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Alternative Mechanisms for Hydroperoxide-Induced DNA Single Strand Breakage

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The results presented in this study point out the existence of similarities as well as differences in the DNA-damaging effects of organic *vs.* inorganic hydroper- oxides in human myeloid leukemia U937 cells. On the one hand, the formation of DNA single strand breaks (SSBs) induced by either hydrogen peroxide (H_2O_2) or *tert*-butylhydroperoxide (tBu-OOH) was prevented by iron chelators, was not affected by antioxidants or glucose omission before and during peroxide exposure and was enhanced by prior catalase depletion. Furthermore, H_2O_2- and tBu-OOH-induced DNA strand scission were also detected after treatment at 0° C. On the other hand, H_2O_2, but not tBu-OOH or cumene hydroperoxide (cum-OOH), produced DNA strand scission in isolated nuclei and post-lysed DNA samples. In addition, lowering the basal intracellular calcium concentration with ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) markedly reduced the DNA-damaging efficiency of tBu-OOH while promoting only a slight decline in the number of DNA SSBs induced by H_2O_2. Taken together, these results are consistent with the commonly held theory that DNA damage caused by H_2O_2 is mediated by the formation of hydroxyl radicals. tBu-OOH-induced DNA single strand breakage appears to involve both the formation of H_2O_2 and a rise in cytosolic calcium ions.

Keywords: Hydroperoxide, DNA damage, calcium ions

INTRODUCTION

The term oxidative stress describes a situation in which the antioxidant defence system is overwhelmed by the rate of formation of reactive oxygen species. Under these conditions, the delicate redox balance is altered and a number of different cellular dysfunctions are generated. Oxidative stress is undoubtedly a relevant process in a number of different pathologies[1–3] and, as a consequence, the effects at the cellular and molecular levels produced by a variety of sources of reactive oxygen intermediates are being actively investigated. Among the agents utilized in these studies are H_2O_2 and the organic hydroperoxides *tert*-butylhydroperoxide (tBu-OOH) and cumene hydroperoxide (cum-OOH). Although few studies have attempted to assess the different abilities of the inorganic *vs.* organic hydroperoxides to generate specific deleterious effects, it would appear that tBu-OOH and cum-OOH promote peroxidation of membrane lipids,[4–8] elevation of cytosolic calcium[5,9–11] and mitochondrial damage[6,12–16] more efficiently than H_2O_2. The radical species mediat-

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ing the cytotoxic responses are clearly different in cells exposed to the inorganic and organic hydroperoxides. The hydroxyl radical generated in Fenton-type reactions has been implicated as the final toxic species in cells intoxicated with H_2O_2.[17,18] As to the species involved in the toxicity promoted by organic hydroperoxides, and in particular by tBu-OOH, a role has been suggested for the methyl and *tert*-butoxyl radicals which have been consistently identified in a number of different studies.[19–21] The effects of organic *vs.* inorganic hydroperoxides on DNA homeostasis have also been investigated and the results obtained demonstrate that H_2O_2 as well as tBu-OOH and cum-OOH introduce damage at the DNA level by causing strand scission,[22–28] an event which can be prevented by iron chelators.[25–27] Furthermore, removal of the peroxide results in rapid reversal of DNA strand breakage.[22,24,27] Although the hydroxyl radical species are thought to mediate the DNA strand scission generated by H_2O_2, the identity of the species mediating DNA damage in cells exposed to organic hydroperoxides remains largely unknown. Studies performed in our laboratory would suggest that the methyl and *tert*-butoxyl radicals do not directly or indirectly generate DNA lesions.[28]

The experimental results presented in this study, along with those recently obtained in our laboratory,[28] demonstrate that the mechanisms whereby organic hydroperoxides and H_2O_2 promote the formation of DNA single strand breaks (SSBs) in cultured cells are remarkably different. In particular, we suggest that DNA damage caused by organic hydroperoxides is the result of different processes involving formation of H_2O_2 (possibly at the mitochondrial level) as well as an increase in cytosolic free calcium ions.

MATERIALS AND METHODS

Cell Culture and Treatments

U937 cells (human myeloid leukemia) were grown in RPMI 1640 culture medium (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Seralab, Sussex, UK), penicillin (50 units/ml), and streptomycin (50 μg/ml), at 37°C in T-75 tissue culture flasks (Corning, Corning, NY, USA) gassed with an atmosphere of 95% air-5% CO_2. Under these conditions, U937 cells grow in suspension with a doubling time of approximately 16 h.

