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The mechanism of the nitric oxide-mediated enhancement of *tert*-butylhydroperoxide-induced DNA single strand breakage

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1 Caffeine (Cf) enhances the DNA cleavage induced by *tert*-butylhydroperoxide (tB-OOH) in U937 cells *via* a mechanism involving Ca^{2+}-dependent mitochondrial formation of DNA-damaging species (Guidarelli *et al*., 1997b). Nitric oxide (NO) is not involved in this process since U937 cells do not express the constitutive nitric oxide synthase (cNOS).

2 Treatment with the NO donors S-nitroso-N-acetyl-penicillamine (SNAP, 10 μM), or S-nitrosoglutathione (GSNO, 300 μM), however, potentiated the DNA strand scission induced by 200 μM tB-OOH. The DNA lesions generated by tB-OOH alone, or combined with SNAP, were repaired with superimposable kinetics and were insensitive to anti-oxidants and peroxynitrite scavengers but suppressed by iron chelators.

3 SNAP or GSNO did not cause mitochondrial Ca^{2+} accumulation but their enhancing effects on the tB-OOH-induced DNA strand scission were prevented by ruthenium red, an inhibitor of the calcium uniporter of mitochondria. Furthermore, the enhancing effects of both SNAP and GSNO were identical to and not additive with those promoted by the Ca^{2+}-mobilizing agents Cf or ATP.

4 The SNAP- or GSNO-mediated enhancement of the tB-OOH-induced DNA cleavage was abolished by the respiratory chain inhibitors rotenone and myxothiazol and was not apparent in respiration-deficient cells.

5 It is concluded that, in cells which do not express the enzyme cNOS, exogenous NO enhances the accumulation of DNA single strand breaks induced by tB-OOH *via* a mechanism involving inhibition of complex III.

Keywords: NO-donors; nitric oxide; *tert*-Butylhydroperoxide; DNA single strand breakage; respiratory chain; U937 cells

Introduction

DNA cleavage caused by *tert*-butylhydroperoxide (tB-OOH) requires a source of iron (Coleman *et al*., 1989; Guidarelli *et al*., 1997a; Latour *et al*., 1995) and is mediated by species different from those involved in the cytotoxic response (Coleman *et al*., 1989; Guidarelli *et al*., 1996). Part of these species are represented by H_2O_2 generated within the mitochondria *via* a Ca^{2+}-dependent mechanism (Guidarelli *et al*., 1997b). Ca^{2+}-mobilizing agents which increase the mitochondrial accumulation of the cation elicited a parallel enhancement in the tB-OOH-induced genotoxic response (Clementi *et al*., 1998a; Guidarelli *et al*., 1997b). Similar effects were also observed using respiratory substrates which increase the electron transport and the electrochemical gradient and thus cause mitochondrial Ca^{2+} accumulation (Guidarelli *et al*., 1997c). Importantly, respiration-deficient cells were as sensitive as the parental cell line to DNA cleavage generated by tB-OOH alone, or associated with the Ca^{2+}-mobilizing agents, but were insensitive to the enhancing effects mediated by the respiratory substrates (Guidarelli *et al*., 1997c).

We recently reported that the process of tB-OOH-induced DNA strand scission is also enhanced by exogenously generated nitric oxide (NO) (Guidarelli *et al*., 1998). Although the mechanism involved in this response was not investigated in detail, we provided experimental evidence indicating that the enhancing effects of NO were not mediated by inhibition of DNA repair, a condition which would result in an enhanced net accumulation of DNA lesions, and were not dependent on inhibition of catalase, a condition which would maximize the formation of DNA lesions generated by H_2O_2.

In the present report we demonstrate that U937 cells do not express the constitutive NO synthase (cNOS), thus ruling out the possibility that the formation of endogenous NO mediates the enhancement of the tB-OOH-induced DNA single strand breakage caused by Ca^{2+}-mobilizing agents. In addition, we analyse the molecular events underlying the enhancing effects mediated by exogenously generated NO. Data are presented which document that this effect of NO is causally linked to inhibition of complex III, leading to Ca^{2+}-dependent mitochondrial formation of H_2O_2.

Methods

Materials

Fura-2 AM was purchased from Calbiochem, San Diego, CA, U.S.A. Carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP), ruthenium red (RR), ryanodine (Ry), S-nitroso-N-acetyl-penicillamine (SNAP), S-nitrosoglutathione (GSNO), N^{W}-nitro-L-arginine methylester (L-NAME), L-N-(1-iminoethyl)-ornithine (L-NIO), 2-phenyl-4,4,5,5-tetramethylimidazolin-1-oxyl-3-oxide (PTIO), tB-OOH, Cf, rotenone, myxothiazol, butylated hydroxytoluene (BHT), N,N′-diphenyl-1,4-phenylene-diamine (DPPD) and the remaining chemi-

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cals were from Sigma-Aldrich, Milano, Italy. RPMI 1640 culture medium, Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12 medium were obtained from GIBCO, Grand Island, NY, U.S.A. and foetal bovine serum, penicillin and streptomycin were from Seralab, 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 liquid scintillation fluid were purchased from Nuclepore, Pleasanton, CA, U.S.A. and Beckman, Fullerton, CA, U.S.A., respectively.

