Free Rad. Res., Vol 34, pp. 513-527
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Peroxynitrite Promotes Mitochondrial Permeability Transition-Dependent Rapid U937 Cell Necrosis: Survivors Proliferate with Kinetics Superimposable on those of Untreated Cells

PIERO SESTILI, ILARIA TOMMASINI and ORAZIO CANTONI*

Istituto di Farmacologia e Farmacognosia, Università di Urbino, Via S. Chiara, 27, 61029 – Urbino (PU), Italy

Accepted for publication by Prof A. Azzi

(Received 16 June 2000; In final form 5 September 2000)

A short term exposure to peroxynitrite promotes a time- and concentration-dependent lethal response in U937 cells. The mode of cell death was necrosis and rapid (within minutes) cell lysis was found to occur via a mechanism involving mitochondrial permeability transition. Apoptosis was not detected in cells exposed to low levels of peroxynitrite, or in cells which survived a treatment with toxic amounts of peroxynitrite, neither after the 60 min exposure nor following increasing time intervals of growth in fresh culture medium. Rather, cells treated with peroxynitrite concentrations which were not immediately lethal, as well as the survivors of treatments with toxic levels of peroxynitrite, proliferated with kinetics superimposable on those observed in untreated cells.

Keywords: Peroxynitrite, U937 cells, cell death, necrosis, mitochondrial permeability transition

INTRODUCTION

Nitric oxide (NO) is a free radical that is endogenously produced by the enzyme NO synthase which catalyzes the oxidation of L-arginine, yielding NO and L-citrulline[1,2]. NO regulates various cell functions via cyclic GMP-dependent and independent mechanisms[3,4] and these effects are critical in the physiological regulation of nervous, immune and vascular systems. It is important to note, however, that excessive or inappropriate formation of NO might cause deleterious effects relevant in various human pathologies, such as acute endotoxemia, neurological disorders, atherosclerosis and ischemia/reperfusion[3,5]. Although NO can be directly detrimental to target cells, most of its toxic effects appear to be mediated by peroxynitrite, the coupling product of NO and superoxides[5–7]. At the molecular level, peroxynitrite causes an array of effects, including lipid peroxidation[8], protein nitration and nitrosylation[9], DNA damage[10] and oxidation of thiols[11], which most likely represent upstream events leading to inhibition of mitochondrial respiration[5,12,13], mitochondrial permeability transition[14] and/or other dysfunctions promoting cell death.

* Corresponding author: Tel: +39–0722–2671. Fax: +39–0722–327670. E-mail: cantoni@uniurb.it

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Apoptosis is the most frequently reported mode of peroxynitrite-induced cell death [10,15,16–23]; other studies, however, have shown that peroxynitrite leads to necrosis [24]or to both modes of cell death[25,26]. These discrepancies are a possible consequence of differences in the peroxynitrite concentrations utilized and/or mode of peroxynitrite administration (e.g. as a precursor or as a bolus). Additional factors which might affect the lethal response evoked by peroxynitrite are the composition and the pH of the solutions in which the cells are treated. Indeed, while specific components of the extracellular milieu can interact with peroxynitrite, changes in the pH from physiological to alkaline values can increase the half life of the oxidant, thus prolonging its activity toward target cells[27,28]. Several studies have utilized treatment conditions at pH values ranging between 8.6 and 9[15,20,23]. Finally, an important factor to take into consideration is the cell type. Astrocytes were reported to be more resistant than neurons to the toxic effects mediated by peroxynitrite[5,12]and it is generally believed that cells that produce large amounts of NO after stimulation may have some resistance mechanism against their own peroxynitrite. Thus, it appears that the toxic response and mode of cell death mediated by peroxynitrite varies in different cell types and using different treatment conditions.

The human promonocytic U937 cell line is remarkably more resistant than other cell lines to the toxic effects mediated by various sources of reactive oxygen species[29–31]. This cell line was employed to perform a carefully controlled study of the mode of cell death caused by authentic peroxynitrite. We herein report experimental evidence consistent with the notion that increasing concentrations of peroxynitrite fail to induce apoptosis in U937 cells. A proportion of these cells, however, was found to die by necrosis via a mitochondrial permeability transition-dependent mechanism. This response, and the ensuing cell lysis, was extremely rapid and the cells which survived this treatment did not undergo delayed apoptosis (or necrosis) but rather proliferated with kinetics superimposable on those observed in untreated cells. Thus, an all or nothing mechanism appears to regulate the fate of U937 cells challenged with peroxynitrite: some cells undergo an extremely fast necrotic response, whereas the remaining cells are fully viable and capable of performing energy demanding functions such as proliferation.

MATERIALS AND METHODS

Cell culture

U937 cells were cultured in suspension in RPMI 1640 medium (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, penicillin (50 units/ml) and streptomycin (50 μg/ml) (Sera-Lab Ltd., Crawley Down, England), at 37°C in T-75 tissue culture flasks (Corning, Corning, NY, USA) gassed with an atmosphere of 95% air-5% CO_2.

Chemicals

L-methionine, Trolox, *o*-phenanthroline, N, N′-diphenyl-1,4-phenylene-diamine (DPPD), as well as most of reagent grade chemicals, were obtained from Sigma-Aldrich (Milan, Italy). Rhodamine 123 was from Molecular Probes Europe (Leiden, The Netherlands). Methyl-[^{14}C]-thymidine was from NEN/Dupont (Boston, MA, USA). Cyclosporin A (CsA) was obtained from Sandoz A.G. (Bern, Switzerland). Bongkrekic acid (BA) was from Calbiochem (San Diego, CA, USA).

Synthesis of peroxynitrite and treatment conditions

Peroxynitrite was synthesized by the reaction of nitrite with acidified H_2O_2 as described by Radi et al.[8] and MnO_2 (1 mg/ml) was added to the

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mixture for 30 min at 4 °C to eliminate the excess of H_2O_2. MnO_2 was removed by centrifugation and filtration through 0.45 μm pore microfilters. The solution was frozen at −80°C for 24 h. The concentration of peroxynitrite, which forms a yellow top layer due to freeze fractionation, was determined spectrophotometrically by measuring the absorbance at 302 nm in 1.5 M NaOH ($\varepsilon_{302} = 1670\ M^{-1} \cdot cm^{-1}$).

