Calcium-Dependent Mitochondrial Formation of Species Mediating DNA Single Strand Breakage in U937 Cells Exposed to Sublethal Concentrations of tert-Butylhydroperoxide

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ABSTRACT

Treatment of U937 cells with a sublethal albeit DNA-damaging concentration of tert-butylhydroperoxide (tB-OOH) enhanced mitochondrial Ca$^{2+}$ uptake and ruthenium red (RR), a polycation that inhibits the calcium uniporter of mitochondria, significantly reduced the extent of DNA cleavage generated by the hydroperoxide. Release of Ca$^{2+}$ from the Ryanodine(Ry)/caffeine(Cf)-sensitive stores further increased mitochondrial Ca$^{2+}$ uptake and elicited a parallel enhancement in DNA strand scission induced by tB-OOH that was prevented by both Ry and RR. DNA damage caused by tB-OOH alone or associated with either Cf or RR was prevented by iron chelators, insensitive to antioxidants and repaired with kinetics superimposable with those observed after treatment with H$_2$O$_2$. Cf enhanced the DNA-damaging effects of tB-OOH in permeabilized cells as well, and similar effects were observed upon addition of CaCl$_2$. Cf did not further increase the formation of DNA lesions elicited by tB-OOH in the presence of CaCl$_2$. The enhancing effects of Cf were prevented by RR and ryanodine, whereas those mediated by exogenous calcium were prevented only by RR. DNA strand scission caused by tB-OOH alone or associated with Cf in the permeabilized cell system was severely inhibited by ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′′-tetraacetic acid. The mechanism(s) whereby Ca$^{2+}$ promotes the mitochondrial formation of species that will ultimately result in the formation of DNA lesions was subsequently analyzed using intact as well as permeabilized cells. Hydrogen peroxide was identified to be one of these species.

Hydrogen peroxide generates an array of different lesions within the cell, including damage at the genomic DNA level (Cantoni et al., 1995). These lesions are mainly represented by DNA SSBs because DNA double strand breaks cannot be detected even under conditions in which the oxidant produces an enormous amount of DNA SSBs (Ward et al., 1985 and 1987; Cantoni et al., 1986, 1989, 1992). A large body of experimental evidence indicates that the effects at the DNA level are mediated by the so-called Fenton reaction in which the hydroxyl radical, the ultimate DNA-damaging species, is formed as a consequence of the interaction between the oxidant and divalent iron (Mello Filho and Meneghini, 1984; Mello Filho et al., 1984). Organic hydroperoxides, and in particular model compounds such as tB-OOH or cumene hydroperoxide, have also been shown to promote DNA single strand breakage in the absence of detectable DNA double strand breakage (Guidarelli et al., 1995). Understanding the mechanism whereby tB-OOH generates DNA single strand breakage is of considerable importance since this and other organic hydroperoxides, while inactive as initiators or complete carcinogens (O’Connel et al., 1986; Slaga et al., 1983), are tumor promoters in the skin of SENCAR mice (Taffe et al., 1987). Few studies, however, have investigated the molecular basis for these effects; most importantly, the identity of the species mediating DNA damage in cells exposed to organic hydroperoxides remains largely unexplored. It has been reported that DNA cleavage evoked by tB-OOH is abolished by iron chelators (Guidarelli et al., 1995, 1997; Coleman et al., 1989; Latour et al., 1995), is insensitive to antioxidants (Guidarelli et al., 1995, 1997; Coleman et al., 1989; Latour et al., 1995) and is repaired in a relatively short time (Guidarelli et al., 1995, 1997; Coleman et al., 1989; Sandström, 1991; Baker and He, 1991). The fact that anti-

