Mechanism of the antimycin A-mediated enhancement of t-butylhydroperoxide-induced single-strand breakage in DNA

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Inhibitors of complex III increased the DNA strand scission induced by t-butylhydroperoxide (tB-OOH) and cumene hydroperoxide but did not affect DNA damage induced by H_2O_2. The hypothesis that these effects are selectively linked to inhibition of the electron transport from cytochrome *b* to cytochrome c_1 is validated by the following observations: (1) two complex III inhibitors, antimycin A and 2-heptyl-4-hydroxyquinoline *N*-oxide, enhanced the tB-OOH-induced DNA cleavage over the same concentration range as that in which inhibition of oxygen consumption was observed; (2) the complex III inhibitor-mediated enhancement of tB-OOH-induced DNA damage was abolished by the complex I inhibitor rotenone or by glucose omission, and (3) the enhancing effects of antimycin A were not observed in respiration-deficient cells. The mechanism whereby the complex III inhibitors potentiate DNA cleavage promoted by tB-OOH was subsequently investigated with intact as well as permeabilized cells. H_2O_2, produced at the level of mitochondria via a Ca^{2+}-dependent process, was found to account for the enhancing effects of antimycin A.

INTRODUCTION

The cytotoxic effects of organic hydroperoxides have been widely investigated in recent years. It has been shown that short-chain organic hydroperoxides, and in particular t-butylhydroperoxide (tB-OOH), induce lipid peroxidation [1], depletion of glutathione [2,3], mobilization of mitochondrial Ca^{2+} [4,5], inhibition of glycolytic and non-glycolytic ATP synthesis [6] and mitochondrial damage [5–9]. These events occur as a result of the formation of tB-OOH-derived radical species, such as the methyl and t-butoxyl radicals [10–12], and represent the molecular basis of the lethal effects promoted by tB-OOH.

Much less is known about the genotoxic effects of organic hydroperoxides. It has been reported that tB-OOH introduces damage at the DNA level by causing strand scission [13–19] and that this event is prevented by iron chelators [15–17,19]. The species mediating DNA damage seem to be different from the toxic oxygen- and carbon-centred radical intermediates [15,18]. Furthermore at least some of these DNA-damaging species are generated at the level of mitochondria via a Ca^{2+}-dependent process [20].

In a recent study [18] we demonstrated that the complex III inhibitors antimycin A and 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) increase the magnitude of the DNA scission produced by tB-OOH.

Here we analyse the molecular events underlying the complex III inhibitor-mediated enhancement of tB-OOH-induced DNA single-strand breakage. Results are presented that document that this effect is selective for organic hydroperoxides, occurs only in intact and functional cells and is causally linked to the impairment of the ability to transport electrons from cytochrome *b* to cytochrome c_1. Furthermore we present evidence demonstrating that the mechanism whereby complex III inhibitors potentiate tB-OOH-induced DNA single-strand breakage involves Ca^{2+}-dependent mitochondrial formation of H_2O_2.

MATERIALS AND METHODS

Materials

Fura 2 acetoxymethyl ester and ryanodine (Ry) were purchased from Calbiochem (San Diego, CA, U.S.A.). Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), Ruthenium Red (RR), catalase, tB-OOH, H_2O_2, cumene hydroperoxide, *N*,*N′*-diphenyl-1,4-phenylene diamine (DPPD), butylated hydroxytoluene (BHT), caffeine, HQNO, antimycin A and the remaining chemicals were from Sigma-Aldrich (Milano, Italy). RPMI 1640 culture medium was from Gibco (Grand Island, NY, U.S.A.), and fetal bovine serum, penicillin and streptomycin were from Seralab (Crawley Down, Sussex, U.K.). T-75 tissue culture flasks were purchased from Corning (Corning, NY, U.S.A.). [^{14}C]Thymidine was obtained from NEN–Dupont (Boston, MA, U.S.A.). Polycarbonate filters and the liquid-scintillation fluid were purchased from Nuclepore (Pleasanton, CA, U.S.A.) and Beckman (Fullerton, CA, U.S.A.) respectively.

Cell culture and treatments

Human myeloid leukaemia U937 cells were cultured in suspension in RPMI 1640 culture medium supplemented with 10% (v/v) fetal bovine serum, penicillin (50 i.u./ml) and streptomycin (50 μg/ml) at 37 °C in T-75 tissue culture flasks in a humidified air/CO_2 (19:1) atmosphere.

Respiration-deficient U937 cells were isolated by culturing the cells in RPMI medium containing 400 ng/ml ethidium bromide, 110 μg/ml pyruvate and 5 μg/ml uridine for 6 days with medium changes at days 2 and 4. These cells were unable to consume oxygen in response to glucose (5 mM) or the membrane-permeant NADH-linked substrate pyruvate (5 mM) (results not shown).

Stock solutions of H_2O_2, tB-OOH, cumene hydroperoxide, catalase and RR were freshly prepared in saline A (8.182 g/l NaCl/0.372 g/l KCl/0.336 g/l $NaHCO_3$/0.9 g/l glucose).

