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Opposite effects of nitric oxide donors on DNA single strand breakage and cytotoxicity caused by *tert*-butylhydroperoxide

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1 The effects of three different NO donors on *tert*-butylhydroperoxide (tB-OOH)-induced DNA cleavage and toxicity were investigated in U937 cells.

2 Treatment with S-nitroso-N-acetyl-penicillamine (SNAP, 1–30 μM), while not in itself DNA-damaging, potentiated the DNA strand scission induced by 200 μM tB-OOH in a concentration-dependent fashion. The enhancing effects of SNAP were observed with two different techniques for the assessment of DNA damage. Decomposed SNAP was inactive. S-nitrosoglutathione (GSNO, 300 μM) and (Z)-1-[(2-aminoethyl)-N-(2-ammonioethyl) amino]diazen-1-ium-1,2-diolate (DETA-NO, 1 mM) also increased DNA cleavage generated by tB-OOH and these responses, as well as that mediated by SNAP, were prevented by the NO scavenger 2-phenyl-4,4,5,5-tetramethylimidazolin-1-oxyl-3-oxide (PTIO).

3 SNAP neither inhibited catalase activity nor increased the formation of DNA lesions in cells exposed to H_2O_2. Furthermore, SNAP did not affect the rate of rejoining of the DNA single strand breaks generated by tB-OOH.

4 Under the conditions utilized in the DNA damage experiments, treatment with tB-OOH alone or associated with SNAP did not cause cell death. However, SNAP as well as GSNO markedly reduced the lethal response promoted by millimolar concentrations of tB-OOH and these effects were abolished by PTIO. Decomposed SNAP was inactive.

5 It is concluded that low levels of NO donors, which probably release physiological concentrations of NO, enhance the accumulation of DNA single strand breaks in U937 cells exposed to tB-OOH. This NO-mediated effect appears to (a) not depend on inhibition of either DNA repair (which would increase the net accumulation of DNA lesions by preventing DNA single strand break removal) or catalase activity (which would also enhance the net accumulation of DNA lesions since H_2O_2 is one of the species mediating the tB-OOH-induced DNA cleavage) and (b) be caused by enforced formation of tB-OOH-derived DNA-damaging species. In contrast to these results, similar concentrations of NO prevented cell death caused by millimolar concentrations of tB-OOH. Hence, DNA single strand breakage generated by tB-OOH in the absence or presence of NO does not represent a lethal event.

Keywords: NO-donors; nitric oxide; *tert*-butylhydroperoxide; DNA single strand breakage; cytotoxicity; U937 cells

Introduction

The organic hydroperoxide *tert*-butylhydroperoxide (tB-OOH) generates DNA single strand breakage (Coleman *et al*., 1989; Latour *et al*., 1995) in the absence of detectable DNA double strand breakage (Guidarelli *et al*., 1995) via a mechanism inhibitable by iron chelators and insensitive to antioxidants (Coleman *et al*., 1989; Latour *et al*., 1995; Guidarelli *et al*., 1997a). In a previous study we demonstrated that some of the tB-OOH-derived DNA-damaging species are generated within the mitochondria via a calcium-dependent mechanism (Guidarelli *et al*., 1997c). A subtoxic, albeit DNA-damaging concentration of tB-OOH, was found to release calcium ions from intracellular stores of neutral pH and different from the endoplasmic reticulum-located inositol, 1, 4, 5-trisphosphate- and ryanodine-sensitive calcium stores, and a significant proportion of the cation was cleared by the mitochondria. Mitochondrial calcium plays a pivotal role in the process of DNA strand scission caused by tB-OOH and agents promoting further mitochondrial accumulation of the cation, elicited a parallel enhancement of the tB-OOH-induced genotoxic response (Clementi *et al*., 1998; Guidarelli *et al*., 1997c). H_2O_2 was found to be one of the species that are produced within the mitochondria via the calcium-dependent mechanism. DNA strand scission caused by tB-OOH was also increased by complex III inhibitors which induce formation of H_2O_2 via autooxidation of ubiquinone (Guidarelli *et al*., 1996; 1997b).

