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Delayed Formation of Hydrogen Peroxide Mediates the Lethal Response Evoked by Peroxynitrite in U937 Cells

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ABSTRACT

The toxicity paradigm used in the present study involves exposure of U937 cells to a concentration of authentic peroxynitrite, leading to a rapid necrotic response mediated by mitochondrial permeability transition. We found that addition of catalase after treatment with peroxynitrite specifically prevents the loss of mitochondrial membrane potential and the ensuing lethal response. The protective effects of catalase were mimicked by the cocktail glutathione peroxidase/reduced glutathione. A defensive role of intracellular catalase was implied by experiments showing that catalase-depleted cells are hypersensitive to peroxynitrite and that cells with an increased catalase content, selected for their resistance to H_2O_2, are cross-resistant to peroxynitrite. Further experiments demonstrated that H_2O_2 formation takes place after peroxynitrite exposure. Various approaches using inhibitors of the mitochondrial respiratory chain as well as respiration-deficient cells revealed that the oxidant is produced upon dismutation of superoxides generated at the level of complex III. Interestingly, respiration-deficient cells were found to be resistant to peroxynitrite toxicity, and all those treatments increasing formation of H_2O_2 produced a parallel increase in toxicity. In conclusion, the results presented in this study indicate that peroxynitrite-induced impairment of electron transport from cytochrome *b* to cytochrome *c1* leads to delayed formation of hydrogen peroxide, which plays a pivotal role in the ensuing necrotic response.

Peroxynitrite, the reaction product of nitric oxide (NO) and superoxide, is a potent biological oxidant that mediates tissue injury in diverse pathological conditions, including ischemia-reperfusion injury, immunocomplex-mediated pulmonary edema, acute endotoxemia, neurological disorders, and atherosclerosis (Moncada et al., 1991; Heales et al., 1999). At the cellular level, peroxynitrite causes deleterious effects on various biomolecules; indeed, an extensive literature documents its ability to promote lipid peroxidation (Radi et al., 1991), protein nitration and nitrosylation (Patel et al., 1999), DNA damage (Salgo et al., 1995a; Szabó, 1996; Guidarelli et al., 2000) and oxidation of thiols (Salgo et al., 1995a). Although each of these events, or their combination, can be a cause of important dysfunctions and can lead to apoptosis (Lin et al., 1995; Salgo et al., 1995b; Shin et al., 1996; Szabó, 1996; Lin et al., 1997; Foresti et al., 1999; Oh-hashi et al., 1999; Virág et al., 1999) and/or necrosis (Delaney et al., 1996), it is unclear whether direct molecular damage is the sole mechanism whereby peroxynitrite causes cell death. This is an important point that needs to be clarified, because prevention of direct effects of peroxynitrite can be achieved only via its scavenging or by inhibiting its formation. Because of the very fast decomposition rate of peroxynitrite (half-life < 1 s) at physiological pH values (Hughes, 1999), it seems obvious that the potential cytoprotective strategies are limited to treatments performed before peroxynitrite formation. However, the possibility exists that the oxidant activates a cascade of secondary events promoting the formation of damaging species that cause additional damage. The identification of these pathways would allow the development of alternative pharmacological strategies preventing the amplification of the cellular damage initiated by peroxynitrite.

In a recent study (Guidarelli et al., 2000), we reported that exposure of U937 cells to peroxynitrite causes DNA single-strand breakage and that the extent of this response increases continuously over time for up to 30 min. It is obviously impossible that an agent that very quickly disappears from the culture medium directly generates lesions that accumulate linearly over such a long time. Indeed, peroxynitrite caused inhibition of complex III and experiments using inhibitors acting on specific sites of the respiratory chain produced results suggesting that mitochondrial hydrogen peroxide plays a pivotal role in the ensuing DNA strand scission. It is important to note that the DNA damage measured in that study was represented by DNA single strand breakage and not by secondary DNA fragmentation occurring during apoptosis. Indeed, we reported subsequently

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ABBREVIATIONS: NO, nitric oxide; ATZ, 3-amino-1,2,4-triazole; GpX, glutathione peroxidase; GSH, reduced glutathione; HQNO, 2-heptyl-4-hydroxyquinoline; DHR, dihydrorhodamine 123.

that, using similar experimental conditions, exposure to authentic peroxynitrite does not promote apoptosis (Sestili et al., 2001). To observe U937 cell death, it was necessary to use higher levels of peroxynitrite that triggered a rapid (within 60 min) necrotic response causally linked to mitochondrial permeability transition (Sestili et al., 2001).

We report herein an extension of the above studies showing that delayed formation of H_2O_2 indeed takes place in U937 cells exposed to authentic peroxynitrite. H_2O_2 seems to be produced upon dismutation of superoxides generated at the level of complex III, in a reaction in which ubisemiquinone serves as an electron donor. Finally, and most importantly, H_2O_2 plays a critical role in the lethal response evoked by peroxynitrite and its scavenging, performed after exposure to peroxynitrite, abolishes the ensuing lethal response.

Materials and Methods

Chemicals. Catalase, 3-amino-1,2,4,-triazole (ATZ), glutathione peroxidase (GpX), reduced glutathione (GSH), H_2O_2, antimycin A, menadione, myxothiazol, rotenone, and 2-heptyl-4-hydroxyquinoline (HQNO) as well as most of reagent grade chemicals, were obtained from Sigma-Aldrich (Milan, Italy). Rhodamine 123 and dihydrorhodamine 123 (DHR) were from Molecular Probes Europe (Leiden, The Netherlands).

Cell Culture and Treatments. U937 cells were cultured in suspension in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel), 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) gassed with an atmosphere of 95% air/5% CO_2. H_2O_2-resistant U937 cells (2.5×10^5/ml) were isolated by culturing the cells for 2 days in the presence of 100 μM H_2O_2. After this period, the cell suspension was centrifuged at 500 rpm for 10 min, the pellet was resuspended in fresh culture medium at a density of 2.5×10^5 cells/ml and incubated for a further 2 days in the presence of 200 μM H_2O_2. These cells were then gradually adapted to 300 and 400 μM H_2O_2 using the same selection protocol. Cells adapted to 400 μM H_2O_2 were then subcultured and their sensitivity to the oxidant and peroxynitrite was determined. In those experiments involving catalase depletion, U937 cells (5×10^6/20 ml) were incubated for 6 h at 37°C in RPMI medium containing 10 mM ATZ, an irreversible inhibitor of catalase (Darr and Fridovich, 1986). Respiration-deficient U937 cells were isolated by culturing the cells in RPMI medium containing 400 ng/ml ethidium bromide, 100 μg/ml pyruvate, and 5 μg/ml uridine for 6 days with changes of medium 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).

