Rotenone and pyruvate prevent the *tert*-butylhydroperoxide-induced necrosis of U937 cells and allow them to proliferate

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Abstract Exposure of U937 cells to *tert*-butylhydroperoxide (tB-OOH) led to cyclosporin A-sensitive mitochondrial membrane permeability transition and necrosis. Pyruvate and rotenone, which increase mitochondrial NADH via different mechanisms, prevented these responses and the cells which received these treatments proliferated with kinetics similar to those observed in untreated cells. In contrast with these results, cells rescued by cyclosporin A were unable to proliferate. Thus, mitochondrial NADH plays a pivotal role in preventing upstream events which result in the onset of mitochondrial membrane permeability transition and death in cells exposed to tB-OOH. These events appear to be critical for recovery of the ability of the cells to proliferate.

Key words: *tert*-Butylhydroperoxide; Necrosis; Mitochondrial membrane permeability transition; Mitochondrial NADH

1. Introduction

Exposure of cultured mammalian cells to *tert*-butylhydroperoxide (tB-OOH) promotes an array of toxic events such as depletion of glutathione [1,2], oxidation of pyridine nucleotides [3,4], peroxidation of membrane lipids [5], perturbation of calcium ion sequestration [4], DNA single strand breakage [6–9], inhibition of glycolytic and non-glycolytic ATP synthesis [10–13] and mitochondrial dysfunction [10,12,14–16]. It is now generally accepted that the latter effect plays a pivotal role in the lethal response to a high concentration of tB-OOH since cyclosporin A, an agent which binds to cyclophillin [17], prevents the opening of permeability transition pores as well as the lethal response [12].

We previously reported that U937 cell death caused by a tB-OOH-induced loss of mitochondrial membrane potential and permeability transition is dependent upon the availability of intramitochondrial NADH [18]. Indeed, the lethal response evoked by the hydroperoxide was prevented by NADH-linked substrates (e.g. pyruvate of β-hydroxybutyrate) promoting mitochondrial formation of NADH or by the complex I inhibitor rotenone, which enhances the mitochondrial content of NADH by preventing its utilization in the respiratory chain.

In this study, we report that the mode of cell death associated with the tB-OOH-dependent permeability transition is necrosis and that its prevention mediated by either pyruvate or rotenone is not followed by delayed apoptosis. Rather, the cells surviving these treatments were able to proliferate with kinetics superimposable to those of untreated cells. These results are in contrast with those observed in cells exposed to cyclosporin A, which survived the treatment with tB-OOH but did not recover their ability to proliferate.

2. Materials and methods

2.1. Cell culture and treatments

U937 cells were grown in RPMI 1640 culture medium (Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (Seralab, Sussex, UK), penicillin (50 U/ml) and streptomycin (50 μg/ml), at 37°C in T-75 tissue culture flasks (Corning, NY, USA) in an atmosphere of 95% air-5% Co_2. Stock solutions of tB-OOH, hydrogen peroxide, 3-aminobenzamide and pyruvate were freshly prepared in saline A (0.14 M NaCl, 5 mM KCl, 4 mM $NaHCO_3$), rotenone was 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 did it affect the cytotoxic properties of tB-OOH.

Treatments were performed in saline A, both in the absence or presence of glucose (5 mM). At the treatment stage, the cell density was 2×10^5 cells/ml. Cells were used at a density of 1×10^5/ml only in those experiments involving exposure to H_2O_2.

2.2. Cytotoxicity and growth inhibition assays

After the treatments, the cells were washed with ice-cold glucose-contaning or -free saline A and resuspended in pre-warmed RPMI 1640 culture medium, plated into 35 mm tissue culture dishes and incubated at 37°C for different times. Cytotoxicity and growth inhibition were determined using the trypan blue exclusion assay. Briefly, an aliquot of the cell suspension was diluted 1:1 (v/v) with 0.4% trypan blue and the cells were counted using a haemocytometer. Results are expressed as the percentage of dead cells (ratio of stained cells versus the total number of cells) or the viable cell number, respectively.