Taken together, these results demonstrate that the process of tB-OOH-induced DNA strand scission is the result of a complex series of events which can be modulated via either enforced accumulation of calcium ions or inhibition of complex III, but always result in an increased formation of H_2O_2.

In this study, we demonstrated that U937 cells supplemented with each of three different nitric oxide (NO) donors were more sensitive to the DNA-damaging effects of tB-OOH and displayed resistance to its cytotoxic effects. NO appeared to be responsible for both effects.

Methods

Cell culture and treatments

U937 cells were grown in RPMI 1640 culture medium (GIBCO, Grand Island, NY, U.S.A.) supplemented with

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10% foetal bovine serum (Seralab, Sussex, U.K.), penicillin (50 units ml^{-1}), and streptomycin (50 $\mu g\ ml^{-1}$), at 37°C in T-75 tissue culture flasks (Corning, Corning, NY, U.S.A.) gassed with an atmosphere of 95% air-5% CO_2. Reagent-grade chemicals, tB-OOH, H_2O_2, S-nitroso-N-acetyl-penicillamine (SNAP), S-nitrosoglutathione (GSNO), 2-phenyl-4,4,5,5,-tetramethylimidazolin-1-oxyl-3-oxide (PTIO) were obtained from Sigma-Aldrich (Milan, Italy); (Z)-1-[(2-aminoethyl)-N-(2-ammonioethyl) amino]diazen-1-ium-1,2-diolate (DETA-NO) was obtained from Alexis Corporation (Läufelfingen, Switzerland).

Stock solutions of tB-OOH, H_2O_2 and DETA-NO were freshly prepared in solution A (8.182 g l^{-1} NaCl, 0.372 g l^{-1} KCl, 0.336 g l^{-1} $NaHCO_3$ and 0.9 g^{-1} glucose). GSNO and SNAP were dissolved in water and 95% ethanol, respectively. 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 cytogenotoxic properties of tB-OOH or H_2O_2.

Light-exposed SNAP solution, which decomposed to penicillamine or N-acetylpenicillamine, was prepared by leaving SNAP in solution A at room temperature under a table lamp (100 W) for 1 day.

Cells ($2.5 \times 10^5 ml^{-1}$) were treated for 30 min in solution A (2 ml), washed and either analysed immediately for DNA damage or post-incubated for 6 h in complete medium and then analysed for cell viability. The NO donors were added to the cultures 5 min before the addition of tB-OOH.

Alkaline elution assay

Cells were labelled overnight with [methyl-^{14}C]-thymidine (0.05 $\mu Ci\ ml^{-1}$; NEN/Dupont, Boston, MA, U.S.A.) and incubated for a further 6 h in a medium containing unlabelled thymidine (1 $\mu g\ ml^{-1}$). After treatments, the cells were analysed for DNA damage by the alkaline elution assay, by a procedure virtually identical to that described by Kohn *et al.* (1981) with minor modifications (Cantoni *et al.*, 1986). Briefly, $3.5-4 \times 10^5$ cells were gently loaded onto 25 mm, 2 μm pore polycarbonate filters (Nuclepore, Pleasanton, CA, U.S.A.) and then rinsed twice with 10 ml of ice-cold solution 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 (Merck-Schuchardt, München, FRG)/0.02 M EDTA (free acid)/0.1% sodium dodecylsulphate (pH 12.1), at a flow rate of ca. 30 $\mu l\ min^{-1}$. Fractions were collected at 2 h intervals and counted in 7 ml of liquid scintillation fluid 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).