Treatments were performed in 2 ml of prewarmed saline A (8.182 g/l NaCl, 0.372 g/l KCl, 0.336 g/l $NaHCO_3$ and 0.9 g/l glucose) containing 5×10^5 cells. The cell suspension was inoculated into 15 ml tubes before addition of peroxynitrite. Peroxynitrite was rapidly added on the wall of plastic tubes and mixed for few seconds to equilibrate the peroxynitrite concentration on the cell suspension; to avoid changes in pH due to the high alkalinity of the peroxynitrite stock solution, an appropriate amount of 1 N HCl was also added. In some experiments, at the end of peroxynitrite challenge, cells were centrifuged, washed, resuspended in fresh culture medium and grown for up to 48 h.

Cytotoxicity assay

Cells were exposed to peroxynitrite for the indicated times and analyzed immediately for cytotoxicity using the trypan blue exclusion assay. Briefly, an aliquot of the cell suspension was diluted 1:1 with 0.4% trypan blue and the cells were counted with a hemocytometer.

Lactate dehydrogenase release

Lactate dehydrogenase activity was measured spectrophotometrically as previously described[32] in the treatment medium as well as in the cell lysates (i.e. that released from the cells into the culture medium and that associated with the cells, respectively)

DNA fragmentation analysis by the filter-binding assay

Secondary DNA fragmentation was quantified using the filter binding assay developed by Bertrand et al.[33] with minor modifications[30]. DNA fragmentation was determined as the percent ratio of the [^{14}C]-labelled DNA eluted out of the filter *vs* the total DNA radioactivity in the sample.

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

Cells were embedded into agarose plugs as previously described by Sestili *et al.* [34]. PACE electrophoresis was carried out using a Bio Rad DRIII variable angle system (Bio Rad, Richmond, CA, USA). Briefly, the gels were cast using 1.0% w/v chromosomal grade agarose in 0.5x TBE buffer (composition of the 0.5x concentrated buffer: 44.5 mM Tris HCl, 44.5 mM boric acid, 1 mM Na_2EDTA [pH 8.3]) and run for 24 h at 160 V with a switch time linearly ramped from 10 to 150 sec, 120° reorientation angle, at a constant buffer temperature of 14°C. Gels were stained with ethidium bromide, viewed with an UV transilluminator and photographed.

Comet assay

DNA fragmentation in individual cells was detected using the comet assay[35]. After the treatments, U937 cells were resuspended at 2.0×10^4cells/100 μl in 1.0% low-melting agarose in phosphate-buffered saline (8 g/l NaCl, 1.15 g/l Na_2HPO_4, 0.2 g/l KH_2PO_4, 0.2 g/l KCl) containing 5 mM ethylenediaminetetraacetic acid (EDTA) and immediately pipetted onto agarose-coated slides. The slides were immersed in ice-cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% sarkosyl, 5% dimethyl sulfoxide and 1% Triton X100 [pH 10.0]) for 60

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min. The slides were then placed on an electrophoresis tray with an alkaline buffer (300 mM NaOH and 1 mM EDTA) and left for 20 min to allow the DNA to unwind; electrophoresis was then performed 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 using a Bio Rad DVC 250 confocal laser microscope and the resulting images were taken and processed with a Hamamatsu chilled CCD 5985 (Hamamatsu Italy S.p.a., Milan, Italy) coupled with the NIH Image 1.61 software. Under the conditions utilized, apoptotic cells can be recognized on the basis of comet shape[36]

Mitochondrial membrane potential

U937 cells ($2x10^5$/ml) were exposed for 5 min to 0 or 0.5 μM CsA and then treated with 1.2 mM peroxynitrite for 15 min in glucose- containing Saline A at 37°C. Cells were centrifuged, washed and rhodamine 123 (10 μg/ml) was added to the cells for 10 min. The cells were then washed twice with phoshate buffer saline and resuspended in 100 μl of the same buffer; 20 μl (8.0×10^4 cells) of this cell suspension was stratified on a slide and excited with an argon laser. Laser exposure was limited to brief image acquisition intervals (≤ 3 s) to minimize photo-bleaching of rhodamine 123. The analysis was performed with a Bio Rad DVC 250 confocal microscope and the resulting images were taken with a Hamamatsu 5985 CCD camera and digitally recorded on a Macintosh computer. Mitochondrial retention of rhodamine 123 was taken as an index of mitochondrial polarization[37].

Statistical analysis

All data in table and figures are expressed as means ± S.E.M. For comparison between two groups the Student's unpaired *t* test was used.

RESULTS

Peroxynitrite induces rapid U937 cell lysis

Treatment for 60 min with increasing concentrations of peroxynitrite results in a significant decrease in the number of viable cells, as measured by the trypan blue exclusion assay (Figure 1A). Since this response was associated with the appearance of very few trypan blue positive cells (Figure 1B), cell lysis occurring during the 60 min incubation seems to account for the loss of viable cells mediated by peroxynitrite (Figure 1C). This conclusion is supported by experiments showing release of lactate dehydrogenase, a parameter indicative of cell lysis (not shown). It is also important to note that rapid cell lysis was not caused by the high local peroxynitrite concentrations at the inoculation site; to avoid this problem, the peroxynitrite alkaline solution was added in the wall of the test tube, along with an appropriate amount of HCl to maintain a physiological pH, and then the cell suspension was vigorously mixed to rapidly equilibrate the peroxynitrite concentration in the extracellular milieu. Control experiments were also performed in which peroxynitrite and HCl were first added to saline A and then this solution was poured on tubes containing the U937 cell pellet; using these experimental conditions, results identical to the ones reported in Figure 1 were obtained (not shown).

The time-dependence of the effects of peroxynitrite (1.2 mM) is illustrated in Figure 2A-C. It is apparent that the kinetics underlying the decrease of the number of viable cells (A) and increase of missing cells (C) were first order with respect to time, whereas the number of trypan blue positive cells was always extremely low (B).

