The respiratory-chain poison antimycin A promotes the formation of DNA single-strand breaks and reduces toxicity in U937 cells exposed to t-butylhydroperoxide

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Antimycin A at levels that abolish oxygen consumption had a slight, although statistically significant, inhibitory effect on the toxicity elicited by t-butylhydroperoxide in U937 cells. The protective effect was observed after 6 h of post-treatment incubation, but was no longer apparent after 24 h. Unexpectedly, these events were associated with a marked accumulation of DNA single-strand breaks produced by low levels of t-butylhydroperoxide. Both an oxygen- and a carbon-centred radical were found to arise during treatment with t-butylhydroperoxide, and their formation was significantly lowered by antimycin A. Thus inhibition of electron transport at the level of complex III appears (a) to decrease the formation of toxic species which mediate, at least partially, the lethal effects elicited by t-butylhydroperoxide, and (b) to enhance the formation of DNA-damaging species generated at low concentrations of t-butylhydroperoxide. Rotenone and cyanide, which respectively inhibit complexes I and IV, did not affect DNA damage elicited by t-butylhydroperoxide. These results suggest that DNA single-strand breaks do not mediate the toxicity of t-butylhydroperoxide, and that specific mitochondrial functions might modulate the formation of toxic and of DNA-damaging species generated by organic hydroperoxides.

INTRODUCTION

It is well known that the mitochondrial electron transport chain is a site of oxyradical generation and thus represents a potentially important endogenous source of oxidative damage [1]. Antimycin A is an inhibitor of electron transport from cytochrome *b* to cytochrome c_1 (complex III), and is commonly used to investigate the contribution of reactive oxygen species formed by the mitochondrial respiratory chain to the cytotoxicity exerted by specific toxins [2,3]. A recent study [4] provided experimental evidence suggesting that the mitochondrial electron transport chain plays an important role in the reduction of t-butylhydroperoxide (tB-OOH) to free radical(s) and, in particular, identified a species inhibitable by antimycin A. It is possible that hydroperoxide-related effects are indirect and caused by free-radical metabolites. In this study we present evidence that inhibition of complex III by antimycin A produces opposite effects on the cyto- and geno-toxicity of tB-OOH in cultured U937 cells. Indeed, the protection observed against toxicity generated by the hydroperoxide appears to be associated with a pronounced increase in the formation of DNA single-strand breaks (SSBs).

MATERIALS AND METHODS

Cell culture and treatments

U937 cells were grown in RPMI 1640 culture medium (Gibco, Grand Island, NY, U.S.A.) supplemented with 10 % (v/v) fetal bovine serum (Seralab), penicillin (50 units/ml) and streptomycin (50 μg/ml) at 37 °C in T-75 tissue culture flasks (Corning, Corning, NY, U.S.A.) in an atmosphere of 95 % air/5 % CO_2. Reagent-grade chemicals, tB-OOH and inhibitors of the mitochondrial respiratory chain were obtained from Sigma (St. Louis, MO, U.S.A.).

Stock solutions of tB-OOH and KCN 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). Antimycin A, 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) and thenoyltrifluoroacetone (TTFA) were dissolved in 95 % (v/v) ethanol. At the treatment stage, the final ethanol concentration was never higher than 0.05 %. Under these conditions, ethanol was neither toxic nor DNA-damaging, nor did it affect the cyto- and geno-toxic effects of tB-OOH. Cells (2.5×10^5/ml) were treated for 30 min in saline A (2 ml), washed with pre-chilled saline A and processed for cytotoxicity or DNA damage assays either immediately or after various periods of incubation.

Cytotoxicity assay

Cytotoxicity was determined using the Trypan Blue exclusion assay. Briefly, an aliquot of the cell suspension was diluted 1:1 (v/v) with 0.4 % Trypan Blue and the cells were counted using a haemocytometer. Viability was expressed as the ratio of the percentage of unstained treated cells to that of unstained untreated cells.

ESR spin trapping measurements

U937 cells (2.5×10^5/ml) were incubated at 37 °C for 30 min in saline A in the presence of tB-OOH, the spin trapping agent 5-diethoxyphospho-5-methyl-1-pyrroline n-oxide (DEPMPO) (50 mM) and, where indicated, antimycin A (5 μM). DEPMPO was synthesized as previously described [5]. A Bruker 300 ESR spectrometer, operating at 9.5 GHz, was used. Measurements were made at 37 °C, using a standard flow dewar for temperature

Abbreviations used: tB-OOH, t-butylhydroperoxide; SSBs, single-strand breaks; SSF, strand scission factor; DEPMPO, 5-diethoxyphospho-5-methyl-1-pyrroline *N*-oxide; TTFA, thenoyltrifluoroacetone; HQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide.