Comet assay

DNA single strand breakage in individual cells was detected by the comet assay (Singh *et al.*, 1988), with minor modifications. After treatments, U937 cells were resuspended at 2.0×10^4 cells 100 μl^{-1} in 1.0% low melting agarose in phosphate-buffered saline (PBS; 8 g l^{-1} NaCl, 1.15 g l^{-1} Na_2HPO_4 (0.2 g l^{-1} KH_2PO_4 and 0.2 g l^{-1} KCl) containing 5 mM EDTA and immediately pipetted onto agarose-coated slides. The slides were immersed in ice-cold lysing solution (2.5 M NaCl, 100 mM Tris, 1% sarkosyl, 5% dimethyl sulphoxide and 1% Triton X100 (pH 10.0)) for 60 min. The slides were placed on an electrophoretic tray with an alkaline buffer (300 mM NaOH and 1 mM EDTA) for 20 min to allow the DNA to unwind; electrophoresis was then conducted at 300 mA for 20 min in the same alkaline buffer maintained at 14°C. The slides were then washed and stained with ethidium bromide. The DNA was visualized with a Bio Rad DVC 250 confocal laser microscope (Bio Rad, Richmond, CA, U.S.A.) and the resulting images were taken and processed with a Hamamatsu chilled CCD 5985 camera (Hamamatsu Italy S.p.a., Milan, Italy) coupled with NIH Image 1.60 software. The measurements from 50 to 75 randomly selected cells per treatment condition were averaged. The extent of DNA damage at the level of the single cell was quantified by calculating the ratio between comet tail area and nucleus area (tail nucleus ratio) according to the method of Müller *et al.* (1994).

Cytotoxicity assay

After the treatments, the cells were washed with solution A and resuspended in pre-warmed RPMI 1640 culture medium before being plated into 35-mm tissue culture dishes and incubated at 37°C for 6 h. Cytotoxicity was determined by 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 with a haemocytometer. Results are expressed as the percentage of dead cells (ratio of stained cells vs the control number of cells).

Catalase activity

Cells were rinsed twice in solution A, resuspended in the same medium at a density of 1×10^7 cells ml^{-1} and finally sonicated three times on ice with a Heat Systems XL sonifer (Heat Systems, Farmingdale, NY, U.S.A.) operating at 20 W for 15 s. The resulting homogenates were centrifuged for 5 min (18,000 *g*) at 4°C. Catalase activity was assayed spectrophotometrically in the supernatant by the method of Aebi (1984).

Results

The NO donor SNAP potentiates DNA strand scission induced by tB-OOH

The effect of NO on DNA cleavage produced by the organic hydroperoxide tB-OOH in cultured U937 cells was investigated. In these experiments, the cells were exposed for 30 min to 200 μM tB-OOH, in the absence or presence of increasing concentrations of SNAP, and the level of DNA single strand breakage was then assessed by the alkaline elution assay. Treatments were performed in a glucose-containing saline and SNAP was given to the cultures 5 min before addition of the organic hydroperoxide. As illustrated in Figure 1a, SNAP increased the extent of DNA strand scission generated by a sublethal concentration of tB-OOH (see below). This effect was concentration-dependent and maximal accumulation of DNA lesions was found at more than 3 μM SNAP. Importantly,

under the treatment conditions utilized in the above experiments, SNAP was neither cytotoxic (Figure 4a) nor DNA-damaging (Figure 1). The enhancing effects of SNAP were also investigated by the comet assay, a technique which allows the measurement of DNA single strand breakage in individual cells (Singh *et al*., 1988). Figure 1b shows representative photomicrographs of cells treated as in the experiments described in Figure 1a. There were no comets in cells untreated or exposed for 30 min to 10 μM SNAP. The comet resulting from treatment with 200 μM tB-OOH was much less pronounced than that observed after exposure to the organic hydroperoxide associated with SNAP. Figure 1c shows the extent of DNA strand scission detected after these treatments and the results are in line with those obtained after alkaline elution analysis (Figure 1a).

Thus, a NO donor enhances the accumulation of DNA single strand breaks in cells exposed to tB-OOH.

