Biochemical Pharmacology, Vol. 58, pp. 1743–1750, 1999.

ISSN 0006-2952/99/$–see front matter
PII S0006-2952(99)00271-3

Apoptosis and Necrosis following Exposure of U937 Cells to Increasing Concentrations of Hydrogen Peroxide: The Effect of the Poly(ADP-ribose)polymerase Inhibitor 3-Aminobenzamide

Letizia Palomba,§ Piero Sestili,*§ Marta Columbaro,† Elisabetta Falcieri‡ and Orazio Cantoni*‖*

*Istituto di Farmacologia e Farmacognosia and Centro di Farmacologia Oncologica Sperimentale, Universita di Urbino, Urbino; †Laboratorio di Biologia Cellulare e Microscopia Elettronica, Istituti Ortopedici Rizzoli, Bologna; and ‡Istituto di Anatomia e Fisiologia, Universita di Urbino, Urbino, Italy

ABSTRACT. A 3-hr exposure of U937 cells to hydrogen peroxide (H_2O_2) followed by a 6-hr posttreatment incubation in fresh culture medium promotes apoptosis or necrosis, depending on the oxidant concentration. Addition of 3-aminobenzamide (3AB) during the recovery phase prevented necrosis and caused apoptosis. 3AB did not, however, affect the apoptotic response of cells treated with apogenic concentrations of H_2O_2. Cells exposed for 3 hr to 1.5 mM H_2O_2, while showing some signs of suffering, maintained a normal nuclear organization and good organelle morphology. At the biochemical level, the oxidant promoted the formation of Mb-sized DNA fragments and rapidly depleted both the adenine nucleotide and non-protein sulphydryl pools, which did not recover during posttreatment incubation in the absence or presence of 3AB. These results allow a novel interpretation of the concentration-dependent switch from apoptosis to necrosis. We propose that H_2O_2 activates the apoptotic response at the early times of peroxide exposure and that this process can be completed, or inhibited, during the posttreatment incubation phase. Inhibition of apoptosis leads to necrosis and can be prevented by 3AB via a mechanism independent of inhibition of poly(ADP-ribose)polymerase. As a corollary, the necrotic response promoted by high concentrations of H_2O_2 in U937 cells appears to be the result of specific inhibition of the late steps of apoptosis. BIOCHEM PHARMACOL **58**;11:1743–1750, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. hydrogen peroxide; 3-aminobenzamide; apoptosis; necrosis

The study of the mechanisms of apoptosis is an emerging area of research with important biological implications and, as a consequence, has attracted the interest of a large number of laboratories [1, 2]. Much less attention has been given to understanding the process of necrosis, which is considered to be a degenerative response of the cell to overwhelming damage. In other words, the general opinion is that necrosis is a passive, non-specific, and obligatory event in direct opposition to apoptosis, which is typically an active process. This would imply that once the cell has received an insult potentially leading to necrosis, loss of cell viability will necessarily occur and, as a corollary, prevention of the necrotic response can only be achieved via reduction of the extent of the insult.

We recently demonstrated, however, that this is not necessarily always true. Indeed, the necrotic response of U937 cells treated for 3 hr with 1 mM H_2O_2¶ and postincubated for 6 hr in drug-free medium was prevented by the addition of the PARP inhibitor 3AB during the posttreatment incubation phase [3]. Under these conditions, 3AB is obviously unable to exert direct protective or scavenging effects and can only modulate secondary responses triggered by the insult inflicted on the cells by H_2O_2. It must therefore be concluded that, at least in this specific experimental system, a biochemical pathway leading to necrosis exists and can be modulated by 3AB. The second parallel observation made was that 3AB did not simply prevent necrosis induced by H_2O_2, but also led these cells into apoptosis. As a consequence, the same treatment can be a triggering event for both apoptosis and necrosis, a finding which introduces a new concept which should be

§ These authors contributed equally to the work described in this report.

‖ Corresponding author: Dr. Orazio Cantoni, Istituto di Farmacologia e Farmacognosia, Università di Urbino, Via S. Chiara 27, 61029, Urbino, Italy. Tel. 011-39-0722-2671; FAX 011-39-0722-327670; E-mail: cantoni@fis.uniurb.it

Received 12 June 1998; accepted 21 June 1999.

¶ *Abbreviations:* H_2O_2, hydrogen peroxide; 3AB, 3-aminobenzamide; PARP, poly(ADP-ribose)polymerase; TBE, Tris-borate-EDTA; PACE, programmable, autonomously controlled electrode electrophoresis; and NPSH, non-protein sulphydryls.

taken into account in interpreting the apoptosis–necrosis switch that is observed after exposure to increasing concentrations of different agents, including H_2O_2 [4–7] and redox-cycling quinones [8]. Thus, further research is needed in order to better define the relationships existing between apoptosis and necrosis in oxidatively injured cells.

In this study, we present experimental results indicating that the necrotic response evoked by high concentrations of H_2O_2 in U937 is the result of a specific and reversible inhibition of the late steps of the apoptotic programme.

MATERIALS AND METHODS

Cell Line and Cell Culture

U937 cells were cultured in suspension in RPMI 1640 medium (GIBCO), supplemented with 10% fetal bovine serum, penicillin (50 units/mL), and streptomycin (50 μg/mL) (Sera-Lab), at 37° in T-75 tissue culture flasks (Corning) gassed with an atmosphere of 95% air–5% CO_2. Stock solutions of hydrogen peroxide were freshly prepared in distilled water. 3AB was dissolved directly in the culture medium. Because the cytotoxicity of H_2O_2 toward cultured cells is dependent on cell density [9], a constant density of 3×10^5 cells/mL was used at the H_2O_2 treatment stage. Under these conditions, a 1.5-mM concentration of the oxidant corresponds to 5 μmol/million cells.