Abbreviations used: BHT, butylated hydroxytoluene; $[Ca^{2+}]_i$, cytosolic Ca^{2+} ion concentration; DPPD, *N*,*N′*-diphenyl-1,4-phenylene diamine; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; HQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide; RR, Ruthenium Red; Ry, ryanodine; SSBs, single-strand breaks; tB-OOH, t-butylhydroperoxide.

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DPPD, BHT, Ry, FCCP, antimycin A and HQNO were dissolved in 95 % (v/v) ethanol, and *o*-phenanthroline was dissolved in DMSO. At the treatment stage the final concentration of ethanol or DMSO was never higher than 0.05 %. Under these conditions ethanol or DMSO was neither toxic nor DNA-damaging, nor did it affect the cyto-genotoxic properties of H_2O_2, tB-OOH or cumene hydroperoxide. Treatment with the hydroperoxides was performed as detailed below and, under the conditions utilized in this study, cell death (as measured by Trypan Blue or lactate dehydrogenase release assays) was never detectable immediately after the exposure to peroxide as well as after up to 24 h of incubation in fresh culture medium after treatment.

Cytosolic Ca^{2+} ion ($[Ca^{2+}]_i$) measurements

Cells were harvested, washed three times by centrifugation and resuspended in Krebs–Ringer–Hepes medium containing 125 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 6 mM glucose and 25 mM Hepes/NaOH, pH 7.4. Cell suspensions were loaded with the Ca^{2+}-sensitive dye fura 2 acetoxymethyl ester (3 μM final concentration) for 30 min at 25 °C in Krebs–Ringer–Hepes medium and kept at 37 °C until use. Cell aliquots (4×10^6 cells) were washed three times and resuspended in saline A, transferred to a thermostatically controlled cuvette in a Perkin Elmer LS-50 fluorimeter and maintained at 37 °C under continuous stirring. The various drugs interfering with Ca^{2+} homoeostasis here employed (tB-OOH and FCCP) were added as indicated in the figures and maintained throughout the experiment. In the experiments in which antimycin A was employed, preincubations with this drug were for 5 min before the beginning of the recording. Traces were recorded and analysed as previously described [21]. The results shown are traces representative of ten highly consistent experiments.

Oxygen consumption

U937 cells were washed once in saline A and then resuspended in the same medium at a density of 10^7 cells/ml. Oxygen consumption was measured with 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 rate of oxygen utilization was monitored under constant stirring for 3 min (basal respiration). Antimycin A or HQNO was then added and once again respiration was measured for 3 min. The rate of oxygen utilization was calculated as described by Robinson and Cooper [22].

Measurement of DNA single-strand breaks (SSBs) by alkaline elution

The cells were labelled overnight with [^{14}C]thymidine (0.05 μCi/ml) and incubated for a further 6 h in a medium containing unlabelled thymidine (1 μg/ml). At this stage the cells (2.5×10^5/ml) were either treated in saline A or permeabilized and treated for 10 min in permeabilization buffer. In some experiments the formation of DNA SSBs was measured in isolated nuclei or post-lysed DNA samples that had been prepared as previously described [19]. Permeabilization of the cells was achieved by adding digitonin (10 μM; 12.5 μg per 10^5 cells) to a medium consisting of 0.25 M sucrose, 0.1 % BSA, 10 mM $MgCl_2$, 10 mM K^+/Hepes and 5 mM KH_2PO_4, pH 7.2, at 37 °C. Under these experimental conditions, digitonin permeabilizes the plasma membrane but leaves mitochondrial membranes intact [23]. After the treatments the cells were washed with pre-chilled saline A and analysed immediately for DNA damage by using the alkaline elution technique, that was performed with a procedure virtually identical with that described in [24] with minor modifications [25]. Briefly, $(3.5–4) \times 10^5$ cells were gently loaded on 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 % (w/v) SDS/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 % (v/v) tetraethyl ammonium hydroxide/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 counted in 7 ml of liquid-scintillation fluid containing 0.7 % (v/v) 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 values were calculated from the resulting elution profiles by determining the absolute logarithm 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). In untreated cells, 85 ± 5 % (mean $\pm$ S.E.M.) of the DNA was retained in the filters after 8 h of elution.

RESULTS

Concentration dependence of the complex III inhibitor-mediated enhancement of tB-OOH-induced DNA SSBs

In a previous study [18] we demonstrated that 5 μM antimycin A and 50 μM HQNO have similar enhancing effects on DNA single-strand breakage elicited by 75 μM tB-OOH. Figure 1 illustrates these effects in greater detail by showing that the effect of the complex III inhibitors is concentration-dependent in the concentration ranges 0.1–1 μM and 5–25 μM for antimycin A (Figure 1A) and HQNO (Figure 1C), respectively. Antimycin A potentiated DNA strand scission caused by another organic hydroperoxide, cumene hydroperoxide (Table 1). In contrast, no potentiating effects of antimycin A or HQNO were apparent in cells treated with H_2O_2 (Figures 1A and 1C). It is interesting to note that both complex III inhibitors impaired oxygen consumption over the same concentration range as that in which the enhancement of DNA single-strand breakage was detected (Figures 1B and 1D).

tB-OOH and cumene hydroperoxide, unlike H_2O_2, fail to produce DNA lesions in isolated nuclei or post-lysed DNA samples [19]. The results reported in Table 1 confirm and extend these findings by showing that DNA SSBs were not induced in post-lysed DNA samples or nuclei, regardless of whether antimycin A (or HQNO) was present or absent during treatment with the organic hydroperoxides.