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regulation. The samples were placed into the ESR cavity in a 100 μl flat cell. The spectrometer settings for the samples containing DEPMPO were: incident microwave power, 20 mW; modulation amplitude, 0.1 mT; time constant, 160 ms; scan time, 320 s; scan range, 11.0 mT.

Oxygen consumption

U937 cells were washed once in saline A and then resuspended in the same medium at a density of 1×10^7 cells/ml. Oxygen consumption was measured using a Y.S.I. oxygraph equipped with a Clark electrode (model 5300; Yellow Springs Instruments Co., Yellow Springs, OH, U.S.A.). The cell suspension (3 ml) was transferred to the polarographic cell and the oxygen levels were monitored for 3 min under constant stirring (basal respiration). Antimycin A was then added, and once again respiration was measured for 3 min. The rate of oxygen utilization was calculated as described in [6].

Alkaline elution assay

Cells were labelled overnight with [^{14}C]thymidine (0.5 μCi/ml) and incubated for a further 6 h in medium containing unlabelled thymidine (1 μg/ml). Cells were then exposed for 30 min to increasing concentrations of tB-OOH in saline A in the presence or absence of 5 μM antimycin A, and analysed for DNA damage. The filter elution assay was carried out using a procedure virtually identical to that described in [7], with minor modifications [8]. Briefly, $(3.5–4.0) \times 10^5$ cells were gently loaded on to 25 mm-diam., 2 μm-pore-size, polycarbonate filters (Nuclepore, Pleasanton, CA, U.S.A.) 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% SDS and 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 (Merck-Schuchardt, Munchen, Germany)/0.02 M EDTA (free acid)/0.1% SDS (pH 12.1), at a flow rate of approx. 30 μl/min. Fractions were collected at 2 h intervals and radioactivity was counted in 7 ml of Lumagel containing 0.7% acetic acid. DNA remaining on the filters was recovered by heating for 1 h at 60 °C in 0.4 ml of 1 M HCl followed by the addition of 0.4 M 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 M 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 AND DISCUSSION

Experiments were carried out to detect free-radical intermediates arising from tB-OOH metabolism in U937 cells, using ESR spectroscopy coupled with the spin trapping technique [9]. Spin traps react with unstable free radicals to form more stable nitroxide radical adducts. In the present study we employed as spin trapping agent the newly synthesized β-phosphorylated cyclic nitrone DEPMPO, which has been shown to efficiently trap various free radicals, giving well resolved and long-lived oxygen-centred spectra [10].

Cells were treated for 30 min with tB-OOH in a glucose-containing saline in the presence of the spin trap DEPMPO and, as shown in Figure 1(b), two radical species were detected; the

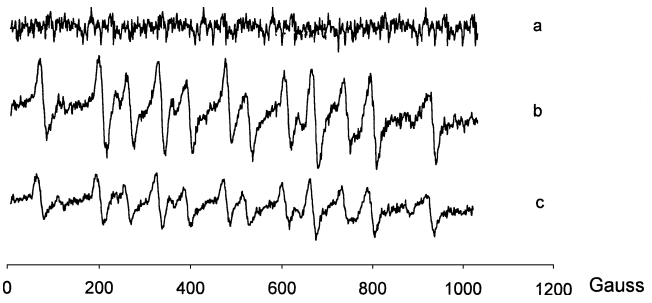

Figure 1 ESR spectra of radical adducts generated by tB-OOH

DEPMPO radical adducts were measured in U937 cells (2.5×10^5/ml) treated for 30 min at 37 °C in saline A as follows: (**a**) control; (**b**) 600 μM tB-OOH; (**c**) 600 μM tB-OOH plus 5 μM antimycin A. The two radical adducts (see the text) are characterized by the following hydrogen, nitrogen, and phosphorus hyperfine splitting constants: $a_H = 22.2$ mT, $a_N = 1.5$ mT, and $a_P = 4.801$ mT for the first species; $a_H = 1.39$ mT, $a_N = 1.39$ mT, and $a_P = 4.609$ mT for the second.