Cytotoxicity Assay

Cells were inoculated into 35-mm tissue culture dishes and exposed for 3 hr to increasing concentrations of H_2O_2. After the treatments, the cells were washed with saline A (0.14 M NaCl, 5 mM KCl, 4 mM $NaHCO_3$, and 5 mM glucose), resuspended in prewarmed culture medium, plated in 35-mm culture dishes, and incubated at 37° for 6 hr. Cytotoxicity was determined 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. Results are expressed as the percentage of dead cells (ratio of stained cells vs the total number of cells).

DNA Fragmentation Analysis by the Filter-binding Assay

Secondary DNA fragmentation was quantified using the filter-binding assay developed by Bertrand *et al.* [10], with minor modifications [3]. DNA fragmentation was determined as the percent ratio of the ^{14}C-labelled DNA eluted out of the filter versus the total DNA radioactivity in the sample.

DNA Fragmentation Analysis by Conventional Gel Electrophoresis

Genomic DNA was isolated from 3.5×10^6 U937 cells. Cells were sedimented in a 15-mL conical tube at 400 *g* for 10 min (4°), and the pellet was washed three times with 5 mL prechilled PBS (0.121 M NaCl, 10 mM NaH_2PO_4, 1.5 mM KH_2PO_4, 3 mM KCl) containing 5 mM EDTA, pH 8.3. Cells were lysed with 100 μL of buffer containing 10 mM Tris/HCl, 25 mM EDTA, 100 mM NaCl, 0.2% SDS, 0.5 mg/mL proteinase K, and the cell suspension was left in a 55° water bath for 2 hr. Two percent agarose (low-melt agarose, BioRad) was melted in TBE buffer (89 mM Tris base, 89 mM sodium borate, 2 mM Na_2EDTA [pH 8.3]), and 50 μL was added to the cell lysate. The mixture was quickly dispensed into a plug former on ice. The plugs were analyzed by electrophoresis using a 1.5% agarose gel in TBE for 4 hr at 50 V. The gel was incubated with DNase-free RNase (20 μg/mL) at 37° for 4 hr before staining with ethidium bromide and then photographed under a UV transilluminator.

DNA Fragmentation Analysis by Programmable, Autonomously Controlled Electrode (PACE) Electrophoresis

Cells prelabelled with [methyl-^{14}C]thymidine (0.05 μCi/mL) were embedded into agarose plugs as previously described by Sestili *et al.* [11]. PACE electrophoresis was carried out using a BioRad DRIII variable angle system. Briefly, the gels were cast using 1.0% w/v chromosomal grade agarose in 0.5× TBE buffer (composition of the 0.5× concentrated buffer: 44.5 mM Tris–HCl, 44.5 mM boric acid, 1 mM Na_2EDTA [pH 8.3]) and run for 24 hr at 160 V with a switch time linearly ramped from 10 to 150 sec, 120° reorientation angle, at a constant buffer temperature of 14° (gel in Fig. 2D), or according to the three-block assay (gel in Fig. 5) described in Sestili *et al.* [11]. Gels were stained with ethidium bromide, viewed with a UV transilluminator, and photographed. The distribution of radioactivity in the gel was determined using a BioRad GS 250 photostimulable storage phosphor imager.

Electron Microscopy

After the treatments, the cells were sedimented and immediately fixed with 2.5% glutaraldehyde in PBS for 1 hr. The cells were then rinsed, postfixed with 1% osmium tetroxide in veronal buffer, alcohol-dehydrated, and embedded in araldite. Preliminary light microscopy observations were performed on semi-thin sections stained with 1% toluidine blue. Thin sections, collected on Formvar-carbon-coated nickel grids, were stained with uranyl acetate and lead citrate. Observations were carried out with a Philips CM10 electron microscope at 80 KV.

NPSH Assay

NPSH levels were determined using the 5, 5′-dithiobis-(2-nitrobenzoic acid) method, as previously described [12]. The concentration of NPSH in the samples was determined by comparison with standard solutions of GSH. Protein

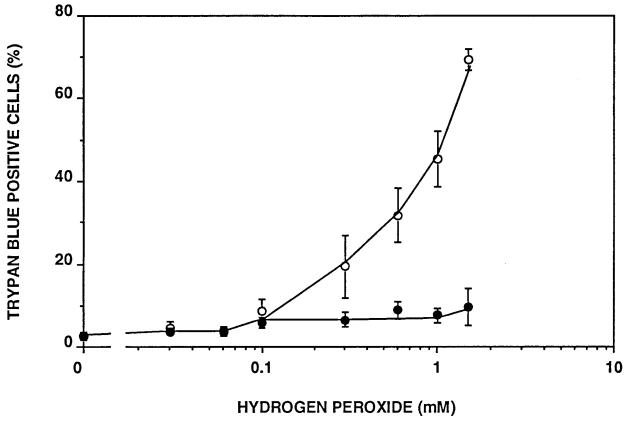

FIG. 1. The effect of 3AB on H_2O_2-induced U937 cell death. The cells were treated with increasing concentrations of H_2O_2 for 3 hr and were then postincubated for 6 hr in the absence (open circles) or presence (closed circles) of 1 mM 3AB. The number of dead cells was estimated at the end of the posttreatment incubation using the trypan blue dye exclusion assay. The results are the means ±SEM of at least three separate experiments.

contents were assayed as described by Lowry *et al.* [13], using BSA as standard.