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ABBREVIATIONS: SSBs, DNA single strand breaks; tB-OOH, tert-butylhydroperoxide; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′′-tetraacetic acid; Tg, thapsigargin; Iono, ionomycin; Cf, caffeine; FCCP, carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone; RR, ruthenium red; Ry, ryanodine; SOD, superoxide dismutase; BHT, butylated hydroxytoluene; DPPD, N,N′-diphenyl-1,4-phenylene-diamine; o-pt, o-phenanthroline; EDTA, ethylenediaminetetraacetic acid; [Ca$^{2+}$], intracellular free Ca$^{2+}$ concentration; SERCA, sarcoplasmic/endoplasmic reticulum Ca$^{2+}$-ATPase; IP$_3$, inositol 1, 4, 5-trisphosphate; ER, endoplasmic reticulum.
oxidants abolished cell death induced by tB-OOH without preventing the formation of DNA lesions (Coleman et al., 1989; Guidarelli et al., 1997) suggests that the species involved in the cyto- and geno-toxic responses are different. Consistent with this possibility are our recent results showing that the complex III inhibitor antimycin A reduces the toxicity of tB-OOH as well as the formation of tB-OOH-derived radical species (methyl and tert-butoyl radicals), while markedly enhancing the accumulation of DNA SSBs (Guidarelli et al., 1996). These findings collectively suggest the existence of similarities between the genotoxic effects of tB-OOH and those of H2O2 and imply that iron-dependent processes play a major role in the formation of free-radical intermediate(s) of an as yet unknown nature, which could ultimately generate DNA lesions in cells challenged with tB-OOH. Recent results from our laboratory, however, indicate that DNA cleavage caused by tB-OOH is a rather complicated process in which the formation of DNA-damaging species or, more likely, of their precursors, requires additional mechanisms operating in the cytosol and is modulated by calcium ions. Indeed, tB-OOH, unlike H2O2, was unable to elicit strand scission in purified DNA or isolated nuclei (Guidarelli et al., 1997). Furthermore, the DNA damaging efficiency of tB-OOH was remarkably decreased when the basal cytosolic calcium concentrations were lowered with EGTA, whereas only a slight decline in the number of DNA SSBs was observed in cells treated with H2O2 (Guidarelli et al., 1997). These data indicate that the biochemical pathways leading to DNA damage in cells exposed to organic or inorganic hydroperoxides coincide only in part.

In our study we investigated the nature of the involvement of calcium ions in the formation of DNA SSBs induced by tB-OOH. The results obtained indicate that tB-OOH releases calcium ions from intracellular stores and that a significant proportion of the cation is cleared by the mitochondria. Mitochondrial calcium plays a pivotal role in the process of DNA strand scission promoted by this organic hydroperoxide. H2O2 was found to be one of the species that are produced within the mitochondria and which mediate part of the DNA single strand breakage elicited by tB-OOH in cultured U937 cells. The relative amount of DNA lesions mediated by H2O2 would appear to be dependent on the intramitochondrial calcium content.

**Materials and Methods**

**Materials.** Fura-2 AM, Tg and Iono, were purchased from Calbiochem, San Diego, CA. Cr, FCCP, RR, Ry, catalase, SOD, tB-OOH, H2O2, menadione and the remaining chemicals were from Sigma-Aldrich, Milano, Italy. RPMI 1640 culture medium was from GIBCO, Grand Island, NY, and fetal bovine serum, penicillin and streptomycin were from Seralab, Sussex, UK. T-75 tissue culture flasks were purchased from Corning, Corning, NY. [14C]-thymidine was obtained from NEN/Dupont, Boston, MA. Polycarbonate filters and liquid scintillation were purchased from Nucleapore, Pleasanton, CA and Beckman, Fullerton, CA, respectively.

**Cell culture and treatments.** Human myeloid leukemia U937 cells were cultured in suspension in RPMI 1640 culture medium supplemented with 10% fetal bovine serum, penicillin (50 U/ml), and streptomycin (50 μg/ml), at 37°C in T-75 tissue culture flasks in a humidified atmosphere of 95% air-5% CO2.

Stock solutions of H2O2, tB-OOH, RR, Cr, catalase and SOD were freshly prepared in Saline A (8.182 g/liter NaCl, 0.372 g/liter KCl, 0.336 g/liter NaHCO3 and 0.9 g/liter glucose). Ry, FCCP, BHT, DPPD and Tg were dissolved in 95% ethanol. Menadione, Iono and o-pt were dissolved in dimethyl sulfoxide. At the treatment stage the final ethanol or dimethyl sulfoxide concentrations were never higher than 0.05%. Under these conditions ethanol or dimethyl sulfoxide was neither toxic nor DNA-damaging, nor did it affect the cyto-genotoxic properties of H2O2 or tB-OOH. Treatment with the hydroperoxides was performed as detailed below and, under the conditions used in this study, cell death, as measured by trypan blue or lactate dehydrogenase release assays, was never detectable immediately after the peroxide exposure as well as after up to 24 hr of posttreatment incubation in fresh culture medium. Cells treated for 30 min with 200 μM tB-OOH, or 50 μM H2O2, and then allowed to grow in fresh culture medium were able to proliferate with a rate similar to that observed in untreated cells.