measured hyperfine splitting constants (see legend to Figure 1) assigned the species to the DEPMPO adduct of the methyl and t-butoxyl radicals. The t-butoxyl radical is generated following the single reduction of tB-OOH, and the methyl radical is formed via the subsequent β-scission of untrapped t-butoxyl radicals, a reaction amply described in the literature [4,11]. This is the first reported demonstration of such species using DEPMPO, and the results obtained in this model system confirm those reported using other spin trapping agents. The same experiment was repeated in the presence of 5 μM antimycin A, and this inhibitor resulted in a significant inhibition of tB-OOH-derived radicals (Figure 1c). Figure 2(A) shows that the production of these species was a direct function of the peroxide concentration, and that antimycin A, under all these conditions, prevented the formation of an equal amount of radical species. As a consequence, the extent of the inhibitory response, as measured by the percentage decrease in radical formation, was an inverse function of the peroxide concentration (Figure 2B).

These results confirm and extend the findings from a recent study [4] showing that the electron transport chain is responsible for the reduction of tB-OOH to free radical(s) and, in particular, that antimycin A is capable of preventing the formation of the methyl and t-butoxyl radicals. Our results also lead to the interesting conclusion that, at low concentrations of tB-OOH (150–300 μM), the tB-OOH-derived radicals are generated almost entirely via an antimycin A-inhibitable mechanism. Additional, possibly cytochrome *P*-450-dependent [12], mechanism(s) appear to be involved in the formation of these radicals in cells challenged with higher levels of the peroxide.

tB-OOH induces killing of U937 cells in a concentration- and time-dependent fashion (Figures 3A and 3B). Addition of antimycin A (5 μM) during the 30 min of exposure to the hydroperoxide, while abolishing oxygen consumption (results not shown; basal respiration measured in a glucose-containing saline was 13.1 ± 2.9 nmol O_2/min per 10^7 cells), also produced a slight, although statistically significant, inhibition of the cytotoxic response elicited by tB-OOH. Cytoprotection was also observed using another complex III inhibitor, HQNO (results not shown). Importantly, the cytoprotection afforded by antimycin A was apparent only after 6 h of post-treatment incubation (Figure 3A), since at 24 h (Figure 3B) the toxicity curves obtained in the absence and presence of antimycin A were virtually superimposable. These results indicate that an inhibitor of complex

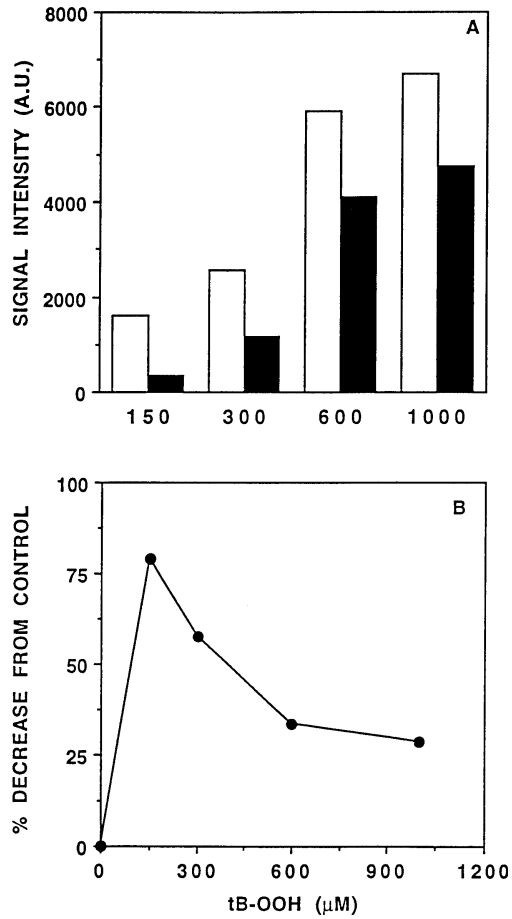

Figure 2 Effect of antimycin A on the production of radical species in cells exposed to tB-OOH

Cells were exposed for 30 min to increasing concentrations of tB-OOH in the absence (open bars) or presence (closed bars) of 5 μM antimycin A and then analysed by ESR spectroscopy. Experimental results are expressed as signal intensity in absorbance units (A.U.) and were obtained from two independent experiments (**A**). Panel (**B**) illustrates the same data plotted as percentage inhibition of signal intensity.