2.3. DNA fragmentation analysis by programmable, autonomously controlled electrodes (PACE) gel electrophoresis

Cells were embedded into agarose plugs as previously described [19], except that the final cell density in the plugs was adjusted to 5×10^6/ml.

PACE electrophoresis was carried out using a Bio-Rad DR III apparatus (Bio-Rad, Richmond, CA, USA). The gels were cast using 1% (w/v) pulsed field-grade agarose in 0.5×Tris-borate-EDTA buffer (44.5 mM Tris base, 44.5 mM boric acid, 1 mM EDTA, pH 8.3). The switch time was linearly ramped from 10 to 70 s over 12 h of run time at 150 V with a 120° reorientation angle. The temperature of the running buffer (0.5×Tris-borate-EDTA buffer) was maintained at 12°C.

Gels were stained with ethidium bromide and photographed under an UV transilluminator.

2.4. DNA fragmentation analysis by the filter binding assay

Secondary DNA fragmentation was quantified using the filter binding assay developed by Bertrand et al. [20]. DNA fragmentation was determined as the fraction of the ^{3}H-labelled DNA in the lysate plus the EDTA wash versus total ^{3}H-labelled DNA.

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Abbreviations: tB-OOH, *tert*-butylhydroperoxide; PACE, programmable, autonomously controlled electrode

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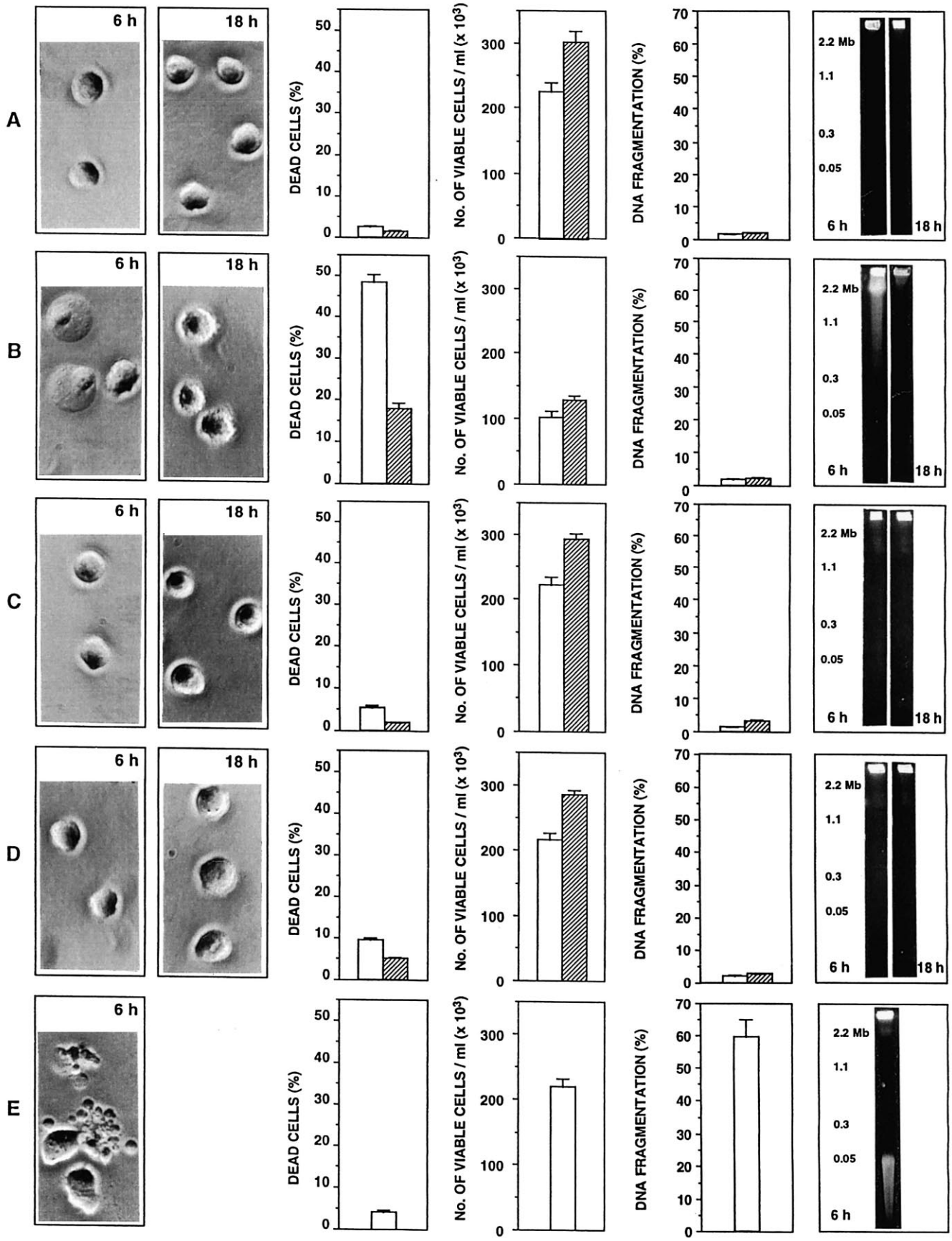
A
B
C
D
E
6 h
18 h
DEAD CELLS (%)
No. OF VIABLE CELLS / ml (x 10³)
DNA FRAGMENTATION (%)
2.2 Mb
1.1
0.3
0.05
6 h
18 h