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. (1991) and MnO_2 (1 mg/ml) was added to the 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 ($\epsilon_{302} = 1670$ M^{-1} cm^{-1}). Stock solutions of peroxynitrite were routinely checked for the presence of H_2O_2 by the method of Webster (1975), using an oxygen electrode (see below). H_2O_2 concentration was always below the detection limit of the instrument (0.25–0.5 μM). 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 1N HCl was also added.

Catalase Activity. Cells were rinsed twice in saline A, resuspended in the same medium at a density of 1×10^6 cells/ml and finally sonicated 3 times on ice with a Branson sonifier operating at 20 W for 15 s. The resulting homogenates were centrifuged for 5 min at 18,000*g* at 4°C. Catalase activity was assayed spectrophotometrically in the supernatant by the method of Aebi (1984).

Cytotoxicity Assay. Cells were exposed to peroxynitrite for 5 min, centrifuged, postincubated for 55 min in the appropriate medium and analyzed for cytotoxicity 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 with a hemocytometer. In some experiments, the cells were exposed to H_2O_2 for 60 min in saline A and immediately analyzed as described above.

Polarographic Determination of Hydrogen Peroxide Concentration. The levels of H_2O_2 in the extracellular medium were determined as described by Webster (1975) using an oxygen electrode (mod. 5300 Oxygen Monitor, YSI, Inc., Yellow Springs, OH) which monitors the amount of O_2 released from the decomposition of the oxidant in the presence of an excess of catalase. The cells (2.5×10^5/ml), previously exposed for increasing times (5–15 min) to a wide range (100 μM-1.2 mM) of peroxynitrite concentrations, were injected into the thermostatic reaction cell (total volume 3 ml) of the oxygen meter. After equilibration of the samples, 50 μl of a catalase solution were microinjected into the cell (final concentration: 100 units of catalase/ml) and the rate of production of oxygen was continuously recorded for up to 5 min. Calibration curves were obtained by measuring the level of O_2 released after addition of increasing concentrations (0.25–10 μM) of standard solutions of H_2O_2.

Rhodamine 123 Mitochondrial Uptake, DHR Oxidation, and Confocal Imaging. Peroxynitrite-pretreated cells (5 min) were postincubated in the presence of either 10 μM DHR (10 min) or fresh saline A (15 min) in which rhodamine 123 (11 μM) was added during the last 5 min of incubation. After accurate washings, the cells were resuspended in 100 μl of phosphate-buffered saline (0.121 M NaCl, 10 mM NaH_2PO_4, 1.5 mM KH_2PO_4, and 3 mM KCl); 20 μl (50.000 cells) of this cell suspension was stratified on a slide, and cellular fluorescence was then imaged using a confocal laser microscope (DVC 250; Bio-Rad, Hercules, CA) equipped with a Hamamatsu 5985 (Hamamatsu Italy, Milan, Italy) chilled charge-coupled device camera. Cells were illuminated with the 488-nm line of the argon laser and the fluorescence emitted was monitored at $\lambda > 515$ nm. The laser intensity, the shutter aperture, and the exposure/integration settings were kept constant to allow quantitative comparisons of relative fluorescence intensity of cells between treatment groups. Laser exposure was limited to brief image acquisition intervals ($\leq$5 s) to minimize photo-oxidation of DHR and/or photo-bleaching of rhodamine 123. Confocal images were digitally acquired and processed for fluorescence determination at the single cell level on a Macintosh G4 computer using the public domain NIH Image 1.63 program (http://rsb.info.nih.gov/nih-image/). Mean fluorescence values were determined by averaging the fluorescence of at least 50 cells per treatment condition per experiment.

Measurement of Oxygen Consumption. The cells were washed once in saline A and then resuspended in the same medium at a density of 1×10^7 cells/ml. Oxygen consumption was measured using a YSI oxygraph equipped with a Clark electrode. The cell suspension (3 ml) was transferred to the polarographic cell and the rate of oxygen utilization was monitored under constant stirring for 3 min (basal respiration). The rate of oxygen utilization was calculated as described by Robinson and Cooper (1970).

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

The lethal response evoked by peroxynitrite in U937 cells was examined using different experimental conditions. In these experiments, the cells were exposed for 5 min to 1.2 mM peroxynitrite in a glucose-containing saline (saline A), centrifuged, rinsed twice with the same saline and postincubated for 55 min either in saline A or in complete culture medium. The first experimental condition—unlike the second—caused an appreciable toxicity (53.2 ± 0.9%), as measured by the trypan blue exclusion assay. The following lines of evidence support the notion that a heat-labile component of the serum was responsible for the observed cytoprotection: 1) post-treatment incubation in serum-free culture medium resulted in a lethal response identical to that observed in cells postincubated in saline A (data not shown); 2) post-treatment incubation in culture medium supplemented with heat-inactivated serum was not cytoprotective (data not shown); and 3) addition of 10% serum to the saline used during recovery abolished the lethal response that was otherwise observed in its absence (data not shown).

In Fig. 1A, it can be seen that exogenous catalase (10 U/ml) was also cytoprotective when added to saline A after peroxynitrite exposure, whereas the boiled enzyme (10 U/ml) was inactive. Catalase, however, was not effective when added during the 5-min treatment with peroxynitrite but omitted during recovery (data not shown); this is an expected finding because stock solutions of peroxynitrite are routinely checked for the presence of H_2O_2 (see *Materials and Methods*).

