The role of intracellular oxidation in death induction (apoptosis and necrosis) in human promonocytic cells treated with stress inducers (cadmium, heat, X-rays)

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Treatment of U-937 human promonocytic cells with the stress inducers cadmium chloride (2 h at 200 μM), heat (2 h at 42.5 °C) or X-rays (20 Gy), followed by recovery, caused death by apoptosis and stimulated caspase-3 activity. In addition, all stress agents caused intracellular oxidation, as measured by peroxide and/or anion superoxide accumulation. However, while pre-incubation with antioxidants (N-acetyl-L-cysteine or butylated hydroxyanisole) inhibited the induction of apoptosis by cadmium and X-rays, it did not affect the induction by heat-shock. Pre-incubation for 24 h with the GSH-depleting agent L-buthionine-[S,R]-sulfoximine (BSO) switched the mode of death from apoptosis to necrosis in cadmium-treated cells. By contrast, BSO only caused minor modifacions in the rate of apoptosis without affecting the mode of death in heat- and X-rays-treated cells. BSO potentiated peroxide accumulation in cells treated with both cadmium and X-rays. However, while the accumulation of peroxides was stable in the case of cadmium, it was transient in the case of X-rays. Moreover, the administration of antioxidants during the recovery period sufficed to prevent necrosis and restore apoptosis in BSO plus cadmium-treated cells. Cadmium and X-rays caused a decrease in intracellular ATP levels, but the decrease was similar in both apoptotic and necrotic cells. Taken together, these results demonstrate that (*i*) stress inducers cause intracellular oxidation, but oxidation is not a general requirement for apoptosis; and (*ii*) the duration of the oxidant state seems to be critical in determining the mode of death.

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Abbreviations. BHA Butylated hydroxyanisole. – BSO L-buthionine-[*S,R*]-sulfoximine. – DAPI 4,6-Diamidino-2-phenylindole. – DHE Dihydroethidium. – H_2DCFDA Dichlorodihydrofluorescein diacetate. – HSP Heat-shock protein. – NAC N-acetyl-L-cysteine. – PARP Poly(ADP-ribose) polymerase. – PI Propidium iodide. – ROS Reactive oxygen species.

Introduction

The inducers of the stress response are a group of physical and chemical agents, such as hyperthermia, heavy metals, inhibitors of energy metabolism, and occasionally radiation, which stimulate the synthesis and accumulation of heat-shock proteins (HSPs) (Lindquist and Craig, 1998). The accumulation of some HSPs (e.g., HSP70 and HSP27) protects the cells from the adverse consequences of prolonged stress, allowing their survival. However, it is known that the stress inducers may also cause cell death, either apoptotic or necrotic (Vilaboa et al., 1997; Habebu et al., 1998; Vayssier et al., 1998). During apoptosis the cells undergo nuclear and cytoplasmic shrinkage, the chromatin is condensed and partitioned into multiple fragments, and the cells are broken into multiple membrane-surrounded bodies (apoptotic bodies). However, the plasma membrane retains the integrity during the process, preventing the release of the cell content into the environment. By contrast, necrosis is characterized by cell swelling and lysis of intracellular organella, and the disintegration of the plasma membrane. Although the morphological characteristics of apoptosis and necrosis are well defined, their regulation and the factors that decide the selection of the mode of death are insufficiently known.

One of the most complex aspects in the regulation of cell death is the role of intracellular oxidation. It was earlier proposed that oxidation could be a general mediator of

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apoptosis (Buttke and Sandstrom, 1994). In fact, (*i*) exposure to reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) or nitric oxide (NO), induces apoptosis in different cell types (Lennon et al., 1991; Albina et al., 1993); (*ii*) many apoptotic inducers which are not ROS themselves cause intracellular oxidation – e.g., growth factor deprivation, glucocorticoids, UV irradiation, and some cytotoxic drugs (Benchekroun et al., 1993; Hockenbery et al., 1993; Greenlund et al., 1995; Slater et al., 1995; Gorman et al., 1997); (*iii*) overexpression of Bcl-2 reduces both ROS generation and apoptosis induction by different stimuli (Hockenbery et al., 1993; Kane et al., 1993); (*iv*) apoptotic inducers often cause a rapid depletion of intracellular reduced glutathione (GSH), the most important antioxidant system (Ghibelli et al., 1998); and (*v*) prolonged treatments with the GSH-depleting agent L-buthionine-[S,R]-sulfoximine (BSO) potentiates the harmful effects of cytotoxic agents (for reviews see (Bailey, 1998; and Anderson, 1998)). In addition, it seems that the intensity of oxidation may be a determinant for the mode of death. For instance, exposure to H_2O_2 provoked apoptosis or necrosis, depending on the concentration used (Lennon et al., 1991), and administration of low H_2O_2 concentrations switched the mode of death from apoptosis to necrosis in drug-treated lymphoma cells (Shacter et al., 2000). However, other observations indicated that at least some forms of apoptosis may take place under very low oxygen tensions, in which ROS generation is expected to be absent or greatly reduced (Jacobson and Raff, 1995; Muschel et al., 1995), or in the presence of antioxidants (Jacobson and Raff, 1995), suggesting that intracellular oxidation is not a universal trigger for apoptosis. Moreover, low ROS concentrations may prevent apoptosis and promote proliferation in some cell models ((Del Bello et al., 1999) and references therein).

We have recently reported that DNA-damaging agents and stress inducers caused death by apoptosis in U-937 human promonocytic cells (Galán et al., 2000b). In addition, it was observed that the toxic action of the heavy metal cadmium was dependent on intracellular oxidation, and was greatly increased by depletion of GSH content (Galán et al., 2000a). However, we do not know whether this is a common characteristic of all stress inducers. To investigate this problem, in the present work we comparatively examined the capacity of heat-shock (the most typical stress inducer), cadmium and X-rays to cause cell death and ROS accumulation, and the capacity of ROS scavengers and BSO to modulate the stress-provoked cell death, in U-937 cells.