Complex III inhibitor-mediated enhancement of DNA SSBs requires active electron transport

The effect of antimycin A or HQNO on DNA damage promoted by tB-OOH was further investigated to assess whether this response was the result of interference with specific mitochondrial functions, i.e. inhibition of the electron transport from cytochrome *b* to cytochrome c_1.

To test this hypothesis we first investigated the glucose dependence of the complex III inhibitor-mediated enhancement of DNA SSBs induced by tB-OOH, an approach based on the notion that the rate of electron transport is glucose-dependent.

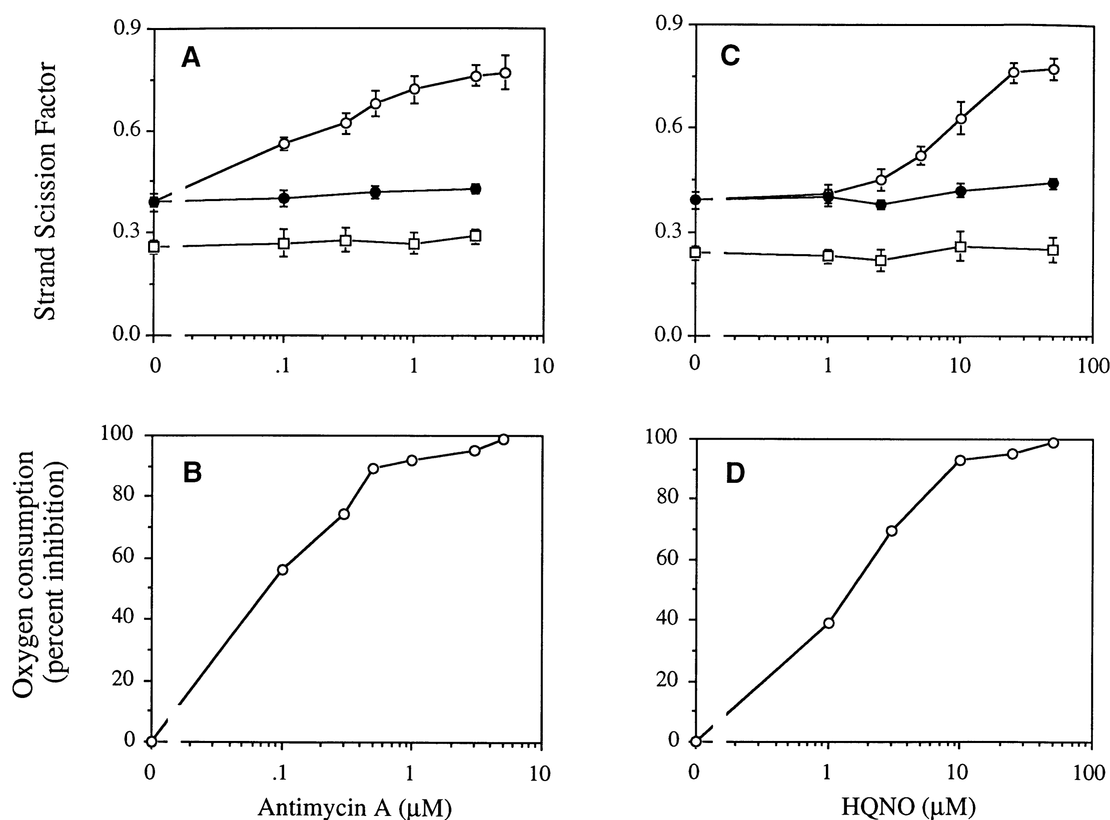

Figure 1 Effect of increasing concentrations of antimycin A or HQNO on U937 cell oxygen consumption and on tB-OOH-induced DNA single-strand breakage

Respiration-proficient (○,□) and respiration-deficient (●) U937 cells were treated for 30 min in saline A with 75 μM tB-OOH (○,●) or 37.5 μM H2O2 (□), in the absence or presence of increasing concentrations of either antimycin A (**A**) or HQNO (**C**). The complex III inhibitors were given to the cultures 5 min before addition of the peroxide. The cells were then rinsed and processed by alkaline elution for the measurement of DNA single-strand breakage, as detailed in the the Materials and methods section. Results are means ± S.E.M. for three or four separate experiments. Oxygen consumption was measured by monitoring the oxygen levels for 3 min after the addition of 5 mM glucose and for a further 3 min after the addition of increasing concentrations of antimycin A (**B**) or HQNO (**D**). Results represent the means of two separate experiments with basically identical outcomes.