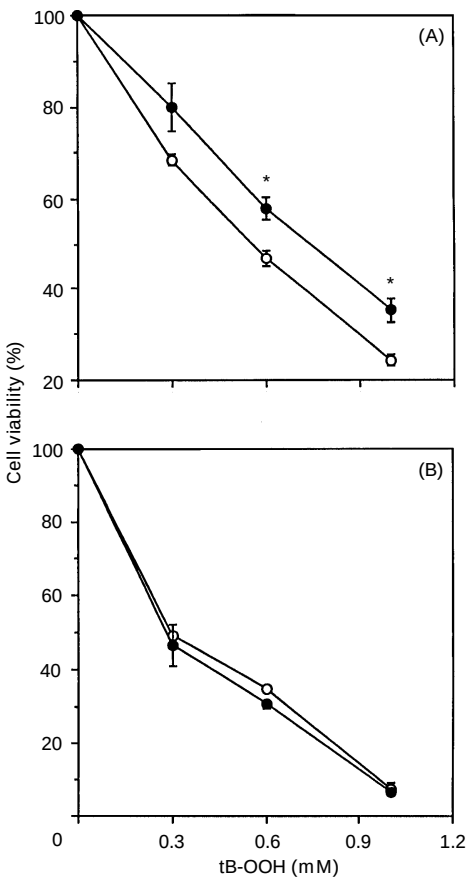

Figure 3 Effect of antimycin A on tB-OOH-induced cytotoxicity

U937 cells were plated at a density of 2.5×10^5 cells/ml in saline A and exposed for 30 min to increasing concentrations of tB-OOH in the absence (○) or presence (●) of 5 μM antimycin A. Treatments were terminated by dilution in 10 ml of saline A, centrifugation and resuspension in fresh pre-warmed growth medium. After incubation for 6 h (**A**) or 24 h (**B**), the number of viable cells was estimated using the Trypan Blue dye exclusion test. Results are means $\pm$ S.E.M. of at least three separate experiments, each performed in duplicate. $^*P < 0.01$ compared with tB-OOH alone.

III, which selectively blocks electron flow from cytochrome *b* to cytochrome c_1, decreases the lethal effects of tB-OOH, suggesting that the toxicity of this hydroperoxide is mediated by at least two mechanisms, one of which involves the activity of complex III. It is important to emphasize, however, that in this study the antimycin A-inhibitable pathway contributed only a small proportion of the overall cytotoxicity exerted by tB-OOH and appeared to be dispensable for the long-term expression of tB-OOH-induced cytotoxicity, since the protective effect afforded by the respiratory chain poison was lost after 24 h of post-challenge incubation. In short, the decreased formation of radical species mediated by antimycin A delays, but does not prevent, the killing of U937 cells exposed to tB-OOH.

tB-OOH has been shown to induce DNA SSBs in cultured mammalian cells [13–15]. In the present study we utilized the sensitive alkaline elution technique and found that tB-OOH is an efficient DNA SSB inducer in U937 cells. Indeed, as illustrated in Figure 4(A), treatment with increasing concentrations of tB-OOH progressively increased the rate of DNA elution. This observation led us to the conclusion that one or more DNA-damaging species are produced via tB-OOH metabolism and that the generation of these species increases as a function of the peroxide concentration. The effect of antimycin A on the formation of DNA SSBs in cells exposed to tB-OOH was also investigated and, as illustrated in Figure 4(A), the inhibitor produced a remarkable enhancement in the accumulation of DNA SSBs. Figure 4(B) shows the SSF values calculated from the DNA elution profiles obtained in experiments similar to that depicted in Figure 4(A). It would appear that the extent of DNA fragmentation is not a linear function of the peroxide concentration but, rather, that different mechanisms are involved in the process of SSB formation at doses below or above 75 μM tB-OOH. This was observed both in the absence and in the presence of antimycin A, which seems to selectively enhance only those SSBs generated by levels of tB-OOH of 75 μM or less. HQNO also enhanced DNA strand scission provoked by tB-OOH (Figure 5), and the extent of this response was remarkably similar to that observed with antimycin A. Taken together, these results are consistent with the possibility that different species might mediate the genotoxic event. The formation of these lesions, however, was prevented by phenanthroline (25 μM) under conditions involving concomitant exposure to different