All the chemicals utilized in this study were of the highest purity available from the suppliers that are indicated below. Stock solutions of H_2O_2, tBu-OOH and cum-OOH were freshly prepared in Saline A (8.182 g/l NaCl, 0.372 g/l KCl, 0.336 g/l $NaHCO_3$ and 0.9 g/l glucose), 1,2-bis-(*o*-aminophenoxy)-ethane-N,N,N′,N′ tetraacetic acid tetra-(acetoxymethyl)-ester (BAPTA/AM) (Calbiochem-Novabiochem, San Diego, CA, USA), butylated hydroxytoluene (BHT) and N,N′-diphenyl-1,4-phenylene-diamine (DPPD) were dissolved in 95% ethanol and *o*-phenanthroline in dimethyl sulfoxide (DMSO). At the treatment stage the final ethanol/DMSO concentrations were never higher than 0.05%. Under these conditions ethanol and DMSO were neither toxic nor DNA-damaging, nor did they affect the cyto-genotoxic properties of H_2O_2, tBu-OOH or cum-OOH. EGTA and 3-amino-1, 2, 4-triazole were dissolved in 1N NaOH (adjusted to pH 7.4 with 1N HCl) or in complete RPMI 1640, respectively. H_2O_2, tBu-OOH, cum-OOH, BHT, DPPD, DMSO, *o*-phenanthroline and EGTA were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Cells (2.5×10^5/ml) were treated for 30 min in Saline A (2 ml), washed with pre-chilled Saline A and analyzed immediately for DNA damage using the alkaline elution technique. In some experiments the formation of DNA SSBs was measured in isolated nuclei or post-lysed DNA samples.

Cells were labelled overnight with [^{14}C]-thymidine (NEN/Dupont, Boston, MA, USA) (0.05 μCi/ml) and incubated for a further 6 h in a medium containing unlabelled thymidine (1 μg/ml). These cells were either exposed to the hydroperoxides or utilized for the preparation of isolated nuclei or post-lysed DNA samples.

Nuclei were isolated as previously described.[29] Briefly, pre-labelled U937 cells were centrifuged and resuspended in nucleus buffer (150 mM NaCl, 1 mM KH_2PO_4, 5 mM $MgCl_2$, 1 mM EGTA, and 0.1 mM dithiothreitol, pH 6.4) at 0°C. These cells were centrifuged again and resuspended in 1/10th volume of ice-cold nucleus buffer; 9/10th volume of 0°C nucleus buffer containing 0.3% Triton X-100 was then added and the mixture incubated for 10 min at 0°C. The nuclei were pelletted by centrifugation (1200 rpm for 5 min), resuspended in nucleus buffer at 37°C and examined microscopically after being stained with trypan blue to confirm their permeability.

The nuclei ($4.5–5 \times 10^5$) were then treated for 20 min in nucleus buffer at 37°C and analyzed immediately for DNA damage.

Post-lysed DNA samples were prepared as follows: pre-labelled cells ($4.5–5 \times 10^5$) were loaded onto 25 mm, 2 μm pore polycarbonate filters (Nuclepore, Pleasanton, CA, USA) and rinsed twice with 10 ml ice cold Saline A. The cells were then lysed with 5 ml of 2% sodium dodecyl sulfate, 0.025 M ethylenediaminetetraacetic acid (tetrasodium salt) (EDTA), pH 10.1, rinsed with 10 ml of Saline A and finally incubated for 20 min in the same solution containing H_2O_2, tBu-OOH or cum-OOH.

Measurement of DNA SSBs by Alkaline Elution

The level of DNA SSBs (frank DNA SSBs and SSBs resulting from alkaline labile lesions) was measured immediately after the treatments using the alkaline elution technique, carried out using a procedure virtually identical to that developed by Kohn and coworkers[30] with minor modifications.[31] Briefly, $4.5–5 \times 10^5$ cells were gently loaded onto 25 mm, 2 μm pore polycarbonate filters and then rinsed twice with 10 ml of ice-cold Saline A containing 5 mM EDTA (disodium salt). Cells were lysed with 5 ml of 2% sodium dodecylsulfate, 0.025 M EDTA (tetrasodium salt), pH 10.1. The same procedure was used for isolated nuclei. Lysates were rinsed with 7 ml of 0.02 M EDTA (tetrasodium salt) and the DNA was eluted overnight in the dark with 1.5% tetraethyl ammonium hydroxide (Merck-Schuchardt, München, FRG)/0.02 M EDTA (free acid)/0.1% sodium dodecyl sulfate (pH 12.1), at a flow rate of ca. 30 μl/min. Fractions were collected at two-hour intervals and counted in 7 ml of Lumagel (Beckman, Fullerton, CA, USA) containing 0.7% glacial acetic acid. DNA remaining on the filters was recovered by heating for 1 h at 60°C in 0.4 ml of 1N HCl followed by the addition of 0.4 N NaOH (2.5 ml) and was again determined by scintillation counting. DNA was also recovered from the interior of the membrane holders after vigorous flushing with 3 ml of 0.4 N NaOH. This solution was processed for scintillation counting as described above. Strand Scission Factor (SSF) values were calculated from the resulting elution profiles by determining the absolute log. of the ratio of the percentage of DNA retained in the filters of the drug-treated sample to that retained from the untreated control sample (both after 8 h of elution).