Cell culture and treatments

U937 human myeloid leukaemia cells were cultured in suspension in RPMI 1640 culture medium supplemented with 10% foetal bovine serum, penicillin (50 units ml^{-1}) and streptomycin (50 μg ml^{-1}), at 37°C in T-75 tissue culture flasks in a humidified atmosphere of 95% air-5% CO_2. CHP100 human neuroblastoma cells were grown in a 1 : 1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12 medium supplemented with 15% heat-inactivated foetal clone III, gentamycin (0.1 mg ml^{-1}), L-glutamine (2 mM).

Respiration-deficient U937 cells were isolated by culturing the cells in RPMI medium containing 400 ng ml^{-1} ethidium bromide, 110 μg ml^{-1} pyruvate and 5 μg ml^{-1} 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 to the membrane-permeant NADH-linked substrate pyruvate (5 mM) (not shown).

Stock solutions of tB-OOH, ATP and RR were freshly prepared in saline A (8.182 g l^{-1} NaCl, 0.372 g l^{-1} KCl, 0.336 g l^{-1} $NaHCO_3$ and 0.9 g l^{-1} glucose). Cf and Trolox were dissolved in distilled water or 1 M $NaHCO_3$, respectively. SNAP, GSNO, Ry, rotenone, myxothiazol, BHT, DPPD and FCCP were dissolved in 95% 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-genotoxic properties of tB-OOH. 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 peroxide exposure or after up to 24 h of post-treatment incubation in fresh culture medium.

Alkaline elution assay

Cells were labelled overnight with [*methyl*-^{14}C]-thymidine (0.05 μCi ml^{-1}) and incubated for a further 6 h in a medium containing unlabelled thymidine (1 μg ml^{-1}). After treatments, the cells were analysed for DNA damage by the alkaline elution assay, using a procedure virtually identical to that described by Kohn *et al.* (1981) with minor modifications (Cantoni *et al.*, 1986). Briefly, $3.5-4\times10^5$ cells were gently loaded onto 25 mm, 2 μm pore polycarbonate filters and then rinsed twice with 10 ml of ice-cold saline A containing 5 mM ethylenediaminetetraacetic acid (EDTA, disodium salt). Cells were lysed with 5 ml of 2% sodium dodecylsulphate, 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 dodecylsulphate (pH 12.1), at a flow rate of ca. 30 μl min^{-1}. Fractions were collected at 2 h 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 h at 60°C in 0.4 ml of 1 N HCl followed by the addition of 0.4 N NaOH (2.5 ml) and was again determined by scintillation counting. DNA was also recovered from the interior of the membrane holders after vigorous flushing with 3 ml of 0.4 N NaOH. This solution was processed for scintillation counting as described above. Strand scission factor 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).

Measurements of cyclic GMP levels

Cell suspensions were incubated for 15 min at 37°C in saline A supplemented with 0.6 mM 3-isobutyl-1-methylxanthine, with or without 200 μM of either one of the NOS inhibitors L-NAME or L-NIO, in the presence or absence of the NOS substrate L-arginine (500 μM). NOS activity was stimulated by treatment with either tB-OOH, Cf or a mixture of the two for 15 min at 37°C. As a control, the cyclic GMP formed upon stimulation for 5 min with the NO donor S-nitroso-N-acetylpenicillamine (30 μM) was also measured. Reactions were terminated by addition of ice-cold trichloroacetic acid (final concentration: 7.5%). After ether extraction, cyclic GMP levels were measured using a radioimmunoassay kit (Du Pont, Boston, MA, U.S.A.) and normalized on cellular proteins, determined using the bicinchoninic acid assay (BCA protein assay reagent; Pierce).

Conversion of L[^{3}H]arginine to L[^{3}H] citrulline

The procedure utilized is basically identical to the one described by Bredt & Snyder (1990). Briefly, the cells were harvested, washed with cold phosphate buffer and the pellet was resuspended in a homogenization buffer containing 20 mM HEPES (pH 7.2) supplemented with 320 mM sucrose, 1 mM dithiothreitol, 1 mM EDTA and a cocktail of protease inhibitors. The samples were immediately processed by adding 25 μl of cell extract (300–400 μg of protein) to 90 μl of reaction buffer (25 mM Tris, pH 7.4, supplemented with 3 μM tertrahydrobiopterin, 1 μM FAD, 1 μM FMN, 100 nM calmodulin, 1 mM NADPH, 50 μM labelled arginine) containing L[^{3}H]arginine (0.5 μCi/sample^{-1}) in the presence of 10 μl of 6 mM $CaCl_2$. The reaction was carried out for 10 min at 37°C and then stopped by addition of 2 ml of cold 50 mM HEPES, pH 5.5, supplemented with 5 mM EDTA. The solution was then applied to Dowex 50X8-400 columns converted to the basic form. L[^{3}H]citrulline not retained by the ion exchange resin, was immediately eluted and quantified with a Beckman β-counter after addition of the scintillation cocktail.