Two lines of evidence support the notion that the observed effects are entirely mediated by peroxynitrite: i) decomposed peroxynitrite- or vehicle-treated cells remained viable; ii) the peroxynitrite scavengers Trolox (1 mM) or

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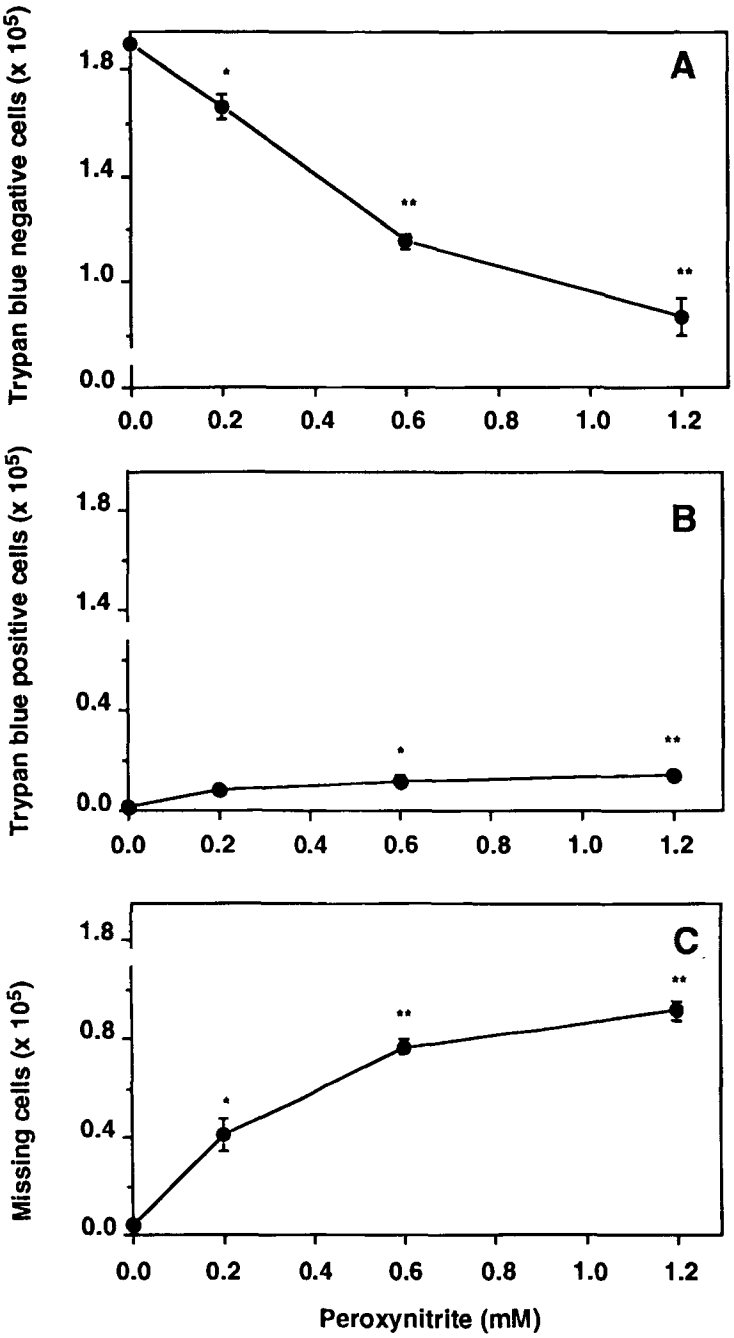

FIGURE 1 Concentration dependence for peroxynitrite-induced U937 cell death. The cells were exposed to increasing concentrations of peroxynitrite for 60 min and immediately analyzed for cytotoxicity by the trypan blue exclusion assay. Results are expressed as: A) number of viable (unstained) cells; B) number of dead (stained) cells and C) number of missing cells. The latter parameter was calculated by subtracting the number of trypan blue positive and negative cells measured in the peroxynitrite-treated cultures from the total cell number estimated in the untreated cultures. Data are expressed as mean ± S.E.M. from at least 10 separate experiments. $^{*}p<0.001$ and $^{**}p<0.0001$ as compared to control cells (unpaired *t* test)

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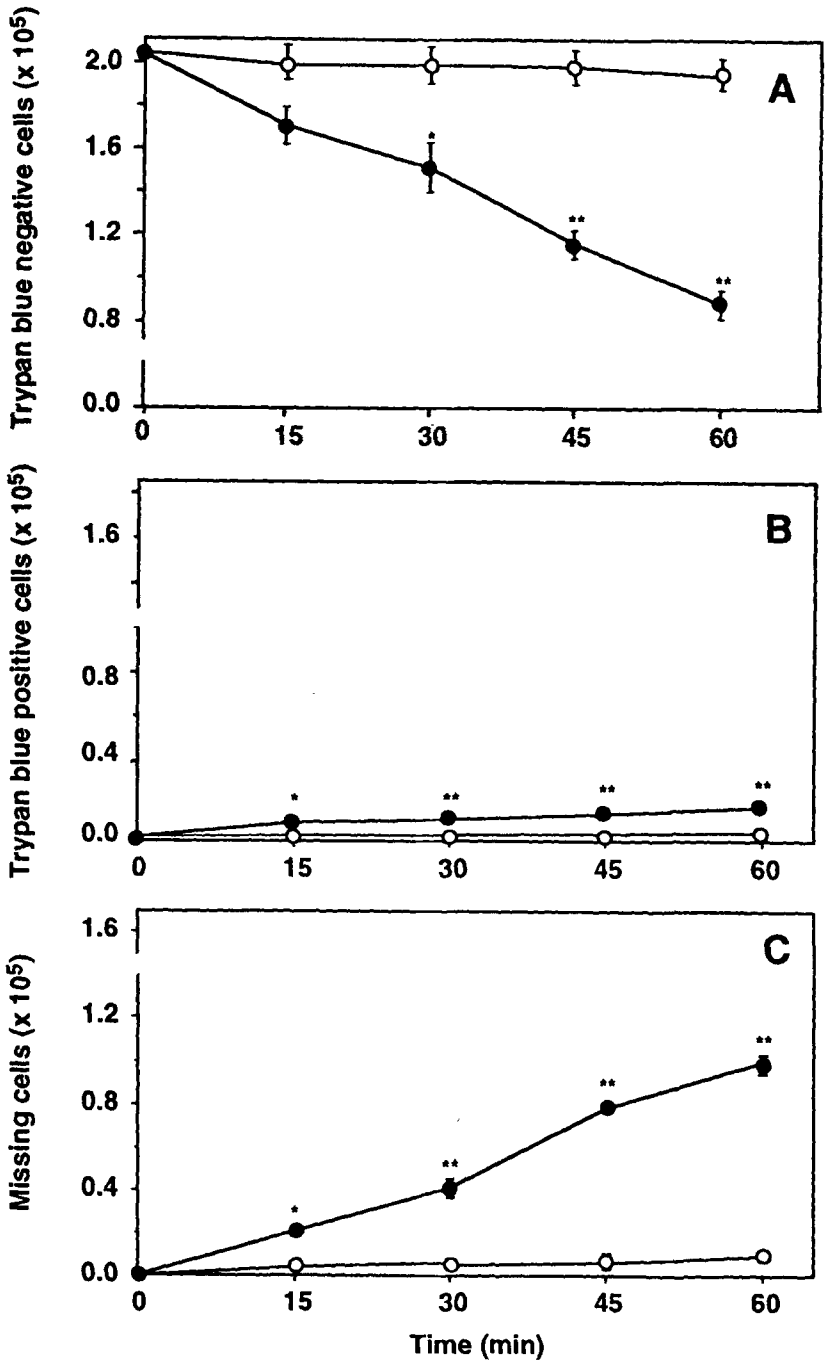