Determination of Adenine Nucleotides

Adenine nucleotides were assayed by the method of Stocchi *et al.* [14]. Briefly, after treatment cells were transferred into a 15-ml Falcon tube and washed twice with cold saline A. One milliliter of cold 2.5% perchloric acid was added to each tube and, after a 10-min incubation in an ice bath, the solution was removed and the extraction procedure repeated. Cell extracts were neutralized with K_2CO_3 and the precipitate was removed by centrifugation. The nucleotide-containing supernatants were filtered through 0.22-μm pore microfilters and analyzed for ATP and NAD^+ content by reversed-phase HPLC using a Supelcosil LC-18 column (Supelco).

RESULTS

Concentration-dependent Induction of Apoptosis and Necrosis in U937 Cells Exposed to H_2O_2

U937 cells were exposed to increasing concentrations of H_2O_2 for 3 hr and then postincubated for 6 hr in fresh culture medium before determination of viability by trypan blue exclusion. At 0.3 mM, but not at lower levels, the oxidant caused a significant loss of viability, and this effect was further augmented by higher concentrations (Fig. 1). Figure 2A shows the extent of secondary DNA fragmentation as a function of the H_2O_2 concentration. The bell-shaped curve relating these two parameters is characterized by an ascending portion at levels below 0.1 mM, by a plateau between 0.1 and 0.3 mM, and by a descending portion above 0.6 mM. These results are in general agreement with those obtained by conventional gel electrophoresis and PACE, indicating that concentrations of H_2O_2 below 0.3 mM promote the formation of DNA laddering (Fig. 2C) and ≤50 Kb double-stranded DNA fragments (Fig. 2D), and that these effects progressively disappear at higher levels. The latter information can be better appreciated by the results illustrated in Fig. 2B, in which the radioactivity associated with the ≤50 Kb region was estimated using a photostimulable storage phosphor imaging system. The ≤50 Kb band increased at levels from 0.1 to 0.3 mM H_2O_2, decreased at 0.6 mM, and disappeared at 1 mM. It is noteworthy that the DNA from cells treated

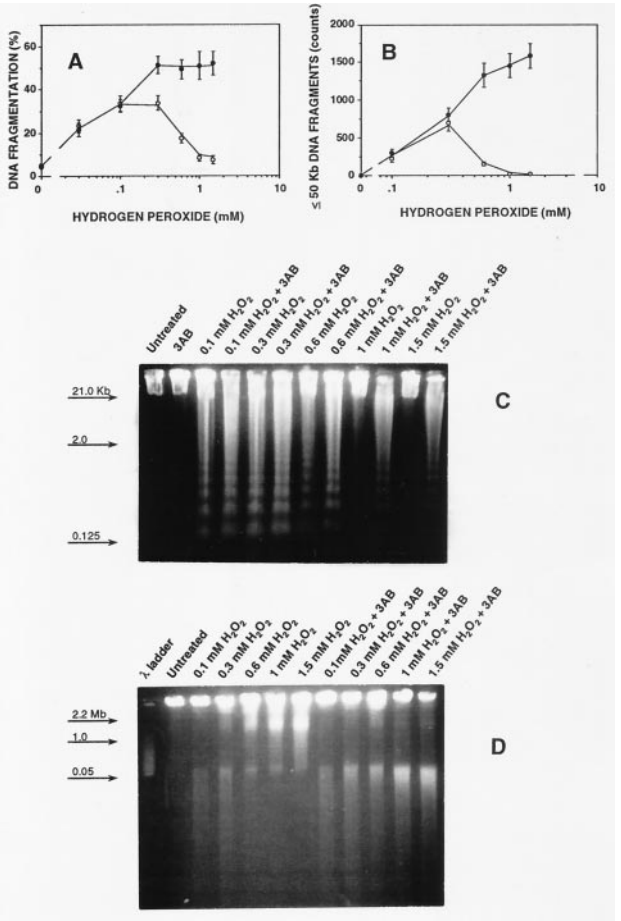

FIG. 2. Concentration-dependent induction of apoptosis and necrosis in U937 cells exposed to H_2O_2 and the modulation of these responses afforded by 3AB. The cells were treated as described in the legend to Fig. 1 and then analyzed for DNA fragmentation. (A) Results obtained using the filter-binding assay. The H_2O_2-treated cells were postincubated in the absence (open circles) or presence (closed circles) of 1 mM 3AB. Each point represents the mean ±SEM of 3–5 separate experiments. (B) Quantitation of the DNA associated with the ≤50 Kb region of gels similar to that shown in (D). The amount of ^{14}C-labelled DNA was determined by photostimulable storage phosphor imaging, and the data represent the means ±SEM of 3–4 separate experiments. Symbols are as in (A). (C) and (D) Photographs of UV light-illuminated ethidium bromide-stained gels obtained by conventional electrophoresis (C) or PACE (D) containing total cellular DNA (representative of three experiments with similar outcomes).

with 0.6, 1.0, and 1.5 mM H_2O_2 (Fig. 2D) displayed a pattern of degradation indicative of necrosis [11, 15].