RESULTS

Specific Requirements for Organic vs. Inorganic Hydroperoxide-Induced DNA Single Strand Breakage

Inorganic and organic hydroperoxides induce DNA SSBs in cultured U937 cells, although with different levels of efficiency. As illustrated in Figure 1A, studies on the concentration-dependence of peroxides on DNA SSB formation enstablished the order H_2O_2 > tBu-OOH > cum-OOH. Treatments were for 30 min in a glucose-containing saline (Saline A) and, under these conditions, none of the hydroperoxides were cytotoxic, as measured by either trypan blue exclusion or lactate dehydrogenase release assays (not shown). A more dramatic difference was observed when the DNA-damaging effects of the hydroperoxides were investigated in post-lysed DNA samples (Figure 1B) or intact nuclei

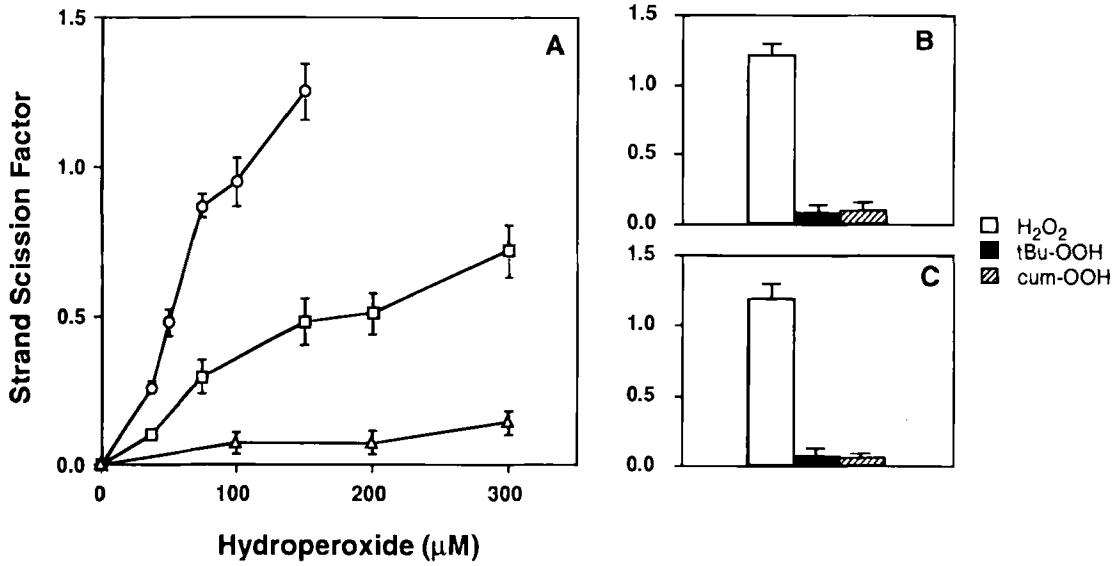

FIGURE 1 Hydroperoxide-induced DNA single strand breakage in intact U937 cells, post-lysed DNA or isolated nuclei. (**A**) Cells were exposed for 30 min in glucose-containing Saline A to increasing concentrations of H_2O_2 (circles), tBu-OOH (squares) or cum-OOH (triangles). (**B**) Cells were lysed onto polycarbonate filters and, after accurate washing, were exposed for 20 min in glucose-containing Saline A to 100 μM H_2O_2, 150 μM tBu-OOH or 200 μM cum-OOH. (**C**) Nuclei were isolated from U937 cells as detailed in the Methods section and then treated for 20 min in nuclei buffer with 50 μM H_2O_2, 150 μM tBu-OOH or 200 μM cum-OOH. The level of DNA SSBs was measured immediately after the treatments using the alkaline elution technique. The SSF values were calculated from the resulting elution profiles, as detailed in the Methods section. Results represent the mean ± S.E.M. calculated from 3–5 separate experiments.

(Figure 1C), conditions under which only H_2O_2 was capable of producing DNA SSBs.

These results strongly suggest that different mechanisms are involved in the process of SSB-formation in cells exposed to inorganic *vs.* organic hydroperoxides. Furthermore, it would appear that the organic peroxides generate DNA SSBs only in intact and functional cells.