**[Ca++]** Measurements. Cells were harvested, washed three times by centrifugation and resuspended in Krebs Ringer Heps medium containing 125 mM NaCl, 5 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 2 mM CaCl2, 6 mM glucose, 25 mM Heps-NaOH (pH 7.4). Cell suspensions were loaded with the Ca++-sensitive dye fura-2 AM (3 μM final concentration) for 30 min at 25°C in Krebs Ringer Heps medium and kept at 37°C until use. Cell aliquots (4 x 106 cells) were washed three times and resuspended in saline A, transferred to a thermostatted cuvette in a Perkin Elmer (Norwalk, CT) LS-50 fluorimeter and maintained at 37°C under continuous stirring. The various drugs interfering with Ca++ homeostasis here employed (Tg, Iono, tB-OOH, FCCP and Cr) were added as indicated in the figures and maintained throughout the experiment. In the experiments in which Ry was used, preincubations with this drug were for 5 min before the beginning of the recording. Traces were recorded and analyzed as previously described (Gryniewicz et al., 1985). The results shown are traces representative of 8 to 10 highly consistent experiments.

**Measurement of DNA SSBs by alkaline elution.** The cells were labeled overnight with [14C]-thymidine (0.05 μCi/ml) and incubated for a further 6 hr in a medium containing unlabelled thymidine (1 μCi/ml). At this stage the cells (2.5 x 107/ml) were either treated in saline or permeabilized and treated for 10 min in permeabilization buffer. Permeabilization was achieved by adding digitonin (10 μM, 12.5 μg/106 cells) to a medium consisting of 0.25 M sucrose, 0.1% bovine serum albumin, 10 mM MgCl2, 10 mM K-Hepes, 5 mM KH2PO4, pH 7.2 at 37°C. Under these experimental conditions, digitonin permeabilizes the plasma membrane but leaves mitochondrial membranes intact (Fiskum et al., 1986). After the treatments the cells were resuspended with prechilled saline A and analyzed immediately for DNA damage using the alkaline elution technique that was carried out using a procedure virtually identical to that described in (Kohn et al., 1981) with minor modifications (Cantoni et al., 1986). Briefly, 3.5 to 4 x 106 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 dodecyl sulfate, 0.025 M EDTA (tetrasodium salt), pH 10.1. 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/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 2-hr intervals and counted in 7 ml of liquid scintillation containing 0.7% glacial acetic acid. DNA remaining on the filters was recovered by heating for 1 hr 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 NaOH. This solution was processed for scintillation counting as described above. Strand scission factor 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 hr of elution).
Results

TB-OOH-induced DNA strand scission is associated with a rise in cytosolic calcium ion concentration and with an increased mitochondrial calcium uptake. A number of studies had previously demonstrated that TB-OOH enhances the intracellular concentration of free calcium ions ([Ca$^{2+}$])$_i$ (Thor et al., 1984; Sakaida et al., 1991; Livingston et al., 1992). Similarly, we found that a short treatment with 200 µM TB-OOH elevated [Ca$^{2+}$]$_i$ in U937 cells (fig. 1). This [Ca$^{2+}$]$_i$ increase was due to release from internal stores, since the experiments were performed utilizing nominally calcium-free medium (saline A). Furthermore, similar results (not shown) were obtained using 10 µM EGTA-containing saline A (estimated extracellular [Ca$^{2+}$] = 0.28 M). The Ca$^{2+}$ pool mobilized by TB-OOH appeared to be of neutral pH, because it was dischargeable by the protonophore Iono (fig. 1A), and insensitive to cell pretreatment with either the sarcoplasmic/endoplasmic reticulum Ca$^{2+}$-ATPase (SERCA) blocker Tg (fig. 1A), the IP$_3$-generating agonist ATP (not shown), or the Ry receptor agonist Cj (fig. 2B). These observations collectively indicate that TB-OOH mobilizes Ca$^{2+}$ from pools that are different from the ER-located, SERCA-containing, IP$_3$- and Ry-sensitive Ca$^{2+}$ stores. Application of the protonophore FCCP (10 µM) 5 min after peroxide addition revealed that, under these conditions, mitochondrial calcium uptake is a major route for clearance of the released calcium ions (fig. 1B). To explore the relationships existing between this event and the formation of DNA lesions promoted by TB-OOH we tested the effect of RR, an inhibitor of mitochondrial calcium uptake (Carafoli, 1987). As illustrated in figure 1C, 25 µM RR markedly reduced the extent of DNA strand scission caused by TB-OOH. In these experiments, the inhibitor was given for 5 min before, and maintained during, the 30-min exposure to the hydroperoxide. RR was ineffective when present only during the pre-exposure phase (not shown). In figure 1D, it can be seen that the inhibitor was also ineffective when added to the cultures 10 min (or more) after treatment with TB-OOH, a result that may well be explained by the fact that most of the DNA SSBs are generated within 10 min of exposure to TB-OOH (inset to fig. 1D). It is important to emphasize that, under the experimental conditions used in this study, TB-OOH was not cytotoxic (not shown, see “Materials and Methods”). The protective effects afforded by RR cannot be ascribed to iron-
chelating or radical-scavenging mechanisms, since under the same experimental conditions in which it inhibited DNA strand scission induced by tB-OOH, RR did not affect the DNA-damaging response evoked by 50 μM H₂O₂ (fig. 1C).