FIGURE 2 Time dependence for peroxynitrite-induced U937 cell death. U937 cells were exposed to 0 (open circles) or 1.2 mM peroxynitrite (closed circles) in Saline A for increasing time intervals and immediately analyzed for cytotoxicity by the trypan blue exclusion assay. Results shown in panels A, B and C are expressed as in Figure 1. Data represent the mean ± S.E.M. from at least 10 separate experiments. $^{*}p<0.001$ and $^{**}p<0.0001$ as compared to control cells (unpaired *t* test)

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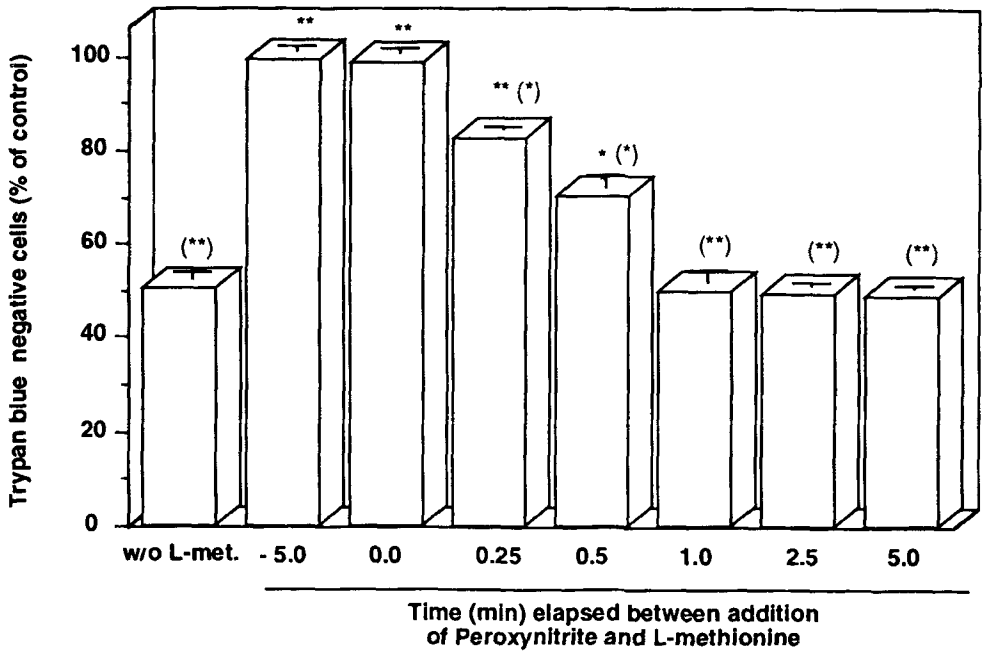

FIGURE 3 Effect of varying the time of L-methionine addition on the cytotoxic response elicited by peroxynitrite in U937 cells. Cells were treated for 60 min with 1.2 mM peroxynitrite in the absence or presence of 20 mM L-methionine. The abscissa axis indicates the time elapsed between the addition of peroxynitrite and that of L-methionine. Cytotoxicity was determined immediately after treatments by the trypan blue exclusion assay. Data are expressed as means ± S.E.M. from 5 separate experiments. $^{**}p<0.0001$ as compared to cells exposed to peroxynitrite alone; $(^{*})$ $p<0.001$ and $(^{**})$ $p<0.0001$ as compared to cells simultaneously treated with peroxynitrite and L-methionine (unpaired *t* test)

L-methionine (20 mM) prevented the lethal response evoked by 1.2 mM peroxynitrite (Table I). In the latter experiments Trolox or L-methionine were added to the cultures 5 min prior to the oxidant addition. In Figure 3 it can be seen that L-methionine was also effective when added together with peroxynitrite, although cytoprotection was found to disappear when the peroxynitrite scavenger was given to the cultures 1 min after peroxynitrite. Experiments identical to the ones illustrated in Figure 3 were performed using 0.2 and 0.6 mM peroxynitrite, with similar outcomes (not shown).

Thus, the above results demonstrate that increasing concentrations of peroxynitrite cause rapid U937 cell lysis.

TABLE I The effects of Trolox and L-methionine on the lethal response evoked by peroxynitrite

Treatment[a]	*Trypan blue negative cells (%)*[b]
–	99.1 ± 0.93
Peroxynitrite	50.3 ± 1.63
Peroxynitrite + 1 mM Trolox	98.7 ± 0.91*
Peroxynitrite + 20 mM L-methionine	99.3 ± 1.04*
Decomposed peroxynitrite	97.1 ± 1.01*
Vehicle[c]	97.3 ± 0.88*

a. *The cells were treated for 60 min in saline* A as detailed in the Methods section. Drugs were added to the cultures 5 min prior to addition of peroxynitrite (1.2 mM). Cytotoxicity was measured using the trypan blue exclusion assay.
b. Results represent the mean ± S.E.M. calculated from 3–5 separate experiments. *$p< 0.0001$ vs. treatment with peroxynitrite alone (unpaired *t* test).
c. Cells were treated with an amount of NaOH identical to that contained in peroxynitrite stock solutions, and immediately buffered with 1N HCl to pH 7.4.