The Effect of Antioxidants, Iron Chelators and Depletion of Catalase on DNA SSBs Induced by H_2O_2 and tBu-OOH

Previous results obtained in our and other laboratories demonstrated that DNA single strand breakage caused by H_2O_2 and tBu-OOH in CHO cells[27] as well as in cultured hepatocytes,[25] while not affected by antioxidants, can be prevented by metal chelators such as *o*-phenanthroline. It is important to note that *o*-phenanthroline is membrane-permeant and an excellent, although non-specific, iron chelator.

The results illustrated in Table I are consistent with the above findings and indicate that also in U937 cells *o*-phenanthroline abolishes DNA strand

TABLE I The effect of antioxidants and iron chelators on hydroperoxide-induced DNA single strand breakage[a]

Treatment	SSF
H_2O_2	.60 ± .06
H_2O_2 + DPPD	.58 ± .07
H_2O_2 + BHT	.63 ± .08
H_2O_2 + *o*-phenanthroline	.02 ± .001*
tBu-OOH	.62 ± .06
tBu-OOH + DPPD	.63 ± .02
tBu-OOH + BHT	.60 ± .05
tBu-OOH + *o*-phenanthroline	.03 ± .002*

[a]Cells were exposed for 30 min in Saline A to 50 μM H_2O_2 or 200 μM tBu-OOH in the absence or presence of 10 μM DPPD, 200 μM BHT or 25 μM *o*-phenanthroline, after pre-incubation for 5 min.

The level of DNA SSBs was measured immediately after the treatments using the alkaline elution technique. Results represent the mean ± S.E.M. calculated from 3–5 separate experiments, and were significantly different from those for DNA damage generated by the hydroperoxide alone at *$p < 0.001$ (unpaired *t* test).

scission caused by both H_2O_2 and tBu-OOH, and that the antioxidants DPPD and BHT do not affect the DNA-damaging response. In these studies, the two hydroperoxides were utilized at concentrations producing similar levels of DNA damage, i.e. 50 μM H_2O_2 and 200 μM tBu-OOH. Thus, while strongly suggesting that iron plays a pivotal role in the formation of DNA lesions in cells exposed to tBu-OOH, the above results may also imply a role for other metals such as copper.

A 6 h treatment with 10 mM 3-amino-1, 2, 4-triazole decreased the catalase activity of U937 cells to a level 80% lower than that observed in unmanipulated cells (7.2 ± 0.3 Sigma Units/10^6 cells). Importantly, under the same experimental conditions, the activity of the enzyme glutathione peroxidase was not affected (122.65 ± 11.3 and 118 ± 8.7 mU/10^6 cells in untreated and treated cells, respectively). When the catalase-depleted cells were subsequently exposed to concentrations of tBu-OOH higher than 75 μM, the formation of DNA SSBs was significantly augmented (Figure 2). The extent of enhancement of DNA strand scission was greater in catalase-depleted U937 cells challenged with H_2O_2 (inset to Figure 2). In principle, these effects of 3-amino-1, 2, 4-triazole could either depend on catalase depletion, which would augment intracellular H_2O_2 and