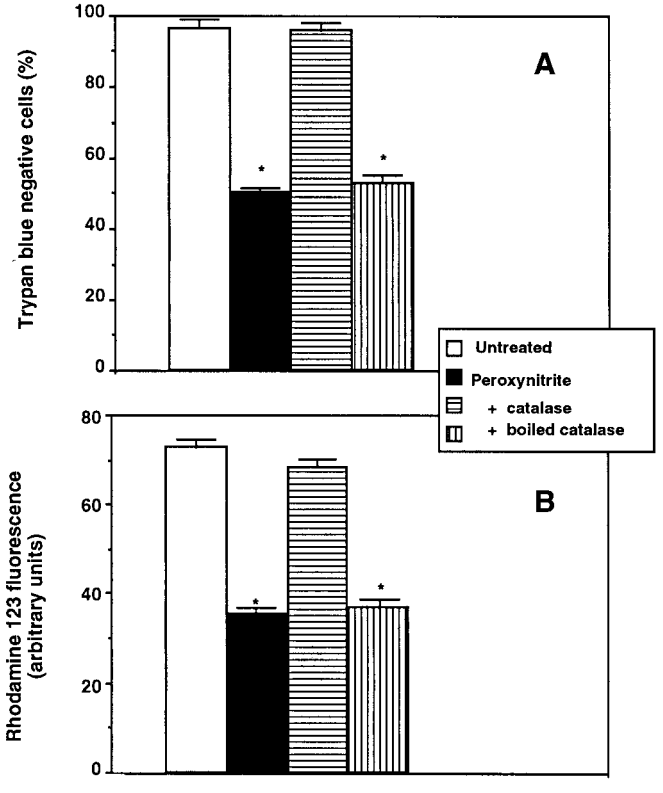

Fig. 1. Catalase prevents mitochondrial depolarization and cytotoxicity induced by peroxynitrite. A, cells were exposed for 5 min to 1.2 mM peroxynitrite, centrifuged, and then postincubated for a further 55 min in fresh saline A in the absence or presence of either catalase (10 U/ml) or boiled catalase (10 U/ml). Cells were then analyzed immediately for cytotoxicity using the trypan blue exclusion assay. Results represent the means ± S.E.M. from at least three separate experiments. *, $p < 0.001$ compared with untreated cells. B, cells were treated as detailed in A and postincubated for 15 min in fresh saline A. Rhodamine 123 (11 μM) was added to the cultures during the last 5 min of post-treatment incubation. After accurate washing, the cells were observed with a confocal microscope equipped with a charge-coupled device camera and images were digitally recorded with a Macintosh computer. Rhodamine 123 fluorescence was then quantified as detailed under *Materials and Methods* section. Results represent the means ± S.E.M. from at least four separate experiments. *, $p < 0.001$ compared with untreated cells.

The uptake of rhodamine 123 was next investigated with the aim of assessing whether catalase prevents the effects of peroxynitrite on mitochondrial transmembrane potential (Sestili et al., 2001). In these experiments, the cells were first exposed for 5 min to 1.2 mM peroxynitrite and then postincubated in fresh saline A for an additional 15 min with or without catalase. Rhodamine 123 was added to the cultures in the last 5 min of post-treatment incubation. The cultures were then visualized with a confocal microscope and subjected to image analysis to quantify fluorescence at the single cell level. These experiments allowed us to calculate the average fluorescence intensity values, expressed as arbitrary units, reported in Fig. 1B. The fluorescence associated with the cells treated with peroxynitrite was remarkably lower than that of untreated cells. Interestingly, the effects of peroxynitrite were sensitive to catalase but insensitive to the boiled enzyme. It should be also noted that catalase did not affect the uptake of rhodamine 123 in cells that were not exposed to peroxynitrite (data not shown). In addition, catalase was not effective when added only during the 5-min exposure to peroxynitrite (data not shown).

Thus, these results demonstrate that exogenous catalase prevents mitochondrial permeability transition and cytotoxicity caused by peroxynitrite. The observation that heat-inactivated catalase was not effective suggests strongly that cytoprotection is causally linked to its enzymatic activity. The experimental results illustrated in Fig. 2 are consistent with this notion because the cocktail GpX (1 U/ml)/GSH (2 mM), unlike each of its components given separately, mimicked the protective effects afforded by catalase.

The question therefore arises as to whether intracellular catalase also plays a protective role in this toxicity paradigm. To obtain information in this direction, we compared the sensitivity to peroxynitrite of control or catalase-depleted U937 cells. A 6-h treatment with 10 mM ATZ decreased catalase activity to 25% of control levels (Fig. 3, inset). As shown in Fig. 3, these cells were hypersensitive to peroxynitrite and the dose-modifying factor, calculated from the ratio of the IC_{50} values for control (1.105 ± 0.089 mM) versus catalase-depleted (0.113 ± 0.009 mM) cells, was close to 10. Under these conditions, exogenous catalase was also cytoprotective (Fig. 3).

Important evidence that lends further experimental support for a central role of intracellular catalase in preventing peroxynitrite cytotoxicity is provided by the results illustrated in Fig. 4. H_2O_2-resistant U937 cells (Fig. 4, inset A) were found to be cross-resistant with peroxynitrite (main graph). Furthermore, H_2O_2-resistant cells displayed significantly greater catalase activity than control cells (Fig. 4, inset B) and addition of exogenous catalase abolished the toxicity mediated by peroxynitrite in H_2O_2-resistant cells (Fig. 4). Thus, both intracellular and extracellular catalase effectively mitigate U937 cell death mediated by authentic

peroxynitrite; this suggests that H_2O_2 plays a pivotal role in this toxicity paradigm.

Inasmuch as previous studies from our laboratory demonstrated that the deleterious effects of H_2O_2 are an inverse function of cell density (Cantoni et al., 1986, Sestili et al., 1996), we investigated whether the same effects could also be observed in cells exposed to peroxynitrite. We therefore performed experiments in which the cells were first treated for 5 min with 1.2 mM peroxynitrite and then subcultured in fresh saline A at different densities. We found that post-treatment incubation at 10^4, 2.5×10^5, or 1×10^6 cells/ml gradually decreased the level of cell killing elicited by peroxynitrite. Under these conditions, the percentage of trypan blue negative cells was 32.1 ± 0.46, 54.4 ± 2.0, or 90.2 ± 2.28, respectively. An additional experiment was perfomed in which 2.5×10^5 cells/ml pretreated for 5 min with 1.2 mM peroxynitrite were cocultured for 55 min with an equal number of cells that were either untreated or catalase-depleted by previous exposure to ATZ (10 mM for 6 h). Cytotoxicity was observed only in the second experimental condition (3.4×10^5 viable cells/ml).