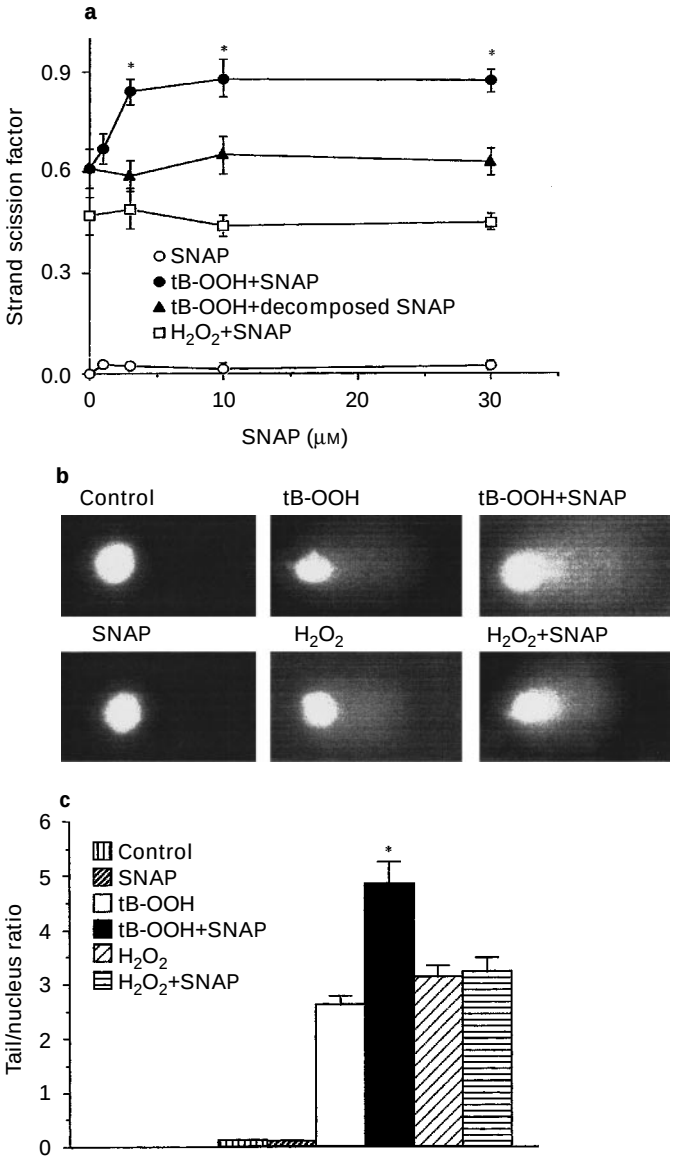

Figure 1 SNAP enhanced DNA single strand breakage caused by tB-OOH in U937 cells. (a) Cells were exposed for 5 min to increasing concentrations of SNAP and for additional 30 min to 200 μM tB-OOH or 50 μM H_2O_2. The effect of increasing concentrations of SNAP alone was also investigated. Finally, (a) also shows the results obtained after exposure to tB-OOH and light-decomposed SNAP. The level of DNA single strand breaks was measured immediately after the treatments by the alkaline elution technique, as detailed in the Methods section. Results represent the mean and vertical lines s.e.mean calculated from three separate experiments and were significantly different from DNA damage generated by tB-OOH alone at $^*P<0.0001$ (unpaired t test). (b) Representative photomicrographs of microgel-electrophoresed U937 cells treated under experimental conditions similar to those detailed in (a) and analysed for DNA damage by the comet assay. tB-OOH and H_2O_2 were used at 200 and 50 μM, respectively. SNAP was used at 10 μM. (c) Analysis of DNA single strand breakage with the comet assay in cells treated as in (b). Results represent the mean $\pm$ s.e.mean calculated from three separate experiments.$^*P<0.0001$ as compared with cells exposed to tB-OOH alone (unpaired t test).

NO is the species responsible for the enhanced accumulation of DNA lesions in cells exposed to tB-OOH

Figure 1a indicates that after 24 h of exposure of SNAP to a table lamp the enhancing effects of SNAP were abolished. In addition, 300 μM GSNO and 1 mM DETA-NO increased the formation of tB-OOH-induced DNA single strand breaks with an efficacy comparable to that observed after exposure to 10 μM SNAP (Figure 2). Finally, the results presented in Figure 2 demonstrate that 50 μM PTIO, a NO scavenger, while not affecting the DNA-damaging response evoked by 200 μM tB-OOH, fully prevented the enhancing effects mediated by 10 μM SNAP, 300 μM GSNO or 1 mM DETA-NO.

These results strongly suggest that the enhancing effects of SNAP were mediated by NO and not by other decomposition products.