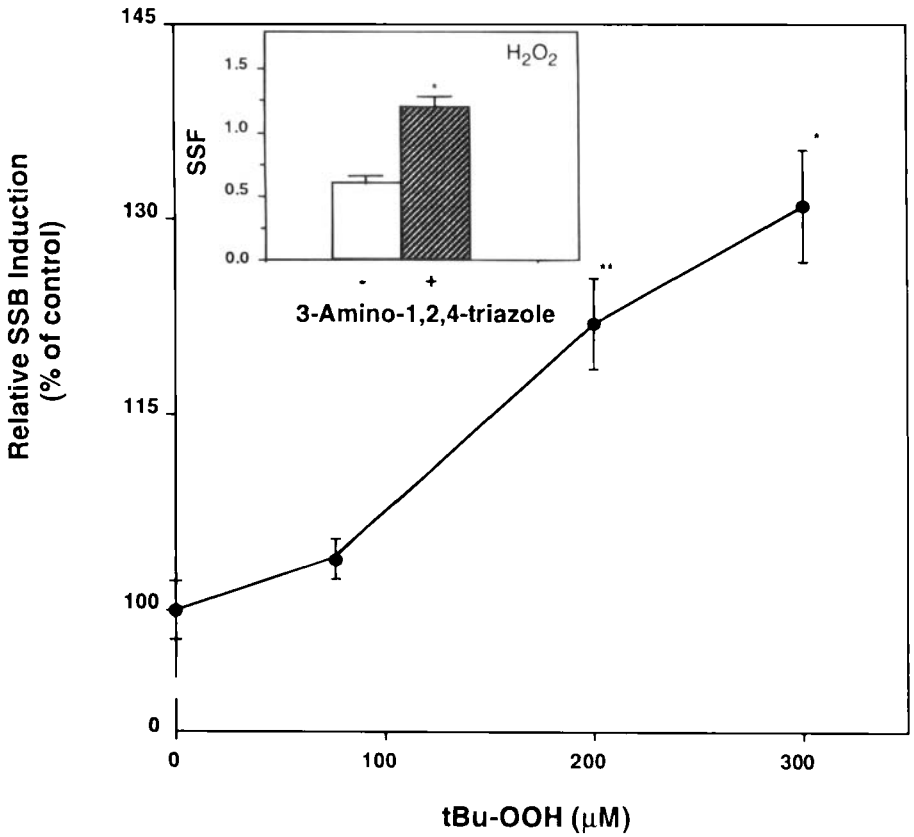

FIGURE 2 Effect of catalase depletion on DNA single strand breakage induced by H_2O_2 or tBu-OOH. Cells were incubated in complete culture medium for 6 h with 10 mM 3-amino-1, 2, 4-triazole, treated for further 30 min in Saline A with increasing concentrations of tBu-OOH and then analyzed for DNA single strand breakage by the alkaline elution technique. Results represent the mean ± S.E.M. calculated from 3–5 separate experiments, and are expressed as a percentage of the SSF obtained with tBu-OOH alone. The inset shows the SSF values obtained with control or catalase-depleted cells exposed for 30 min to 50 μM H_2O_2. Results were significantly different from those for DNA damage generated by the hydroperoxide alone at *$p < 0.001$; **$p < 0.002$ (unpaired t test).

thus the formation of DNA- damaging species, or depend on a less specific event such as inhibition of repair. We investigated this second possibility by measuring the kinetics of DNA SSB-removal in control or 3-amino-1, 2, 4-triazole pre-treated cells that were exposed for 30 min to 200 μM tBu-OOH and then allowed to repair in fresh pre-warmed medium, in the absence or presence of the catalase inhibitor. The results illustrated in Table III indicate that the $t_{1/2}$ values that were measured under the four different experimental conditions were basically identical. Thus, the increased accumulation of DNA SSBs observed in 3-amino-1, 2, 4-triazole pre-treated cells challenged with tBu-OOH is not dependent on inhibition of DNA repair but, rather, appears to be the consequence of an increased production of the net amount of DNA SSBs.

Taken together, the above results indicate that H_2O_2 mediates at least part of the DNA single strand breakage generated by tBu-OOH. The fact that antioxidants do not modify the DNA-damaging efficiency of inorganic or organic hydroperoxides strongly suggests that it is unlikely that products of lipid peroxidation are involved in the formation of DNA lesions promoted by these agents.

The Effect of Glucose or Temperature of Exposure on DNA SSBs Induced by H_2O_2 or tBu-OOH

The formation of DNA SSBs was measured in U937 cells treated with either 50 μM H_2O_2 or 200 μM tBu-OOH for 30 min in Saline A, in the presence or absence of 5 mM glucose. As illustrated in Table II, the level of DNA damage generated by either of the two oxidants was not affected by glucose omission during a 15 min interval prior to treatment as well as during the 30 min of peroxide exposure. Thus, it is likely that energy-independent reactions mediate the formation of at least part of the DNA lesions generated by H_2O_2 or tBu-OOH.

TABLE II The effect of glucose and temperature of exposure on hydroperoxide-induced DNA single strand breakage[a]

Treatment	SSF
H_2O_2 in glucose-containing saline A (37 °C)	.60 ± .06
H_2O_2 in glucose-free saline A (37 °C)	.61 ± .07
H_2O_2 in glucose-containing saline A (0 °C)	.66 ± .08
tBu-OOH in glucose-containing saline A (37 °C)	.62 ± .06
tBu-OOH in glucose-free saline A (37 °C)	.65 ± .07
tBu-OOH in glucose-containing saline A (0 °C)	.59 ± .05

[a]Cells were incubated for 15 min in glucose-free or glucose-containing (5 mM) saline A and then treated for 30 min with either 50 μM H_2O_2 or 200 μM tBu-OOH. Also shown are the results from experiments in which the cells were treated with each of the hydroperoxides at 0°C (30 min.).

The level of DNA SSBs was measured immediately after treatments by the alkaline elution techique. Data represent the means ± S.E.M. of the SSF values calculated from 3 separate experiments.