Nitrite measurements

The nitrite accumulated in the culture medium was measured using the Griess reaction, as described by Green *et al.* (1982). Standard curves with increasing concentrations of sodium nitrite were set up in parallel.

$[Ca^{2+}]_i$ 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 AM (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 thermostatted cuvette in a Perkin Elmer LS-50 fluorimeter and maintained at 37°C under continuous stirring. Traces were recorded and analysed and the $[Ca^{2+}]_i$ as well as the amounts of Ca^{2+} released from the mitochondria were quantified as previously described (Grynkiewicz *et al*., 1985).

Data analysis

All data are reported as means ± s.e.mean. Statistical analysis was conducted using the two-tailed Student's *t*-test for paired samples where appropriate, with a probability value less than 0.05 regarded as significant. The number of replicates is listed in the Figure and Table legends.

Results

Lack of involvement of NO in DNA cleavage generated by tB-OOH alone or associated with caffeine in U937 cells

The role of NO in DNA cleavage generated in U937 cells by tB-OOH alone or associated with Cf, a drug known to release Ca^{2+} from the intracellular stores *via* the Ry receptor (Pozzan *et al*., 1994), was investigated. In these experiments, the cells were exposed to 200 μM tB-OOH for 30 min in saline A and then analysed by the alkaline elution technique. As illustrated in Table 1, the hydroperoxide promoted the formation of a significant level of DNA single strand breaks. Treatment of the cultures with 10 mM Cf 5 min prior to the addition of tB-OOH and during the 30 min exposure to the hydroperoxide markedly enhanced the DNA-damaging response. The results illustrated in Table 1 also indicate that the NOS inhibitors L-NAME (0.2–3 mM) or L-NIO (200–500 μM) and the NO scavenger PTIO (50 μM) had hardly any effect on the DNA cleavage generated by tB-OOH alone or in combination with Cf.

The lack of effect of the NOS inhibitors or NO scavengers is readily explained by the observation that U937 cells do not express cNOS. This inference is supported by a number of different observations. Indeed, treatment with tB-OOH and Cf, administered alone or combined, yielded cyclic GMP values no different from those of untreated cells (Table 2). A similar lack of effect of tB-OOH and Cf was observed when the medium was supplemented with L-arginine (not shown). 0.5 μM ionomycin was also ineffective. In control experiments, addition of the NO donor SNAP (30 μM) gave appreciable increases in cyclic GMP formation. NOS activity was also determined by measuring the accumulation of nitrite in the culture medium or the conversion of L-arginine to L-citrulline. The nitrite concentration in the culture medium was quite low and was insensitive to L-NAME (200 μM) as well as to tB-OOH, Cf or a combination of both agents (Table 3). The experiments analysing the conversion of L-arginine to L-citrulline gave similar outcomes. When the same parameters were measured using the cNOS expressing CHP100 cell line (Corasaniti *et al*., 1992), the basal L-citrulline levels were more than one order of magnitude higher than those observed in U937 cells and, more

Table 2 The effect of various treatments on cyclic GMP formation in U937 cells

Treatment	*Cyclic GMP formation* (pmol/mg protein min^{-1})
Control	1.35 ± 0.14
200 μM tB-OOH	1.32 ± 0.25
10 mM Cf	1.79 ± 0.29
200 μM tB-OOH + 10 mM Cf	1.78 ± 0.10
0.5 μM ionomycin	1.35 ± 0.28
30 μM SNAP	12.23 ± 0.51*

U937 cell suspensions were pre-incubated for 15 min at 37°C in saline A supplemented with 600 mM 3-isobutyl-1-methylxanthine. cyclic GMP formation was stimulated by exposing cell suspensions to the various drugs for 15 min at 37°C. Results represent the means ± s.e.mean calculated from three separate experiments and were significantly different from control at $*P<0.0001$ (unpaired *t*-test).

Table 1 NOS inhibitors or NO scavengers do not prevent the DNA-damaging response evoked by tB-OOH alone or associated with Cf