Materials and methods

Cell culture and drug treatments

U-937 promonocytic leukemia cells (Sundström and Nilsson, 1976) were grown in RPMI 1640 supplemented with 10% (v/v) heat-inactivated fetal calf serum and 0.2% sodium bicarbonate and antibiotics in a humidified 5% CO_2 atmosphere at 37 °C. Butylated hydroxyanisole (BHA), N-acetyl-L-cysteine (NAC), DL-buthionine-[S,R]-sulfoximine (BSO) and dihydroethidium (DHE) were obtained from Sigma Química, Madrid, Spain. Dichlorodihydrofluorescein diacetate (H_2DCFDA) was obtained from Molecular Probes, Inc., Eugene, OR, USA. Cadmium chloride was obtained from Merck, Darmstadt, Germany. The caspase inhibitors Z-Val-Ala-Asp (OMe)-CH_2F (Z-VAD-FMK) and acetyl-Asp-Glu-Val-Asp aldehyde (Ac-DEVD-CHO) were obtained from Enzyme Systems Products, Dublin, CA, USA, and Bachem Feinchemikalien AG, Bubendorf, Switzerland, respectively. NAC, DHE and Z-VAD-FMK were dissolved in dimethyl sulfoxide at 3 M, 10 mM and 20 mM, respectively; BHA and H_2DCFDA in ethanol at 0.5 M and 5 mM, respectively; and Ac-DEVD-CHO in distilled water at 10 mM. All these solutions were stored at – 20 °C. BSO and cadmium chloride were freshly prepared in distilled water at 0.1 M. For heat-shock, the cultures were placed in a bath at 42.5 °C. For recovery after treatments, the cells were either directly placed under standard culture conditions (in the case of heat-shock or X-radiation), or washed with pre-warmed (37 °C) RPMI medium and then cultured under standard conditions (in the case of cadmium chloride). Control cells were subjected to the same manipulations, except for treatment with the stress inducers.

GSH depletion

GSH depletion was achieved by incubating the cells for 24 hours with 1 mM BSO, as earlier described by Ghibelli et al. (1995) using U-937 cells. Under these conditions BSO reduced the intracellular GSH level by more than 90%, as determined with a Bioxytech GSH-400 kit (OXIS International, Inc., Portland, OR, USA), but it did not affect cell proliferation or viability. For experiments in combination with other agents, cells pre-incubated with BSO were treated with cadmium chloride, heat or X-radiation and allowed to recover, always in the presence of BSO. In some experiments BSO was removed during the period of cadmium treatment to avoid possible drug-metal interactions, but this modification did not significantly alter the results.

Determination of apoptosis and necrosis

To analyze changes in nuclear morphology, cells were collected by centrifugation, washed with PBS, resuspended in PBS and mounted on glass slides. After fixation in 70% (v/v) ethanol, the cells were stained for 20 min at room temperature with 1 μg/ml 4,6-diamidino-2-phenylindole (DAPI) (Serva, Heidelberg, Germany) and examined by fluorescence microscopy. Apoptosis was characterized by chromatin condensation followed by partition into multiple bodies. Within the experimental time periods used in this work, non-apoptotic, primary necrotic cells still exhibited diffuse and uniform chromatin staining, as untreated cells.

To measure loss of DNA, cells were collected by centrifugation and incubated for 30 min in PBS containing 0.5 mg/ml RNase A. After the addition of 50 μg/ml propidium iodide (PI) and permeabilization with 0.1% (w/v) Nonidet P-40, the cells were analyzed by flow cytometry. Late apoptotic cells and apoptosis-derived necrotic cells (secondary necrosis) exhibited sub-G_1 PI incorporation (hypo-diploid cells). Within the experimental time periods used in this work, non-apoptotic, primary necrotic cells did not exhibit significant loss of DNA, nor significant alterations in the cell cycle distribution in relation to untreated cells.

The criterion currently used to examine necrosis was the loss of membrane integrity, as measured by massive influx of either trypan blue or PI in non-permeabilized cells. In the first case, cells were incubated for 5 min with 0.2% (w/v) trypan blue and examined by microscopy using a Neubauer haemocytometer. Under these conditions, only necrotic cells were clearly stained. In the second case, cells were washed with PBS and incubated for 10 min at room temperature in 500 μl of a buffer consisting of 10 mM HEPES (pH 7.4), 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 1.8 mM $CaCl_2$, containing 20 μg of PI and 3 μl of FITC-conjugated human annexin V, a molecule which specifically binds phosphatidylserine (Annexin V-FITC kit, Bender MedSystems, Vienna, Austria). The cells were then analyzed by flow cytometry using appropriate colour filters to determine the PI-derived reddish orange fluorescence (emission peak 590 nm) and the FITC-derived greenish fluorescence (emission peak 530 nm). Under these conditions necrotic cells exhibited great green and red fluorescence, due to massive penetration of both annexin V and PI. By contrast, apoptotic cells could exhibit green fluorescence, due to phosphatidylserine translocation from the inner to the outer layer of the plasma membrane, but null or moderate red fluorescence (Martin et al., 1995; Bedner et al., 1999).

Measurement of reactive oxygen species

The intracellular accumulation of ROS was determined using the fluorescent probes DHE and H_2DCFDA. DHE preferentially measures

O_2^- (Benov et al., 1998). H_2DCFDA was commonly used to measure H_2O_2 (LeBel et al., 1992), but it is now accepted that this probe is also sensitive to other peroxides (Ischiropoulos et al., 1999). With this aim, 1 h prior to treatment with the stress inducers the cells were resuspended in RPMI medium without FCS and red phenol, and loaded with either 5 μM H_2DCFDA or 2 μM DHE. The probes were maintained during the treatment and recovery periods. The fluorescence was then measured by flow cytometry. Control cells were subjected to the same manipulation, except for treatment with the stress inducers.