Table 1 Hydroperoxide-induced DNA single-strand breakage in intact U937 cells, post-lysed DNA samples or isolated nuclei and its modulation by antimycin A

Intact cells were exposed to 3 μM antimycin A for 5 min in glucose-containing saline A and then treated for a further 30 min with 150 μM tB-OOH or 300 μM cumene hydroperoxide (cumene-OOH). For experiments on isolated nuclei, cells were lysed on polycarbonate filters and after accurate washing were exposed for 20 min to each of the hydroperoxides in the absence or presence of 3 μM antimycin A. For experiments on post-lysed DNA samples, nuclei were isolated from U937 cells as detailed in the the Materials and methods section and then treated for 20 min with the hydroperoxides in the absence or presence of 3 μM antimycin A. The level of DNA SSBs was measured immediately after the treatments by using the alkaline elution technique. The SSF values were calculated from the resulting elution profiles, as detailed in the the Materials and methods section. Results are means ± S.E.M. for three to five separate experiments, and were significantly different from those for DNA damage generated by the hydroperoxides alone at $^{*}P < 0.01$ (unpaired t test).

		Strand scission factor	
Treatment	Target	− Antimycin A	+ Antimycin A
tB-OOH	Intact cells	0.48 ± 0.07	0.85 ± 0.05*
tB-OOH	Isolated nuclei	0.07 ± 0.006	0.06 ± 0.005
tB-OOH	Post-lysed DNA samples	0.05 ± 0.005	0.07 ± 0.006
Cumene-OOH	Intact cells	0.14 ± 0.04	0.28 ± 0.02*
Cumene-OOH	Isolated nuclei	0.08 ± 0.009	0.09 ± 0.007
Cumene-OOH	Post-lysed DNA samples	0.06 ± 0.006	0.05 ± 0.004

Indeed, U937 cells consume approx. 3 or 12 nmol of O_2/min per 10^7 cells in the absence or presence of glucose respectively. The formation of DNA SSBs in U937 cells treated with 75 μM tB-OOH for 30 min in saline A, in the presence or absence of glucose (5 mM), was then assessed. It was interesting to observe that, although the level of DNA damage generated by the oxidant was not affected by glucose, the antimycin A (3 μM)- or HQNO (50 μM)-mediated enhancement of DNA SSBs elicited by tB-OOH could be detected only in the presence of glucose (Table 2). Even more interestingly, the enhancing effects of the two complex III inhibitors were prevented by 0.5 μM rotenone, an inhibitor of complex I. DNA damage induced by tB-OOH both in the absence and the presence of glucose, or by tB-OOH associated with either of the complex III inhibitors in the absence of glucose, was insensitive to rotenone.

The effect of the complex III inhibitors on DNA cleavage induced by tB-OOH was also investigated in respiration-deficient U937 cells. Neither antimycin A (Figure 1A) nor HQNO (Figure C) enhanced this response in the absence of a functional mitochondrial respiratory chain.

Mitochondrial formation of tB-OOH-derived DNA-damaging species is Ca^{2+}-dependent

Pilot work performed in our laboratory provided experimental evidence indicating that a significant proportion of the cytosolic

Table 2 Effect of glucose on tB-OOH-induced DNA single-strand breakage and its modulation by antimycin A or HQNO

Cells were exposed for 30 min in saline A (with or without 5 mM glucose) to 75 μM tB-OOH in the absence or presence of 3 μM antimycin A or 50 μM HQNO. The complex III inhibitors were given to the cultures 5 min before addition of the peroxide. Also shown is the effect of 0.5 μM rotenone. The level of DNA SSBs was measured immediately after the treatments by using the alkaline elution technique. Results are means ± S.E.M. for three to five 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).

Treatment	Strand scission factor	
	− Glucose	+ Glucose
tB-OOH	0.36 ± 0.06	0.34 ± 0.05
tB-OOH + rotenone	0.33 ± 0.04	0.35 ± 0.06
tB-OOH + antimycin A	0.38 ± 0.03	0.66 ± 0.07**
tB-OOH + antimycin A + rotenone	0.35 ± 0.04	0.33 ± 0.05
tB-OOH + HQNO	0.35 ± 0.04	0.69 ± 0.06*
tB-OOH + HQNO + rotenone	0.34 ± 0.03	0.35 ± 0.05

Table 3 Antimycin A enhances DNA single-strand breakage induced by tB-OOH via a mechanism involving mitochondrial Ca^{2+} accumulation

Intact U937 cells were exposed for 5 min to 0 or 3 μM antimycin A and then treated for a further 30 min with 200 μM tB-OOH. Where indicated, 25 μM RR, 20 μM Ry or 10 mM caffeine was added to the cultures 5 min before antimycin A. Treatment with caffeine, antimycin A, RR or Ry did not produce DNA SSBs. The level of DNA SSBs was measured immediately after the treatments by using the alkaline elution technique. Results are means ± S.E.M. for three or four separate experiments and were significantly different from those for DNA damage generated by tB-OOH alone or associated with antimycin A at $^*P < 0.002$, $^{**}P < 0.01$ (unpaired t test). For experiments on permeabilized cells, digitonin-permeabilized cells were treated for 10 min with 200 μM tB-OOH either alone or associated with 1 μM antimycin A in the absence or presence of 200 nM RR, 100 μM $LaCl_3$, 20 μM Ry or 30 μM $CaCl_2$. Treatment with antimycin A, RR, $LaCl_3$, Ry or $CaCl_2$ alone did not produce DNA SSBs. The results are means ± S.E.M. for three or four separate experiments and were significantly different from those for DNA damage generated by tB-OOH alone or associated with antimycin A at $^*P <$ 0.002 (unpaired t test).