These results confirm that H_2O_2 plays a pivotal role in cell killing induced by peroxynitrite; consequently, formation of H_2O_2 should take place after a pulse-treatment with peroxynitrite. The occurrence of this event was investigated using the fluorescent probe DHR (10 μM), which accumulates in the mitochondria and fluoresces when oxidized by various species, including H_2O_2 (Emmendörffer et al., 1990) or peroxynitrite (Kooy et al., 1994). However, it is important to note that these experiments involved a 5-min treatment with peroxynitrite followed by a 10-min postincubation in fresh saline A containing the fluorescent probe, a condition that does not allow peroxynitrite to directly oxidize DHR. After treatments, the cultures were processed as in the experiments illustrated in Fig. 1B. The results shown in Fig. 5A indicate that pre-exposure to peroxynitrite markedly enhances fluorescence above control values and that enzymatically active catalase, unlike the boiled enzyme, prevents this response.

Thus, these results are in line with the above premise and consistent with the notion that exposure to peroxynitrite promotes delayed formation of H_2O_2. The question therefore arises as to whether some of the peroxide leaked out of the cells. This possibility is consistent with the observation that exogenous catalase affords cytoprotection in this toxicity paradigm. However, polarographic measurements performed in the saline in which the cells had been exposed for increasing time intervals (5–15 min) to a wide range of peroxynitrite concentrations (100 μM-1.2 mM) failed to reveal detectable amounts of H_2O_2 (data not shown). Similar results were obtained in catalase-depleted cells treated with 100 μM peroxynitrite (data not shown), a condition resulting in a lethal response comparable with that mediated by 1.2 mM peroxynitrite in naive cells (Fig. 3). It is important to note that the technique used to measure extracellular H_2O_2 has a detection limit of 0.25/0.5 μM H_2O_2 (see *Materials and Methods*). Indeed, we were able to reproducibly measure 0.65 μM H_2O_2 in cells exposed for 10 min to 150 μM menadione. These results therefore suggest that H_2O_2 generated by peroxynitrite is largely retained within the cells and, consequently, it seems unlikely that the small fraction of the oxidant that is

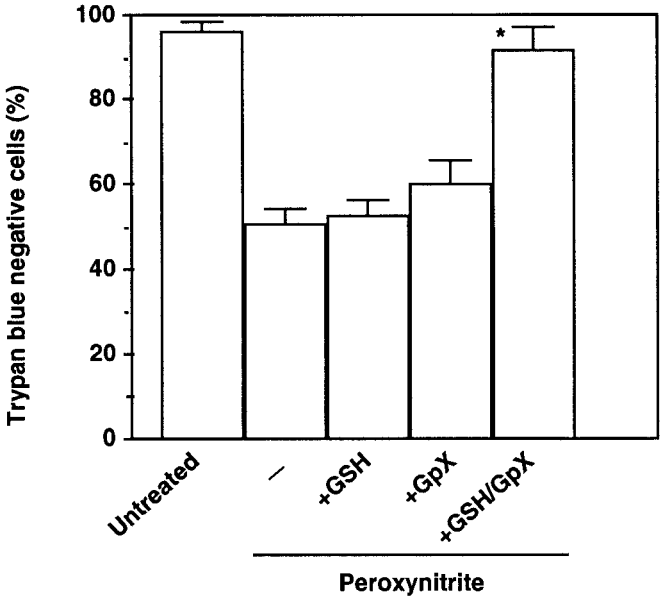

Fig. 2. The effect of GSH, GpX, or a combination of the two agents on peroxynitrite-induced cytotoxicity. Cells were exposed for 5 min to 1.2 mM peroxynitrite, centrifuged, and then postincubated for a further 55 min in saline A in the absence or presence of GSH (2 mM), GpX (1 U/ml), or both GSH and GpX. Cells were then analyzed for cytotoxicity using the trypan blue exclusion assay. Results represent the means ± S.E.M. from at least four separate experiments. *, $p < 0.001$ compared with cells treated with peroxynitrite and subsequently postincubated in saline A.

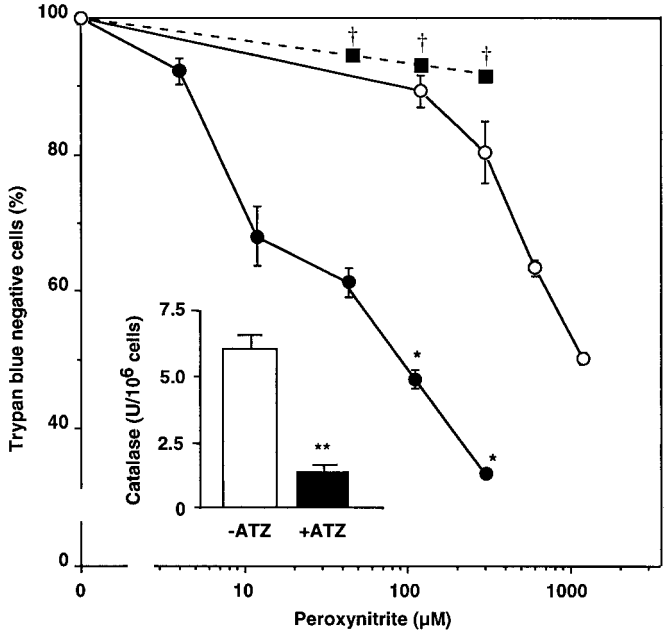

Fig. 3. The effect of catalase depletion on peroxynitrite-induced U937 cell death. Cells were incubated in complete culture medium for 6 h with 0 (open symbols) or 10 mM ATZ (closed symbols) and subsequently treated for 5 min with increasing concentrations of peroxynitrite in saline A. The cells were then centrifuged and postincubated for a further 55 min in fresh saline A. The dashed line shows the effect of exogenous catalase (10 U/ml), added after exposure to peroxynitrite, to catalase-depleted cells. Cytotoxicity was determined immediately after the treatments using the trypan blue exclusion assay. Results represent the mean ± S.E.M. from 5–6 separate experiments. *, $p < 0.001$ compared with cells that were not previously exposed to ATZ. †, $p < 0.001$ compared with catalase-depleted cells. Also shown (inset) are the catalase levels measured before peroxynitrite exposure in untreated cells and in cells supplemented with ATZ (**, $p < 0.0001$).

being released plays a significant role in the ensuing lethal response.