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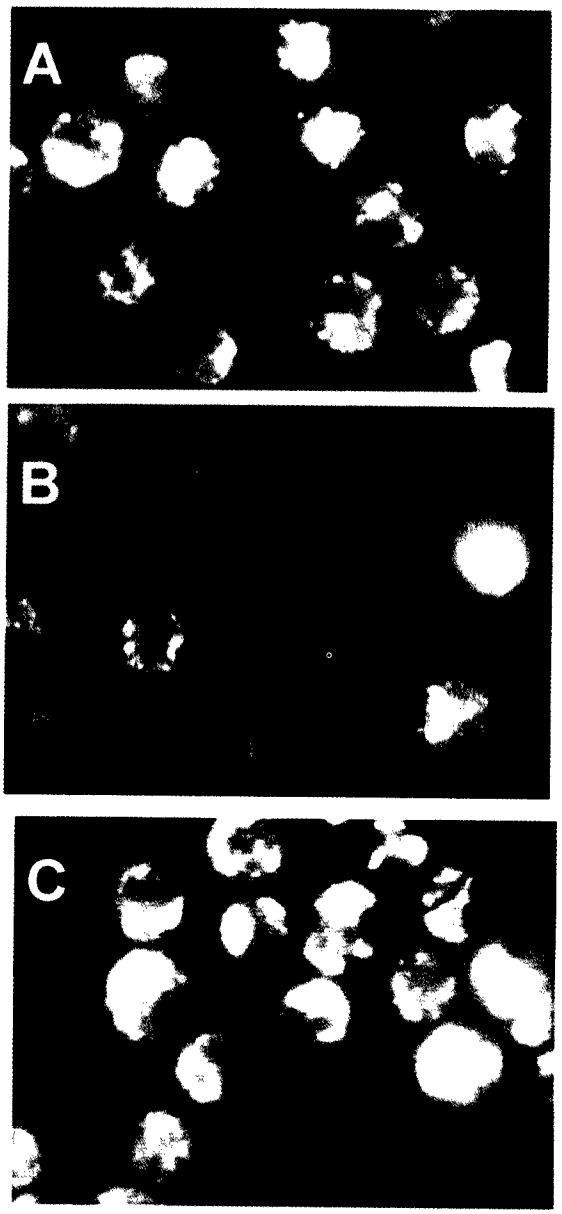

FIGURE 4 CsA prevents the peroxynitrite-induced mitochondrial depolarization. Representative micrographs of U937 cells incubated for 5 min in the absence (B) or presence of 0.5 μM CsA (C) and then treated for a further 15 min with 1.2 mM peroxynitrite. After the treatments, the cells were centrifuged, washed, and incubated for 10 min in fresh culture medium containing rhodamine 123 (10 μg/ml). After accurate washing, the cells were observed with a confocal microscope equipped with a CCD camera and images were digitally recorded with a Macintosh computer. Rhodamine 123 is taken up by the mitochondria and retained as a function of the electrochemical and proton gradient. As the potential dissipates the marker is progressively excluded. Also shown (A) is a representative micrograph of untreated cells

Mechanism of peroxynitrite-dependent U937 cell death

The uptake of rhodamine 123 was investigated with the aim of assessing the effects of peroxynitrite on mitochondrial transmembrane potential. A 10 min exposure to 1.2 mM peroxynitrite induced a marked loss of mitochondrial rhodamine uptake in some cells, whereas other cells were found to fluoresce as untreated cells. Typical images of peroxynitrite-treated cells are shown in Figure 4B (control in A). Interestingly, the effects of peroxynitrite were sensitive to CsA (0.5 μM C), an inhibitor of mitochondrial permeability transition[38]. Similar results (not shown) were obtained when CsA was replaced with BA (50 μM), a ligand of the adenine nucleotide translocator of the inner mitochondrial membrane[39]. Finally, the loss of mitochondrial rhodamine uptake induced by peroxynitrite was also prevented by the antioxidant DPPD (10 μM) and by the membrane-permeant iron chelator *o*-phenanthroline (25 μM) (not shown).

The above findings, in conjunction with the observation that the toxicity induced by 1.2 mM peroxynitrite was also blunted by CsA, BA, DPPD or *o*-phenanthroline (Figure 5), strongly suggest that peroxidation of membrane lipids and mitochondrial permeability transition play a pivotal role in peroxynitrite-induced U937 cell death. Experiments in which peroxynitrite was used at 0.2 or 0.6 mM provided similar qualitative results (not shown).

The mode of U937 cell death induced by peroxynitrite is necrosis

The fact that cell lysis occurs soon after peroxynitrite exposure strongly suggests that the mode of cell death is necrosis. Consistently, visual inspection of the cells challenged with peroxynitrite revealed that the morphological alterations were mainly represented by swelling of the cells followed by loss of membrane integrity (not shown). In some circumstances, it was

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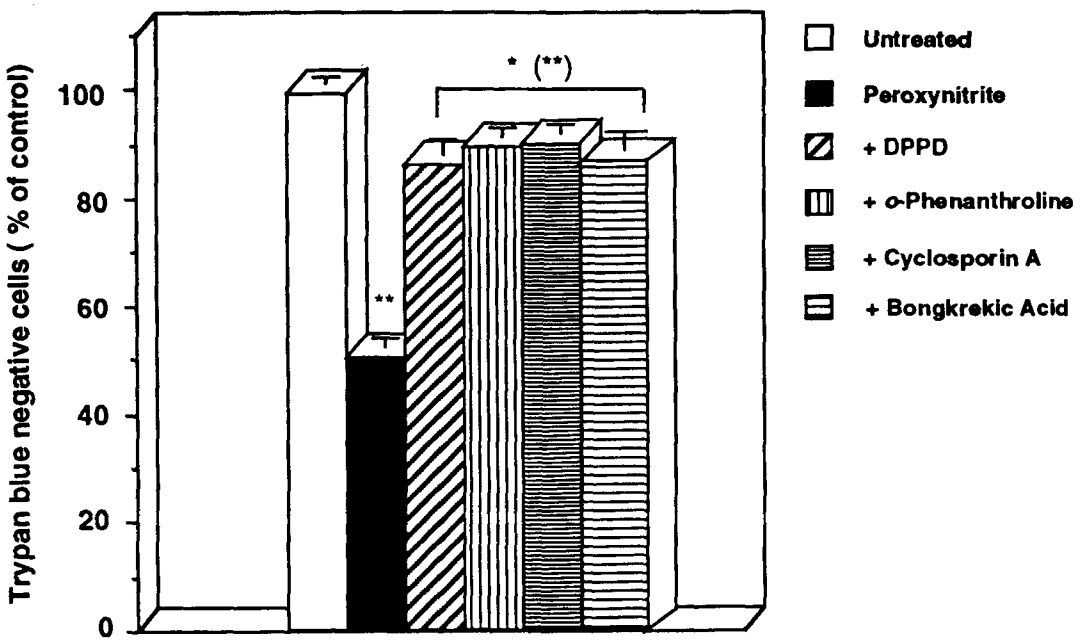