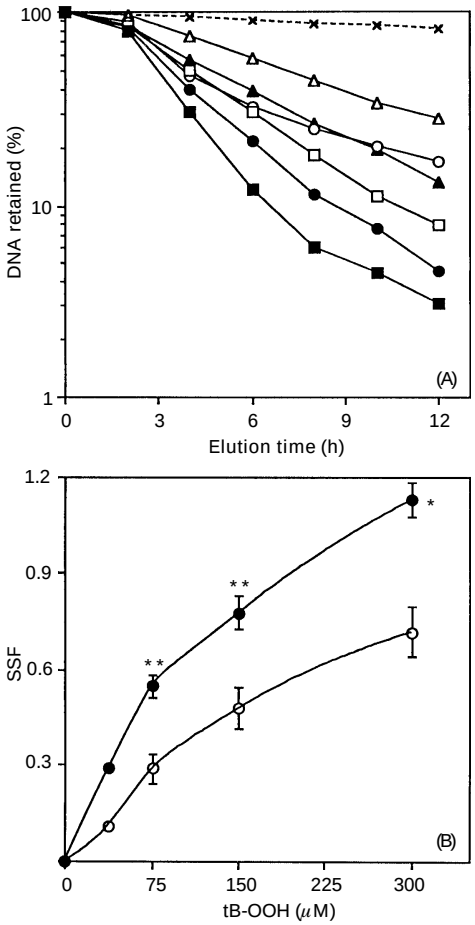

Figure 4 Alkaline elution analysis of DNA SSBs in U937 cells treated with tB-OOH

Cells were treated for 30 min with increasing concentrations of tB-OOH in the absence (open symbols) or presence (closed symbols) of 5 μM antimycin A. The level of DNA SSBs was measured, immediately after treatment, by the alkaline elution technique. (**A**) Elution profiles resulting from a typical experiment. ×, Control; △, ▲, 75 μM tB-OOH; ○, ●, 150 μM tB-OOH; □, ■, 300 μM tB-OOH. (**B**) SSF values (means ± S.E.M.) calculated from three to five experiments similar to that illustrated in (**A**). $^{*}P < 0.001$; $^{**}P < 0.01$ compared with tB-OOH alone.

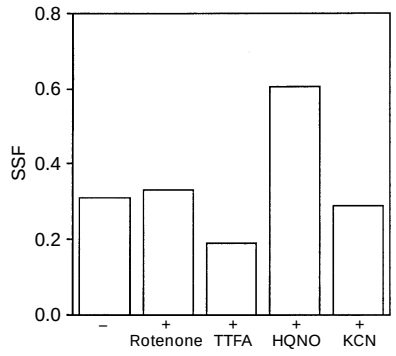

Figure 5 Effect of inhibitors of mitochondrial electron transport on DNA SSBs induced by tB-OOH

Cells were treated for 30 min with 75 μM tB-OOH in the absence or presence of 0.5 μM rotenone, 250 μM TTFA, 50 μM HQNO or 500 μM KCN. The level of DNA SSBs was measured, immediately after treatment, by the alkaline elution technique. Data represent the means of two separate experiments.

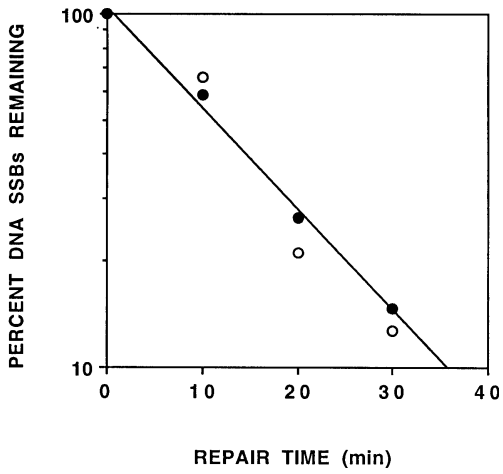

Figure 6 Effect of antimycin A on the repair of DNA SSBs in U937 cells treated with tB-OOH/antimycin A

Cells were exposed for 30 min to 150 μM tB-OOH and analysed for DNA SSBs either immediately or after post-treatment incubation in the absence (○) or presence (●) of 5 μM antimycin A for various time intervals. Data represent the means of two separate experiments.

doses of the peroxide alone or associated with antimycin A (results not shown). Thus, although different DNA-damaging species might be involved in the process of tB-OOH-induced DNA fragmentation, their formation is always iron-dependent. In addition, the same requirement was found for DNA damage elicited by the peroxide in the presence of antimycin A.