Treatment	*Strand scission factor*
200 μM tB-OOH	0.59 ± 0.06
200 μM tB-OOH + 200 μM L-NIO	0.57 ± 0.05
200 μM tB-OOH + 500 μM L-NIO	0.58 ± 0.07
200 μM tB-OOH + 200 μM L-NAME	0.58 ± 0.04
200 μM tB-OOH + 600 μM L-NAME	0.61 ± 0.09
200 μM tB-OOH + 1 mM L-NAME	0.59 ± 0.08
200 μM tB-OOH + 3 mM L-NAME	0.54 ± 0.05
200 μM tB-OOH + 50 μM PTIO	0.58 ± 0.08
200 μM tB-OOH + 10 mM Cf	0.92 ± 0.07*
200 μM tB-OOH + 10 mM Cf + 200 μM L-NIO	0.94 ± 0.05*
200 μM tB-OOH + 10 mM Cf + 500 μM L-NIO	0.97 ± 0.09*
200 μM tB-OOH + 10 mM Cf + 200 μM L-NAME	0.89 ± 0.07*
200 μM tB-OOH + 10 mM Cf + 600 μM L-NAME	0.91 ± 0.09*
200 μM tB-OOH + 10 mM Cf + 1 mM L-NAME	0.99 ± 0.05*
200 μM tB-OOH + 10 mM Cf + 3 mM L-NAME	0.84 ± 0.11*
200 μM tB-OOH + 10 mM Cf + 50 μM PTIO	0.94 ± 0.11*

U937 cells were exposed for 5 min to 0 or 10 mM Cf and then treated for a further 30 min with tB-OOH. Where indicated, L-NAME or L-NIO were added to the cultures 5 min prior to Cf or tB-OOH. Treatment with Cf, L-NAME, L-NIO or PTIO did not produce DNA cleavage. The level of DNA strand scission was measured immediately after the treatments using the alkaline elution technique. Results represent the means ± s.e.mean calculated from three to four separate experiments and were significantly different from those for DNA damage generated by tB-OOH alone at $*P<0.001$ (unpaired *t*-test).

Table 3 The effect of various treatments on the conversion of L-arginine to L-citrulline and nitrite formation in U937 and CHP100 cells

Treatment	*L-citrulline* (pmol/mg protein min^{-1})	NO_2^- (μM)
U937 cells		
Control	143	1.04
200 μM L-NAME	130	0.98
200 μM tB-OOH	152	1.08
10 mM Cf	139	0.99
200 μM tB-OOH + 10 mM Cf	158	1.10
CHP 100 cells		
Control	1996	5.71
200 μM L-NAME	250	1.3
10 mM Cf	NT	18.09
10 mM Cf + 200 μM L-NAME	NT	3.42

The cells were treated as detailed above for 30 min at 37°C in saline A. The conversion of L[^{3}H]arginine to L[^{3}H]citrulline and the nitrite levels in the culture medium were determined as detailed in the Methods section. Results represent the mean values calculated from two separate experiments. NT = not tested.

importantly, were dramatically diminished by L-NAME (200 μM) (Table 3). Consistently, the nitrite levels in the culture medium were found to decrease in the presence of L-NAME and increase in a L-NAME-inhibitable fashion after exposure to Cf (10 mM) (Table 3).

Exogenous NO enhances DNA cleavage generated by tB-OOH via *a mechanism which does not involve formation of peroxynitrite*

The NO donor SNAP (10 μM) enhances DNA single strand breakage caused by tB-OOH in U937 cells (Figure 1A). Under both experimental conditions, DNA strand scission was abolished by the membrane-permeant iron chelator *o*-phenanthroline (25 μM), was insensitive to the antioxidants DPPD (10 μM), BHT (200 μM) and Trolox (1 mM) and was slightly mitigated by the peroxynitrite/hydroxyl radical scavenger uric acid (1 mM). The peroxynitrite scavenger L-methionine (20 mM) was not effective. The results illustrated in Figure 1B indicate that the rates of repair of alkaline elution-detected DNA breaks were superimposable in cells in which the initial damage was induced by tB-OOH alone or associated with 10 μM SNAP.

The formation of DNA-damaging species in cells exposed to tB-OOH alone or associated with SNAP is dependent on mitochondrial Ca^{2+} accumulation

The representative traces illustrated in Figure 2 indicate that 30 μM SNAP neither modified the basal mitochondrial calcium content nor affected the changes in $[Ca^{2+}]_i$ as well as the extent of mitochondrial calcium accumulation in response to tB-OOH (200 μM) alone. Table 4 summarizes the results obtained in ten separate experiments which strongly support the above observations. Similar results were obtained using SNAP concentrations in the 1–10 μM range (not shown).

Figure 3A shows that RR (25 μM), an inhibitor of the calcium uniporter of mitochondria (Carafoli, 1987), significantly reduced the DNA damage induced by tB-OOH (200 μM) and suppressed the enhancing effects promoted by 10 μM SNAP. Similar results were obtained using 300 μM GSNO. Ry, at concentrations (20 μM) inhibiting the efflux of

