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Differential Effects of Catalase on Apoptosis Induction in Human Promonocytic Cells. Relationships with Heat-Shock Protein Expression

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ABSTRACT

The administration of the H_2O_2-specific scavenger catalase attenuated the generation of apoptosis by the antitumor drugs etoposide, camptothecin, doxorubicin, and cisplatin in U-937 human promonocytic cells. By contrast, the antioxidant potentiated the generation of apoptosis by the inducers of the stress response, heat shock and cadmium, in this and other myeloid cell types. Catalase also increased the heat shock-provoked stimulation of caspase-3 and -9 activities, as well as the release of cytochrome c from mitochondria to the cytosol. The potentiation of cell death by catalase correlated with its capacity to inhibit the stress response, as demonstrated by the suppression of 70- or 27-kDa heat-shock protein expression and the inhibition of heat-shock transcription factor 1 binding activity. Conversely, the toxicity of catalase plus heat shock was attenuated when the cells were preconditioned with a soft heating, which elevated the 70-kDa heat-shock protein levels. By contrast with catalase, the antioxidants superoxide dismutase and probucol did not inhibit heat-shock protein expression or affect apoptosis in U-937 cells. Finally, it was observed that the antitumor drugs did not activate the stress response in U-937 cells and that catalase failed to inhibit HSP expression and to potentiate apoptosis in heat shock-treated RPMI 8866 lymphoblastic cells. Taken together, these results provide the first demonstration of a proapoptotic action of catalase, suggest that H_2O_2 is a critical regulator of both apoptosis and the stress response, and corroborate the antiapoptotic action of heat-shock proteins in myeloid cells.

The possibility that intracellular oxidation may function as a common mediator of apoptosis induction has attracted great attention in the recent years. This proposal is supported by different observations, such as the capacity of reactive oxygen species (ROS) to cause cell death (Albina et al., 1993; Nosseri et al., 1994) and, conversely, that of antioxidant agents to prevent cell death (McGowan et al., 1996; Troyano et al., 2001); the capacity of apoptotic inducers other than ROS to cause intracellular oxidation (Hockenbery et al., 1993; Slater et al., 1995; Gorman et al., 1997); and the increased susceptibility to apoptosis obtained by decreasing the level of reduced glutathione, the most important intracellular defense against oxidation (for review, see Bailey, 1998). More specifically, some studies have emphasized the pivotal importance of hydrogen peroxide (H_2O_2) for death induction. Much of the evidence supporting this conclusion was obtained by analyzing the protective action of catalase, an H_2O_2-specific scavenger. For instance, addition of exogenous catalase usually attenuated apoptosis induction, even when superoxide dismutase (SOD; an anion superoxide-specific scavenger) did not (Gorman et al., 1997; Ikeda et al., 1999; Katschinski et al., 2000). Conversely, treatment with the catalase inhibitor aminotriazole increased the incidence of apoptosis (Jing et al., 1999; Palomba et al., 1999); the comparison of different cell lines or subclones revealed an inverse relationship between the level of endogenous catalase and the susceptibility to apoptosis (Sagara et al., 1998; Jing et al., 1999). Despite this, the relationship between oxidation and cell death is not always clear. For instance, H_2O_2 was able to stimulate cell proliferation and survival, or to prevent the execution of apoptosis, under some experimental conditions (Del Bello et al., 1999; Lee and Um, 1999; Shacter et al., 2000). Moreover, because of the multiple action of ROS, it

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ABBREVIATIONS: ROS, reactive oxygen species; HSP, heat-shock protein; DAPI, 4,6-diamidino-2-phenylindole; mAb, monoclonal antibody; HSP70, 70-kDa heat-shock protein; HSP27, 27-kDa heat-shock protein; pAb, polyclonal antibody; cisplatin, *cis*-diamminedichloroplatinum(II) (CDDP); PI, propidium iodide; PBS, phosphate-buffered saline; SOD, superoxide dismutase; HSE, heat-shock element; PKC, protein kinase C; HSF1, heat-shock factor 1.

may be conceived that antioxidant enzymes could indirectly facilitate apoptosis if they are able to inhibit the expression of protective proteins [e.g., heat-shock proteins (HSPs)], as has been described previously (Nishizawa et al., 1999; Ozaki et al., 2000).

To reexamine the importance of catalase as an apoptosis-regulatory enzyme, in the present work we analyzed the manner in which exogenous catalase could modulate the toxicity of apoptotic inducers with different mechanisms of action in U-937 human promonocytic cells. The treatments included the antitumor drugs etoposide, doxorubicin, camptothecin, and cisplatin, and the activators of the stress response, heat shock and cadmium chloride. In contrast with earlier reports, our results demonstrate that catalase may either prevent or potentiate cell death, depending on the agent used. Such a differential action of catalase seems to be the consequence, at least in part, of its capacity to modulate the expression of HSPs.