FIGURE 5 Effect of DPPD, *o*-phenanthroline, CsA or BA on the lethal response evoked by peroxynitrite in U937 cells. The cells were exposed for 5 min to saline A, 10 μM DPPD, 25 μM *o*-phenanthroline, 0.5 μM CsA or 50 μM BA and then treated for a further 60 min with 1.2 mM peroxynitrite. Cytotoxicity was determined immediately after treatments by the trypan blue exclusion assay. Data are expressed as means ± S.E.M. calculated from 3–5 separate experiments. ** $p<0.0001$ and * $p<0.01$ as compared to untreated cells; (**) $p<0.0001$ as compared to cells treated with peroxynitrite alone (unpaired *t* test)

possible to observe that this sequence of events was completed within 3–5 min. Experimental support for the notion that the mode of cell death is necrosis also derives from experiments in which U937 cells were treated with 1.2 mM peroxynitrite for 30 min (Figure 6B, B1 and B2) and then analysed for DNA fragmentation using three different techniques. The PACE technology did not provide evidence that peroxynitrite promotes formation of discrete 50 kb paired DNA fragments (B1) that are generated during the apoptotic DNA degradation[34]; rather, the smeared fragments that were obtained (B1) are typically observed in necrotic cells[34,40]. As expected, under the same conditions, secondary DNA fragmentation was not detected using the filter binding assay (B2). These results should be compared with those provided by the same analyses performed in U937 cells exposed to an apoptotic regimen (20 μM etoposide for 30 min followed by growth in fresh medium for 6h). As illustrated in Figure 6, this treatment caused a large band of ≤ 50 kB DNA fragments (E1) as well as extensive secondary DNA fragmentation (E2).

The last experimental approach utilized to detect apoptotic cells involved the use of a single-cell microgel electrophoresis technique, the comet assay. As previously reported, a comet shape characterized by a very small "head" and a large, pear-shaped, "tail" can be observed only in apoptotic cells[36]. These comets (a typical image is shown in Figure 6E) were frequently observed in preparations obtained from etoposide-treated cells. In the same preparations non apoptotic cells were also detected and these cells produced images similar to the ones obtained with untreated cells (Figure 6A). The typical

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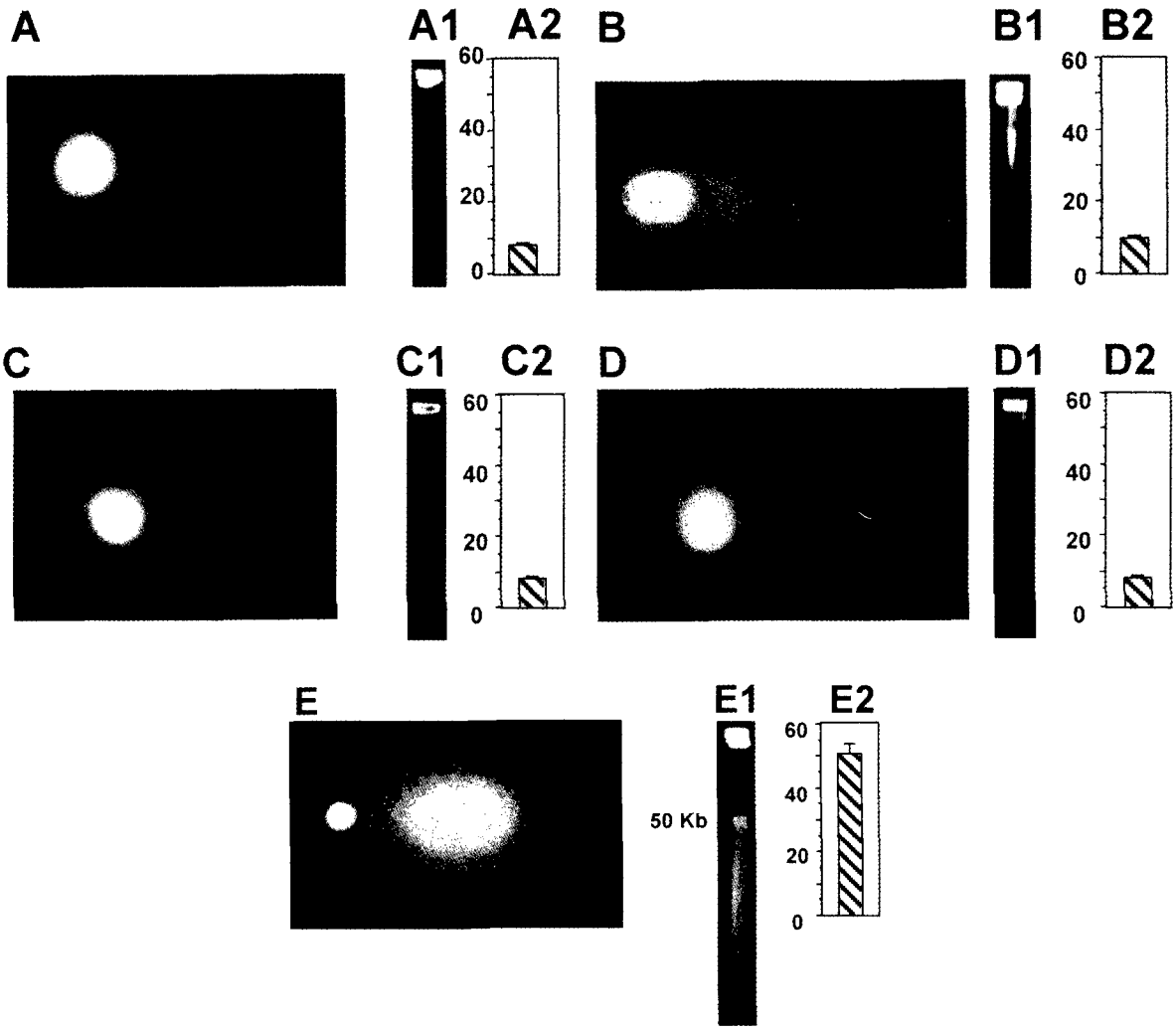