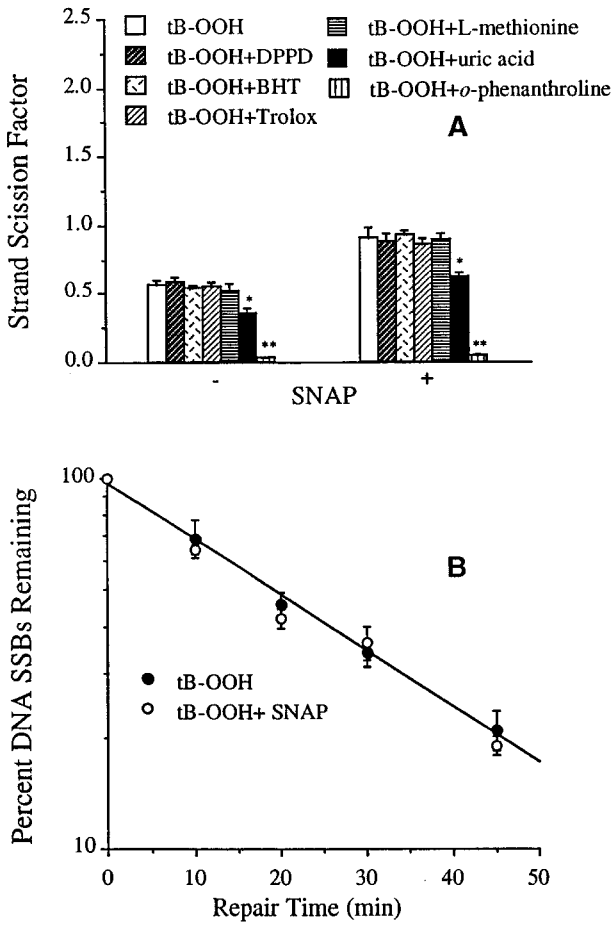

Figure 1 Characterization of the DNA lesions generated by tB-OOH in the presence of the NO donor SNAP. (A) The cells were treated for 5 min with 10 μM SNAP and then treated for a further 30 min with 200 μM tB-OOH. DPPD (10 μM), BHT (200 μM), Trolox (1 mM), *o*-phenanthroline (25 μM), uric acid (1 mM) or L-methionine (20 mM) was added to the cultures 5 min prior to addition of SNAP. The level of DNA strand scission was measured immediately after the treatments. Results represent the means ± s.e.mean calculated from three separate experiments and were significantly different from DNA damage generated by tB-OOH alone or associated with SNAP at $^*P<0.001$, $^{**}P<0.0001$ (unpaired *t*-test). SNAP significantly ($P<0.001$) enhanced DNA cleavage generated by tB-OOH. (B) The cells were exposed for 30 min to 200 μM tB-OOH alone or associated with 10 μM SNAP and then allowed to repair in fresh pre-warmed RPMI medium containing 10% foetal bovine serum. The NO donor was added to the cultures 5 min prior to addition of the hydroperoxide. The level of DNA strand scission was measured by alkaline elution immediately after the treatments as well as after various time intervals of repair. Data points are the means ± s.e.mean calculated from three separate experiments.

Ca^{2+} from the Ry receptor (Guidarelli *et al*., 1997b), did not modify DNA damage induced by tB-OOH alone or associated with SNAP or GSNO. In addition, the enhancing effects of SNAP were not additive with those of Cf or ATP (Figure 3B).

The NO-mediated enhancement of the tB-OOH-induced DNA-damaging response requires an active electron flow through complex I as well as from the reduced coenzyme Q to cytochrome c_1

The results illustrated in Figure 4 indicate that the complex I inhibitor rotenone (0.5 μM), as well as the inhibitor of the electron flow from the reduced coenzyme Q to cytochrome c_1, myxothiazol (5 μM), prevented the SNAP- as well as the

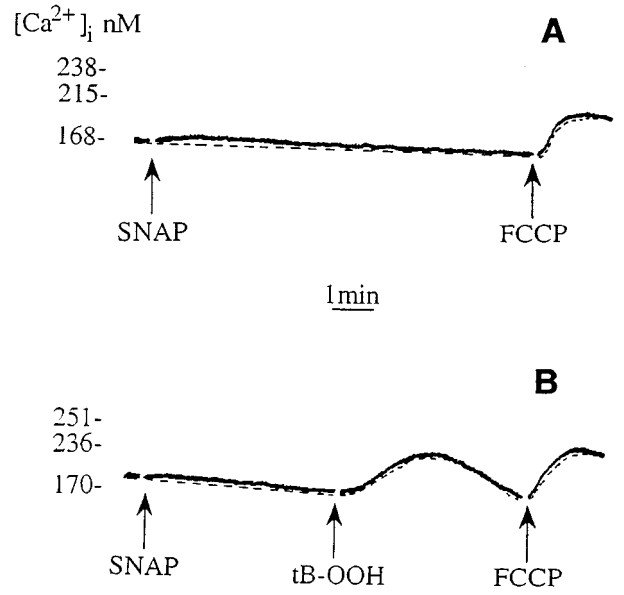

Figure 2 tB-OOH promotes mitochondrial Ca^{2+} accumulation and this process is not affected by NO. Fura-2 loaded U937 cells were incubated for 5 min in the absence (continuous trace) or presence (broken trace) of 30 μM SNAP. The cells were then challenged with FCCP (10 μM) given alone (A) or after a 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 ten consistent experiments.