Materials and Methods

Chemicals. All components for cell culture were obtained from Invitrogen, Inc. (Carlsbad, CA). Cadmium chloride was obtained from Merck (Darmstadt, Germany); 4,6-diamidino-2-phenylindole (DAPI) was obtained from from Serva (Heidelberg, Germany), and RNase A was obtained from Roche Diagnostics S.L. (Barcelona, Spain). Digitonin, caspase-3 substrate I (*N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide), caspase-9 substrate II (Leu-Glu-His-Asp-*p*-nitroanilide), and the caspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone, were obtained from Calbiochem (Darmstadt, Germany). Mouse anti-human HSP70 mAb (clone C92F3A-5, which specifically recognizes the stress-inducible form of HSP70) and mouse anti-human HSP27 mAb were obtained from StressGen Biotechnologies Corp. (Victoria, Canada); mouse anti-human Bcl-2 mAb, rabbit anti-human Bax pAb, and rabbit anti-human nPKCδ pAb were obtained from Santa Cruz Biotechnology, Inc (Santa Cruz, CA); rabbit anti-human Bcl-X_L pAb was obtained from BD Transduction Laboratories (Lexington, KY); mouse anti-pigeon cytochrome *c* mAb was obtained from BD PharMingen (San Diego, CA); mouse anti-chick α-tubulin mAb was obtained from from Sigma Química (Madrid, Spain); and peroxidase-conjugated rabbit anti-mouse IgG and peroxidase conjugated goat anti-rabbit IgG were obtained from Dakopatts (Copenhagen, Denmark). All other reagents were obtained from Sigma Química.

Cells and Treatments. U-937 (Sundström and Nilsson, 1976) and THP-1 (Tsuchiya et al., 1980) human promonocytic cells, HL-60 human promyelocytic cells (Collins et al., 1977), and RPMI 8866 B lymphoblastic cells (Lampson and Levy, 1980) were routinely 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. Stock solutions of etoposide (20 mM) and camptothecin (10 mM) were prepared in dimethyl sulfoxide and doxorubicin (20 mM) and cadmium chloride (100 mM) in distilled water. All these solutions were stored at −20°C. SOD (7300 U/ml) and *cis*-diamminedichloroplatinum(II) (cisplatin; 3.3 mM) were dissolved in distilled water; DAPI (10 μg/ml) and propidium iodide (PI; 1 mg/ml) were dissolved in phosphate-buffered saline (PBS). All these solutions were stored at 4°C. Probucol was freshly prepared at 100 mM in ethanol just before use. Catalase was commercially purchased as an ammonium sulfate suspension. Typically, the cells were seeded at 2×10^5 cells/ml 16 h before treatments. For treatment with the antitumor drugs, the cells were subjected to continuous incubation with the desired concentration of the drugs. For cadmium experiments, the cells were pulse-treated for 2 h with 200 μM cadmium chloride, then washed with prewarmed (37°C) RPMI medium and allowed to recover under standard culture conditions. For heat shock, cells were placed in a bath for 2 h at 42.5°C and then allowed to recover under standard culture conditions. Catalase was applied 30 min in advance to the treatments. As controls, cells were subjected to the same manipulations as treated cells, in the absence of cadmium and heating.

Determination of Apoptosis and Necrosis. Distinctive characteristics of apoptotic cells were a marked reduction in volume (cell shrinkage), changes in nuclear morphology, and reduction in DNA content. To analyze 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 DAPI and examined by fluorescence microscopy. Apoptosis was characterized by chromatin condensation followed by partition into multiple bodies. To measure DNA content, cells were collected by centrifugation and permeabilized by incubation for 30 min at 37°C in PBS containing 0.1% (w/v) Nonidet P-40 and 0.5 mg/ml RNase A. After the addition of 50 μg/ml PI, the cells were analyzed by flow cytometry. Cells with sub-G_1 (hypodiploid) DNA content were considered apoptotic. Distinctive characteristics of genuine, "primary" necrosis are cell swelling and the rapid loss of plasma membrane integrity, as revealed by the free penetration of trypan blue or PI without prior cell permeabilization (Troyano et al., 2001; our unpublished observations).

Caspase Activity Assays. Samples of 4×10^6 cells were collected by centrifugation, washed twice with ice-cold PBS, resuspended in 50 μl of ice-cold lysis buffer [1 mM dithiothreitol, 0.03% Nonidet P-40 (v/v), in 50 mM Tris pH 7.5], kept on ice for 30 min, and finally centrifuged at 14,000*g* for 15 min at 4°C. Samples containing aliquots of the supernatants (corresponding to 10 μg of total protein), 8 μl of the appropriate caspase substrate (*N*-acetyl-Asp-Glu-Val-Asp-*p*-nitroanilide for caspase-3 and Leu-Glu-His-Asp-*p*-nitroanilide for caspase-9), and PBS to complete 200 μl were prepared in triplicate in 96-well microtiter plates, and incubated for 1 h at 37°C. The absorption was measured by spectrometry at 405 nm.