The experimental results illustrated in Table II also indicate that H_2O_2 and tBu-OOH are both capable of inducing DNA single strand breakage at 0°C. It is important to emphasize, however, that a major problem exists in comparing the results from these experiments with those obtained in cells treated at physiological temperature. Indeed, the level of DNA single strand breakage detected after exposure of cultured cells for a fixed period of time to agents producing radical species is not only a function of the total level of breaks that are

Table III The effect of 3-amino-1, 2, 4-triazole on the repair of tBu-OOH-induced DNA single strand breaks[a]

Conditions of repair	$T_{1/2}$ (min)
Cells not pre-treated with 3-amino-1, 2, 4-triazole	
Repair without 3-amino-1, 2, 4-triazole	16.8
Repair with 3-amino-1, 2, 4-triazole	17.2
Cells pre-treated with 3-amino-1, 2, 4-triazole	
Repair without 3-amino-1, 2, 4-triazole	17.4
Repair with 3-amino-1, 2, 4-triazole	16.9

[a]Cells were incubated in complete culture medium for 6 h with 0 or 10 mM 3-amino-1, 2, 4-triazole, treated for further 30 min in Saline A with 200 μM tBu-OOH and then analyzed for DNA single strand breakage either immediately or after post-incubation for increasing time intervals in the absence or presence of the catalase inhibitor. The kinetics of DNA SSB-removal observed under these different experimental conditions were first order with respect to time and the $T_{1/2}$ values (indicating the time in minutes necessary for repair of 50% of the initial damage) were calculated from linear regression analysis. Results represent the means of two separate experiments.

produced, but also depends on the proportion of those breaks that have been rejoined during the treatment.[22,24,32] In contrast, treatments at 0° C most likely lead to the accumulation of DNA lesions in the absence of repair. Thus, although the assessment of the steady state level of DNA single strand breakage at a fixed time of exposure may give an indication of the responsiveness, at the molecular level, to the insult elicited by the hydroperoxide at the two temperatures, we cannot establish the relative DNA-damaging efficiency of the peroxides under these different experimental conditions. Nevertheless, we can conclude that both H_2O_2 and tBu-OOH cause DNA strand scission at 37° C and 0° C, and thus suggest that energy-independent reactions mediate the formation of at least part of the DNA lesions generated by H_2O_2 or tBu-OOH.

The Role of Calcium Ions in the Induction of DNA SSBs by H_2O_2 and tBu-OOH

The formation of DNA SSBs was measured in U937 cells treated with either H_2O_2 or tBu-OOH for 30 min in Saline A, in the presence or absence of increasing concentrations of the membrane-permeant calcium chelator BAPTA/AM. As illustrated in Figure 3A, the level of DNA damage generated by either of the two oxidants was severely inhibited by BAPTA/AM concentrations of 5 and 10 μM and abolished by a 25 µM level of the calcium chelator. Figure 3B shows the effect of a pre/co-exposure to the membrane-impermeant calcium chelator EGTA (2.5–10 mM) on hydroperoxide-induced DNA single strand breakage. Under these conditions, EGTA lowers the intracellular calcium levels and abolishes both the tBu-OOH[9] and thapsigargin[33]-evoked rise in cytosolic calcium. We found that EGTA afforded a slight protection against DNA damage induced by H_2O_2 and that this effect was never greater than 10%, even at the highest EGTA concentration tested (10 mM). Inhibition of DNA SSB-formation was remarkably higher using tBu-OOH and, as illustrated in Figure 3B, 10 mM EGTA produced a 40% decrease in this response.

It is important to note that treatments were performed in a nominally calcium free extracellular milieu. Addition of 1 mM $CaCl_2$, however, did not effect the DNA-damaging responses elicited by tBu-OOH or H_2O_2 (not shown).

The interpretation of the results reported in this section is not straightforward. Indeed, on the one hand using BAPTA/AM we obtained experimental evidence suggesting that a rise in calcium ions is a critical event for the formation of both H_2O_2—and tBu-OOH-induced DNA SSBs whereas, on the other hand, the results obtained with EGTA indicate that calcium mediates DNA damage triggered by tBu-OOH but plays a marginal role in the genotoxic response to H_2O_2. It is however important to point out that the effects of BAPTA/AM on DNA strand scission induced by H_2O_2 could be the consequence of nonspecific effects such as iron chelation. We therefore find it reasonable to conclude that the process of calcium mobilization is important in the genotoxic response to organic hydroperoxides and has marginal effects on DNA damage evoked by H_2O_2. The fact that DNA strand scission caused by tBu-OOH was independent on the presence of extracellular calcium would suggest that calcium ions are released from intracellular stores.