Table 4 Effects of SNAP, tB-OOH or a combination of the two agents on the FCCP-sensitive mitochondrial Ca^{2+} pool

Treatment	*FCCP-induced $[Ca^{2+}]_i$ increase (% over basal)*
Control	27.8±0.15
200 μM tB-OOH	44.3±0.23*
10 μM SNAP	28.9±0.19
200 μM tB-OOH+10 μM SNAP	45.8±0.24*

Fura-2-loaded U937 cells were incubated for 10 min in saline A (control) or SNAP, prior to FCCP (30 μM administration. In the samples in which the effect of tB-OOH was analysed, the organic hydroperoxide was added 5 min before FCCP administration. Results represent the means±s.e.mean calculated from ten separate experiments and were significantly different from controls at $*P<0.0001$ (unpaired *t*-test).

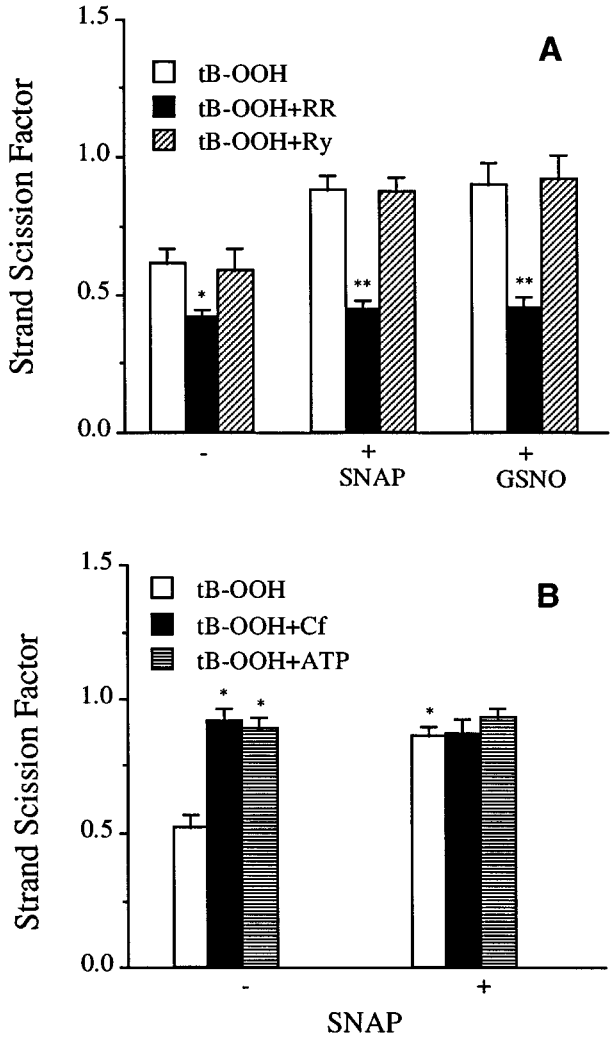

Figure 3 SNAP, or GSNO, enhances DNA single strand breakage caused by tB-OOH *via* a mechanism involving mitochondrial Ca^{2+} accumulation. (A) Cells were exposed for 5 min in saline A to 0 or 25 μM RR or 20 μM Ry, for an additional 5 min to 10 μM SNAP, or 300 μM GSNO, and then treated for a further 30 min with 200 μM tB-OOH. Treatment with RR or Ry in the absence or presence of NO donors did not produce DNA single strand breakage. Results represent the means±s.e.mean calculated from three separate experiments and were significantly different from DNA damage generated by tB-OOH alone at $*P<0.01$ or associated with NO-donors at $**P<0.001$ (unpaired *t*-test). (B) Cells were exposed for 5 min in saline A to 0 or 10 mM Cf or 1 mM ATP, for an additional 5 min to 10 μM SNAP and then treated for a further 30 min with 200 μM tB-OOH. Treatment with Cf or ATP in the absence or presence of SNAP did not produce DNA single strand breakage. Results represent the means±s.e.mean calculated from three separate experiments and were significantly different from DNA damage generated by tB-OOH alone $*P<0.001$ (unpaired *t*-test).

GSNO-mediated enhancement of the tB-OOH-induced DNA-damaging response. Furthermore, SNAP or GSNO failed to potentiate the DNA strand scission evoked by tB-OOH in respiration-deficient cells.

Discussion

Previous studies performed in our laboratory demonstrated that a portion of the tB-OOH-derived DNA-damaging species are generated within the mitochondria *via* a Ca^{2+}-dependent process (Guidarelli *et al*., 1997b). ATP (Clementi *et al*., 1998a) or Cf (Guidarelli *et al*., 1997b), which enhance the mitochondrial accumulation of the cation, promoted a parallel increase in the formation of DNA lesions. Since we recently found that exogenously generated NO also potentiates DNA cleavage induced by tB-OOH in U937 cells (Guidarelli *et al*., 1998), the involvement of NO in the process of DNA strand scission induced by tB-OOH with or without Cf was investigated. We found that the NOS inhibitors L-NAME (0.2–3 mM) or L-NIO (200–500 μM) did not prevent the DNA-damaging response evoked by tB-OOH either alone or associated with Cf (Table 1). Furthermore, using a number of experimental approaches, no evidence of cNOS activity was found in U937 cells (Tables 2 and 3).