Target	Treatment	Strand scission factor	
		− Antimycin A	+ Antimycin A
Intact cells	tB-OOH	0.63 ± 0.04	0.89 ± 0.03
	tB-OOH + RR	0.49 ± 0.04**	0.48 ± 0.04*
	tB-OOH + Ry	0.62 ± 0.06	0.94 ± 0.07
	tB-OOH + caffeine	0.89 ± 0.02*	0.90 ± 0.02
Permeabilized cells	tB-OOH	0.20 ± 0.03	0.37 ± 0.03
	tB-OOH + RR	0.19 ± 0.02	0.23 ± 0.01*
	tB-OOH + $LaCl_3$	0.20 ± 0.02	0.22 ± 0.02*
	tB-OOH + Ry	0.21 ± 0.02	0.36 ± 0.01
	tB-OOH + $CaCl_2$	0.34 ± 0.02*	0.36 ± 0.01

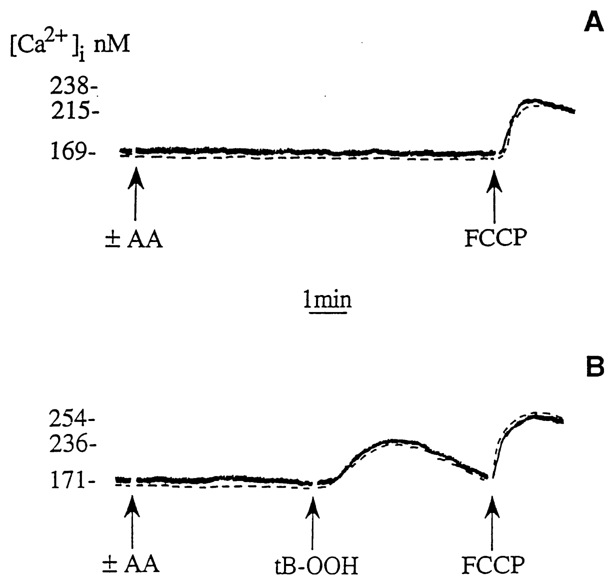

Figure 2 tB-OOH promotes mitochondrial Ca^{2+} accumulation and this process is not affected by antimycin A

Fura 2-loaded U937 cell suspensions were supplemented with saline A and incubated for 5 min at 37 °C in the absence (continuous trace) or presence (broken trace) of antimycin A (AA; 3 μM). The cells were then challenged with FCCP (10 μM) given alone (**A**) or after 5 min of pretreatment with tB-OOH (200 μM; **B**). The numbers at the left indicate $[Ca^{2+}]_i$ values. The traces are representative of 10 consistent experiments.

Ca^{2+} released by sublethal concentrations of tB-OOH was cleared by the mitochondria [20]. In the same study, mitochondrial Ca^{2+} was found to have a pivotal role in the process of strand scission of genomic DNA brought about by the hydroperoxide.

The results illustrated in Figure 2 confirm the above findings and demonstrate that antimycin A (3 μM) neither modified the basal mitochondrial Ca^{2+} content (Figure 2A) nor affected the changes in $[Ca^{2+}]_i$ or the extent of mitochondrial Ca^{2+} accumulation in response to tB-OOH (200 μM) alone (Figure 2B). In Table 3 it is shown that RR (25 μM), which inhibits the Ca^{2+} uniporter of mitochondria [26], significantly decreased the DNA damage induced by tB-OOH (200 μM) and suppressed the enhancing effects promoted by antimycin A. The effect of RR was not the consequence of possible interactions with the Ry receptor because 20 μM Ry did not modify DNA damage induced by tB-OOH alone or associated with antimycin A. We have also demonstrated [20] that caffeine, which releases Ca^{2+} from stores different from those affected by tB-OOH, further enhanced mitochondrial Ca^{2+} uptake as well as the formation of DNA lesions. Caffeine (10 mM) did not further enhance the DNA cleavage generated by tB-OOH in combination with antimycin A (Table 3).

Experiments performed in permeabilized cells led to outcomes in line with the above results. Indeed, the results in Table 3 indicate that the enhancing effects of the complex III inhibitor (1 μM) were prevented by concentrations of RR as low as 200 nM and by La^{3+} ions (100 μM), which are known to competitively inhibit mitochondrial Ca^{2+} uptake [27]. As observed in intact cells, Ry was inactive. The addition of Ca^{2+} ions (30 μM) to permeabilized cells also enhanced DNA damage induced by tB-OOH [20] and antimycin A did not further enhance this DNA-damaging response in Ca^{2+}-supplemented cells.