Mechanism of NO-enhanced tB-OOH-induced DNA damage

Once it had been established that NO promotes an increased accumulation of tB-OOH-induced DNA single strand breaks,

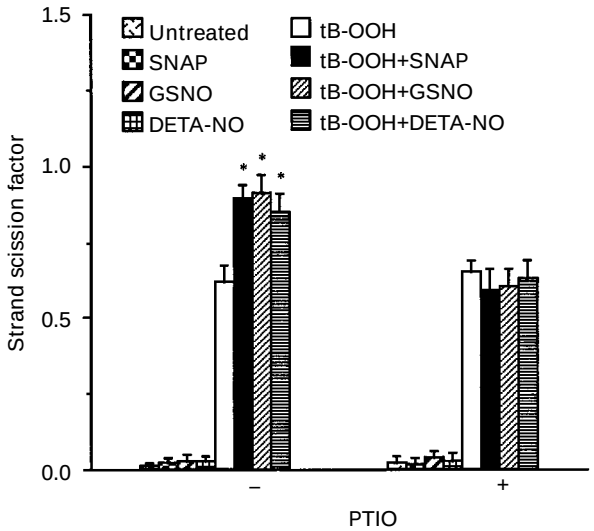

Figure 2 Three different NO donors enhanced DNA single strand breakage caused by tB-OOH in U937 cells and the NO scavenger PTIO prevented these responses. Cells were exposed for 5 min in solution A to 0 or 50 μM PTIO, for an additional 5 min to 10 μM SNAP, 300 μM GSNO or 1 mM DETA-NO and then treated for a further 30 min with 200 μM tB-OOH. The effect of the NO donors alone, both in the absence and presence of PTIO was also investigated. The level of DNA single strand breaks was measured immediately after the treatments by the alkaline elution technique. Results represent the mean $\pm$ s.e.mean calculated from three separate experiments and were significantly different from DNA damage generated by tB-OOH alone, $^*P<0.0001$ (unpaired t test).

it was important to determine whether this response was due to increased formation of DNA lesions, or resulted from either inhibition of DNA repair or impairment of the cellular defences against the tB-OOH-derived DNA-damaging species. We therefore examined the kinetics of removal of DNA single strand breaks generated by tB-OOH. For this purpose the cells were exposed to 200 μM tB-OOH for 30 min and then incubated in fresh prewarmed medium in the absence or presence of 10 μM SNAP. The extent of DNA cleavage was assessed immediately after the treatment with tB-OOH and at later times to establish the kinetics of repair of the DNA lesions. As illustrated in Figure 3, the rates of repair of alkaline elution-detected DNA single strand breaks were superimposable, regardless of whether the post-treatment incubation was performed in the absence or presence of the NO donor. Thus, these results rule out the possibility that the mechanism whereby NO promotes enhanced accumulation of DNA lesions could involve inhibition of DNA repair. Consistent with this conclusion are the results indicating that SNAP did not potentiate the DNA cleavage induced by H_2O_2 as measured by both the alkaline elution (Figure 1a) and comet (Figure 1b–c) assays.

In a previous study we demonstrated that part of the DNA cleavage generated by tB-OOH was mediated by H_2O_2 (Guidarelli *et al*., 1997c). We therefore investigated whether the mechanism whereby SNAP potentiates the DNA strand scission induced by tB-OOH involves inactivation of catalase with a concomitant increase in H_2O_2 - mediated DNA single strand breakage. The results presented in Table 1 indicate that 10 μM SNAP alone, or associated with tB-OOH, did not produce significant changes in catalase activity.

Taken together, the above results strongly suggest that NO specifically increases the formation of tB-OOH-derived DNA-damaging species.