Measurement of ATP levels

To estimate the intracellular ATP content, aliquots of 106 cells were collected in a pre-heated (70 °C) buffer consisting of 100 mM Tris-HCl and 4 mM EDTA, pH 8, and heated for 2 min at 100 °C. After freezing on ice, the samples were centrifuged and the supernatants stored at − 70 °C. The ATP content was determined using an ATP Bioluminescence Assay Kit CLSII (Roche Diagnostics, Barcelona, Spain), following the procedure indicated by the manufacturer.

Determination of PARP cleavage

Poly (ADP-ribose) polymerase (PARP) cleavage was examined by immunoblot, using 6.5% polyacrylamide minigels and rabbit anti-human PARP polyclonal antibody (Roche Diagnostics). All other conditions were as previously described (Galán et al., 2000a).

Results

Cell death

U-937 cells were pulse-treated with heat (2 h at 42.5 °C), cadmium chloride (2 h at 200 μM) or X-rays (20 Gy), and then allowed to recover, following the experimental scheme indicated in Fig. 1. Under these conditions the three agents induced HSP70 expression ((Vilaboa et al., 1997) and results not shown), indicating that they may be strictly considered stress inducers in this cell model. In addition, the treatments caused death by apoptosis, as determined by the presence of condensed and fragmented chromatin (Fig. 2). According to this criterion, apoptosis was already detected at 3 h, reaching 50–60% apoptotic cells at 6 h of recovery (Fig. 2A). The expression of other apoptotic markers, namely nucleosome-sized DNA fragmentation (as measured by gel electrophoresis) and sub-

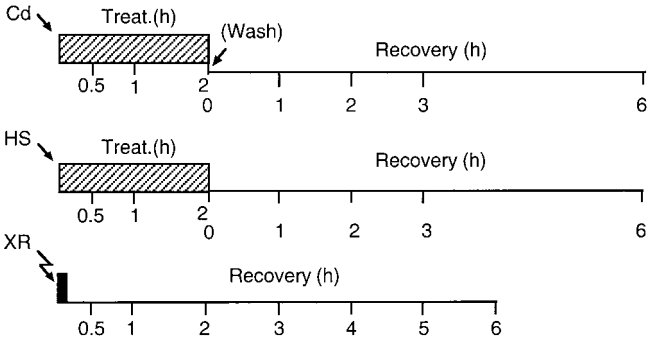

Fig. 1. Experimental scheme used for treatments with cadmium, heat-shock and X-rays. U-937 cells were treated for 2 h with either cadmium chloride (Cd, 200 μM) or heat (HS, 42.5 °C), and then allowed to recover; or subjected to X-radiation (XR, 20 Gy) and allowed to recover. Since X-radiation lasted only 6 min, this treatment was considered to be punctual. Hence, 0.5, 1 and 2 h of treatment with Cd and HS corresponded to 0.5, 1 and 2 h of recovery in the case of XR; and 1, 2 and 3 h of recovery in the case of Cd and HS corresponded to 3, 4 and 5 h of recovery in the case of XR.

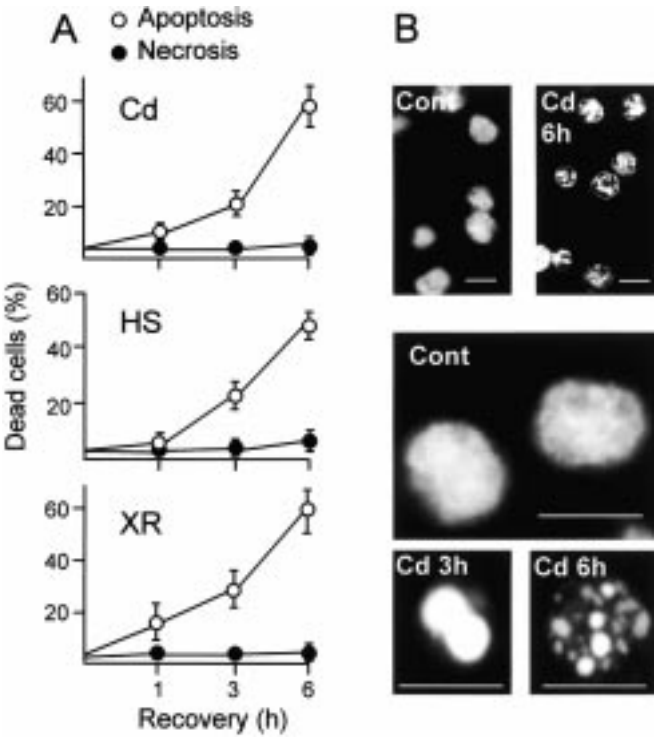

Fig. 2. Induction of cell death by cadmium, heat-shock and X-rays. (**A**) Frequency of apoptotic cells, as determined by the presence of condensed and fragmented chromatin, and of necrotic cells, as determined by trypan blue uptake, at the indicated times of recovery. The data represent the mean ± S.D. of three experiments with similar results. (**B**) Examples of nuclei of untreated cells (Cont), with diffuse and uniformly stained chromatin, and of cadmium-treated cells at the indicated times of recovery, with condensed and fragmented chromatin, as determined by DAPI staining and microscopy examination. Bars, 10 μm. All other conditions were as in Fig. 1.

G_1 DNA content (as measured by PI staining and flow cytometry), was demonstrated in a preceding work (Galán et al., 2000b) and hence is omitted here. Under the used conditions, the stress treatments failed to cause primary necrosis, as evidenced by trypan blue exclusion at 6 h of recovery (Fig. 2A). Nevertheless, after prolonged recovery periods (15 h and thereafter) abundant cell debris and trypan blue-stained cells could be observed. This probably reflects apoptosis-derived "secondary" necrosis, a situation commonly observed in cell cultures due to the lack of phagocytic cells. Hence, 6 h was the maximum recovery period usually adopted for further experiments.