DISCUSSION

The results presented in this study indicate that the mechanism(s) involved in the formation of DNA SSBs in U937 cells challenged with inorganic or organic hydroperoxides is/are remarkably different. We have first provided experimental evidence indicating that tBu-OOH (or cum-OOH) generates DNA strand scission in intact cells but has no effect in isolated nuclei or partially purified DNA (Figure 1). In marked contrast, H_2O_2 was found to be a potent DNA-damaging agent using each of these three different preparations (Figure 1). The results obtained with H_2O_2 can be readily explained on

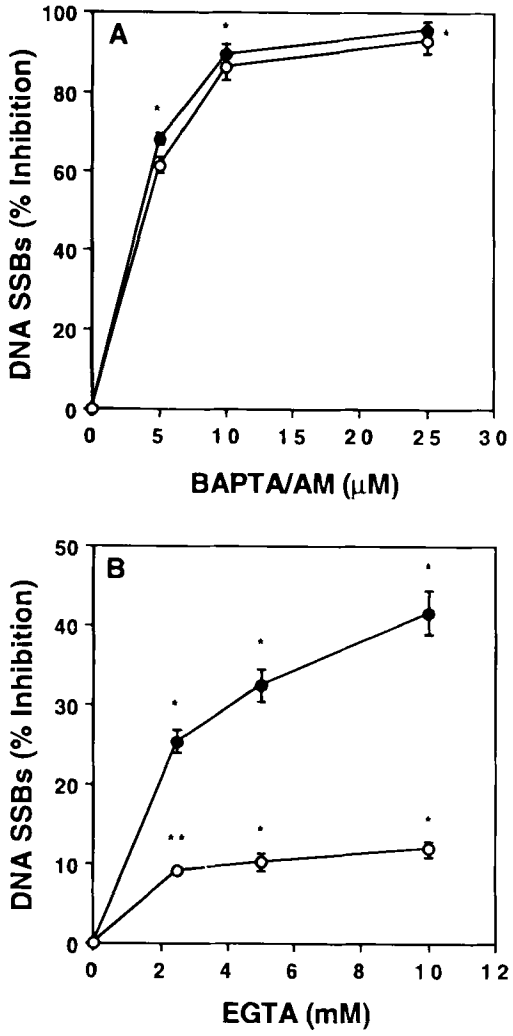

FIGURE 3 Effect of calcium chelators on DNA single strand breakage induced by H_2O_2 or tBu-OOH. Cells were exposed for 5 min in glucose-containing Saline A to increasing concentrations of either BAPTA/AM or EGTA and then treated for further 30 min with 50 μM H_2O_2 (open symbols) or 200 μM tBu-OOH (closed symbols). Data represent the means ± S.E.M. of the percent inhibition of DNA single strand breakage induced by each of the hydroperoxides. The level of DNA SSBs was measured immediately after the treatments using the alkaline elution technique. Results represent the mean ± S.E.M. calculated from 3–5 separate experiments, and were significantly different from those for DNA damage generated by the hydroperoxide alone at *$p < 0.001$; **$p < 0.01$ (unpaired t test).

the basis of the commonly held theory that DNA damage caused by H_2O_2 is a consequence of the formation of hydroxyl radical species generated via the interaction of the oxidant with transition metals (the Fenton reaction). The results showing that *o*-phenanthroline abolishes the formation of DNA SSBs elicited by the oxidant (Table I) are therefore consistent with the above mechanism.

The experiments performed with the organic hydroperoxides lead to a different conclusion, since the formation of DNA SSBs was detected only in intact and functional cells. This would suggest that these lesions are not simply the result of chemical interactions and/or reactions. In apparent contradiction with this conclusion are the results reported in Table II, which indi-

cate that glucose omission did not reduce the extent of tBu-OOH-induced DNA damage. In a previous study[34] we demonstrated that glucose omission markedly reduced the DNA strand scission caused by the redox-cycling quinone menadione, an effect that was paralleled by a reduced formation of superoxides. Thus, the above results would suggest that the formation of at least part of the DNA lesions generated by tBu-OOH are not energy-dependent. The results obtained by incubating the cells with the hydroperoxide at 0° C (Table II) are also consistent with this conclusion.

In summary, it would appear that tBu-OOH generates DNA single strand breakage only in intact cells, although this process does not seem to require energy-dependent reactions. We previously suggested that it is unlikely that the methyl and *tert*-butoxyl radicals generated during tBu-OOH metabolism mediate the formation of DNA lesions.[28] This conclusion was based on the fact that the complex III inhibitor antimycin A, while reducing the formation of the methyl and *tert*-butoxyl radicals as well as the cytotoxic response, promoted a remarkably increased generation of DNA SSBs. Experiments in progress (not shown) demonstrate that metyrapone also prevents the formation of tBu-OOH-derived radical species and the toxicity of the hydroperoxide, although the extent of DNA single strand breakage remains unaffected. Thus, it would appear that in cells exposed to tBu-OOH the resulting formation of DNA damage and toxicity are separate events. As a corollary, different species must be involved in these two processes.