H_2O_2 mediates the enhancing effects of antimycin A on tB-OOH-induced DNA cleavage

The role of H_2O_2 in the antimycin A-mediated enhancement of DNA strand scission caused by tB-OOH was investigated in digitonin-permeabilized cells. In these experiments the cells were treated for 10 min with 200 μM tB-OOH in the absence or presence of 1 μM antimycin A. In Table 4 it can be seen that

Table 4 Catalase prevents the antimycin A-mediated enhancement of the tB-OOH-induced DNA strand scission

Digitonin-permeabilized cells were treated for 10 min with 200 μM tB-OOH in the absence or presence of 1 μM antimycin A and/or 10 Sigma units/ml catalase. 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 by using the alkaline elution technique. Results are means $\pm$ S.E.M. for three to five separate experiments and were significantly different from those for DNA damage generated by tB-OOH alone at *$P < 0.0001$ or **$P < 0.02$ (unpaired t test) and by the hydroperoxide associated with antimycin A at †$P < 0.0001$.

Treatment	Strand scission factor	
	− Antimycin A	+ Antimycin A
tB-OOH	0.21 ± 0.01	0.44 ± 0.012*
tB-OOH + catalase	0.18 ± 0.009**	0.26 ± 0.01**†
tB-OOH + boiled catalase	0.20 ± 0.02	0.45 ± 0.012*

Table 5 Effect of antioxidants or iron chelators on DNA strand scission induced by tB-OOH associated with antimycin A

Antimycin A-supplemented cells were treated for 30 min with 75 μM tB-OOH in the absence or presence of 10 μM DPPD, 200 μM BHT or 25 μM *o*-phenanthroline. Antimycin A (3 μM) and 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 treatment. Results are means $\pm$ S.E.M. for three to five separate experiments, and were significantly different from those for DNA damage generated by tB-OOH/antimycin A at *$P < 0.001$ (unpaired t test).

Treatment	Strand scission factor
tB-OOH + antimycin A	0.69 ± 0.03
tB-OOH + antimycin A + DPPD	0.68 ± 0.01
tB-OOH + antimycin A + BHT	0.65 ± 0.05
tB-OOH + antimycin A + *o*-phenanthroline	0.04 ± 0.003*

catalase (10 Sigma units/ml) was able to decrease significantly the extent of DNA strand scission caused by tB-OOH and afforded a much greater protection against DNA cleavage caused by the hydroperoxide in the presence of antimycin A. Under this second experimental condition, catalase almost completely inhibited the enhancing effect of antimycin A, although it is important to note that the difference between the residual damage and that caused by tB-OOH alone was still statistically significant. The addition of equivalent amounts of heat-inactivated catalase was ineffective in decreasing DNA strand scission caused by tB-OOH, either alone or associated with antimycin A.

Consistent with this conclusion are the results from experiments aimed at defining the kinetics of removal of DNA SSBs generated by tB-OOH, both in the absence (200 μM tB-OOH) and presence (75 μM tB-OOH) of 3 μM antimycin A. Under these conditions tB-OOH produces similar levels of initial damage. After treatment, the cells were allowed to repair in fresh, pre-warmed medium for increasing periods and the level of DNA SSBs was measured immediately after the damage and at later times. The kinetics of repair of alkaline elution-detected DNA breaks were superimposable in cells exposed to the organic hydroperoxide regardless of whether the initial damage was induced in the presence or absence of antimycin A ($t_{\frac{1}{2}}$ values 16.2 ± 1.5 and 15.7 ± 1.9 min respectively; means $\pm$ S.E.M., $n = 3$). Interestingly, these kinetics were basically identical with those observed in cells in which the initial damage was generated by 50 μM H_2O_2 ($t_{\frac{1}{2}}$ 15.8 ± 1.4 min; mean $\pm$ S.E.M., $n = 3$).

Finally, experimental evidence was collected that indicates that iron has a pivotal role in the induction of DNA SSBs mediated by the cocktail tB-OOH/antimycin A. Indeed, a low concentration of *o*-phenanthroline (25 μM) fully protected the cells against DNA SSB formation elicited by 75 μM tB-OOH associated with 3 μM antimycin A (Table 5). In contrast, the antioxidants DPPD and BHT did not affect this DNA-damaging response. Importantly, DNA strand scission induced by tB-OOH or H_2O_2 is also abolished by *o*-phenanthroline and is insensitive to antioxidants [19].

DISCUSSION

Antimycin A and HQNO selectively enhance the formation of DNA SSBs elicited by organic hydroperoxides (Figures 1A and 1C) and this effect does not seem to be related to non-specific interactions between the peroxides and the inhibitors and/or some subcellular component (Table 1). Rather, the effects of antimycin A or HQNO seem to depend on their ability to interfere with a biological function of the cell, most probably the respiratory chain, by preventing the electron flow from cytochrome b to cytochrome c_1. This conclusion finds experimental support in a number of different observations.