Importantly, under these conditions, H₂O₂ did not produce significant changes in [Ca²⁺]ᵢ (not shown). Finally, the results shown in figure 1C demonstrate that the effect of RR was not a consequence of possible interactions with Cf-sensitive intracellular Ca²⁺ release channels (Ry receptors, Berridge, 1993) because 20 μM Ry, while able to abolish the elevation in [Ca²⁺]ᵢ promoted by 10 mM Cf (fig. 2A), did not modify DNA damage generated by either H₂O₂ or tB-OOH (fig. 1C).

**Caffeine enhances mitochondrial calcium uptake as well as the extent of DNA cleavage in cells exposed to tB-OOH.** We next investigated whether release of Ca²⁺ from intracellular stores other than those mobilized by tB-OOH could further enhance the DNA-damaging effects of the hydroperoxide as well as its effects on mitochondrial Ca²⁺ uptake. For this purpose we used Cf that, at high concentrations, had previously been shown to promote the efflux of calcium ions from the ER-located, Tg-sensitive Ca²⁺ pool via opening of the Ry receptor in a number of different cell lines (Berridge, 1993). This was also true in U937 cells because addition of 10 mM Cf resulted in a transient increase in [Ca²⁺]ᵢ (fig. 2A), an effect abolished by prior Tg-treatment (not shown). Cf-induced Ca²⁺ release was followed by enhanced mitochondrial calcium accumulation (fig. 2A). Addition of tB-OOH 5 min after the application of Cf further enhanced mitochondrial calcium uptake (fig. 2B). Interestingly Cf, although not producing DNA strand scission (not shown), markedly enhanced the extent of DNA cleavage caused by tB-OOH (fig. 2C). RR abolished this response and the extent of DNA damage detected under these conditions was identical to that observed after treatment with tB-OOH and RR (compare figs. 1C and 2C). Ry also reduced the Cf-mediated enhancement of tB-OOH-induced DNA single strand breakage (fig. 2C) leading to the same level of DNA damage generated by tB-OOH alone (fig. 1C). Consistent with these results, Ry was found to prevent the increase in [Ca²⁺]ᵢ and mitochondrial calcium accumulation elicited by Cf (fig. 2A) as well as the effects of Cf on mitochondrial calcium accumulation mediated by tB-OOH (fig. 2B).

The mitochondrial calcium uptake-based mechanism either does not alter the identity of the DNA-damaging species produced by tB-OOH or results in the formation of different species with similar reactivities mediating similar types of DNA lesions. Previous studies demonstrated that lipid peroxidation products may display DNA-damaging properties (Ochi and Cerutti, 1987; Brambilla et al., 1986). These species, however, did not appear to mediate DNA cleavage generated by tB-OOH in U937 cells, because this response was not inhibited by antioxidants under the same experimental conditions in which these agents prevented cell death caused by the hydroperoxide (Coleman et al., 1989; A. Guidarelli, P. Sestili, O. Cantoni, unpublished). Iron chelators suppressed the formation of DNA lesions as well as the toxicity induced by tB-OOH (Guidarelli et al., 1995). The results illustrated in table 1 confirm the above findings and indicate that DNA SSBs generated by the hydroperoxide in the presence of Cf were also insensitive to antioxidants and abolished by the membrane-permeant iron chelator o-pt. Similar results were obtained in cells treated with the combination tB-OOH/RR (table 1) or with H₂O₂ (Guidarelli et al., 1997). In other experiments, the cells were treated for 30 min with 200 μM tB-OOH, alone or...
TABLE 1
The effect of antioxidants or iron chelators on DNA strand scission induced by tB-OOH in the absence or presence of caffeine or ruthenium red