Fig. 1. The mode of cell death induced by tB-OOH is necrosis and its prevention promoted by pyruvate or rotenone does not lead U937 cells into apoptosis. U937 cells (2×10^5/ml) were treated for 30 min in glucose-containing saline A with 1 mM tB-OOH in the absence (B) or presence of 5 mM pyruvate (C) or 0.5 μM rotenone (D) and then post-incubated in complete culture medium. After 6 and 18 h, the cells were photographed (400× magnification), counted and analyzed for cell viability (by the trypan blue exclusion assay) or secondary DNA fragmentation (using both the filter binding assay and PACE electrophoresis). Also shown are the results obtained with untreated U937 cells (A) or cells treated for 6 h with a combination of 1 mM H_2O_2 and 1 mM 3-aminobenzamide (E). Results shown in the bar graphs (samples taken after 6 h, open bars; samples taken after 18 h, striped bars) represent the mean ± S.E.M. of at least three separate determinations, each performed in duplicate. The microphotographs and the gels are representatives of three experiments with similar outcomes.
←

3. Results and discussion

It is well known that the progression through necrotic or apoptotic cell death is characterized by alterations in cell morphology and specific nuclear changes [21]. In particular, microscopic examination of morphological changes, in conjunction with the analysis of DNA fragmentation by pulsed field and conventional gel electrophoresis, can provide information about the mode of cell death. The morphological alterations induced in U937 cells by a 30 min exposure to 1 mM tB-OOH, followed by growth in fresh culture medium for 6 h, were mainly represented by swelling of the cells followed by loss of nuclear material (Fig. 1B), with no evidence of chromatin condensation or fragmentation. The onset of these morphological changes occurred after 4 h of post-treatment incubation (not shown). Conventional electrophoretic analysis of the DNA from cells treated for 30 min with 1 mM tB-OOH and then grown for 6 h in fresh medium indicated the absence of internucleosomal DNA cleavage (not shown). Consistently, no DNA fragmentation was detected (Fig. 1B). Analysis of large DNA fragments by PACE gel electrophoresis revealed that treatment with tB-OOH did not result in the formation of the discrete, 50 kb paired DNA fragments which are produced during apoptotic cell death [21–23], but rather generated the smeared fragments which are indicative of necrotic cell death [22]. Under these conditions, approximately 50% of the cells had lost their ability to exclude trypan blue. When these cells were grown for a further 12 h, the relative number of viable cells increased and a much lower proportion of dead cells could be detected in the culture medium, as a result of proliferation of the undamaged cells and lysis of the damaged cells. Most of these cells displayed a normal morphology and DNA fragmentation was not detected using both the filter binding assay and PACE gel electrophoresis (Fig. 1B). These results should be compared with those illustrated in Fig. 1A, which were obtained using untreated cells. It was interesting to observe that the biochemical and morphological parameters measured in cells exposed to tB-OOH in the presence of pyruvate (Fig. 1C) or rotenone (Fig. 1D) were similar to those of untreated cells (Fig. 1A). Fig. 1C and D also shows that tB-OOH was not toxic for pyruvate- or rotenone-supplemented cells after 6 h of post-treatment incubation and that