Effects of 3AB on the Apoptotic and Necrotic Responses Evoked by H_2O_2

Addition of 1 mM 3AB during the 6-hr recovery phase prevented the loss of viability elicited by 0.3–1.5 mM H_2O_2 (Fig. 1). Importantly, however, the cytoprotection afforded by 3AB was only apparent since the inhibitor, while abolishing necrosis, led the cells into apoptosis. Indeed, under these experimental conditions, we were able to detect secondary DNA fragmentation (Fig. 2A), DNA laddering (Fig. 2C), and the formation of ≤50 Kb DNA fragments (Fig. 2, B and D). Interestingly, the extent of secondary DNA fragmentation (Fig. 2A) and the accumulation of ≤50 Kb DNA fragments (Fig. 2B) measured in 3AB-supplemented cells linearly increased after exposure to concentrations of H_2O_2 ranging from 0.03 to 1.5 mM. Finally, 3AB did not affect the extent of secondary DNA fragmentation (Fig. 2A), DNA laddering (Fig. 2C), or the formation of ≤50 Kb DNA fragments (Fig. 2, B and D) evoked by concentrations of H_2O_2 lower than 0.3 mM. 3AB alone neither caused loss of viability (Fig. 1) nor delayed apoptosis (Fig. 2, A–C).

Morphological Features of Cells Exposed to a High Concentration of H_2O_2

Electron microscopic analysis of untreated U937 cells showed a large round nucleus with dispersed chromatin and a large nucleolus (Fig. 3a). A 3-hr exposure to 1.5 mM H_2O_2 led to the appearance of diffuse cytoplasmic vacuolization (Fig. 3, b and c), and this response was both observed in the majority of the cells and associated with preservation of the other cellular compartments. The nucleus and chromatin appeared to be normally organized, except for some occasional swelling of the perinuclear cisternae. The mitochondria, while slightly hydrated, showed a good morphology (Fig. 3c). A 6-hr posttreatment incubation in fresh culture medium promoted dramatic changes in these cells, most of which displayed typical morphological features of necrosis such as disruption of the cytoplasm and appearance of remnants of swollen organelles (Fig. 3d). The nucleus, however, maintained its arrangement and the spatial organization of its components. The morphology of cells that were postincubated for 6 hr in the presence of 1 mM 3AB was indicative of apoptosis. Indeed, a large number of apoptotic cells and apoptotic bodies were found by light microscopy on semithin sections (not shown). Electron microscopic analysis allowed the detection of cells with an initial chromatin margination (Fig. 4a), which is indicative of a very early apoptotic stage. Late apoptotic cells characterized by deep cytoplasmic rearrangements, micronuclei, and nucleolar residues scattered throughout the cytoplasm (Fig. 4b) were also identified.

Effects of a High Concentration of H_2O_2 on the Formation of Double-Stranded DNA Fragments and Adenine Nucleotide or NPSH Intracellular Levels

Treatment with 1.5 mM H_2O_2 produced a time-dependent formation of double-stranded DNA fragments, as measured by the highly sensitive PACE three-block assay (Fig. 5). About 20% of the DNA was eluted after a 3-hr treatment and the size of these fragments was >2.2 Mb (inset to Fig. 5). Treatment with 1.5 mM H_2O_2 also produced a dramatic decline in cellular ATP (Fig. 6A) and NAD^+ (Fig. 6B), whose levels remained extremely low during the posttreatment incubation in fresh culture medium. Addition of 3AB did not ameliorate the status of the adenine nucleotide pool. Figure 6C shows that 1.5 mM H_2O_2 also depleted the NPSH pool, which remained low during recovery; once again, this response was not affected by 3AB.

DISCUSSION

It is now well established that hydrogen peroxide [4–7] and redox-cycling quinones [8] induce apoptosis or necrosis, depending on the severity of the insult inflicted on the cells. In the present study, we present results that are consistent with this notion, since the mode of U937 cell death mediated by H_2O_2 was found to be apoptosis at ≤0.1 mM and necrosis at ≥1 mM. At intermediate concentrations the oxidant induced apoptosis as well as necrosis. Experimental support for this observation is given by the results shown in Fig. 2A–D, indicating that low levels of H_2O_2 produced secondary DNA fragmentation, DNA laddering, and the formation of ≤50 Kb paired DNA fragments, and in Fig. 4a, b, showing the characteristic morphological features of apoptosis. These effects gradually disappeared at higher concentrations of the oxidant (Fig. 2A–D) and were accompanied by a parallel loss of cell viability, as assessed by trypan blue exclusion (Fig. 1) and by electron microscopy, indicating that under these conditions the mode of cell death was necrosis (Fig. 3d). We also found that 3AB prevents the necrotic response of cells exposed to concentrations of H_2O_2 higher than 0.3 mM (Fig. 1) and switches these cells into apoptosis (Fig. 2A–D and Fig. 4a, b). 3AB, however, did not affect the apoptotic response triggered by low concentrations of H_2O_2 (Fig. 2). The fact that 3AB neither affected the fate of those cells treated with apogenic concentrations of the oxidant nor increased the extent of the resulting apoptotic response indicates that the inhibitor does not directly or indirectly affect the pathways involved in the execution of the apoptotic programme.