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SSF</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>tB-OOH (200 μM)</td>
<td>0.54 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>200 μM tB-OOH + 10 μM DPPD</td>
<td>0.53 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>200 μM tB-OOH + 200 μM BHT</td>
<td>0.50 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>200 μM tB-OOH + 25 μM o-pt</td>
<td>0.03 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>200 μM tB-OOH + 10 mM Cf</td>
<td>0.86 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>200 μM tB-OOH + 10 mM Cf + 10 μM DPPD</td>
<td>0.88 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>200 μM tB-OOH + 10 mM Cf + 200 μM BHT</td>
<td>0.85 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>200 μM tB-OOH + 10 mM Cf + 25 μM o-pt</td>
<td>0.05 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>200 μM tB-OOH + 25 μM RR</td>
<td>0.38 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>200 μM tB-OOH + 25 μM RR + 10 μM DPPD</td>
<td>0.41 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>200 μM tB-OOH + 25 μM RR + 200 μM BHT</td>
<td>0.40 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>200 μM tB-OOH + 25 μM RR + 25 μM o-pt</td>
<td>0.04 ± 0.01</td>
<td></td>
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</table>

* CELLS were treated for 30 min with tB-OOH alone or associated with Cf or RR, in the absence or presence of the indicated concentrations of DPPD, BHT or o-pt. Cf, RR as well as the antioxidants or iron chelators were added to the cultures 5 min before addition of the hydroperoxide.

The level of DNA SSBs was measured by the alkaline elution technique immediately after the treatments. Results represent the mean ± S.E.M. calculated from three to five separate experiments and were significantly different from those for DNA damage generated by the peroxide alone or associated with Cf or RR at < P < .0001 (unpaired t test).

associated with 10 mM Cf, and the level of DNA SSBs was measured immediately after the treatments as well as at different time intervals of posttreatment incubation in fresh culture medium. The results illustrated in figure 3 clearly indicate that the DNA SSBs induced under these two experimental conditions are removed with kinetics that are basically identical. Interestingly, the same kinetics of DNA SSB-removal were observed when the initial damage was induced by tB-OOH associated with 25 μM RR or by a 30-min exposure to 50 μM H₂O₂.

**Calcium-dependent modulation of DNA single strand breakage in permeabilized cells exposed to tB-OOH.** Previous studies from our laboratory demonstrated that tB-OOH is an efficient inducer of DNA SSBs in intact cells but fails to generate strand scission in partially purified DNA or in isolated nuclei (Guidarelli et al., 1997).

The results illustrated in figure 4A indicate that exposure (10 min) to a 200 μM concentration of this hydroperoxide induced DNA damage in digitonin-permeabilized cells, although less efficiently than in intact cells (about 2.5 times). The permeabilized cell system has a number of advantages because it allows the use of membrane-impermeant substrates and/or inhibitors and therefore appears to be an ideal condition for investigating the mechanism by which tB-OOH generates DNA single strand breakage.

Under these experimental conditions EGTA suppressed DNA damage induced by tB-OOH (fig. 4A) whereas addition of CaCl₂, which in itself did not produce DNA cleavage (not shown), induced a concentration-dependent enhancement of the level of DNA strand scission generated by the hydroperoxide (fig. 4B). Maximal accumulation of tB-OOH-induced DNA SSBs was observed using 30 μM CaCl₂. Cf enhanced DNA strand scission evoked by tB-OOH also in permeabilized cells and once again the accumulation of these DNA lesions was remarkably reduced by EGTA (fig. 4A). Interestingly, Cf did not further enhance DNA damage generated by tB-OOH associated with CaCl₂ (fig. 4B). Importantly, addition of increasing concentrations of CaCl₂ did not affect DNA single strand breakage induced by H₂O₂ (fig. 4B).

Figure 4C shows that neither RR nor Ry significantly affected DNA strand breakage caused by tB-OOH in permeabilized cells. However, as low as 200 nM RR—but not Ry (20 μM)—suppressed the enhanced DNA cleavage that was observed when treatment with the hydroperoxide was associated with the addition of 30 μM CaCl₂. However, RR (200 nM) as well as Ry (20 μM) prevented the Cf-mediated enhancement of tB-OOH-induced DNA single strand breakage.

H₂O₂ mediates part of the DNA strand scission caused by tB-OOH/Ca²⁺. Previous reports indicated that mitochondrial production of superoxide anions and hydrogen peroxide is sensitive to mitochondrial Ca²⁺ content (Cadenas and Boveris, 1980) and, in particular, it was recently suggested (Valle et al., 1993; Castilho et al., 1995) that mitochondrial calcium accumulation leads to an enhanced formation of H₂O₂ in mitochondria exposed to tB-OOH. It is therefore possible that mitochondrial calcium uptake enhances the accumulation of DNA lesions in cells treated with tB-OOH via enforced mitochondrial formation of superoxides and hydrogen peroxide. To test this hypothesis we took advantage of the permeabilized cell system described above with the specific aim of assessing the effects of catalase and/or SOD on DNA damage induced by tB-OOH both in the absence and presence of CaCl₂. As illustrated in figure 5, 10 Sigma U/ml of bovine catalase were able to significantly reduce the extent of DNA strand scission caused by 200 μM tB-OOH and afforded a much greater protection against DNA cleavage caused by the hydroperoxide in the presence of 30 μM CaCl₂. Under this second experimental condition, however, catalase markedly reduced but did not abolish the...