The conclusion that intracellular H_2O_2 is critical in the peroxynitrite-induced lethal response is consistent with the possibility that cytoprotection afforded by extracellular catalase is associated with internalization of the enzyme. This event, although in apparent contrast with the well established notion that enzymes are not readily taken up by cultured cells, might take place via at least two separate mechanisms, receptor-mediated endocytosis and fluid-phase endocytosis. We found that, under the conditions used in toxicity studies, exposure to catalase did not affect the U937 cell catalase content (data not shown). Furthermore, agents interfering with one or more steps of endocytosis, like monensin (1–10 μM), did not modify the toxicity induced by peroxynitrite and, more importantly, failed to prevent the cytoprotective effects mediated by catalase (data not shown). Thus, cytoprotection afforded by extracellular catalase seems to be mediated by scavenging of intracellular H_2O_2.

Experiments were then performed with the aim of assessing whether formation of H_2O_2 takes place at the level of the respiratory chain. As indicated in Fig. 5B, peroxynitrite failed to promote fluorescence in respiration-deficient cells. In addition, the catalase-sensitive peroxynitrite-dependent DHR fluorescence response mediated in respiration-proficient cells was abolished by the complex I inhibitor rotenone (0.5 μM) as well as by myxothiazol (5 μM), an agent that prevents the electron flow from the reduced coenzyme Q to cytochrome *c1* (Rich et al., 1990). Antimycin A (1 μM) and HQNO (10 μM), which are known to inhibit complex III at the same site (Van Ark and Berden, 1977), did not affect the DHR fluorescence response evoked by 1.2 mM peroxynitrite. It is important to note that none of the above respiratory chain inhibitors produced detectable changes in DHR oxidation when given alone to the cultures (data not shown).

The effects of antimycin A and HQNO were next tested against a low concentration (100 μM) of peroxynitrite that fails to promote conversion of DHR to rhodamine. It was found that both complex III inhibitors potently stimulated this fluorescence response. The enhancing effects of antimycin A were sensitive to catalase, rotenone, or myxothiazol, but insensitive to boiled catalase (Fig. 6). These results are therefore consistent with the notion that delayed formation

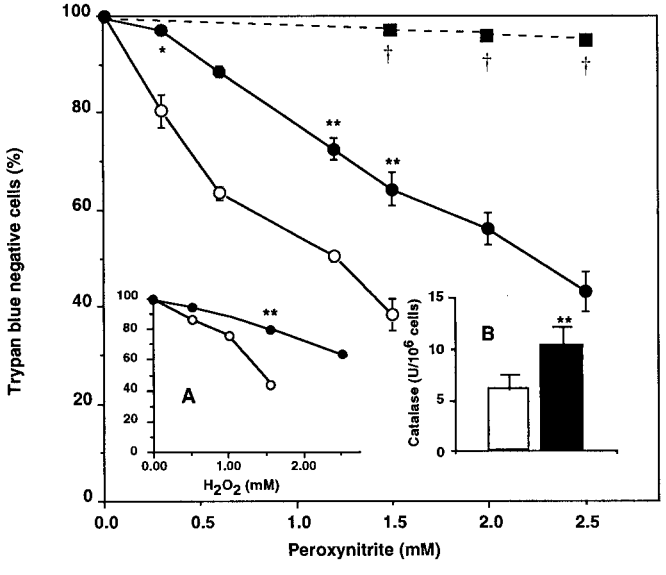

Fig. 4. The effect of the H_2O_2-resistant phenotype on the lethal response evoked by peroxynitrite. Parental (○) or H_2O_2-resistant (●) U937 cells were exposed for 5 min to increasing concentrations of peroxynitrite and then postincubated for a further 55 min in fresh saline A at 37°C. The dashed line shows the effect of catalase (10 U/ml) in H_2O_2-resistant cells. Cytotoxicity was determined immediately after the treatments using the trypan blue exclusion assay. Also shown as insets are the cytotoxic effect of hydrogen peroxide (60 min treatment in saline A) in parental (open symbols) or H_2O_2-resistant (closed symbols) U937 cells (inset A) and the levels of catalase in the two cell lines [parental U937 cells (□) and H_2O_2-resistant U937 cells (■)] (B). Results represent the means ± S.E.M. from at least four separate experiments. *, $p < 0.001$ and **, $p < 0.0001$ compared with parental U937 cells. †, $p < 0.001$ compared with H_2O_2-resistant U937 cells.