Caspase activity

It has been reported that caspase-3 activity mediates the execution of apoptosis in different models (for review see (Porter and Jänike, 1999)). Moreover, caspase inactivation may prevent apoptosis and drive the cells into necrosis (Ueda et al., 1998; Samali et al., 1999). For these reasons, we wanted to analyze the capacity of the stress inducers to activate caspase-3, as measured by PARP cleavage, and the capacity of caspase inhibitors to modulate cell death. The results are represented in Fig. 3. Treatment with the stress inducers caused a late cleavage of PARP (116 kDa), coincident with the expression of apoptotic markers, to give an 85 kDa fragment, characteristic of

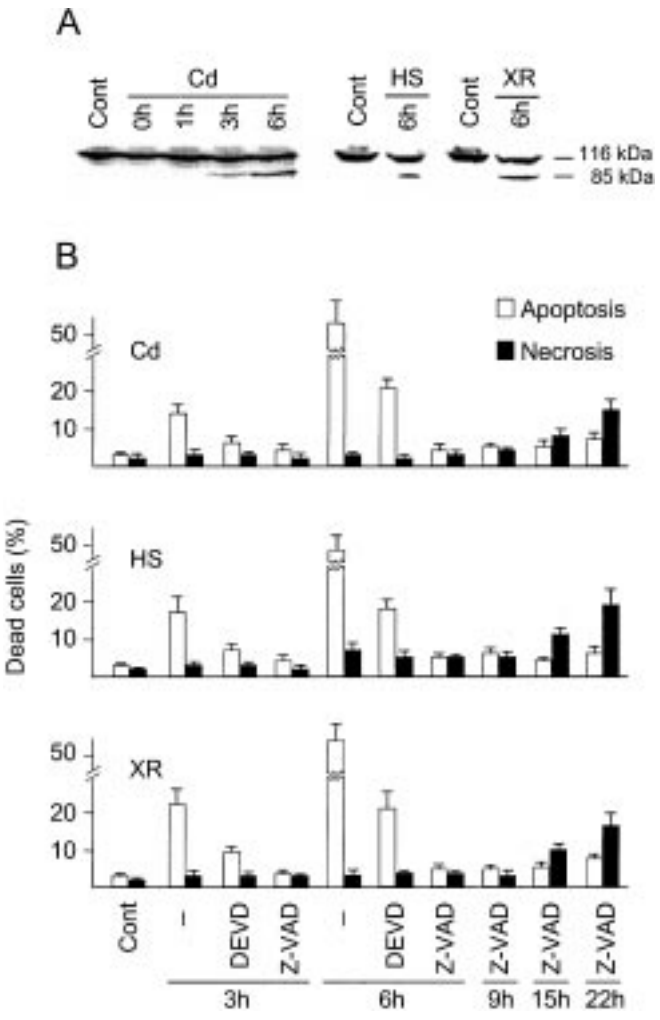

Fig. 3. PARP cleavage and effect of caspase inhibitors. (**A**) PARP cleavage at the indicated times of recovery after cadmium, heat and X-rays, as determined by immunoblot. The positions of the whole protein (116 kDa) and the major cleavage fragment (85 kDa) are indicated. The experiment was repeated twice with similar results. (**B**) Effect of Ac-DEVD-CHO (200 μM) and Z-VAD-FMK (50 μM) on the stress-provoked cell death. The caspase inhibitors were applied at the beginning of treatment with heat and X-rays, and at the beginning of recovery in the case of cadmium. The resuls are the mean ± S.D. of at least three determinations.

apoptosis (Kaufmann et al., 1993; Lazebnik et al., 1994) (Fig. 3A). Apoptosis was attenuated by the caspase-3-specific inhibitor Ac-DEVD-CHO, and almost totally suppressed by the non-specific inhibitor Z-VAD-FMK, at least until 22 h of recovery (Fig. 3B). Under these conditions necrosis was slightly initiated at 15 or 22 h (Fig. 3B).

ROS accumulation and effect of antioxidants

It was earlier reported that the cytotoxic agents cause a rapid increase in H_2DCFDA-derived fluorescence in myeloid cells, suggesting that peroxides may mediate induction of apoptosis (Gorman et al., 1997). For this reason, we measured peroxide accumulation in response to treatment with the stress inducers. The results in Fig. 4 indicate that the H_2DCFDA-derived fluorescence was increased by treatment with cadmium and X-rays, while it was not increased and was even reduced by heat-shock, when compared to untreated cells. The same negative result was obtained at different times of heating (from 15 min to 2 h) and recovery after heat-shock (from 1 to 3 h) (results not shown). Pre-heated H_2DCFDA was still fully responsive to treatment with cadmium and X-rays (results no shown), excluding a possible inactivation by heat of the fluorescent probe. In spite of this, all stress treatments, including heat-shock, augmented the DHE-derived fluorescence, suggesting an increased accumulation of O_2^- (Fig. 4).

To investigate whether intracellular oxidation mediates the stress-provoked apoptosis, we examined the capacity of the non-specific ROS scavengers BHA and NAC to prevent cell death. These antioxidants were selected since they were able to inhibit the induction of the stress response by heat in U-937 cells (Galán et al., 2000a). The antioxidants were applied 2 h prior to treatment with the apoptotic inducers, and maintained during the treatment and recovery periods. In these experiments NAC had to be omitted in the case of cadmium, due to the reactivity of Cd^{2+} ions with -SH groups. The results in Table I indicate that the antioxidants reduced the frequency of apoptotic cells in cultures treated with cadmium and X-rays, but not in heat-treated cultures. These results indicate that intracellular oxidation mediates the induction of apoptosis by cadmium and X-rays, but not by heat-shock.