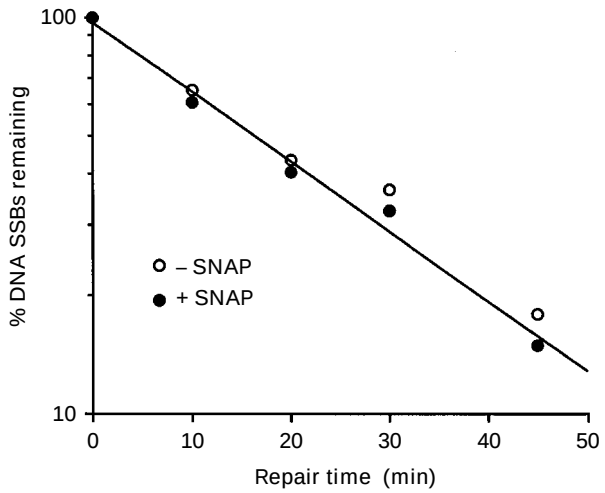

Figure 3 SNAP does not affect the rate of repair of DNA single strand breaks induced by tB-OOH. Cells were exposed for 30 min to 200 μM tB-OOH and analysed for DNA single strand breakage either immediately or after post-treatment incubation in the absence or presence of 10 μM SNAP for various time intervals. Data represent the means of two separate experiments.

Table 1 Effect of SNAP, tB-OOH or a combination of the two agents on U937 cell catalase activity

Treatment[a]	*Catalase activity*[b] *15 min*	*30 min*
Control	7.21 ± 0.42	7.32 ± 0.17
tB-OOH 200 μM	6.94 ± 0.23	7.35 ± 0.57
SNAP 10 μM	7.23 ± 0.7	7.49 ± 0.71
tB-OOH 200 μM + SNAP 10 μM	7.55 ± 0.24	7.25 ± 0.20

[a]Cells were treated in solution A and catalase activity was measured after 15 or 30 min of incubation. [b]Catalase activity was determined spectrophometrically with the method of Aebi (1984); data are expressed as Sigma units/10^6 cells and represent the, mean ± s.e.mean of at least three seperate experiments, each performed in duplicate.

NO prevents the lethal response evoked by tB-OOH

We first assessed whether the enhancement of the tB-OOH-induced genotoxic response was associated with changes in cell viability. Interestingly, treatment with 200 μM tB-OOH (the concentration used in DNA damage experiments) in the absence or presence of 10 μM SNAP did not cause loss of viability, as measured at the end of the 30 min exposure or after post-treatment incubation in fresh culture medium for up to 24 h (not shown).

The effect of the NO donors SNAP and GSNO on the lethal response evoked by tB-OOH was also investigated. For this purpose the cells were treated as in DNA damage experiments, except that the organic hydroperoxide was used at 1.5 mM. Following treatments, the cells were rinsed and post-incubated in fresh culture medium for 6 h. As illustrated in Figure 4a, exposure to 1.5 mM tB-OOH resulted in 65% cell death and this response was significantly mitigated by concentrations of SNAP in the range of 1–10 μM. Cytoprotection was not improved by increasing the concentration of SNAP to 30 μM or 300 μM (not shown). Interestingly, the SNAP-mediated protective effects were prevented by 50 μM PTIO and disappeared when the NO donor was decomposed by prolonged light exposure. Figure 4b shows the results of experiments similar to those illustrated in Figure 4a except that GSNO was used in the place of SNAP. The outcome of these experiments is basically superimposable to that obtained with SNAP. Finally, under the experimental conditions utilized in this study, neither SNAP (Figure 4a) nor GSNO (Figure 4b) was toxic for cultured U937 cells.

Thus, these results indicate that the toxicity brought about by millimolar levels of tB-OOH is markedly reduced by NO.

Discussion

Our study demonstrates that NO is a potent enhancer of the genotoxic response evoked by tB-OOH in cultured U937 cells. By using two separate and unrelated techniques for the assessment of DNA damage, we provide experimental evidence indicating that an exogenous source of NO increases the accumulation of DNA single strand breaks in cells exposed to tB-OOH (Figure 1a,b,c). The specificity of this response is emphasized by the observation that the NO donor SNAP enhances DNA strand scission caused by tB-OOH in a concentration-dependent fashion (Figure 1a) but does not affect the DNA-damaging response evoked by hydrogen peroxide (Figure 1a,b,c).