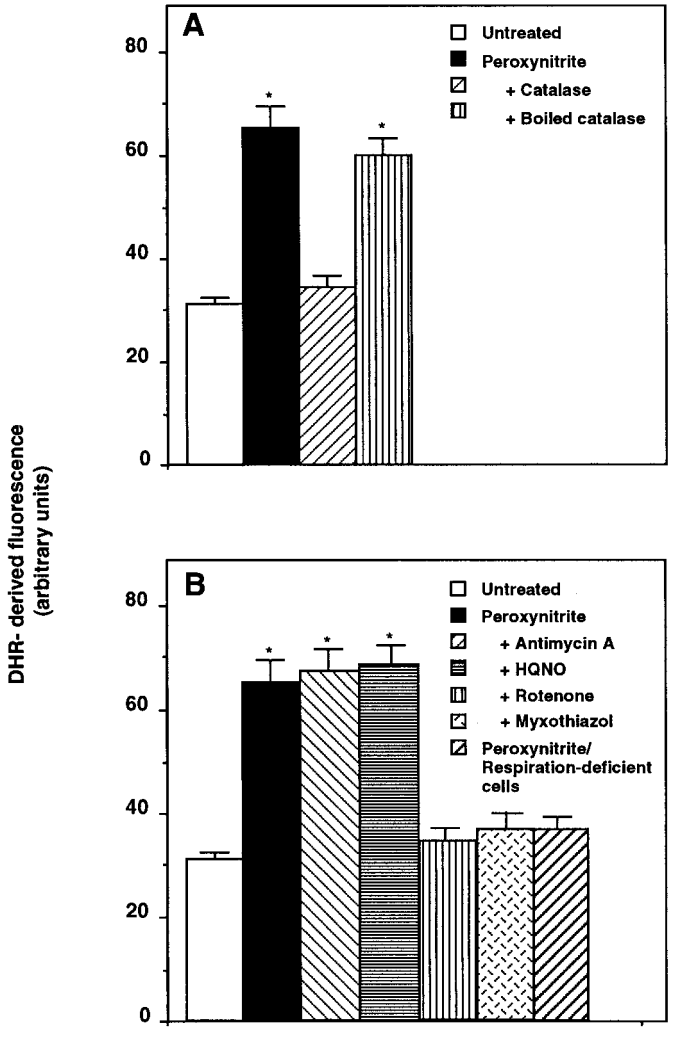

Fig. 5. The effect of catalase or various respiratory chain inhibitors on the DHR-derived fluorescence in cells pre-exposed to peroxynitrite. A, the cells were exposed for 5 min to 1.2 mM peroxynitrite, centrifuged, and postincubated for a further 10 min in fresh saline A containing 10 μM DHR in the absence or presence of catalase (10 U/ml) or boiled catalase (10 U/ml). The DHR-derived fluorescence was then monitored by confocal microscopy; images were then digitally recorded and analyzed as detailed under *Materials and Methods*. Results represent the means ± S.E.M. from at least four separate experiments. *, $p < 0.001$ compared with untreated cells. B, the cells were exposed for 5 min to 1.2 mM peroxynitrite, centrifuged, and postincubated as detailed in A in the absence or presence of antimycin A (1 μM), HQNO (10 μM), rotenone (0.5 μM), or myxothiazol (5 μM). Also shown is the effect of peroxynitrite in respiration-deficient cells. The DHR-derived fluorescence was quantified as detailed in A. Results represent the means ± S.E.M. from four separate experiments. *, $p < 0.001$ compared with untreated cells.

of H_2O_2 takes place in the mitochondrial respiratory chain of U937 cells pretreated with peroxynitrite.

The last set of experiments was designed to assess whether the effects of the respiratory chain inhibitors on H_2O_2 formation were paralleled by changes in peroxynitrite-dependent cytotoxicity. In these experiments, the cells were exposed for 5 min to increasing concentrations of peroxynitrite and postincubated for 55 min in fresh saline A containing each of the above inhibitors. As illustrated in Fig. 7, cytotoxicity was prevented by the complex I inhibitor rotenone. It can also be noted that bona fide complex III inhibitors, although not being in and of themselves cytotoxic (data not shown), increased toxicity, in particular using low concentrations of peroxynitrite. Furthermore, respiration-deficient U937 cells were resistant to the lethal response evoked by peroxynitrite and addition of rotenone (or antimycin A, not shown) did not produce detectable effects. Thus, these results provide an important indication that H_2O_2 generated at the level of the respiratory chain mediates toxicity in U937 cells exposed to peroxynitrite.

Discussion

Peroxynitrite is a highly toxic reactive nitrogen species that very rapidly decomposes at physiological pH values (Hughes, 1999). Thus it was assumed that, using toxicity paradigms in which peroxynitrite is administered as a bolus, the oxidant directly generates potentially lethal lesions within few seconds (i.e., before its disappearance). The results presented in this study are in conflict with this interpretation because they clearly show that the toxicity triggered by a brief exposure of U937 cells to a high concentration of authentic peroxynitrite promotes delayed formation of toxic levels of H_2O_2. The need of using high concentrations of peroxynitrite emphasizes the extraordinary resistance of these cells (Sestili et al., 2001) to the insult elicited by a toxic species, which can be, upon stimulation, generated in very large amounts. Indeed, U937 cells are a promonocytic cell line and exposure to specific cytokines leads to expression of inducible NOS and formation of peroxynitrite. These cells are obviously well equipped to defend themselves from their own peroxynitrite.

In this study we report that exposure to 1.2 mM peroxynitrite (5 min in a glucose-containing saline) was dependent on the composition of the extracellular milieu in which the cells were incubated to allow the expression of the lethal response (55 min). In particular, post-treatment incubation in saline A caused an about 50% cell death that was no more apparent when the saline was replaced with complete culture medium (data not shown). Further experiments revealed details on the identity of the cytoprotective factor that was identified as a heat labile component of the serum (data not shown). This factor was most probably represented by catalase because addition of the pure enzyme to saline A mimicked the protective effects afforded by the serum component (Fig. 1A) and prevented the loss of mitochondrial membrane potential (Fig. 1B) that precedes—and is causally linked to—the lethal response initiated by peroxynitrite (Sestili et al., 2001). The specificity of these responses is emphasized by the observation that the effects of catalase disappeared when the enzyme was boiled before its addition to the culture medium.

These results therefore provide an important indication that the initial treatment with peroxynitrite triggers delayed formation of toxic levels of H_2O_2 that can be destroyed by extracellular catalase. It is important to emphasize that this

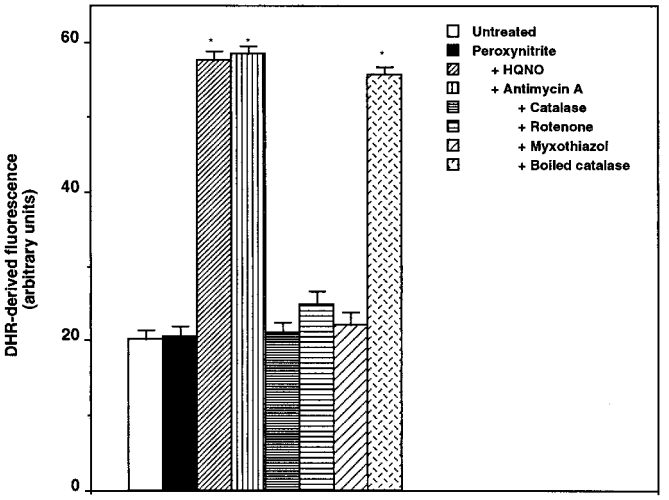

Fig. 6. The effect of antimycin A or HQNO on the DHR-derived fluorescence in U937 cells exposed to a low concentration of peroxynitrite. Cells were exposed for 5 min to 100 μM peroxynitrite, centrifuged, and then postincubated for a further 10 min in fresh saline A containing 10 μM DHR in the absence or presence of either HQNO (10 μM) or antimycin A (1 μM). The effects of catalase (10 U/ml), rotenone (0.5 μM), myxothiazol (5 μM), or boiled catalase in peroxynitrite (100 μM)-treated cells supplemented with antimycin A is also shown. The DHR-derived fluorescence was determined as detailed in the legend to Fig. 5. Results represent the means ± S.E.M. from four separate experiments. *, $p < 0.001$ compared with untreated cells.