Protein Extraction, Subcellular Fractionation, and Immunoblot Assays. To obtain total cellular protein extracts, samples of 3×10^6 cells were collected by centrifugation, washed with PBS, and lysed by 5-min heating at 100°C followed by sonication in Laemmli buffer. To obtain cytosolic extracts for determination of cytochrome *c* release, samples of 3×10^6 cells were collected by centrifugation, resuspended in 100 μl of ice-cold PBS containing 80 mM KCl, 250 mM sucrose, and 200 μg/ml digitonin, and kept on ice for 5 min. After centrifugation, the pellet was discarded and the supernatant was kept for further assays. In all cases, the detection of specific proteins in either the total cellular extracts or the cytosolic fraction was carried out by immunoblot, as described previously (Galán et al., 2000a).

Gel Retardation Assays. Preparation of nuclear extracts was carried out as described by Schreibert et al. (1988). Preparation of double strand oligonucletides containing "heat-shock element" (HSE) and Sp1 consensus binding sites was carried out as described by Galán et al. (2000a) and López-Rodríguez et al. (1995), respectively. Oligoprobe labeling, binding reactions, determination of binding specificity, and electrophoretic separation were performed and measured as described previously (Galán et al., 2000a).

Results

Cell Death. We reported previously that continuous treatment with the antitumor drugs etoposide (10 μM), camptothecin (0.4 μM), doxorubicin (7.5 μM), and cisplatin (100 μM) and pulse-treatment with heat (2 h at 42.5°C) and cadmium chloride (2 h at 200 μM), followed by recovery, rapidly caused death by apoptosis in U-937 human promonocytic cells (Galán et al., 2000b; Troyano et al., 2001). Hence, these conditions were used to analyze the capacity of catalase to modulate cell death. Catalase was applied at 500 U/ml, fol-

lowing the indications of other laboratories (Katschinski et al., 2000) and our own observations (Fig. 1C). It was observed that catalase inhibited apoptosis in cultures treated with the antitumor drugs, as indicated by the decrease in the frequency of cells with condensed/fragmented chromatin (Fig. 1A) and with sub-G_1 DNA content (Fig. 1B) and by reduced cell volume (results not shown). By contrast, the antioxidant did not reduce and even augmented the frequency of apoptotic cells in heat shock- and cadmium-treated cultures (Fig. 1, A and B; results not shown). Incubation of nonpermeabilized cells with trypan blue followed by microscopic examination or with PI followed by flow cytometry analysis revealed that under the experimental conditions used, the frequency of necrotic cells remained negligible (below 7%, near their frequency in untreated, control cultures).

Because the potentiation by catalase of the stress-provoked cell death was unexpected, we wanted to corroborate this finding using other cell types. As indicated in Fig. 1D, the antioxidant greatly augmented the frequency of apoptosis in heat shock-treated THP-1 human promonocytic cells and, to a lower extent, in heat shock-treated HL-60 human promyelocytic cells. However, catalase attenuated the generation of apoptosis in heat shock-treated RPMI 8866 lymphoblastic cells.

Cytochrome *c* Release, Caspase Activities, and Bcl-2 Protein Family Levels. It is known that most cytotoxic agents, including stress inducers, activate the mitochondrial pathway of apoptosis, which involves the release of cytochrome *c* from the mitochondria to the cytosol, and the sequential cleavage/activation of caspase-9 and caspase-3 (for review, see Adrain and Martin, 2001). Hence, we wanted to examine these events in U-937 cells subjected to heat shock with and without catalase. The results are indicated in Fig. 2. Immunoblot assays revealed that cytochrome *c*, which was