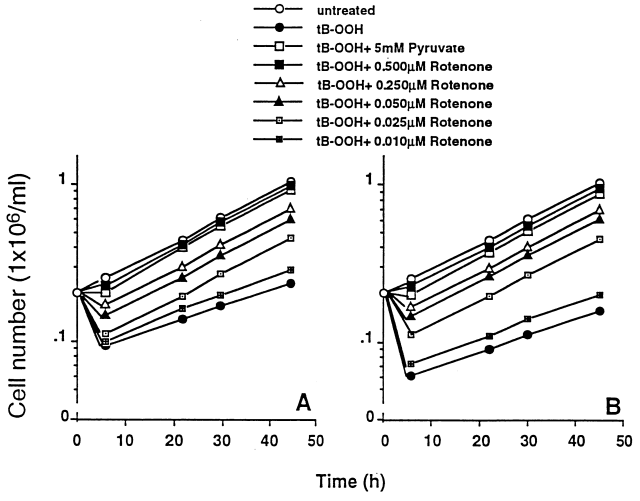

Fig. 2. Cell counts of U937 cells at various time intervals after treatment with tB-OOH in the absence or presence of rotenone and pyruvate. Cells were grown in liquid suspension culture after exposure for 30 min in glucose-containing (A) or glucose-free (B) saline A to 1 mM tB-OOH in the absence or presence of 5 mM pyruvate or increasing concentrations of rotenone. Pyruvate or rotenone, at the concentrations utilized in this study, did not affect cell growth (not shown). Results are presented as means of three experiments, each performed in duplicate. Variation between experiments was less than 5% and S.E.M.s were omitted for clarity.

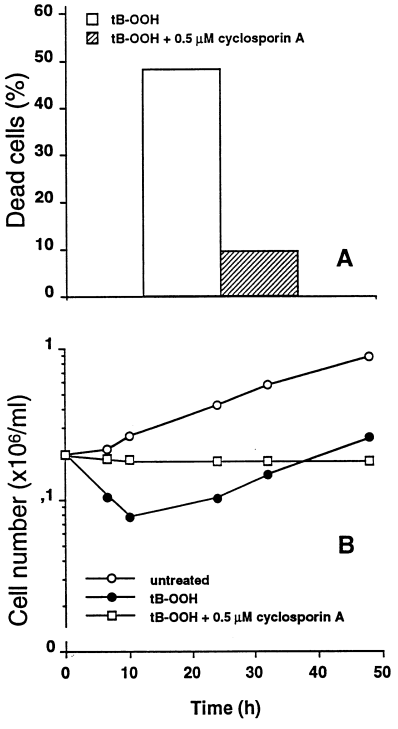

Fig. 3. Acute lethal response and proliferation rate of U937 cells after treatment with tB-OOH in the absence or presence of cyclosporin A. U937 cells (2×10^5/ml) were treated for 30 min in glucose-containing saline A with 1 mM tB-OOH, in the absence or presence of 0.5 μM cyclosporin A, and then post-incubated in complete culture medium. The number of viable and dead cells was analyzed by the trypan blue exclusion assay after increasing time intervals. In (A), it is shown that cyclosporin A suppresses the acute (6 h post-treatment incubation) lethal response evoked by tB-OOH. In (B), it is shown that the cells rescued by cyclosporin A were unable to proliferate. Results are presented as means of two separate experiments, each performed in duplicate.

the relative number of viable cells further increased after 12 h of growth.