Taken together, these results lead to the conclusion that U937 cells do not express the enzyme cNOS and therefore rule out the possibility that NO participates in reactions leading to the formation of tB-OOH-derived DNA-damaging species.

The addition of the NO donor SNAP, however, enhanced the accumulation of DNA single strand breaks in U937 cells

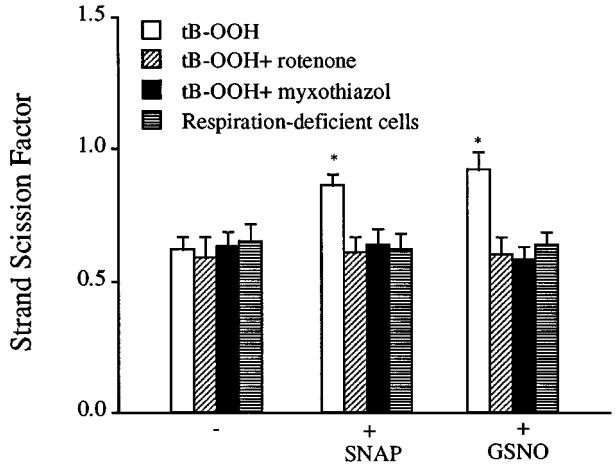

Figure 4 SNAP, or GSNO, enhances DNA single strand breakage caused by tB-OOH *via* a mechanism involving inhibition of electron transport in the respiratory chain. Respiration-proficient or -deficient cells were exposed for 30 min to 200 μM tB-OOH in saline A in the absence or presence of either 10 μM SNAP or 300 μM GSNO. The effect of 0.5 μM rotenone or 5 μM myxothiazol on these treatments in respiration-proficient cells was also investigated. In these experiments, tB-OOH was added after a 5 min pre-incubation with the NO donor and/or rotenone or myxothiazol. The level of DNA strand scission was measured immediately after the treatments. Results represent the means$\pm$s.e.mean calculated from three separate experiments and were significantly different from DNA damage generated by tB-OOH alone at $^*P<0.001$ (unpaired *t*-test).

exposed to tB-OOH (Figure 1A). We previously reported that the increased accumulation of DNA lesions mediated by NO in cells exposed to tB-OOH is not dependent on inhibition of DNA repair, since addition of SNAP to the culture medium of cells that had been pre-exposed to the hydroperoxide did not affect the rate of DNA strand break removal. The results illustrated in Figure 1B indicate that the rates of repair of alkaline elution-detected DNA breaks in cells in which the initial damage was induced by tB-OOH alone or associated with 10 μM SNAP were also superimposable. This would suggest remarkable similarities in the identity of the DNA lesions generated under these two experimental conditions. Consistent with this notion are the results indicating that the DNA strand scission caused by tB-OOH alone or associated with SNAP was abolished by the membrane-permeant iron chelator *o*-phenanthroline (25 μM) and insensitive to the anti-oxidants DPPD (10 μM), BHT (200 μM) and Trolox (1 mM) (Figure 1A). It is important to note that Trolox was previously shown to be a potent inhibitor of DNA strand scission caused by peroxynitrite in intact rat thymocytes (Salgo *et al*., 1995). DNA strand scission caused by tB-OOH alone or associated with SNAP was also insensitive to the peroxynitrite scavenger L-methionine and mitigated by an additional peroxynitrite scavenger, uric acid. It is important to note, however, that uric acid is also a potent scavenger of reactive oxygen species. The fact that uric acid was equally potent in reducing DNA cleavage generated by tB-OOH and by the cocktail tB-OOH/SNAP, in cells which do not express cNOS, strongly suggests that scavenging of reactive oxygen species rather than peroxynitrite is the mechanism whereby uric acid reduces DNA strand scission. Thus the formation of DNA lesions, regardless of whether SNAP was present or absent during treatment with tB-OOH, was iron-dependent and insensitive to anti-oxidants as well as to peroxynitrite scavengers. As a consequence, peroxynitrite is not involved in the mechanism whereby exogenous NO potentiates the formation of tB-OOH-derived DNA-damaging species. This conclusion is not surprising and indeed we had predicted the lack of involvement of peroxynitrite on the basis of our previous results obtained in cytotoxicity studies (Guidarelli *et al*., 1998). We reported that, under experimental conditions identical to those utilized in the present study, tB-OOH alone or associated with SNAP was not toxic for cultured U937 cells. In addition, we presented data indicating that SNAP prevented cell death caused by lethal (millimolar) concentrations of tB-OOH. We have taken these results as an indication that peroxynitrite, which is highly toxic for the cells, was not produced under our experimental conditions.