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**Fig. 3.** Kinetics of DNA SSB-rejoining after treatment with tB-OOH in the absence or presence of caffeine or ruthenium red. The cells were exposed for 30 min to 200 μM tB-OOH alone or associated with either 10 mM Cf or 25 μM RR and then allowed to repair in fresh prewarmed RPMI medium containing 10% fetal bovine serum. In other experiments the cells were exposed for 30 min to 50 μM H₂O₂. The level of DNA SSBs was measured immediately after the treatments as well as after various time intervals of repair. Data points are the means of two separate experiments. The kinetics of DNA SSB removal illustrated appear to be first order with respect to time and linear regression analysis of the data points obtained under the different experimental conditions leads to the estimated t₁/₂ values that are reported in the figure.
enhancing effects of calcium ions. However, catalase prevented DNA strand scission induced by H$_2$O$_2$ (50 μM) or by the redox-cycling quinone menadione (25 μM) (fig. 5, inset). Importantly, the protective effects of catalase disappeared when the enzyme was boiled prior to addition to the cultures. SOD (200 U/ml) neither affected the accumulation of DNA lesions in cells challenged with tB-OOH alone or combined with CaCl$_2$, nor did it modulate the protective effects elicited by catalase (fig. 5).

**Discussion**

The results presented in this study define a previously unexpected role of mitochondria in the formation of lesions at the level of genomic DNA. In particular, we report experimental evidence demonstrating Ca$^{2+}$-dependent mitochondrial formation of species, mainly represented by H$_2$O$_2$, which mediate DNA cleavage triggered by tB-OOH. In addition, we demonstrate that agents increasing mitochondrial Ca$^{2+}$ accumulation evoke a parallel enhancement of the tB-OOH-induced genotoxic response.

Our results demonstrate that treatment with a subtoxic albeit DNA-damaging concentration of tB-OOH promotes a transient elevation in [Ca$^{2+}$] (fig. 1A) and that a significant proportion of the cation was cleared by the mitochondria (fig. 1B). The cation was released from internal stores that are of neutral pH (fig. 1A) and different from the ER-located, SERCA-containing, IP$_3$- and Ry-sensitive Ca$^{2+}$ stores (not shown and fig. 2B).

The first experimental evidence providing a link between mitochondrial calcium uptake and the formation of DNA lesions generated by tB-OOH was that RR significantly reduced the DNA-damaging response evoked by the hydroperoxide (fig. 1C). It is important to note that RR, although being a potent inhibitor of the calcium uniporter of mitochondria (Carafoli, 1987), can also inhibit the calcium efflux from the Ry receptor (Berridge, 1993). Furthermore, a number of different studies have reported that RR can exert antioxidant and scavenging as well as redox properties (Bello et al., 1984; Bernardes et al., 1986; Vercesi et al., 1988; Weiss et al., 1994). These possibilities, however, were ruled out by the observation that Ry and RR did not modify the extent of the DNA-damaging responses evoked by tB-OOH and H$_2$O$_2$, respectively (fig. 1C). Thus, the effect of RR on the formation of DNA SSBs induced by tB-OOH appears to be specifically associated with inhibition of mitochondrial calcium uptake. This inference is further supported by the experimental results that will be discussed below.

As a second approach to investigate the effect of an elevation in [Ca$^{2+}$] in general and more specifically of mitochondrial calcium accumulation on the DNA-damaging effects of the hydroperoxide, we assessed the effects of release of Ca$^{2+}$ from intracellular stores other than those mobilized by tB-OOH. On the basis of the results discussed above we used Cf, which specifically releases Ca$^{2+}$ from the Ry receptor (Meissner, 1994; fig. 2A). The results obtained indicate that