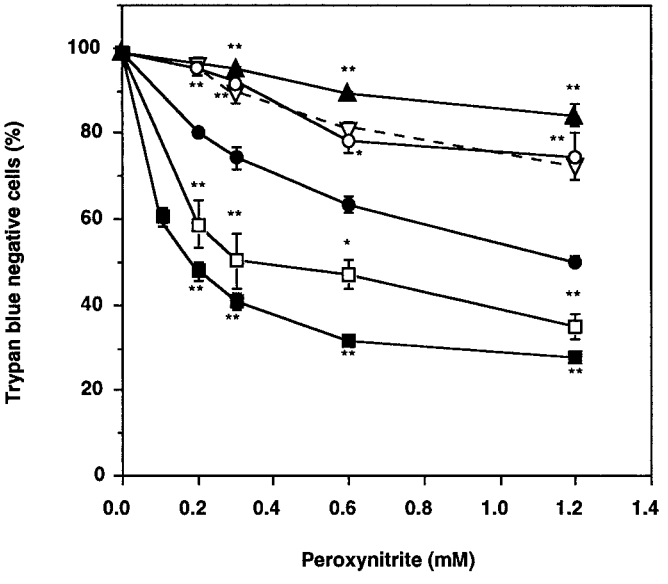

Fig. 7. The effects of selected inhibitors of the mitochondrial respiratory chain and of experimentally induced respiration deficiency on peroxynitrite-induced cytotoxicity. U937 cells were exposed for 5 min to increasing concentrations of authentic peroxynitrite, centrifuged, and then postincubated for a further 55 min in fresh Saline A in the absence (●) or presence of 1 μM antimycin A (□), 10 μM HQNO (■), or 0.5 μM rotenone (▲). Also shown is the lethal response evoked by peroxynitrite in respiration-deficient cells in the absence (○) or presence (△, dashed line) of 0.5 μM rotenone. Cytotoxicity was analyzed immediately after the treatments using the trypan blue exclusion assay. Results represent the means ± S.E.M. from five separate experiments. *, $p < 0.001$; **, $p < 0.0001$ compared with respiration-proficient U937 cells exposed to peroxynitrite alone.

inference is also based on the information obtained in additional studies testing the possibility that the H_2O_2 present in the peroxynitrite stock solution was responsible for the observed catalase-sensitive toxicity. As stated under *Materials and Methods*, different preparations of peroxynitrite were routinely checked for the presence of H_2O_2; using a polarographic method that detects micromolar levels of the oxidant negative results were always obtained. Furthermore, addition of catalase during the peroxynitrite exposure phase did not result in cytoprotection (data not shown).

Thus, it would seem that a flux of toxic levels of H_2O_2, accessible to extracellular catalase, is produced after exposure to peroxynitrite. The observation that the effects of catalase were mimicked by addition of a different H_2O_2-scavenging enzymatic system, namely GpX/GSH (Fig. 2), is consistent with this possibility.

Two separate lines of evidence suggest that also intracellular scavenging of H_2O_2 is an important mechanism counteracting the lethal response evoked by peroxynitrite. First, depletion of catalase caused a tremendous increase (10-fold decrease in IC_{50} values) in toxicity that was once again abolished by exogenous catalase added after peroxynitrite exposure. Second, cells resistant to H_2O_2 (Fig. 4, inset A)—with an increased catalase content (Fig. 4, inset B)—displayed cross resistance to peroxynitrite (Fig. 4).

The results thus far discussed support the hypothesis that delayed formation of H_2O_2 plays a critical role in the lethal response initiated by a bolus of peroxynitrite. An additional indication in this direction is given by the observation that toxicity is remarkably different under conditions in which cells exposed for 5 min to peroxynitrite are postincubated at different densities. The relative amount of cells surviving this treatment was an inverse function of cell density (see *Results*). Other experiments revealed that addition of untreated cells to peroxynitrite-pretreated cells abolished the ensuing toxicity; this response was dependent on the catalase content of the cells that did not receive peroxynitrite because catalase-depleted U937 cells failed to induce cytoprotection. These results collectively support the notion that H_2O_2 plays a pivotal role in this toxicity paradigm because similar experiments using reagent H_2O_2 or H_2O_2 generated by ascorbate led to identical outcomes (Sestili et al., 1996).

Having established the pivotal role of H_2O_2 as an intermediate toxic species resulting from exposure to peroxynitrite, experimental evidence indicating that H_2O_2 is indeed being generated was next produced. These studies (Fig. 5A) demonstrated a catalase-sensitive oxidation of DHR, that was given to the cultures after exposure to peroxynitrite to prevent its direct oxidation (Kooy et al., 1994). Because boiled catalase was inactive, oxidation of DHR can be attributed to H_2O_2 (Emmendörffer et al., 1990). Under the same conditions, however, we were unable to measure significant amounts of H_2O_2 using a technique that detects 0.25/0.5 μM concentrations of the oxidant and that monitored formation of 0.65 μM H_2O_2 in U937 cells exposed for 10 min to 150 μM menadione (data not shown).

Thus, it seems that exogenous catalase affords cytoprotection by scavenging intracellular H_2O_2. In recent years, several studies (Min et al., 1998; De Bleser et al., 1999; Nemoto et al., 2000) have demonstrated that extracellular catalase prevents various effects mediated by endogenous H_2O_2 and, to our best knowledge, the question of whether the enzyme acted inside or outside the cell has been unanswered. In this study we found that 1) the catalase activity of U937 cells is unaffected by previous exposure to the exogenous enzyme (data not shown) and that 2) inhibitors of endocytosis fail to prevent the cytoprotective effects mediated by exogenous catalase (data not shown). These findings are consistent with the notion that the enzyme is not readily taken up by the cells. Consequently, we speculate that catalase interacts fleetingly with the plasma membrane and promotes diffusion of the peroxide through the membrane, followed by its scavenging. To be firmly established, however, such a sequence of events obviously needs a more detailed investigation.