Effect of BSO on cell death

We recently reported that pre-incubation with the GSH-depleting agent BSO drastically increased the toxicity of cadmium in U-937 cells, causing necrosis instead of apoptosis (Galán et al., 2000a). To analyze whether GSH content also affects the mode of death caused by other stress inducers, cells were incubated for 24 h with 1 mM BSO, prior to treatment with cadmium, heat and X-rays. Fig. 5A shows the frequency of apoptotic and necrotic cells, as determined by chromatin condensation and fragmentation and by trypan blue uptake, respectively. While BSO switched the mode of death from

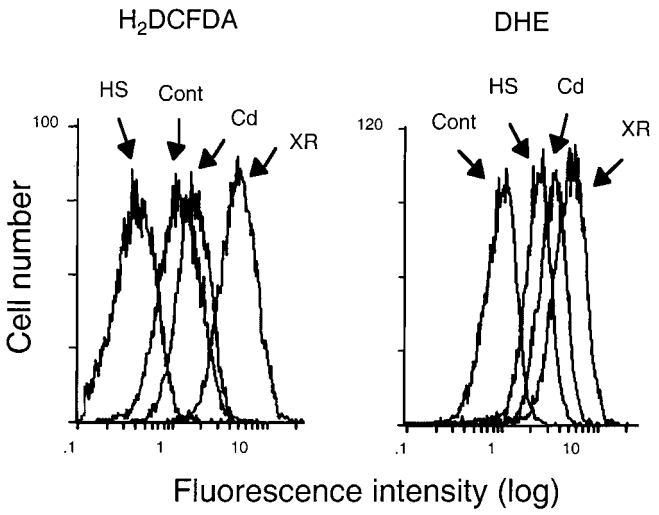

Fig. 4. ROS generation. To determine the intracellular content of peroxides and anion superoxide, cells were loaded with H_2DCFDA and DHE, respectively, and the fluorescence was measured by flow cytometry in the absence of treatment (Cont), at 1 h of treatment with cadmium and heat-shock, and at 1 h of recovery after treatment with X-rays. The experiment was repeated at least three times with similar results. All other conditions were as in Fig. 1.

Tab. I. Effect of antioxidants on the cadmium-, heat- and X-rays-provoked apoptosis.

Treatment	3 h	6 h
Control	N.D.	4 ± 2
Cd	23 ± 2	58 ± 4
Cd + BHA	5 ± 2	22 ± 5
HS	23 ± 3*	49 + 4*
HS + BHA	20 ± 3*	44 ± 5*
HS + NAC	24 ± 4*	54 ± 3*
XR	31 ± 4	61 ± 4
XR + BHA	N.D.	26 ± 5
XR + NAC	11 + 2	26 ± 3

Values (mean ± S.D. of at least three determinations) represent the percentage of apoptotic cells at the indicated times of recovery, as determined by changes in chromatin structure. BHA (200 μM) and NAC (15 mM) were applied 2 h before the stress inducers, and maintained during the treatment and recovery periods. All other conditions were as in Fig. 1. N.D., not determined. *No significant differences between HS and HS + BHA or HS and HS + NAC ($p > 0.10$, Student's *t*-test).

apoptosis to necrosis in cadmium-treated cells, it only caused minor modifications in the frequency of apoptosis, whithout affecting the mode of death, in heat- and X-rays-treated cells. These observations were qualitatively confirmed by double staining with FITC-conjugated annexin V and PI (Fig. 5B). In fact, treatments with heat and X-rays, either with or without BSO, caused annexin V binding and null or moderate PI influx, which is characteristic of apoptosis (Bedner et al., 1999). By contrast, the combination of BSO plus cadmium caused massive PI influx in a fraction of cells. Noteworthily, the permeability to trypan blue and the sharp increase in PI uptake in BSO plus cadmium-treated cells indicated genuine "primary" necrosis, instead of "secondary" necrosis derived from a possible acceleration of apoptosis. In fact, the fraction of cells with sub-G_1 DNA content (characteristic of late apoptosis and secondary necrosis) observed in cadmium-treated cultures was absent in BSO plus cadmium-treated cultures (Fig. 5C); and pre-incubation with BSO prevented the formation of the apoptosis-associated PARP-derived 85-kDa fragment in cadmium-treated cells, but not in heat-treated cells (Fig. 5D).

Effect of BSO on intracellular oxidation

The depletion of intracellular GSH may induce per se a prooxidant state and/or potentiate the oxidation activity of other agents, as measured by increased peroxide accumulation (McGowan et al., 1998). Hence, we asked whether the selective potentiation of cadmium toxicity by BSO, as revealed by necrosis induction, could be associated to the selective increase in oxidation. To investigate this possibility, we re-examined peroxide accumulation at different times of treatment and recovery with cadmium and X-rays, either with or without pre-incubation with BSO. The results are presented in Fig. 6. It was observed that BSO potentiated the increase in H_2DCFDA-derived fluorescence in cells treated with both cadmium and X-rays. The initial increase (0.5 to 1 h of treatment with cadmium, which corresponded to 0.5 to 1 h of recovery with X-rays: see scheme in Fig. 1) was higher in X-rays-treated cells than in cadmium-treated cells, either with or without BSO. However, while the increase persisted along the whole period studied in the case of cadmium, it was transient in the case of X-rays, dropping to values similar to untreated cells at 3–5 h of recovery, either with or without BSO.