Three separate lines of evidence support the notion that the effects of SNAP are mediated by NO. Firstly, the enhancing effects of the NO donor are lost when SNAP which had previously been decomposed by prolonged light exposure, was used (Figure 1a). Secondly, high concentrations of the NO donors GSNO and DETA-NO also increased the tB-OOH-induced DNA cleavage and the extent of these responses was

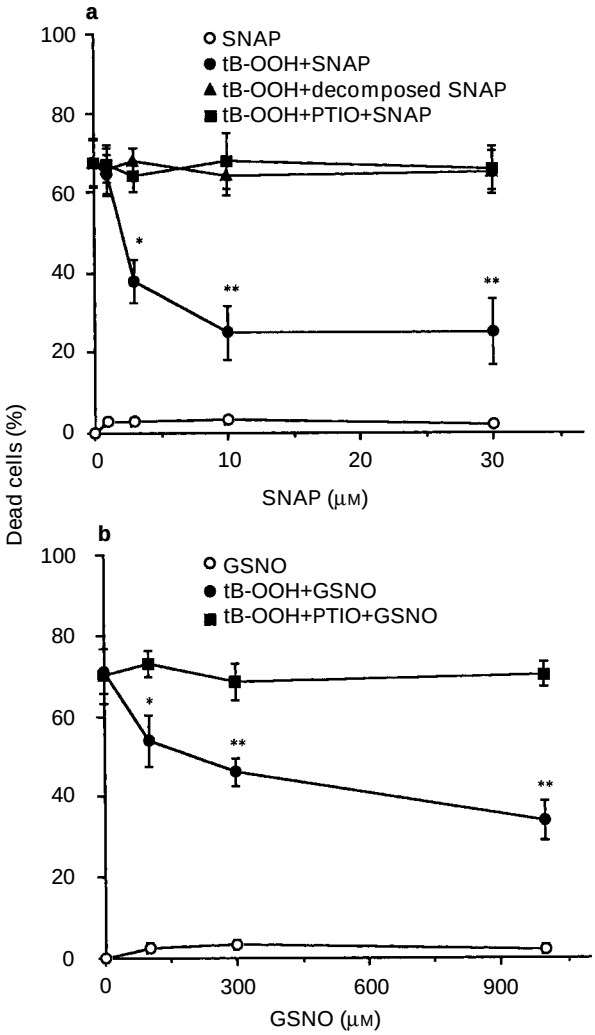

Figure 4 SNAP and GSNO prevented cell killing promoted by tB-OOH. Cells were exposed for 5 min to 0 or 50 μM PTIO, for additional 5 min to increasing concentrations of SNAP (a) or GSNO (b) and then treated for a further 30 min with 1.5 mM tB-OOH. The effect of increasing concentrations of SNAP (or GSNO) alone was also investigated. Finally, (a) also shows the results obtained after exposure to tB-OOH and light-decomposed SNAP. The relative number of dead cells was measured after 6 h of post-challenge growth by use of the trypan blue exclusion assay. Results represent the mean and vertical lines s.e.mean calculated from 3–5 separate experiments and were significantly different from cell killing generated by tB-OOH alone, $^{**}P<0.0001$; $^{*}P<0.001$ (unpaired *t* test).

similar to that observed after exposure to levels of SNAP producing maximal effects (Figure 2). Finally, the specific NO scavenger PTIO, while not affecting DNA strand scission caused by tB-OOH alone, fully prevented the enhancing effects mediated by the three different NO donors.

Thus, these results lead to the conclusion that NO is responsible for the increased accumulation of the tB-OOH-induced DNA lesions detected in cells exposed to the different NO donors.

In principle, this increased DNA damage could either be the result of an enhanced formation of tB-OOH-derived DNA-damaging species or depend on inhibition of strand break-removal. Indeed, as we previously demonstrated (Cantoni *et al*., 1987), the level of DNA single strand breaks detectable at a given time of exposure to a hydroperoxide is not only a function of the net amount of breaks generated, but also depends on the proportion of those breaks that have been repaired during treatment. We ruled out this possibility by demonstrating that SNAP does not affect the kinetics of removal of DNA single strand breaks generated by 200 μM tB-OOH (Figure 3). The results indicating that SNAP does not increase the accumulation of DNA lesions in cells exposed to hydrogen peroxide (Figure 1a,b,c) are also consistent with this conclusion. Thus, under the experimental conditions utilized in this study, NO does not result in inhibition of DNA repair, as was found in other studies (Kwon *et al*., 1991; Lepoivre *et al*., 1991; Laval & Wink, 1994; Wink & Laval, 1994).