First, two agents that inhibit complex III via interaction with the same site [28], namely antimycin A and HQNO, enhance the formation of tB-OOH-induced DNA SSBs in a dose-dependent manner (Figures 1A and 1C) over the same concentration range as that in which oxygen utilization is decreased (Figures 1B and 1D). These results are consistent with the possibility that a cause–effect relationship exists between the inhibition of the electron transport at the complex III level and the elevated genotoxic response. Secondly, antimycin A, as well as HQNO, failed to potentiate DNA strand scission promoted by tB-OOH in the absence of glucose (Table 2). The glucose dependence of this response is well correlated with the notion that the rate of electron transport is also glucose-dependent (see the Results section) and thus implies that the complex III inhibitor-mediated enhancement of DNA SSBs induced by tB-OOH requires active electron transport. This inference is further supported by the observation that the complex I inhibitor rotenone, although not affecting DNA strand scission caused by tB-OOH alone, abolishes the enhancing effects of both antimycin A and HQNO (Table 2). Along the same lines, antimycin A and HQNO separately failed to enhance DNA single-strand breakage induced by tB-OOH in respiration-deficient U937 cells (Figures 1A and 1C). Taken together, these results lead to the conclusion that antimycin A and HQNO enhance DNA damage caused by tB-OOH via their specific interaction with the mitochondrial respiratory chain, i.e. by preventing the electron transport from cytochrome b to cytochrome c_1.

The results presented here also demonstrate that the enhancing effect of antimycin A is Ca^{2+}-dependent because this response was abolished by inhibitors of mitochondrial Ca^{2+} uptake in intact as well as in permeabilized cells (Table 3). Importantly, antimycin A did not affect the extent of mitochondrial Ca^{2+} accumulation evoked by tB-OOH (Figure 2B). DNA strand scission induced by tB-OOH can be enhanced by caffeine [20], an agent that promotes the release of Ca^{2+} from the Ry receptor and the subsequent accumulation of the cation at the mitochondrial level. We now show that caffeine does not further enhance DNA cleavage generated by tB-OOH in antimycin A-supplemented cells (Table 3). Thus it seems that the mitochondrial formation of

tB-OOH-derived DNA-damaging species is Ca^{2+}-dependent and that the extent of this response can be maximally enhanced either via enforced mitochondrial Ca^{2+} uptake (e.g. with caffeine) or via inhibition of complex III.

A series of experiments is also presented that lead to the conclusion that the Ca^{2+}-dependent mechanism whereby antimycin A potentiates the tB-OOH-induced DNA strand scission involves the mitochondrial formation of H_2O_2. This inference is supported by a number of observations that are summarized below. First, DNA damage generated by tB-OOH in the absence [19] or presence of antimycin A, although requiring a source of iron, was not mediated by lipid hydroperoxides and alkenals formed as products of membrane lipid peroxidation (Table 5). Importantly, DNA damage generated by H_2O_2 was also prevented by iron chelators and was insensitive to antioxidants [15–17,19]. Secondly, DNA SSBs induced by tB-OOH alone or associated with antimycin A were removed with superimposable kinetics, identical with those obtained after treatment with H_2O_2. Thirdly, catalase was found to mitigate the DNA-damaging response evoked by tB-OOH alone and to diminish markedly the enhancing effects of antimycin A on DNA cleavage elicited by the hydroperoxide in permeabilized cells (Table 4). It is important to note, however, that a small proportion of the DNA lesions was resistant to the effects of catalase, a result that suggests the possible existence of DNA-damaging intermediates other than H_2O_2.

Taken together, these results suggest strongly that the complex III inhibitor-mediated enhancement of the tB-OOH-induced DNA single-strand breakage is largely dependent on the Ca^{2+}-dependent mitochondrial formation of H_2O_2. It is conceivable that the oxidant is generated via dismutation of the superoxide anion resulting from the oxidation of the ubiquinone site of the mitochondrial respiratory chain. This hypothesis is supported by earlier reports indicating that ubiquinone is a major source of production of reactive oxygen species in the mitochondrial respiratory chain [29,30]. Furthermore the formation of superoxides and hydrogen peroxide mediated by ubiquinone is enhanced by antimycin A and inhibited by rotenone [31–33]. We have demonstrated consistently in this study that the complex III inhibitor-mediated enhancement of tB-OOH-induced DNA SSBs is prevented by rotenone (Table 2). Finally, recent work from the Vercesi group [34,35] demonstrated that in isolated mitochondria tB-OOH promotes the formation of H_2O_2 and that the addition of Ca^{2+} ions markedly potentiates this response.

In conclusion, our results demonstrate that the respiratory chain represents a site in which DNA-damaging species are generated in tB-OOH-treated cells. Complex III inhibitors enhance DNA cleavage generated by tB-OOH and this phenomenon seems to be selective for organic hydroperoxides because it was not observed in cells challenged with reagent H_2O_2. Impairment of the ability to transport electrons from cytochrome b to cytochrome c_1, leading to the Ca^{2+}-dependent formation of hydrogen peroxide at the ubiquinone site, seems at present to be the most plausible explanation for the potentiation of tB-OOH-induced DNA single-strand breakage in complex III-inhibited cells.