20 μM Ry in the presence of 200 μM tB-OOH either alone or associated with 10 mM Cf or 30 μM CaCl$_2$. Treatment with RR or Ry did not produce DNA SSBs. Data represent the means ± S.E.M. of the percent inhibition of DNA single strand breakage induced by tB-OOH alone or associated with caffeine or Ca$^{2+}$ which was statistically significant at *P < .0001, **P < .001 (unpaired t test).
Ca concentration-dependent fashion (fig. 4B). The fact that the cation enhances DNA cleavage caused by tB-OOH in a straightforward demonstration causally linking these two enzymes. The experiments using permeabilized cells provide a mechanism whereby Cf enhances DNA strand scission caused by tB-OOH. The inset shows the effect of catalase on DNA strand scission caused by 50 μM H2O2 or 25 μM menadione. The specificity of the inhibitory effect promoted by catalase is emphasized by the lack of effect of the temperature-inactivated (boiled) enzyme. 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 three to five separate experiments and were significantly different from those for DNA damage generated by menadione, H2O2 and tB-OOH alone or associated with CaCl2 at *P < .0001, **P < .001 or ***P < .01 (unpaired t test).

Cf not only elevated [Ca2+]i, and mitochondrial Ca2+ uptake (fig. 2A) but also further enhanced mitochondrial accumulation of calcium ions promoted by treatment with the hydroperoxide (fig. 2B), as well as the formation of DNA SSBs (fig. 2C). These effects of Cf appeared to be specific and were due to the ability of the drug to release Ca2+ via opening of the Ry receptor, since they were prevented by high doses of Ry (fig. 2A-C), a treatment known to block the opening of the Ry receptor (Meissner, 1994).

Taken together, these results are consistent with the possibility that mitochondrial calcium overload is the sole mechanism whereby Cf enhances DNA strand scission caused by tB-OOH. The experiments using permeabilized cells provide a straightforward demonstration causally linking these two phenomena. Indeed, using digitonin-permeabilized cells we were able to directly deliver Ca2+ in the cytosol and show that the cation enhances DNA cleavage caused by tB-OOH in a concentration-dependent fashion (fig. 4B). The fact that Ca2+ did not modify the DNA-damaging response evoked by H2O2 (fig. 4B) and that its potentiating effects on DNA damage induced by tB-OOH were prevented by as low as 200 nM RR—but not by Ry- (fig. 4C) demonstrates that mitochondrial calcium uptake specifically enhances the formation of DNA-damaging species generated by tB-OOH. The permeabilized cell system was also responsive to Cf that once again potentiated DNA damage caused by tB-OOH (fig. 4A) in a RR- as well as Ry-inhibitable fashion (fig. 4C). Interestingly, addition of exogenous calcium ions did not, however, further increase the accumulation of DNA lesions in cells exposed to tB-OOH and Cf (fig. 4B), strongly suggesting that Ca2+ and Cf act via common mechanisms, i.e., by promoting mitochondrial calcium overload. It is important to emphasize that these experiments also rule out the possibility that the compounds used to modulate the intramitochondrial calcium content, and the extent of the tB-OOH-induced DNA damage, produced changes in the uptake or intracellular distribution of the hydroperoxide that were coincidental with, but not causally related to mitochondrial calcium. Along the same lines, we can exclude that an enhancement in [Ca2+]i per se generates DNA strand scission, making it unlikely that Ca2+-dependent endonucleases mediate the formation of DNA lesions in cells exposed to tB-OOH. The results thus far discussed clearly indicate that the site in which at least some of the species mediating DNA damage induced by tB-OOH are being formed is the mitochondrion and that mitochondrial Ca2+ uptake enhances their formation. It was therefore important to investigate the nature of these species that, on the basis of previous results from our (Guidarelli et al., 1996) and other (Coleman et al., 1989) laboratories, appear to be different from those involved in the cytotoxic response. In this study we report experimental evidence indicating that H2O2 is one of the species that mediate DNA cleavage induced by tB-OOH. It is conceivable that Ca2+ is involved at the level of formation of H2O2 because mitochondrial production of superoxide anions and hydrogen peroxide was shown to be sensitive to mitochondrial Ca2+ content (Cadenas and Boveris, 1980). Furthermore, reports from the Vercesi group (Valle et al., 1993; Castilho et al., 1995) indicate that mitochondrial calcium accumulation leads to an enhanced formation of H2O2 in mitochondria exposed to tB-OOH. Therefore, it is tempting to speculate that at least part of the DNA strand scission caused by tB-OOH occurs as a result of H2O2 formation taking place at the mitochondrial level and that mitochondrial Ca2+ uptake leads to an increased formation of H2O2 and thus to an enhanced DNA strand scission. Our previous results, demonstrating an increased formation of tB-OOH-induced DNA lesions in catalase-depleted cells (Guidarelli et al., 1997), are consistent with this possibility that finds definitive proof in the outcome of the experiments obtained in our study using the permeabilized cell system. Indeed, catalase was found to reduce DNA cleavage caused by tB-OOH and afforded a greater protective effect after treatment with the peroxide in the presence of exogenous calcium ions (fig. 5). However, it is important to note that catalase, although abolishing DNA strand scission caused by H2O2 or by the redox cycling quinone menadione (fig. 5, inset), afforded only a partial protection against DNA damage caused by tB-OOH alone or associated with CaCl2. The fact that SOD neither affected DNA damage induced by tB-OOH nor modulated the protective effects of catalase (fig. 5) rules out the possibility that superoxides migrate into the nucleus before being converted into H2O2. This sequence of events, although unlikely (superoxides readily dismutate either spontaneously or enzymatically), would have explained why catalase failed to completely abolish the formation of DNA lesions under conditions in which the only species responsible for this effect was H2O2.