FIGURE 6 The mode of cell death induced by peroxynitrite is necrosis. The cells were exposed to 1.2 mM peroxynitrite for 30 min (B) and either analyzed immediately, or treated for 60 min and then grown in fresh culture medium for 6 (C) or 24 h (D). Also shown are the results obtained with untreated cells (A) or with cells exposed for 30 min to 20 μM etoposide and then grown for a further 6 h in drug-free culture medium (E). Analyses were performed using the comet assay (A-E), PACE electrophoresis (A1-E1) or the filter binding assay (A2-E2). The photographs shown in A-E and A1-E1 provide typical images observed in at least 3 separate experiments. Results shown in A2-E2 represent the mean ± S.E.M. calculated from 3 experiments, each performed in duplicate

image of an apoptotic cell is remarkably different from those observed in preparations of cells exposed to peroxynitrite for 30 min (Figure 6B) which present a large "tail" of DNA fragments mobilized from the "head". These findings, along with the observation that virtually all the cells treated with peroxynitrite appeared as "tailed cells", are indicative of direct DNA single strand breakage and indeed the notion that peroxynitrite causes early DNA strand scission is very well documented[10]. It is important to note that, although Figure 6 presents the results obtained with 1.2 mM peroxynitrite, lower concentrations of the oxidant (0.01, 0.03, 0.1, 0.2 and

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0.6 mM) also failed to produce biochemical evidence of apoptosis (not shown). Thus, the results reported in this section lead to the conclusion that peroxynitrite does not cause apoptosis in U937 cells but, rather, necrosis.

U937 cells surviving treatment with peroxynitrite proliferate with kinetics superimposable on those of untreated cells

Having established that the mode of cell death caused by a 60 min exposure of U937 cells to peroxynitrite is necrosis, we investigated whether this response was followed by delayed apoptosis. In these experiments the cells were treated for 60 min in saline A with 0.01, 0.03, 0.1, 0.2, 0.4, 0.6 and 1.2 mM peroxynitrite and analyzed for apoptosis using the above criteria after increasing time intervals of growth in fresh culture medium. Under all these conditions, apoptosis was never detected. Figure 6 shows typical results that were obtained in cells treated with 1.2 mM peroxyntrite and indicate the absence of DNA fragmentation after 6 (C, C1 and C2) or 24 (D, D1 and D2) h of post-challenge growth, as measured by the comet assay (C and D), PACE technology (C1 and D1) and by the filter elution assay (C2 and D2). Microscopic analysis revealed that, at these times, virtually all the cells that had been previously treated with peroxynitrite display normal morphology (not shown).

These results therefore suggest that peroxynitrite, under the experimental conditions utilized in this study, promotes a concentration-dependent necrotic response rapidly followed by cell lysis and that the cells surviving this treatment do not subsenquently undergo delayed apoptosis (or necrosis). This effect was further explored by measuring the proliferation rate of cells treated with 0.1, 0.2, 0.6 and 1.2 mM peroxynitrite, as in the above experiments. Under these conditions, untreated U937 cells exihibited a short lag period and then grew in an exponential fashion for at least 48 h (Figure 7). In agreement with the results shown in Figure 1A, levels of peroxynitrite ≥ than 0.2 mM caused an early concentration-dependent decrease in the number of viable cells. Interestingly, however, measurement of the number of viable cells at increasing time intervals after plating of these cells in complete culture medium revealed similar rates of proliferation in cells which survived the treatments with peroxynitrite. The notion that survivors proliferate as efficiently as untreated cells is well emphasized by the finding that superimposable curves describing the time-dependent increase in cell number were obtained after seeding of equal numbers of untreated cells or cells surviving the 60 min exposure to 1.2 mM peroxynitrite (Figure 7, inset). Finally, the possibility that survivors represented a subclone resistant to peroxynitrite was ruled out by the observation that, under conditions identical to those utilized in Fig. 1, the lethal response induced by peroxynitrite was remarkably similar in control cells and in cells which survived a previous treatment with peroxynitrite (not shown). The latter cell populations were obtained by using a protocol involving a 60 min exposure to 1.2 mM peroxynitrite followed by growth in fresh medium for 1, 6, 15, 24 or 48 h.

Taken together these results suggest that peroxynitrite rapidly kills U937 cells and that the survivors promptly recover their ability to proliferate.

DISCUSSION

This paper presents the results of a carefully controlled study on the mode of death induced by authentic peroxynitrite in cultured U937 cells. Particular care was taken in selecting appropriate treatment conditions to minimize events which would otherwise potentially affect the overall cellular responses. These conditions are described in detail in the Methods and Results sections and allowed exposure to peroxynitrite at a physiological pH. We could show that the

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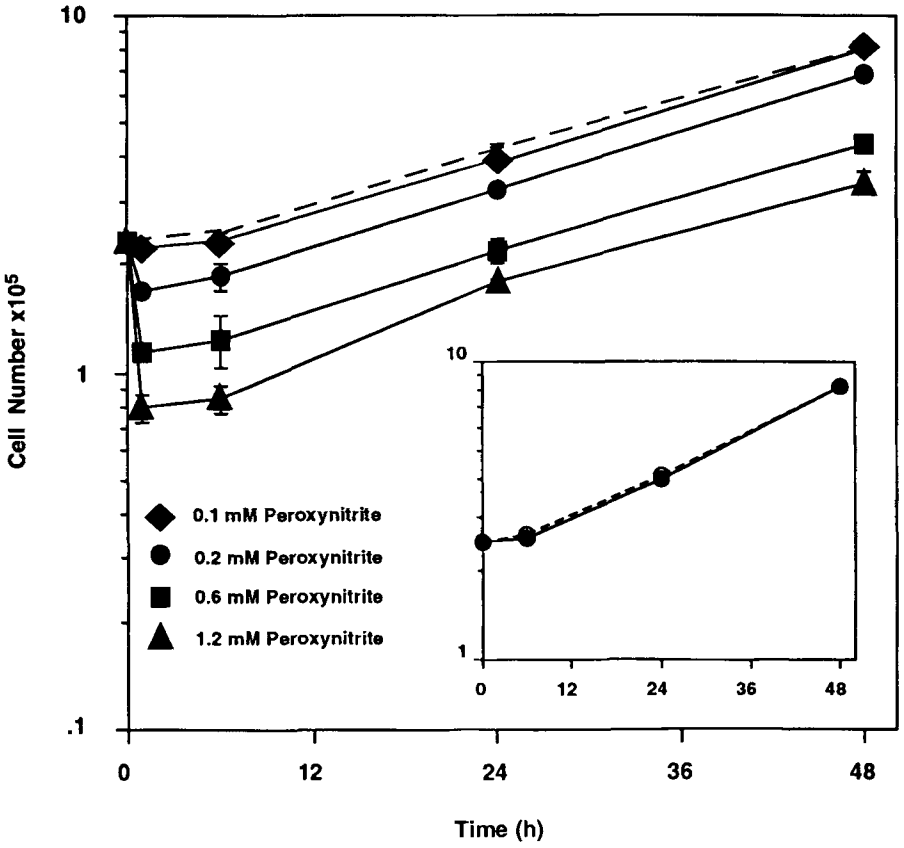