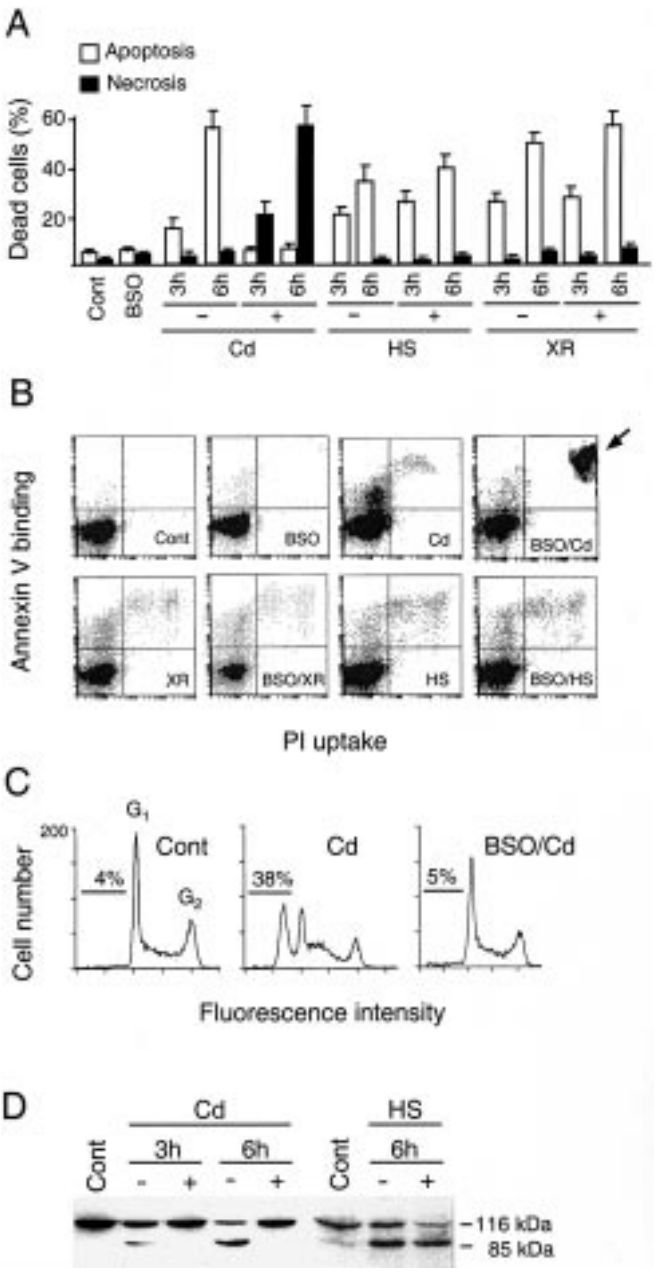

Fig. 5. Modulation by BSO of cell death and PARP cleavage. BSO (1 mM) was applied 24 h before treatment with the stress inducers, and maintained during the treatment and recovery periods. (**A**) Frequency of apoptotic and necrotic cells, as determined by chromatin condensation/fragmentation and by trypan blue uptake, respectively, in untreated cultures (Cont), in cultures incubated with BSO alone, and in cultures treated with cadmium, heat-shock and X-rays, and allowed to recover for 3 or 6 h, with (+) or without (−) BSO. The results represent the mean ± S.D. of at least three determinations. (**B**) Cell distribution according to annexin V binding and PI uptake, as measured by flow cytometry in non-permeabilized cells at 6 h of recovery. The *arrow* at the margin of the BSO/Cd panel indicates cells with massive PI uptake, characteristic of necrosis. (**C**) Cell distribution according to DNA content, as measured by PI incorporation in permeabilized cells at 6 h of recovery. The fraction of cells with sub-G_1 DNA content (apoptotic cells) is indicated in each profile. (**D**) PARP cleavage at the indicated times of recovery after cadmium and heat treatments, either with (+) or without (−) BSO. The experiments in (**B–D**) were repeated twice (**C**, **D**) or three times (**B**) with similar results. All other conditions were as in Figs. 1–3

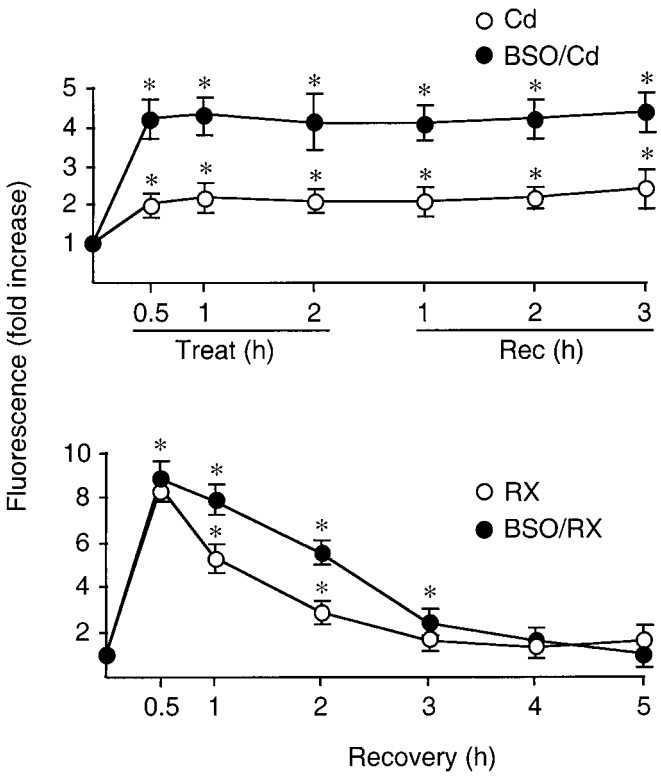

Fig. 6. Modulation by BSO of H_2O_2 accumulation. The graphs show the relative peroxide levels at different times of treatment and recovery with cadmium and X-rays, either alone (Cd, RX) or in combination with BSO (BSO/Cd, BSO/RX), as determined by flow cytometry after loading with H_2DCFDA. The results (mean ± S.D. of at least three determinations) are represented as fold induction in relation to untreated cells, which was given the arbitrary value of one. The treatments with cadmiun and X-rays were performed within the same experiment, although for clarity the results are presented separately. All other conditions were as in Figs. 1, 4 and 5. * $P < 0.05$ versus mean value in untreated cells (Student's *t*-test)