The above results, while confirming our previous data [3] obtained using U937 cells treated with 1 mM H_2O_2, clearly demonstrate that the sole effect of 3AB is prevention of necrosis. It should also be emphasized that this response was observed under experimental conditions in which the inhibitor was given after exposure to the oxidant. This would imply that 3AB abolishes the acute lethal response

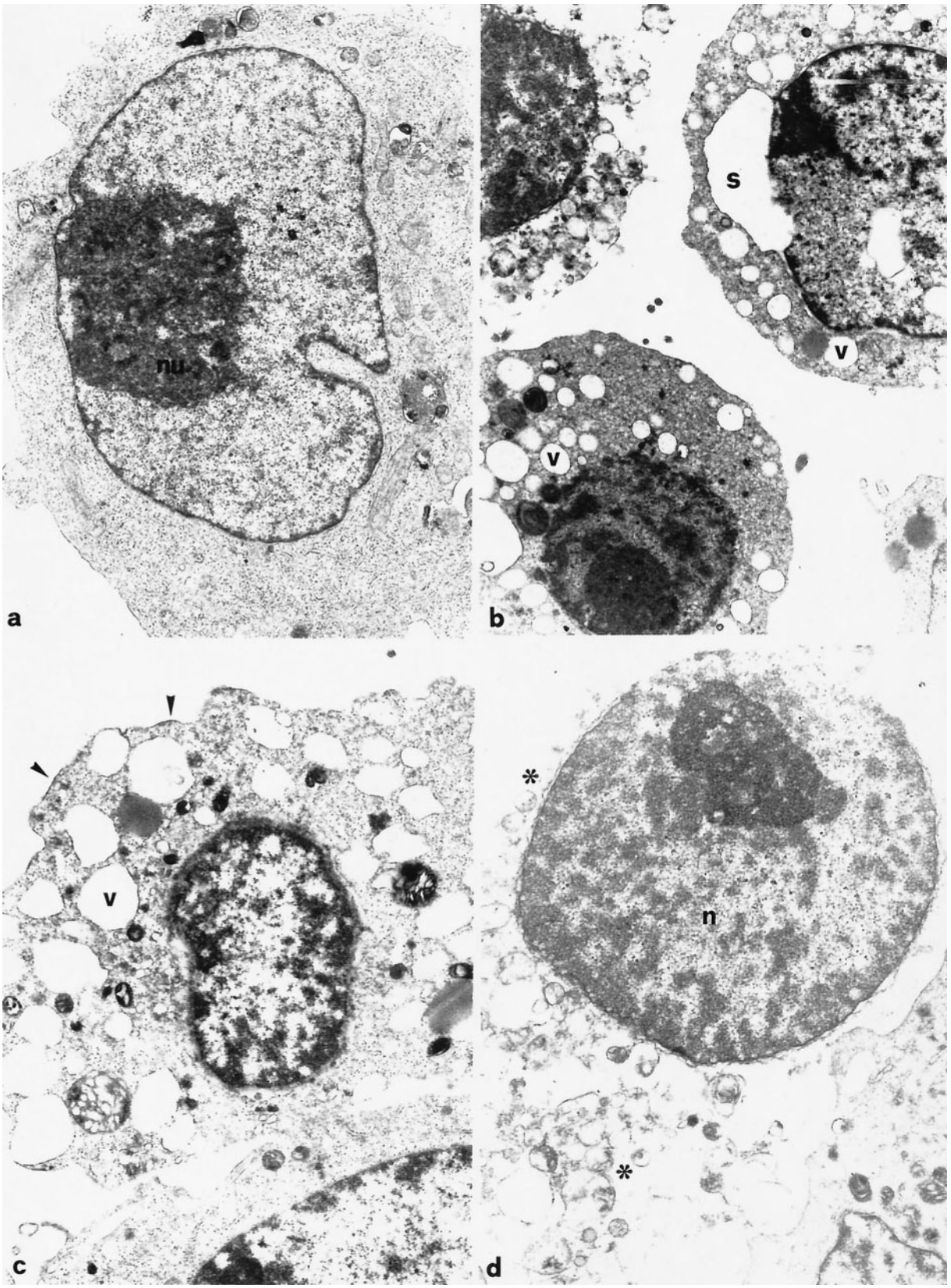

FIG. 3. The morphology of U937 cells treated with a high concentration of H_2O_2. Electron micrographs of: untreated U937 cells (a) nu = nucleolus; cells exposed for 3 hr to 1.5 mM H_2O_2 (b, c); cells treated as in (b, c) and postincubated for 6 hr in fresh culture medium (d). In (b) and (c), a diffuse vacuolization is associated with occasional swelling of perinuclear cisternae (s) and well-preserved plasma membranes (⟩) and cytoplasmic morphology. (d) shows a necrotic cell with a general cytoplasmic disruption (*) and a lasting partial preservation of the nucleus. n = nucleus; V = vacuoles; a = ×13,000; b = ×10,000; c = ×14,000; and d = ×16,000.