The lack of effect of SNAP on DNA strand scission caused by hydrogen peroxide has an additional important implication, since our previous work (Guidarelli *et al*., 1997c) indicated that hydrogen peroxide mediates part of the DNA cleavage generated by tB-OOH. As a consequence, it would appear unlikely that the NO dependent accumulation of tB-OOH induced DNA lesions is caused by inhibition of enzymes involved in the inactivation of hydrogen peroxide. This conclusion is supported by the observation that SNAP did not inhibit catalase activity (Table 1). It is important to note that other studies have found that NO displays profound effects on a number of different haemoproteins (Wink *et al*., 1993; Khatsenko *et al*., 1993; Stadler *et al*., 1994; Gorbunov *et al*., 1997), including catalase (Brown, 1995; Mohazzab *et al*., 1996).

Taken together, the results thus far discussed strongly suggest that NO specifically increases the formation of tB-OOH-derived DNA-damaging species. In a recent study (Guidarelli *et al*., 1997c), we demonstrated that a large proportion of these species is generated at the level of mitochondria via a calcium-dependent process. Since agents like caffeine (Guidarelli *et al*., 1997c) and ATP (Clementi *et al*., 1997) increased the extent of mitochondrial calcium accumulation and promoted a parallel enhancement in the tB-OOH-induced genotoxic response, it is important to investigate whether a similar mechanism mediates the effects of NO. We also found that DNA damage caused by tB-OOH can be enhanced by inhibitors of complex III (Guidarelli *et al*., 1996) via a mechanism involving autooxidation of ubiquinone (Guidarelli *et al*., 1997b). This represents an additional important mechanism which may be responsible for the NO-mediated enhancement of the DNA-damaging activity of tB-OOH, since the inhibitory effects of NO and peroxynitrite in the respiratory chain are well documented (Radi *et al*., 1994; Cassina & Radi, 1996; Poderoso *et al*., 1996).

Finally, the possibility that peroxynitrite directly generates DNA strand scission should also be considered. Indeed, previous studies demonstrated that peroxynitrite is an efficient DNA-damaging agent (Beckman *et al*., 1990; Inoue & Kawanishi, 1995; Epe *et al*., 1996; Szabò, 1996). However, the latter possibility is unlikely since peroxynitrite is expected to cause cell death whereas, under the conditions utilized in the DNA damage experiments, cells retained full viability after exposure to tB-OOH both in the absence and presence of SNAP (not shown).

On the other hand, by using millimolar concentrations of tB-OOH we were able to demonstrate that NO prevents the lethal response evoked by the organic hydroperoxide. Indeed, a PTIO-inhibitable cytoprotective effect was observed with two different NO donors (Figure 4a, b) and the decomposed SNAP was inactive (Figure 4a). These results confirm previous work from other laboratories (Wink & Laval, 1994; Gorbunov *et al*., 1997) indicating that NO protects cells against killing caused by tB-OOH, an effect which is currently ascribed to nitrosylation of haeme-iron and non-haeme-iron catalytic sites (Gorbunov *et al*., 1997).

In conclusion, the results presented in this study are, to our knowledge, the first demonstration that NO enhances the formation of tB-OOH-derived DNA-damaging species. This effect was observed with very low concentrations of NO donors which probably release physiological levels of NO. Furthermore, under the conditions utilized in these experiments, neither tB-OOH nor NO, nor the combination of the two treatments, was cytotoxic. However, NO afforded a significant cytoprotection against treatment with millimolar concentrations of tB-OOH. Hence the DNA single strand breaks generated by tB-OOH both in the absence and presence of NO do not represent a lethal event.

Although the functional significance of our findings remains to be elucidated, the results presented in this study provide novel information which may be of great importance in furthering our understanding of the complex effects of NO and short chain organic hydroperoxides in an array of pathologies.

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