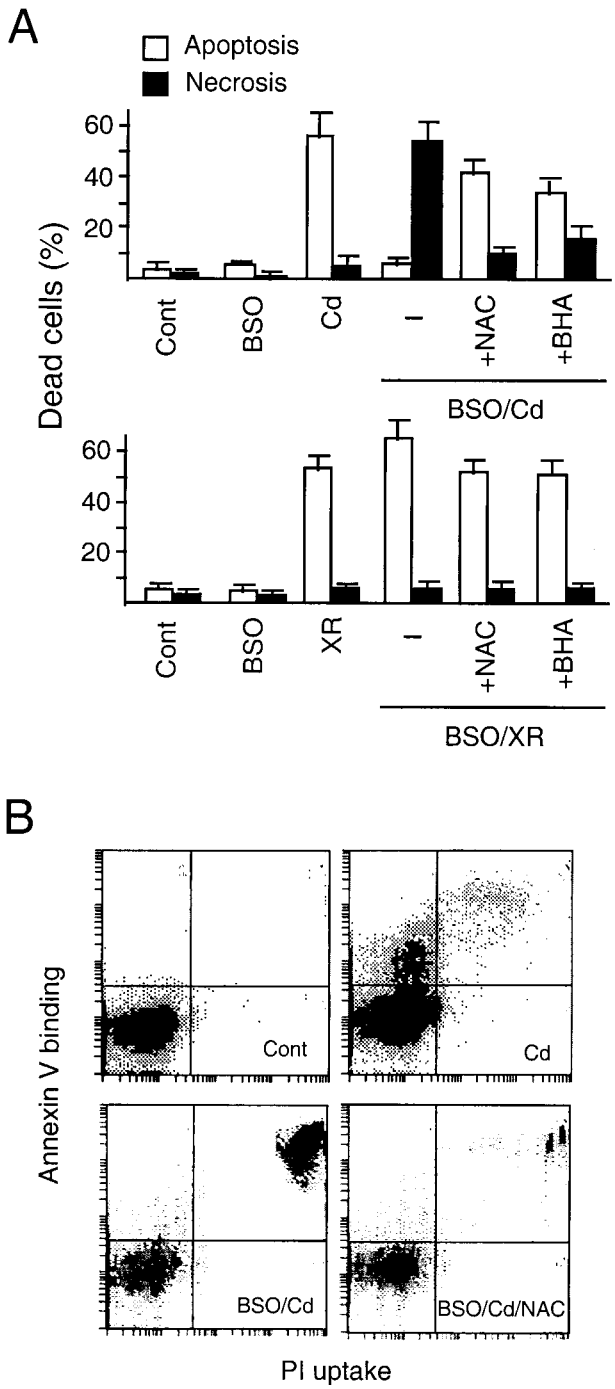

Fig. 7. Effect of post-treatment with antioxidant agents on the stress-provoked apoptosis and necrosis. BHA (200 μM) and NAC (15 mM) were applied after cadmium removal (0 h of recovery) or at 2 h of recovery after X-rays treatment. (**A**) Frequency of apoptosis and necrosis, measured by chromatin condensation/fragmentation and by trypan blue uptake, respectively, in untreated cultures (Cont); in cultures treated with BSO alone; in cultures treated with either cadmium or X-rays alone (Cd, XR); and in cultures treated with BSO plus cadmium or X-rays (BSO/Cd, BSO/XR) and allowed to recover with (+) or without (–) NAC or BHA. The results are the mean ± S.D. of at least three determinations. (**B**) Measurement of annexin V binding and PI uptake in the cadmium experiment, following the conditions indicated in (**A**). The experiment was repeated three times with similar results. All determinations were carried out at 6 h of recovery. All other conditions were as in Figs. 1 and 5.

These results might indicate that the stability of the oxidant state is important in determining the mode of cell death in stress-treated cells. To examine this possibility, new experiments with ROS scavengers were carried out. By contrast to the assays in Table I, in which the antioxidants were applied prior to the stress inducers to completely suppress oxidation, in this occasion they were only applied during the recovery period to preserve the initial oxidation. The results are shown in Fig. 7. It was observed that the administration of NAC or BHA after cadmium removal (0 h of recovery) inhibited necrosis and restored apoptosis in BSO plus cadmium-treated cells, as determined by chromatin condensation/fragmentation and trypan blue uptake (Fig. 7A), and by massive PI uptake (Fig. 7B). By contrast, the administration of NAC or BHA at 2 h of recovery after X-rays treatment, which corresponded to 0 h of recovery with cadmium (see scheme in Fig. 1), had little effect on the frequency of apoptosis in BSO plus X-rays-treated cells (Fig. 7A).

ATP levels

It was reported that the adoption of the apoptotic or necrotic pathways may be determined by the availability of intracellular ATP (Eguchi et al., 1997; Leist et al., 1997). To investigate

whether the generation of necrosis by BSO plus cadmium could be explained by a selective fall in ATP content, we comparatively measured the ATP levels in cells treated with cadmium and X-rays, either with or without pre-incubation with BSO. The results are shown in Fig. 8. It was observed that BSO plus cadmium, which caused necrosis, effectively decreased the ATP level. However, the decrease was similar to that caused by cadmium alone, and not higher than that caused by X-rays with or without BSO, which only caused apoptosis.

Discussion

The results in the present work corroborate and extend earlier observations of our laboratory and also others indicating that cadmium chloride, mild temperature and X-rays, cause death by apoptosis in myeloid cells (Gorman et al., 1999; Galán et al., 2000b; Haimovitz-Friedman et al., 1994). The execution of apoptosis was dependent on caspase-3 activation, as evidenced by PARP cleavage to give the 85-kDa fragment characteristic of apoptosis, and the capacity of caspase inhibitors to inhibit apoptosis. These results are congruent with earlier reports indicating the involvement of caspase-3 in spontaneous and drug-induced apoptosis in myeloid cells (Polverino and Patterson, 1997; Watson et al., 1997).