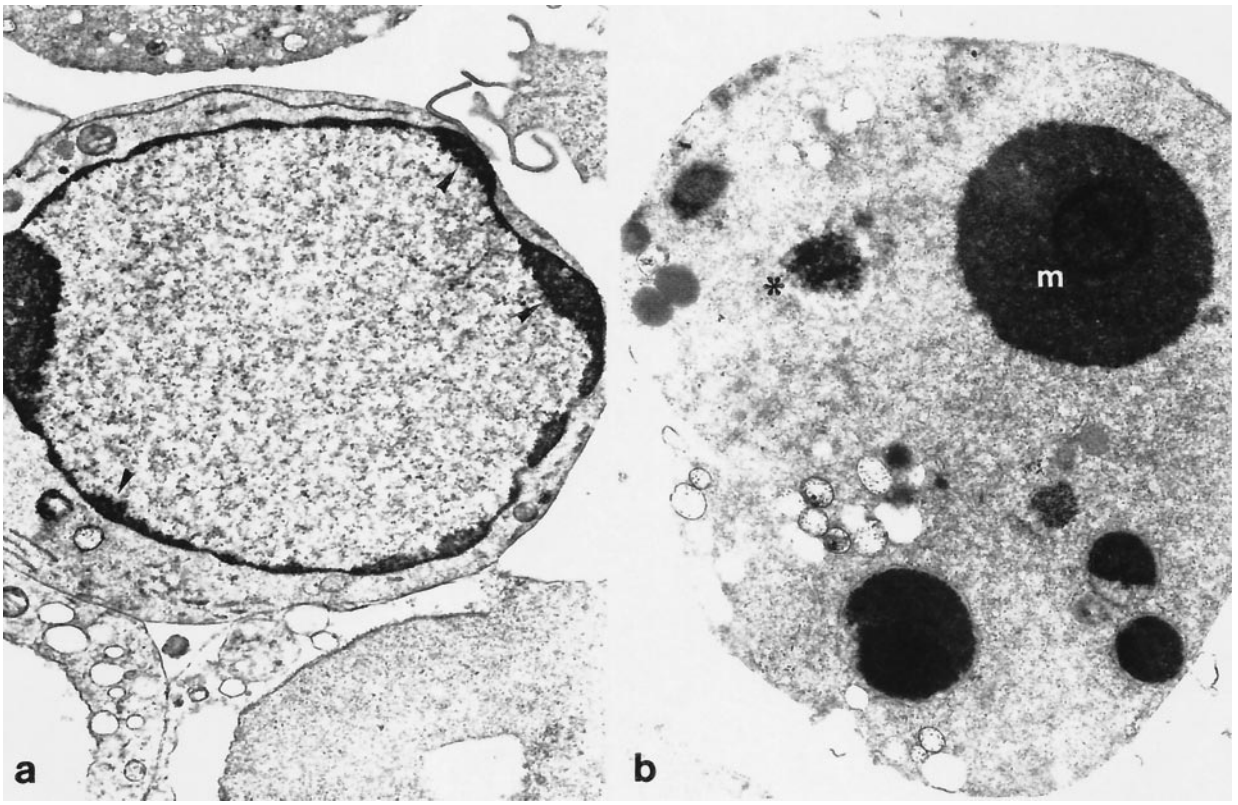

FIG. 4. The effect of 3AB on the morphology of U937 cells treated with a high concentration of H_2O_2. Electron micrographs of cells exposed for 3 hr to 1.5 mM H_2O_2 and postincubated for 6 hr in fresh culture medium supplemented with 1 mM 3AB. (a) Early phase apoptotic cell showing progressive chromatin margination (⟩). (b) Late phase apoptotic cell with micronuclei (m) and dispersed nucleolar remnants (*). a = ×11,000; b = ×14,000.

evoked by H_2O_2 without changing the extent and the nature of the initial damage induced by the oxidant. In another study [11], we reported that a 3-hr exposure to 1 mM H_2O_2 promoted the formation of double-stranded DNA fragments of $> 2 < 5.7$ Mb. Similar results were obtained in the present study using 1.5 mM H_2O_2 (inset to Fig. 5). The extent of DNA strand scission was quantified and found to linearly increase from 0.5 to 3 hr of peroxide exposure (Fig. 5). In principle, this response could be the consequence of either direct or indirect DNA fragmentation. The first possibility, however, is unlikely, since H_2O_2 has been shown to be a potent inducer of DNA single-strand breakage but a very poor inducer of DNA double-strand breakage in an array of investigations [16–19]. The cells treated with 1.5 mM H_2O_2 for 3 hr, while showing some signs of suffering, maintained a normal nuclear organization and good organelle morphology (Fig. 3b). Their NAD^+ and ATP levels, however, were dramatically low (Fig. 6A, B), a finding which suggests that these cells were energy-depleted. Their redox status also appeared to be compromised, since a 40% decline in cellular NPSH was detected (Fig. 6C).

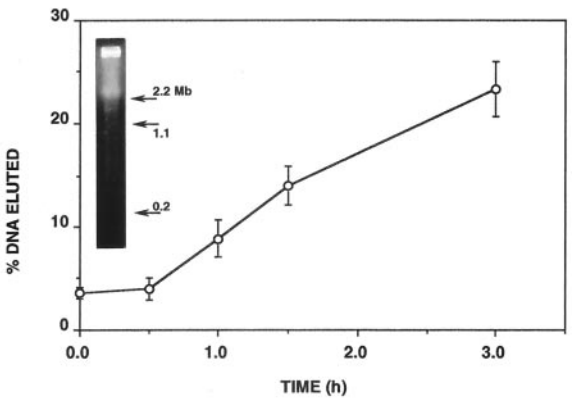

FIG. 5. Kinetics of formation of double-stranded DNA fragments in cells exposed to H_2O_2. The cells were exposed for increasing time intervals to 1.5 mM H_2O_2 and the resulting double-stranded DNA fragments were resolved by the three-block PACE assay. The inset shows a typical distribution pattern of double-stranded DNA fragments from cells treated for 3 hr.