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REFERENCES

1 Masaki, N., Kyle, M. E. and Farber, J. L. (1989) Arch. Biochem. Biophys. **269**, 390–399
2 Korytowski, W., Bachowski, G. J., Geiger, P. G., Lin, F. and Girotti, A. W. (1992) Biochim. Biophys. Acta **1267**, 31–40
3 Geiger, P. G., Lin, F. and Girotti, A. W. (1993) Free Radicals Biol. Med. **14**, 251–256
4 Livingston, F. R., Lui, E. M. K., Loeb, G. A. and Forman, H. J. (1992) Arch. Biochem. Biophys. **299**, 83–91
5 Bellomo, G., Martino, A., Richelmi, P., Moore, G. A., Jewell, S. A. and Orrenius, S. (1984) Eur. J. Biochem. **140**, 1–6
6 Imberti, R., Nieminen, A. L., Herman, B. and Lemasters, J. J. (1993) J. Pharmacol. Exp. Ther. **265**, 392–400
7 Wu, E. Y., Smith, M. T., Bellomo, G. and Di Monte, D. (1990) Arch. Biochem. Biophys. **282**, 358–362
8 Nieminen, A. L., Saylor, A. K., Tesfai, S. A., Herman, B. and Lemaster, J. J. (1995) Biochem. J. **307**, 99–106
9 Masaki, N., Kyle, M. E., Serroni, A. and Farber, J. L. (1989) Arch. Biochem. Biophys. **270**, 672–680
10 O'Donnel, V. and Burkitt, M. J. (1994) Biochem. J. **304**, 707–713
11 Iannone, A., Marconi, A., Zambruno, G., Giannetti, A., Vannini, V. and Tomasi, A. (1993) J. Invest. Dermatol. **101**, 59–63
12 Barr, D. P. and Mason, R. P. (1995) J. Biol. Chem. **270**, 12709–12716
13 Sandstrom, B. E. (1991) Free Radicals Res. Commun. **15**, 79–89
14 Baker, M. A. and He, S. (1991) Free Radicals Biol. Med. **11**, 563–572
15 Coleman, J. B., Gilfor, D. and Farber, J. L. (1989) Mol. Pharmacol. **36**, 193–200
16 Latour, I., Demoulin, J. B. and Buc-Calderon, P. (1995) FEBS Lett. **373**, 299–302
17 Guidarelli, A., Sestili, P., Cossarizza, A., Franceschi, C., Cattabeni, F. and Cantoni, O. (1995) J. Pharmacol. Exp. Ther. **275**, 1575–1582
18 Guidarelli, A., Brambilla, L., Rota, C., Tomasi, A., Cattabeni, F. and Cantoni, O. (1996) Biochem. J. **317**, 371–375
19 Guidarelli, A., Cattabeni, F. and Cantoni, O. (1997) Free Radical Res. **226**, 537–547
20 Guidarelli, A., Clementi, E., Sciorati, C., Cattabeni, F. and Cantoni, O. (1997) J. Pharmacol. Exp. Ther., in the press
21 Grynkiewicz, G., Poenie, M. and Tsien, R. Y. (1985) J. Biol. Chem. **260**, 3440–3450
22 Robinson, J. and Cooper, J. M. (1970) Anal. Biochem. **33**, 390–399
23 Fiskum, G., Craig, S. W., Decker, G. L. and Lehninger, A. L. (1985) Proc. Natl. Acad. Sci. U.S.A. **77**, 3430–3434
24 Kohn, K. W., Ewig, R. A. G., Erickson, L. C. and Zwelling, L. A. (1981) in DNA Repair: A Laboratory Manual of Research Procedures, vol. 1, part B (Friedberg, E. and Hanawalt, P., eds.), pp. 379–401, Marcel Dekker, New York
25 Cantoni, O., Murray, D. and Meyn, R. E. (1986) Biochim. Biophys. Acta **867**, 135–143
26 Carafoli, E. A. (1987) Annu. Rev. Biochem. **56**, 395–433
27 Thomas, C. E. and Reed, D. J. (1988) J. Pharmacol. Exp. Ther. **245**, 501–507
28 van Ark, G. and Berden, J. A. (1977) Biochim. Biophys. Acta **459**, 119–137
29 Boveris, A. and Chance, B. (1973) Biochem. J. **134**, 707–714
30 Turrens, J. F., Alexandre, A. and Lehninger, A. L. (1985) Arch. Biochem. Biophys. **237**, 408–414
31 Cadenas, E. and Boveris, A. (1980) Biochem. J. **188**, 31–37
32 Konstantinov, A. A., Peskin, A. V., Popova, E. Y., Khomutov, G. B. and Ruuge, E. K. (1987) Biochim. Biophys. Acta **894**, 1–10
33 Cino, M. and Del Maestro, R. F. (1989) Arch. Biochem. Biophys. **269**, 623–638
34 Valle, V. G. R., Fagian, M. M., Parentoni, L. S., Meinike, A. R. and Vercesi, A. E. (1993) Arch. Biochem. Biophys. **307**, 1–7
35 Castilho, R. F., Kowaltowski, A. J., Meinike, A. R., Bechara, E. J. H. and Vercesi, A. E. (1995) Free Radicals Biol. Med. **18**, 479–486