The present results also indicate that all stress inducers cause intracellular oxidation, but the importance of oxidation for cell death depends on the used inducer. Oxidation was measured using ROS-sensitive fluorescent probes, namely DHE, which measures O_2^-, and H_2DCFDA, which measures H_2O_2 and other peroxides. The observation that heat-shock increased the DHE-derived fluorescence without concomitant increase in H_2DCFDA-derived fluorescence could be surprising. Nevertheless, other authors also reported the uncoupling of different ROS production using the same technical approach. For instance, Zamzami et al. (1995) observed an increase in DHE-derived fluorescence without increase in H_2DCFDA-derived fluorescence in dexamethasone-treated splenocytes, and conversely Gorman et al. (1997) detected an increase in H_2DCFDA-derived fluorescence without increase in DHE-derived fluorescence in HL-60 promyelocytic cells treated with antitumour drugs. Moreover, our results are in full agreement with those of Reddy and Gangadharam (1992), who observed an increase in O_2^- production without increase in H_2O_2 in heat-shocked murine macrophages, using other detection procedures. Whatever the case, it seems clear that intracellular oxidation mediates the induction of apoptosis by cadmium and X-rays but not by heat-shock, since pre-treatment with the ROS scavengers NAC and BHA only protected the cells against cadmium and X-rays. This does not mean that oxidation is totally irrelevant in heat-shocked cells. In fact, NAC and BHA inhibited the induction of HSP70 expression by heat-shock in U-937 cells, indicating that ROS mediate the trigger of the stress response (Galán et al., 2000a).

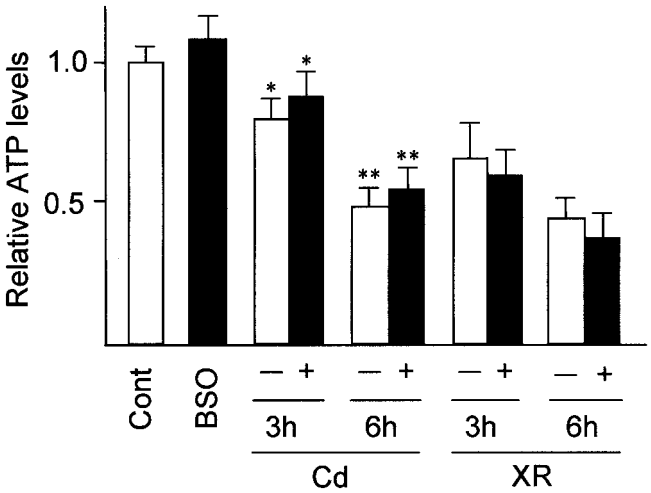

Fig. 8. Modulation of ATP levels. The histogram shows the relative ATP content in untreated cells (Cont), in cells incubated with BSO alone, and in cells allowed to recover after treatment with cadmium and X-rays, either with (+) or without (−) pre-incubation with BSO. The results (mean ± S.D. of at least three experiments) are represented in relation to the control, which was given the arbitrary value of one. The ATP content in controls was 22.1 ± 2.2 nmol/10^6 cells. *Asterisks*: no significant differences ($P > 0.1$, Student's *t*-test) between Cd- and BSO/Cd-treated cells at the same time of recovery.

It has been reported that the GSH-depleting agent BSO increases the harmful effects of several cytotoxic agents, including cadmium, radiation and hyperthermia, in different cell types (for reviews see (Bailey, 1998; Andersen, 1998)). Our results confirm that the BSO-provoked depletion of GSH increases the toxicity of cadmium in U-937 cells, as measured by the switch from apoptosis to necrosis. However, in this cell model BSO causes little if any alteration in the lethality of X-rays and heat-shock. Since GSH directly interacts with Cd^{2+} ions, the BSO-provoked GSH depletion probably decreases the frequency of intracellular GSH-Cd^{2+} interactions, increasing the concentration of free Cd^{2+} and exacerbating the toxicity. This explanation is congruent with reports which indicate that stress agents cause apoptosis or necrosis, depending on the intensity of the treatment (Habebu et al., 1998; Vayssier et al., 1998; Gorman et al., 1999). Nevertheless, our attempts to induce necrosis by increasing the external cadmium chloride concentration up to 1 mM were ineffective (results not shown), probably due to the saturation of the cadmium uptake system (Vilaboa et al., 1995).

Looking for possible factors which could explain the selection between apoptosis or necrosis, we examined the amount of intracellular oxidation, ATP content and caspase activity. It was reported that excessive oxidation may deplete the cells of ATP, preventing the execution of apoptosis (which is an energy-requiring process) and driving the cells into necrosis (Lee and Shacter, 1999). Although in our experiments BSO plus cadmium decreased the ATP content, this decrease may not explain the trigger of necrosis, since it was not greater than that caused by cadmium alone or by X-rays with or without BSO, which only caused apoptosis. Another way in which excessive ROS production could provoke necrosis is by inactivating caspases, since these proteases are strictly required for apoptosis (Ueda et al., 1998; Samali et al., 1999). In fact, caspases contain redox-sensitive cysteine residues in the catalytic domains ((Ueda et al., 1998) and references therein), and the processing of pro-caspase-3 is also thiol-sensitive (Nobel et al., 1997). Although our results partially fit this hypothesis, some restrictions must be made. First, in our experiments the initial increase in intracellular peroxides caused by X-rays, with or without BSO, was higher than that caused by BSO plus cadmium, and in spite of it X-rays never caused necrosis. However, while the increase in peroxide accumulation caused by X-rays was transient, the increase caused by cadmium was

stable, and the late administration of antioxidants (i.e., only during recovery) sufficed to prevent necrosis and restore apoptosis in BSO plus cadmium-treated cells. If we compare the results in Table I, in which the ROS scavengers were applied prior to treatment with the stress inducers, with those in Fig. 7, in which they were applied only during the recovery period, the following conclusion may be drawn: a transient oxidation may suffice to trigger apoptosis; by contrast necrosis requires not only the increase in intensity, but also the permanence of the oxidant state. A second restriction concerns the role of caspase activity. Although BSO inhibited caspase-3 activation by cadmium, as judged by the lack of PARP cleavage (Fig. 5D), caspase inactivation does not suffice to explain the generation of necrosis by BSO plus cadmium. In fact, in our experiments Z-VAD-FMK abrogated apoptosis without increase in the frequency of necrosis during at least 9 h (Fig. 3B), while BSO plus cadmium already caused necrosis at 3 h of recovery (Fig. 5). The possibility that the treatments may affect other redox-sensitive factors critical for apoptosis remains to be determined.

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