FIGURE 7 Cell counts of U937 cells at various time intervals after treatment with peroxynitrite. Cells were incubated for 60 min in the absence (dotted line) or presence of increasing concentrations of peroxynitrite, washed, resuspended in fresh culture medium and grown for up to 48 h. Also shown (inset) is the growth of control (dotted line) or peroxynitrite treated cells (1.2 mM, closed circles) seeded in the culture medium at the same density. The cell number was determined with a hemocytometer. Results are presented as means ± S.E.M. from 5 separate experiments. S.E.M. bars are in some cases covered by symbols

toxic effects observed were entirely mediated by peroxynitrite, since cell death was prevented by peroxynitrite scavengers, whereas addition of the vehicle, or decomposed peroxynitrite, did not cause toxicity (Table I). Furthermore, the effects of peroxynitrite were induced within the first min of treatment, since L-methionine was no more cytoprotective when given 1 min after peroxynitrite (Figure 3). This is consistent with the notion that peroxynitrite decomposes within seconds in a buffer at physiological pH. Other studies utilized toxicity paradigms in which peroxynitrite exposure was performed at an alkaline pH[15,20,23]and it is unclear whether the observed apoptotic response was a consenquence of the increased stability of peroxynitrite in the extracellular milieu, or rather the result of interference with cellular homeostasis and/or responses to the damage inflicted by the oxidant. In the present study, no evidence of secondary DNA fragmentation typical of apoptosis was found in cells exposed to a wide range of peroxynitrite concentrations (0.01 to 1.2 mM) and then post-incubated for increasing time intervals in fresh culture medium (Figure 6 C, C1, C2 and D, D1, D2). This was established by three different

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biochemical criteria involving the use of the comet assay, PACE technology and the filter binding assay.

We found that the mode of cell death mediated by authentic peroxynitrite in U937 cells is necrosis; this response was rather unique in that cell death occurred soon after peroxynitrite addition and was rapidly followed by cell lysis. It is also important to note that careful control experiments illustrated in the Results section ruled out the possibility that rapid cell lysis was caused by the high local peroxynitrite concentrations at the inoculation site. The following lines of evidence are consistent with the notion that the mode of peroxynitrite-induced cell death is necrosis; a) cell lysis occurs soon after peroxynitrite exposure (Figures 1 and 2); b) the morphological alterations induced by peroxynitrite were mainly represented by swelling of the cells followed by loss of membrane integrity (not shown); c) analysis of the DNA of cells treated with peroxynitrite by the PACE technology showed the formation of smeared fragments (Figure 6B1) that are typically observed in necrotic cells[34,40].

It is important to note that, although these biochemical analyses were performed in cells treated with 1.2 mM peroxynitrite, similar qualitative results were otained using lower concentrations of peroxynitrite.

We subsequently found that DPPD prevents the toxicity induced by peroxynitrite (Figure 5) in U937 cells. DPPD is a lipophilic antioxidant and the cytoprotection afforded by this treatment strongly suggests that peroxidation of membrane lipids is a key event in the above cytotoxicity paradigm. The iron chelator *o*-phenanthroline mimicked the effects of DPPD, thus indicating that iron also plays a pivotal role in this lethal response. Finally, CsA, an agent which binds to cyclophillin thereby preventing pore opening in the inner mitochondrial membrane [38], was found to afford cytoprotection (Figure 5) in cells treated with peroxynitrite. Similar results were obtained using BA, an agent which prevents mitochondrial permeabilty transition[39] via its binding to a protein located in the inner mitochondrial membrane, the adenine nucleotide translocator. Thus, these findings are consistent with the notion that peroxidation of membrane lipids and mitochondrial permeability transition are likely mechanisms of the rapid lethal response evoked by peroxynitrite in U937 cells.

An additional indication that mitochondria represent the main target of the peroxynitrite-induced cytotoxicity is given by the results shown in Figure 4. Treatment with peroxynitrite caused a marked reduction of mitochondrial rhodamine 123 uptake which was prevented by CsA (Figure 4C), BA, DPPD or *o*-phenanthroline (not shown). Since it is well established that the uptake of rhodamine 123 into mitochondria is a direct function of the mitochondrial transmembrane potential[37], we conclude that peroxynitrite causes mitochondrial depolarization via a CsA- or BA-sensitive mechanism.

The last set of results presented in this study indicate that the cells which survived the treatment with peroxynitrite did not undergo delayed cell death but rather proliferated as untreated cells (Figure 7). This would indicate that, under the experimental conditions utilized in this study, peroxynitrite either induces immediate necrosis or inflict a reversible damage which does not result in delayed cell death. Importantly, the cells surviving the treatment with peroxynitrite do not represent a sub-clone of peroxynitrite-resistant cells, since the level of cell death induced by the oxidant under standard conditions was identical to that observed using cells selected by a treatment with a toxic concentration of peroxynitrite (see the Results section). The striking differences observed between these subpopulations of U937 cells therefore suggest the existence of a threshold mitochondrial damage which leads to irreversible mitochondrial permeability thansition and necrosis; if this threshold is not reached then the cells survive and rapidly remove the lesions induced by peroxynitrite.

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In conclusion, the findings presented in this study demonstrate that a) the damage inflicted by peroxynitrite to U937 cells causes mitochondrial permeability transition-dependent early necrosis and b) the survivors promptly repair the damage inflicted by the oxidant and proficiently exert energy demanding functions such as cell proliferation.

Acknowledgements

The financial support of Telethon-Italy (Grant no. 1110) is gratefully acknowledged.

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