Experiments aimed at investigating the source of H_2O_2 in cells exposed to a high concentration of peroxynitrite (1.2 mM) revealed that the ensuing DHR fluorescence response was inhibited by rotenone and myxothiazol but was unaffected by two structurally unrelated complex III inhibitors, antimycin A and HQNO (Fig. 5B). Furthermore, peroxynitrite did not cause an increased fluorescence in respiration-deficient cells. Interestingly, antimycin A and HQNO promoted a remarkable oxidation of DHR in cells exposed to a low concentration of peroxynitrite (100 μM) that was otherwise unable to stimulate detectable fluorescence, and this response was sensitive to catalase, rotenone, or myxothiazol (Fig. 6).

The above results therefore indicate that two stoichiometric inhibitors of complex III, antimycin A and HQNO, promote formation of H_2O_2 in cells exposed to an inactive concentration of peroxynitrite. Although these effects were observed at the same concentrations producing a $>$ 95% inhibition of oxygen utilization (data not shown), the possibility that interruption of electron transport in the respiratory chain is causally linked to oxidation of DHR is unlikely for the following reasons: first, other inhibitors such as myxothiazol or rotenone were also used at levels causing a $>$95% inhibition of oxygen consumption but did not promote oxidation of DHR in cells supplemented with 100 μM peroxynitrite (data not shown); second, the enhancing effects mediated by antimycin A seemed to be causally linked to its ability to inhibit complex III because the DHR fluorescence response was suppressed under conditions in which the entry of electrons in the Q cycle was prevented by either rotenone or myxothiazol (Fig. 6).

The results discussed thus far indicate that inhibition of oxygen consumption does not in and of itself promote oxidation of DHR in cells treated with a low concentration of peroxynitrite. Rather, it seems that the enhancing effects of antimycin A or HQNO are caused by specific binding to their specific site in complex III. Because previous studies from this (Guidarelli et al., 2000) and other laboratories (see below) demonstrated that peroxynitrite promotes inhibition of complex III, we postulated that this event was causally linked to oxidation of DHR. The fact that antimycin A and HQNO inhibit complex III at the same level (Van Ark and Berden, 1977), along with the observation that the action of each of the two complex III inhibitors is on a saturable mechanism, suggests strongly that peroxynitrite-dependent inhibition of complex III plays a pivotal role in the oxidation of DHR. Under the conditions that were used in this study, increasing concentrations of peroxynitrite may lead to a progressive impairment in complex III activity, which would explain both the strong enhancement of DHR oxidation me-

diated by antimycin A (or HQNO) in cells treated with a low concentration of peroxynitrite (100 μM) and the lack of effect of antimycin A (or HQNO) after exposure to 1.2 mM peroxynitrite.

Thus, it seems that the mechanism whereby peroxynitrite causes formation of H_2O_2 in intact cells involves inhibition of complex III at a level close to the antimycin A or HQNO binding site. This region represents "the weak site" of the respiratory chain and prevention of the electron flow from cytochrome b_{562} to oxidized coenzyme Q leads to formation of superoxides and hydrogen peroxide (Cadenas and Boveris 1980). Addition of peroxynitrite to submitochondrial particles, isolated mitochondria, or intact cells was reported to cause effects, mostly irreversible, at the level of virtually all the respiratory complexes, with the exception of complex IV. In particular, inhibition of complex I was reported by Radi et al. (1994), Cassina and Radi (1996), Lizasoain et al. (1996), Guidarelli et al. (2000), and Pearce et al. (2001). Inhibition of complex II was detected in the studies performed by Bolaños et al. (1995), Radi et al. (1994), and Cassina and Radi (1996). Inhibition of complex III was found by Bolaños et al. (1995), Lizasoain et al. (1996), Guidarelli et al. (2000), and Pearce et al. (2001). Although these differences might be explained by the different experimental systems and conditions used in these investigations, it is important to note that our previous study (Guidarelli et al., 2000), reporting partial inhibition of complex I and a more severe inhibition of complex III, involved the use of the same cell line and treatment conditions employed in the present investigation. This situation is compatible with electrons reaching the Q cycle, thereby producing superoxides and the inhibitor studies reported in Figs. 5 to 7 further support the notion that complex III is a preferential target of peroxynitrite. Detailed studies must be awaited to explain why Fe-S clusters of complex I are less sensitive than those of complex III to peroxynitrite.

The last set of experiments performed in this study addressed the question of whether the observed changes in H_2O_2 formation mediated by the bona fide respiratory chain inhibitors were paralleled by changes in peroxynitrite toxicity. We found that the lethal response evoked by peroxynitrite was markedly enhanced by antimycin A or HQNO and prevented by rotenone. Furthermore, respiration-deficient U937 cells were highly resistant to toxicity induced by peroxynitrite (Fig. 7). In these cells, rotenone did not afford cytoprotection, a finding that indicates that cells are indeed respiration-deficient and emphasizes the specificity of the protective effects mediated by rotenone in respiration-proficient cells. Thus, these results strongly suggest a cause-effect relationship between the extent of H_2O_2 formation and the lethal response evoked by peroxynitrite.

In conclusion, the results presented in this study demonstrate that delayed mitochondrial formation of H_2O_2 plays a critical role in the mechanism whereby authentic peroxynitrite causes U937 cell death. Whether H_2O_2 is directly toxic for the cells or, rather, represents an intermediate species in a signaling pathway leading to a very rapid necrotic response is currently under investigation.

As stated above, the toxicity mediated by peroxynitrite has traditionally being viewed as the result of a stochastic process of cell damage; indeed, the pharmacological strategies used to mitigate its deleterious effects have thus far been restricted to the use of scavengers of this species (Salgo and Pryor, 1996; Arteel et al., 1999) and to agents inhibiting its formation (e.g., superoxide dismutase mimetics or NO synthase inhibitors) (Pfeiffer et al., 1998; Arteel et al., 1999; Salvemini et al., 1999). The results presented in this study have therefore very important implications because they provide experimental evidence indicating that the lethal response can be modulated by acting downstream of peroxynitrite formation.

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