As reported above, the DNA cleavage generated by tB-OOH is at least in part mediated by the Ca^{2+}-dependent mitochondrial formation of DNA-damaging species (Guidarelli *et al*., 1997b). The latter mechanism was amplified by Ca^{2+}-mobilizing agents promoting mitochondrial clearance of the cation (Clementi *et al*., 1998a; Guidarelli *et al*., 1997b). Enhancement of mitochondrial Ca^{2+} accumulation is therefore a potential mechanism by which NO may increase this response. We found that SNAP neither modified the basal mitochondrial calcium content nor affected the changes in $[Ca^{2+}]_i$ or the extent of mitochondrial Ca^{2+} accumulation in response to tB-OOH alone (Figure 2). However, the inhibitor of the Ca^{2+} uniporter of mitochondria, RR, significantly reduced the DNA damage induced by tB-OOH and suppressed the enhancing effects promoted by the NO donors SNAP or GSNO (Figure 3A). The effect of RR was not the consequence of possible interactions with the Ry receptor since 20 μM Ry did not modify DNA damage induced by tB-OOH alone or associated with SNAP or GSNO. In addition, the enhancing effects of SNAP were not additive with those of other agents (e.g. Cf, ATP) which, *via* different mechanisms (Clementi *et al*., 1998a; Guidarelli *et al*., 1997b), promote mitochondrial Ca^{2+} accumulation (Figure 3B).

Thus these results, while supporting the notion that mitochondrial Ca^{2+} uptake plays a pivotal role in the formation of DNA lesions in cells exposed to tB-OOH alone, or associated with SNAP, also demonstrate that NO potentiates the genotoxic effects of the hydroperoxide *via* a mechanism which does not depend on enforced mitochondrial Ca^{2+} accumulation. The fact that the potentiating effects of SNAP were not additive with those of Cf or ATP strongly suggests that, in the presence of NO, the accumulation of mitochondrial calcium evoked by tB-OOH promotes maximal formation of tB-OOH-derived DNA-damaging species.

This situation is reminiscent of our previous results demonstrating that the complex III inhibitors antimycin A and 2-heptyl-4-hydroxyquinoline N-oxide potentiated tB-OOH-induced DNA strand scission *via* a mechanism involving inhibition of electron transport from cytochrome b to cytochrome c_1 and subsequent Ca^{2+}-dependent formation of hydrogen peroxide at the ubiquinone site (Guidarelli *et al*., 1997d). Consistent with this notion, we found that the complex I inhibitor rotenone prevented the SNAP- as well as the GSNO-mediated enhancement of the tB-OOH-induced DNA-damaging response (Figure 4). Furthermore, SNAP as well as GSNO failed to potentiate the DNA strand scission evoked by tB-OOH in respiration-deficient cells. Thus, it would appear that the effects of the NO donors are remarkably similar to those previously observed with antimycin A and 2-heptyl-4-hydroxyquinoline N-oxide (Guidarelli *et al*., 1997d), since in both cases an active electron transport through complex I was required. The ability of NO to impair electron transport in the respiratory chain has been documented and complex I as well as complexes III and IV were shown to be sensitive to

inhibition, depending on the concentration of NO and on the system utilized in the different investigations (Bolaños *et al*., 1997; Stuehr & Nathan, 1989; Stadler *et al*., 1991; Clementi *et al*., 1998b). The results presented in this study provide indirect experimental evidence suggesting that, under these specific conditions, exogenous NO does not affect complex I but inhibits complex III. Importantly, inhibitors of complexes I and IV fail to enhance the DNA damage induced by tB-OOH (Guidarelli *et al*., 1996). Complex III represents the 'weak site' of the respiratory chain and its inhibition leads to formation of superoxides and H_2O_2 (Cadenas & Boveris, 1980). The results illustrated in Figure 4 provide further experimental support for the notion that NO enhances DNA cleavage generated by tB-OOH *via* inhibition of complex III, since the enhancing effects of SNAP and GSNO were abolished by myxothiazol (0.5 μM). Myxothiazol is an inhibitor of the electron flow from the reduced coenzyme Q to cytochrome c_1 and therefore prevents the formation of ubisemiquinone (Turrens *et al*., 1985). As a corollary, ubisemiquinone appears to be the most likely electron donor in cells supplemented with exogenous NO.

In conclusion, the results presented in this study provide compelling evidence supporting the notion that the mechanism whereby NO potentiates the DNA-damaging response evoked by tB-OOH in U937 cells, which do not express the enzyme NOS, involves inhibition of complex III. Under these conditions, electrons are directly transferred to oxygen, thus promoting Ca^{2+}-dependent formation of superoxides and hydrogen peroxide. Hydrogen peroxide then migrates to the nucleus and generates DNA-damaging species *via* a transition metal-dependent Fenton-type reaction.

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