The data presented in Figure 4 are certainly surprising, and lead to the intriguing conclusion that inhibition of complex III markedly enhances the accumulation of DNA SSBs in U937 cells exposed to tB-OOH. This increased accumulation could either be the result of an increased formation of DNA-damaging species, thereby producing more lesions, or depend on a less specific event such as inhibition of repair. We investigated this second possibility by measuring the effect of antimycin A on the rate of DNA SSB removal in pre-damaged cells. For this purpose, U937 cells were treated for 30 min with 150 μM tB-OOH and then allowed to repair in fresh pre-warmed medium in the absence or presence of antimycin A (5 μM). The results illustrated in Figure 6 indicate that the respiratory-chain poison does not affect the rate of repair of DNA SSBs induced by tB-OOH. Thus the increased accumulation of DNA SSBs observed in cells treated with tB-OOH in the presence of antimycin A appears to be the consequence of an increased production of the net amount of DNA SSBs.

We next addressed the question of whether other inhibitors of mitochondrial electron transport are also capable of enhancing the formation of DNA SSBs induced by tB-OOH in U937 cells. As shown in Figure 5, the complex I inhibitor rotenone (0.5 μM), as well as the complex IV inhibitor cyanide (500 μM), did not affect the extent of DNA damage promoted by 75 μM tB-OOH. The effect of the complex II inhibitor TTFA was also tested, although this agent did not appear suitable for these studies, since it has been previously suggested to act as an iron chelator [16,17]. As expected, TTFA markedly decreased DNA SSBs generated by tB-OOH (Figure 5), and a similar effect was observed using H_2O_2 as a DNA-damaging agent (results not shown).

Taken together, these results indicate that interference with complex I or complex IV does not affect the DNA-damaging response. The results obtained with TTFA are more difficult to interpret, since the protective effect was observed using both tB-OOH and H_2O_2 and is therefore likely to depend on the ability of TTFA to chelate transition metals [16,17]. Indeed, chelation of intracellular iron mediated by phenanthroline abolishes DNA damage induced by tB-OOH ([15]; and results not shown).

The results presented in this study do not allow a straightforward interpretation of the mechanism whereby antimycin A augments the DNA-damaging potential of tB-OOH. It is possible that antimycin A forms a radical species which could damage the DNA, although it appears unlikely that this mechanism is entirely responsible for the enhanced strand scission. Furthermore, HQNO and antimycin A, which inhibit complex III at same site, had similar enhancing effects on the DNA strand scission elicited by tB-OOH. It is therefore more likely that the complex III inhibitors, by preventing electron flow from cytochrome *b* to cytochrome c_1 in peroxide-treated cells, might favour the production of one or more species capable of inducing DNA SSBs. Since at these tB-OOH levels the cytotoxic response was not affected by antimycin A, we might also conclude that this/these species is/are not toxic for U937 cells. As a corollary, these results strengthen the hypothesis put forward by our [18] and other [19] laboratories that DNA SSBs, although of potential relevance in carcinogenesis, are not a toxic event in oxidatively injured cells. Also unclear is the mechanism whereby the lethality promoted by tB-OOH is decreased, or delayed, by antimycin A. An attractive possibility is that electron flow from cytochrome *b* to cytochrome c_1 mediates the formation of additional potentially lethal species. Likely candidates are the carbon- and oxygen-centred radical species that were detected and found to be sensitive to inhibition by antimycin A (Figures 1 and 2). Thus tB-OOH could be directly reduced by either cytochrome c_1 or cytochrome *c*, or by both of these electron transport chain components in mitochondria. It is therefore possible that the toxicity of tB-OOH is the result of the formation of these radical species, and that the mitochondrial respiratory chain represents a site of metabolic activation of the hydroperoxide. This site, as indicated by the results illustrated in Figure 2, seems to be entirely responsible for the activation of low concentrations of tB-OOH, whereas other mechanism(s), which is/are not inhibited by antimycin A, appear to be involved in the production of the same species promoted by higher levels of the hydroperoxide. If our hypothesis turns out to be correct, it can then be speculated that the mitochondrial electron transport chain is a site which can modulate the expression of the cyto- compared with the geno-toxic properties of tB-OOH. In view of the potential importance of these findings in carcinogenesis, further research is needed to validate this hypothesis.

Received 2 January 1996/8 March 1996; accepted 27 March 1996

This investigation was supported by grants from CNR and AIRC (Italy). We gratefully acknowledge the assistance of Professor P. Tordo (Université de Provence, CNRS URA 1412, Marseille, France) for kindly supplying DEPMPO and for helpful discussion on the assignment of the radical adducts.

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