Taken together these results, although demonstrating that H2O2 is the most relevant DNA-damaging species that is produced within the mitochondria via a Ca2+-dependent mechanism, would imply the formation of tB-OOH-derived DNA-damaging species different from H2O2.

Additional results reported in this study, however, provide...
circumstantial evidence suggesting remarkable similarities in the types of DNA lesions generated after treatments with tB-OOH alone or associated with Cf (a condition that magnifies the relative amount of the DNA lesions generated via the calcium-based mechanism), or with the peroxide associated with RR (a condition abolishing the formation of DNA lesions generated via the calcium-based mechanism), thus suggesting that the species responsible for their formation were characterized by similar reactivities. Indeed, the formation of DNA lesions was always iron dependent and insensitive to antioxidants (table 1) and their repair was characterized by superimposable kinetics (fig. 3). Even more interesting was the observation that these kinetics were virtually identical to those detected after treatment with H2O2 (fig. 3), which also generates DNA strand scission inhibitable by iron chelators (Mello Filho et al., 1984; Mello Filho and Meneghini 1984; Guidarelli et al., 1995; Coleman et al., 1989; Latour et al., 1995; Guidarelli et al., 1997) and insensitive to antioxidants (Coleman et al., 1989; Guidarelli et al., 1997). Thus, it may be hypothesized that the H2O2-independent component of DNA strand scission caused by tB-OOH is mediated by different species with reactivities similar to that of the hydroxyl radical, the final DNA-damaging product resulting from the interaction between H2O2 and divalent iron. The nature of these species is not readily apparent from the results presented in this study. Ferryl and per-ferryl radicals as well as complexes of iron and oxygen are possible candidates, because they are all sensitive to iron chelators and their reactivities are remarkably similar to that of the hydroxyl radical. The identification of these species, however, does not appear to be an easy task and their involvement in specific reactions can only be suggested on the basis of indirect experimental evidence. In a recent review on the Fenton reaction Goldstein et al. (1993) well summarized the problems that can arise in these types of studies by stating that “identifying oxidizing intermediates in mammalian cells is almost impossible.”

Conclusions

Our results demonstrate that a sub-toxic concentration of tB-OOH elevates [Ca2+]i, a large proportion of which is promptly cleared by the mitochondria. Intramitochondrial Ca2+ promotes the formation of tB-OOH-derived DNA-damaging species mainly represented by H2O2. The contribution of this mechanism to the overall DNA-damaging response could be remarkably enhanced by Cf. Thus, it may be hypothesized that the DNA-damaging efficiency of organic hydroperoxides is potentially modulated by agents (hormones, drugs, toxins, etc.) which elevate [Ca2+]i, provided that this latter event is associated with mitochondrial clearance of the cation. Finally, tB-OOH also generates DNA-damaging species other than H2O2 resulting in DNA lesions remarkably similar to those generated by the hydroxyl radical.

This study identifies the mechanism whereby tB-OOH induces DNA single strand breakage in cultured mammalian cells and, more generally, provides new insights into the mechanism of oxidative stress associated with organic hydroperoxides. Future research should better define the generality as well as the specific aspects of these effects. It will be important to determine the role of the Ca2+-dependent mitochondrial formation of tB-OOH-derived DNA-damaging species on the tumor promoting properties of the hydroperoxide. These events may also activate specific redox-sensitive signal transduction pathways. Finally, it will be important to investigate the effects at the level of mitochondrial DNA. Indeed, it is reasonable to expect that a large amount of lesions will accumulate in the DNA of those mitochondria in which tB-OOH-derived DNA-damaging species are being formed.

References


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