A recent study[35] demonstrated that copper ions may promote the decomposition of benzoyl peroxide with concomitant formation of benzoyloxyl radicals displaying DNA-damaging properties. The fact that tBu-OOH was unable to generate DNA strand scission in isolated nuclei strongly suggests that it is unlikely that similar radicals mediated DNA strand scission generated in intact cells.

There is evidence that reduction of organic peroxides is accompanied by considerable lipid peroxidation.[4–8] Lipid hydroperoxides and alkenals have been reported to be clastogenic and are products of membrane lipid peroxidation.[36,37] The results presented in Table I, however, demonstrate that inhibiting lipid peroxidation with antioxidants such as DPPD or BHT, while inhibiting cell death (not shown) did not affect the DNA-damaging response to tBu-OOH. Thus, it would appear that products of the peroxidation of membrane lipids do not mediate DNA damage generated by tBu-OOH.

The results presented in Table I indicate that, as observed for H_2O_2, transition metals -possibly iron ions- were necessary for the occurrence of DNA single strand breakage in cells challenged with tBu-OOH. The finding that tBu-OOH-induced DNA strand scission is enhanced under conditions of catalase depletion (Figure 2) is therefore consistent with the possibility that at least part of these lesions were caused by H_2O_2. In order to understand the relevance of the H_2O_2—based mechanism it is first important to understand how H_2O_2 itself is generated in tBu-OOH treated cells. The fact that tBu-OOH did not produce DNA lesions in partially purified DNA or in isolated nuclei would suggest a critical role for some components or organelles present in the cytosol. We speculate that the mitochondrion could be the site in which the DNA-damaging H_2O_2 is being formed in tBu-OOH-treated cells. H_2O_2 could derive from the superoxide anions resulting from ubiquinone oxidation and indeed such a mechanism has been proposed.[12] Furthermore, we have recently found that complex III inhibitors (antimycin A and 2-heptyl-4-hydroxyquinoline N-oxide) enhance the formation of tBu-OOH-induced DNA single strand breakage and that this effect is prevented by rotenone, further increased in catalase-depleted cells and does not occur in respiration-deficient cells (manuscript in preparation), results consistent with the possibility that DNA damage is mediated by H_2O_2 deriving from the oxidation of ubiquinone. Importantly, the kinetics of repair of

DNA SSBs generated by tBu-OOH alone or associated with antimycin A were basically superimposable, strongly suggesting that similar types of DNA lesions were generated under the two experimental conditions. It is conceivable that tBu-OOH can oxidize ubiquinone also in glucose-deprived cells or following treatment at 0° C, which would explain why DNA SSBs were detected under these conditions (Table II).

It is important to point out, however, that H_2O_2—independent mechanisms might also be involved in the process of tBu-OOH-induced DNA damage. The calcium chelator EGTA, while slightly decreasing the formation of DNA SSBs promoted by H_2O_2, was found to markedly reduce the extent of DNA lesions generated by tBu-OOH (Figure 3B), suggesting that Ca^{2+} plays a role in the formation of DNA lesions caused by the organic hydroperoxides. The cation seemed to derive from intracellular stores since the DNA-damaging response promoted by tBu-OOH was the same under conditions in which $CaCl_2$ was absent or present in the extracellular milieu (not shown). The level at which the involvement of calcium ions occurs still remains to be determined. The possibility that Ca^{2+} reaches the nucleus and directly activates endonucleolytic enzymes is remote for a number of reasons. Most important is the fact that the concentrations of tBu-OOH that were used in this study have hardly any effect on cell viability, whereas activation of nucleases is often associated with the onset of cell death. We are therefore examining the possibility that tBu-OOH-induced Ca^{2+} mobilization leads to mitochondrial Ca^{2+} accumulation followed by Ca^{2+}—stimulated H_2O_2 production at the level of the ubiquinone site. The ability of Ca^{2+} to induce electron leakage at the level of the reduced and semiquinone forms of coenzyme Q was recently demonstrated.[12,38]

In conclusion, the experimental results presented in this paper collectively suggest that DNA strand scission generated by organic hydroperoxides is the result of a complex series of biochemical events involving the formation of H_2O_2 and an elevation in cytosolic Ca^{2+}. Circumstantial evidence would suggest that mitochondria play a central role in these responses. The results obtained with reagent H_2O_2 are consistent with the commonly held theory that DNA single strand breakage is caused by hydroxyl radicals generated in reactions of the Fenton type.

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