The results illustrated in Fig. 1E were obtained using U937 cells treated with the combination 1 mM H_2O_2/1 mM 3-aminobenzamide, a condition that induces apoptosis [24]. It is clear that these cells maintained their ability to exclude trypan blue and displayed extensive blebbing as well as DNA fragmentation. Furthermore, under these conditions, the PACE technique revealed the formation of DNA fragments ≤50 kb.

Taken together, these findings strongly suggest that tB-OOH, under the experimental conditions utilized in this study, does not elicit apoptosis but rather necrosis. Both pyruvate and rotenone prevented this necrotic response and the cells did not subsequently undergo apoptosis. Thus, the NADH-linked substrate and the complex I inhibitor rescued the cells from the toxic injury inflicted by tB-OOH.

This effect was further explored by measuring the proliferation rate of cells treated for 30 min with 1 mM tB-OOH in a glucose-free or -containing saline A and then post-incubated for increasing time intervals in fresh growth medium. Under these conditions, U937 cells exhibited a short lag period and then grew in an exponential fashion for at least 48 h (Fig. 2). As previously observed [18], glucose mitigated the lethal response that was detected 6 h after exposure to tB-OOH. In agreement with the above results, 5 mM pyruvate as well as 0.5 μM rotenone abolished the cell killing promoted by exposure to hydroperoxide. Fig. 2 also shows that the cytoprotective effect of the complex I inhibitor was concentration-dependent over a range of 0.01–0.5 μM both in the presence (A) or absence (B) of glucose. Interestingly, measurement of the number of viable cells at increasing time intervals revealed similar rates of proliferation in cells which survived the treatment given by tB-OOH alone or associated with pyruvate or rotenone, both in the absence (B) and presence (A) of glucose.

Taken together, these results demonstrate that pyruvate and rotenone prevent the acute lethal response evoked by tB-OOH and that cells surviving these treatments recover their ability to proliferate. This would indicate that maintenance of adequate intramitochondrial concentrations of NADH allows the cells to fully recover from the insult generated by tB-OOH.

The results illustrated in Fig. 3A indicate that the early necrotic response evoked by tB-OOH is abolished by cyclosporin A (0.5 μM). These cells, however, were unable to proliferate and their number remained basically unchanged during the 48 h of post-treatment growth (Fig. 3B). Since a few dead cells (less than 10%) with the morphological features of necrosis were occasionally found during this period (not shown), it is reasonable to speculate that the net cell number measured during the 48 h of post-challenge growth was the result of proliferation of the undamaged cells and progressive lysis of the damaged cells.

Thus, these results indicate that cyclosporin A prevents the early necrotic response evoked by tB-OOH and that the cells serving this treatment are either unable to proliferate or undergo delayed death. As a consequence, prevention of pore opening is a condition necessary and sufficient for abolishing the acute lethal response evoked by tB-OOH, but necessary and not sufficient for recovery of cell proliferation.

It is now well-established that many agents induce apoptosis or necrosis, depending on the severity of the insult inflicted on the cells. The concept of the concentration-dependent induction of apoptotic or necrotic cell death applies very well to cells challenged with hydrogen peroxide. That H_2O_2 can trigger apoptotic cell death has been clearly demonstrated in a number of reports [25–28] and it is just as clear that the mode of cell death switches from apoptosis to necrosis by increasing the concentration of the oxidant. It may therefore be predicted that by reducing the extent of the insult, the opposite may also occur. We recently demonstrated that the necrotic response of U937 cells exposed to high concentrations of H_2O_2 can also be prevented by inhibitors of poly(ADP-ribose)polymerase via a mechanism which does not involve scavenging of reactive oxygen species or effects on peroxide metabolism [24]. Importantly, prevention of necrosis was associated with a massive apoptotic response [24]. Thus, it is reasonable to suggest that prevention of necrosis can be associated with the onset of apoptosis both via mitigation of the insult received by the cells and via interference with specific functions underlaying the necrotic response. The fact that an increase in intramitochondrial NADH rescues the cells from necrosis induced by tB-OOH and allows for the recovery of functional competence and the ability to proliferate would therefore indicate that this pyridine nucleotide promotes repair of those lesions which may potentially lead the cells into necrosis or apoptosis.

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