Thus, these results collectively indicate that a 3-hr exposure to a high concentration of H_2O_2 promotes formation of Mb-sized DNA fragments and profound alterations in the energy and redox status of the cells, as well as minor morphological alterations. One possible interpretation of these results is that cells suffering from the insult inflicted by the oxidant are initiated to death in a sort of "neutral" fashion which can be directed, at a later stage, into necrosis or apoptosis in the absence or presence of 3AB, respectively. It is important to emphasize, however, that both the adenine nucleotides and the NPSH declined sharply during peroxide exposure and that these biochemical parameters

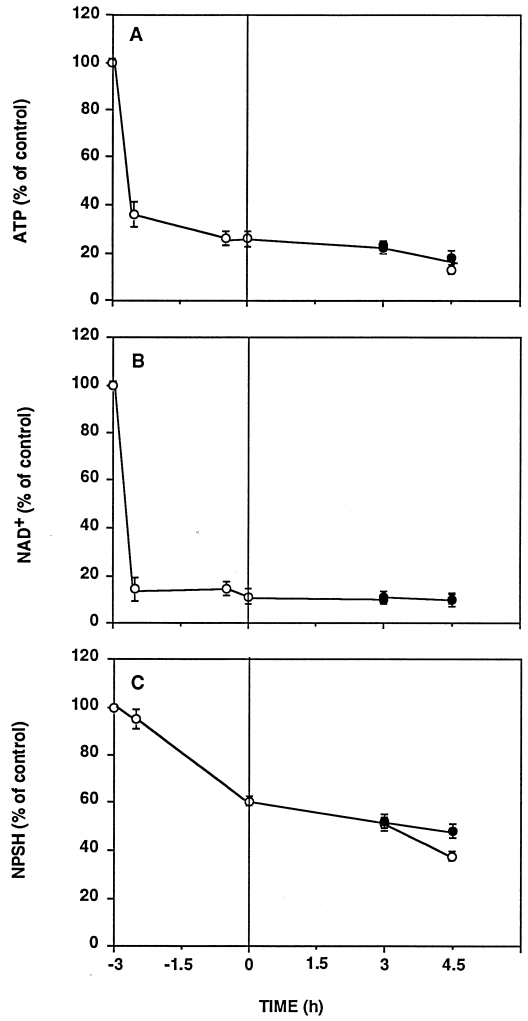

FIG. 6. The effect of 3AB on H_2O_2-induced depletion of adenine nucleotides or NPSH. The cells were treated with 1.5 mM H_2O_2 for 3 hr and then postincubated for increasing intervals in the absence (open circles) or presence (closed circles) of 1 mM 3AB. The vertical line corresponding to time 0 separates the H_2O_2 exposure phase to postincubation with or without 3AB. The levels of ATP (A) or NAD^+ (B) were measured by HPLC. The NPSH (C) content was estimated by the colorimetric assay described in the Methods section. The results are the means ±SEM of at least three separate experiments.

remained extremely low regardless of whether 3AB was absent or present during the 6 hr of posttreatment incubation (Fig. 6A–C). This observation leads to three important conclusions. First, cells depleted of energy and of reducing equivalents are able to execute the late steps of the apoptotic programme. As a corollary, the early steps, in particular those which require ATP, must take place during the early times of peroxide exposure. Secondly, the cells which received 3AB were rescued from necrosis but were nevertheless depleted of energy and of reducing equivalents; as a consequence, these events appear to be unrelated to the mechanism whereby H_2O_2 causes necrosis. Thirdly, these results are consistent with the possibility that the mechanism whereby 3AB blocks the necrotic response involves inactivation of a specific pathway.

We therefore interpret our results as an indication that micromolar as well as millimolar concentrations of H_2O_2 activate apoptosis, and in particular the energy-requiring step(s), at very early times of peroxide exposure. The apoptotic process, however, can be completed during the posttreatment incubation phase only in those cells which received micromolar levels of the oxidant. Millimolar concentrations of H_2O_2 would appear to activate some process inhibiting the late steps of the apoptotic response and, as a consequence, cells receiving these treatments undergo necrosis since they are already committed to death. By removing this obstacle, 3AB allows the expression of the apoptotic response over the entire range of H_2O_2 concentrations utilized in this study. Indeed, the apoptotic response in 3AB-supplemented cells was a direct function of the oxidant concentration (Fig. 2A, B).

Finally, the fact that following the 3-hr challenge with high concentrations of H_2O_2 the NAD^+ levels were always very low and were not affected by the addition of 3AB strongly suggests that its effects were independent of PARP inhibition. It is important to emphasize that this enzyme functions by transferring large amounts of the ADP-ribose moiety of NAD^+ to acceptor proteins [20]. This makes it highly unlikely that significant PARP activity can occur in the presence of very low levels of the adenine nucleotide. On the other hand, the rapid NAD^+ consumption observed immediately after H_2O_2 addition strongly suggests early PARP activation. Since it has been proposed that PARP triggers the early and key steps of the apoptotic programme [21], the possibility exists that PARP activation is one of those energy-requiring processes occurring at the very early times of peroxide exposure which finally lead cells into apoptosis (see above). Thus, under the specific experimental conditions used in the present study, the effects of 3AB appear to be independent of PARP inhibition. It is important to note that these results do not rule out the possibility that 3AB-mediated inhibition of PARP is of importance in toxicity paradigms different from that utilized in this study. Indeed, an extensive body of literature (reviewed in Ref. 22) clearly indicates that PARP activation plays a pivotal role in the process of cell death.

In conclusion, the results presented in this study strongly suggest that, at least under these specific experimental conditions, necrosis is not an obligatory response to overwhelming damage, but rather the result of a direct or indirect inhibition of the late steps of the apoptotic programme triggered by H_2O_2.

The financial support of Telethon—Italy (Grant nr 1110 to O.C.) and Istituti Ortopedici Rizzoli, Bologna (E.F.) is gratefully acknowledged. The authors are indebted to Mr. A. Valmori for skillful photography.

