on results with the ferric ion chelator desferrioxamine, that the toxicity of cumene hydroperoxide closely paralleled that of H_2O_2.[43] In contrast, our work [Sandström and Marklund unpublished experimental observations] with desferrioxamine pretreatment of P31 cells shows that both *tert*-butyl hydroperoxide and H_2O_2 are protected while the cytotoxicity of cumene hydroperoxide is unaffected. This discrepancy in results emphasizes that hydroperoxide cytotoxicity is very complex. Effects can be found at many levels. The difficulty is to separate the effects directly responsible for cell killing from the merely indirect effects. In this situation comparisons of effects at different levels, of different hydroperoxides, could prove valuable.

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