References

1. Corcoran GB, Fix L, Jones DP, Treinen Moslen T, Nicotera P, Oberhammer FA and Buttyan R, Apoptosis: Molecular control point in toxicity. *Toxicol Appl Pharmacol* **128:** 169–181, 1994.
2. Jacobson MD, Reactive oxygen species and programmed cell death. *Trends Biochem Sci* **21:** 83–86, 1996.
3. Palomba L, Sestili P, Cattabeni F, Azzi A and Cantoni O, Prevention of necrosis and activation of apoptosis in oxidatively injured human myeloid leukemia U937 cells. *FEBS Lett* **390:** 91–94, 1996.
4. Lennon SV, Kilfeather SA, Hallett MB, Campbell AK and Cotter TG, Elevations in cytosolic free Ca^{2+} are not required to trigger apoptosis in human leukaemia cells. *Clin Exp Immunol* **87:** 645–647, 1992.
5. Ueda N and Shah SV, Endonuclease-induced DNA damage and cell death in oxidant injury to renal tubular epithelial cells. *J Clin Invest* **90:** 2593–2597, 1992.
6. Hockenbery DM, Oltvai ZN, Yin X, Milliman CL and Korsmeyer SJ, Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell* **75:** 241–251, 1993.
7. Nosseri C, Coppola S and Ghibelli L, Possible involvement of poly(ADP-ribosyl)polymerase in triggering stress-induced apoptosis. *Exp Cell Res* **212:** 367–373, 1994.
8. Dypbukt JM, Ankarcrona M, Burkitt M, Sjoholm A, Strom K, Orrenius S and Nicotera P, Different prooxidant levels stimulate growth, trigger apoptosis, or produce necrosis of insulin-secreting RINm5F cells. The role of intracellular polyamines. *J Biol Chem* **269:** 30553–30560, 1994.
9. Cantoni O, Murray D and Meyn RE, Effect of 3-aminobenzamide on DNA strand-break rejoining and cytotoxicity in CHO cells treated with hydrogen peroxide. *Biochim Biophys Acta* **867:** 135–143, 1986.
10. Bertrand R, Sarang M, Jenkin J, Kerrigan D and Pommier Y, Differential induction of secondary DNA fragmentation by topoisomerase II inhibitors in human tumor cell lines with amplified c-*myc* expression. *Cancer Res* **51:** 6280–6285, 1991.
11. Sestili P, Cattabeni F and Cantoni O, Direct excision of 50 Kb pair DNA fragments produced during apoptotic cleavage of genomic DNA. *FEBS Lett* **396:** 337–342, 1996.
12. Cantoni O, Sestili P, Guidarelli A and Cattabeni F, Development and characterization of hydrogen peroxide-resistant Chinese hamster ovary (CHO) cell variants-II. Relationships between non-protein sulfydryl levels and the induction/stability of the oxidant-resistant phenotype. *Biochem Pharmacol* **47:** 1258–1261, 1994.
13. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement by the Folin phenol reagent. *J Biol Chem* **193:** 265–275, 1951.
14. Stocchi V, Cucchiarini L, Magnani M, Chiarantini L, Palma P and Crescentini G, Simultaneous extraction and reverse-phase high-performance liquid chromatographic determination of adenine and pyridine nucleotides in human red blood cells. *Anal Biochem* **146:** 118–124, 1985.
15. Sestili P, Brandi G, Brambilla L, Cattabeni F and Cantoni O, Hydrogen peroxide mediates the killing of U937 tumor cells elicited by pharmacologically attainable concentrations of ascorbic acid: Cell death prevention by extracellular catalase from co-cultured erythrocytes or fibroblasts. *J Pharmacol Exp Ther* **277:** 1719–1725, 1996.
16. Ward JF, Blakely WF and Joner EI, Mammalian cells are not killed by DNA single-strand breaks caused by hydroxyl radicals from hydrogen peroxide. *Radiat Res* **103:** 383–392, 1985.
17. Ward JF, Evans CL, Limoli CL and Calabro-Jones PM, Radiation and hydrogen peroxide induced free radical damage to DNA. *Br J Cancer Suppl* **8:** 105–112, 1987.
18. Cantoni O, Cattabeni F, Stocchi V, Meyn RE, Cerutti P and Murray D, Hydrogen peroxide insult in cultured mammalian cells: Relationships between DNA single-strand breakage, poly(ADP-ribose) metabolism and cell killing. *Biochim Biophys Acta* **1014:** 1–7, 1989.
19. Sestili P, Cantoni O and Murray D, Evidence for separate mechanisms of cytotoxicity in mammalian cells treated with hydrogen peroxide in the absence or presence of L-histidine. *Biochim Biophys Acta* **1268:** 130–136, 1995.
20. Shall S, Inhibition of DNA repair by inhibitors of nuclear ADP-ribosyl transferase. In: *DNA Repair and Its Inhibition* (Eds. Collins A, Downes CS and Johnson RT), pp 143–191. IRL Press, Oxford, 1994.
21. Simbulan-Rosenthal CM, Rosenthal DS, Iyer S, Boulares AH and Smulson ME, Transient poly(ADP-ribosyl)ation of nuclear proteins and role of poly(ADP-ribose)polymerase in the early stages of apoptosis. *J Biol Chem* **273:** 13703–13712, 1998.
22. Pieper AA, Verma A, Zhang J and Snyder SH, Poly(ADP-ribose) polymerase, nitric oxide and cell death. *Trends Pharm Sci* **20:** 171–181, 1999.