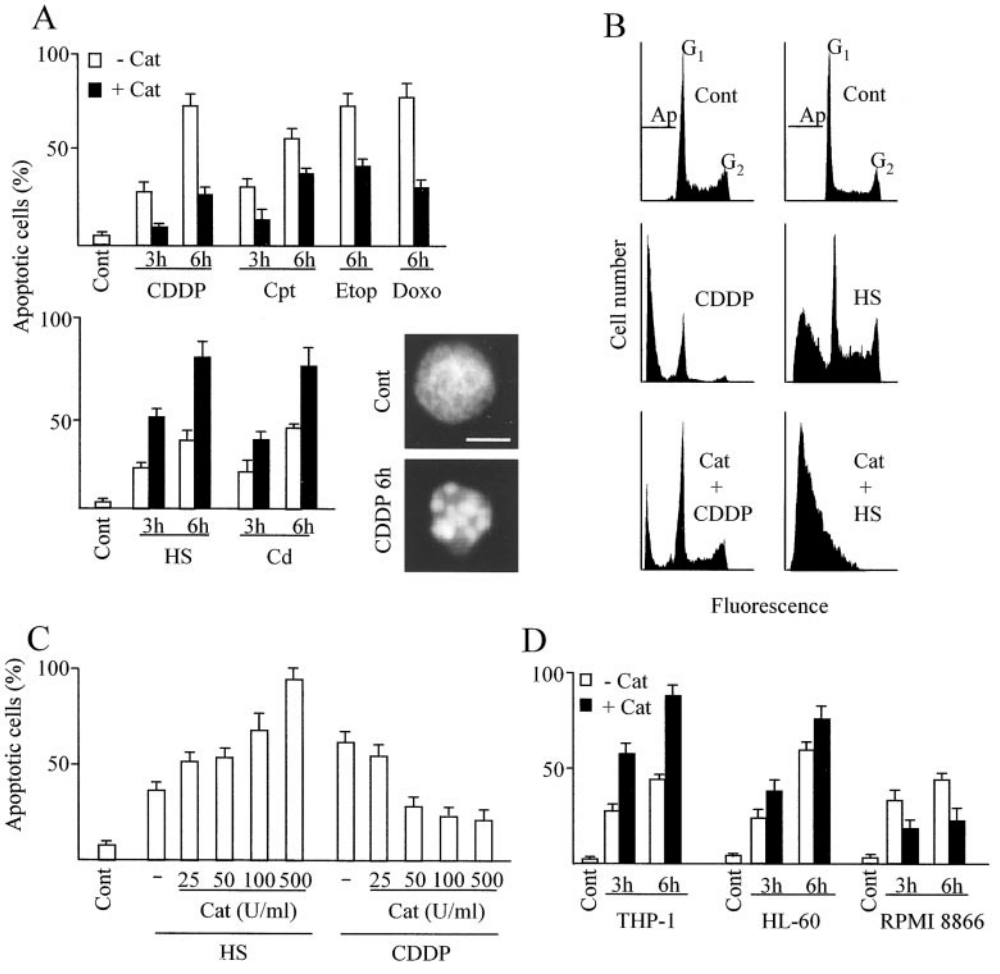

Fig. 1. Modulation of apoptosis induction by catalase. A, frequency of apoptotic cells, as measured by chromatin fragmentation, in untreated U-937 promonocytic cell cultures (Cont); in cultures treated for the indicated time periods with 100 μM cisplatin (CDDP), 0.4 μM camptothecin (Cpt), 10 μM etoposide (Etop), and 7.5 μM doxorubicin (Doxo), in either the absence (− Cat) or the presence (+ Cat) of catalase; and in cultures pulse-treated with cadmium chloride (Cd, 2 h at 200 μM) or heat (HS, 2 h at 42.5°C) and then allowed to recover for the indicated time periods with or without catalase. An example of cells with diffuse chromatin (control) and with fragmented chromatin (apoptotic) is given in the photograph. Bar, 5 μm. B, cell distribution according to their DNA content in untreated U-937 cultures (Cont), in cultures treated for 6 h with cisplatin, and in cultures subjected to heat shock and allowed to recover for 6 h in either the absence or presence of catalase. The position of cells with sub-G_1 DNA content (apoptotic cells) is indicated (Ap). C, frequency of apoptotic cells in U-937 cultures subjected to heat shock and allowed to recover for 6 h, or treated for 6 h with cisplatin, either in the absence (−) or presence of the indicated concentrations of catalase. D, frequency of apoptotic cells in THP-1 promonocytic, HL-60 promyelocytic, and RPMI 8866 lymphoblastic cell cultures treated with heat shock and allowed to recover for the indicated time periods in either the absence or the presence of catalase. Except when indicated, catalase was used at 500 U/ml, added 30 min before treatment with the apoptotic inducers, and maintained during the entire treatment and recovery periods. The results in A, C, and D are the mean ± S.D. of at least three determinations. Differences in treated versus control cells and in catalase-treated versus catalase-untreated cells were always significant ($p < 0.05$, Student's t test).

undetectable in extracts from untreated cells, was slightly increased in extracts from cells treated with heat shock alone and increased to a higher level in extracts from cells treated with heat shock plus catalase (Fig. 2A). In a similar manner, in vitro assays revealed that heat shock alone caused a slight (approximately 100%) increase in caspase-9 activity and a higher (approximately 500%) increase in caspase-3 activity, and that both activities were greatly potentiated by catalase (Fig. 2B). These results were corroborated by immunoblot assays demonstrating PKCδ cleavage to give a fragment of approximately 40 kDa (Fig. 2B, inset), a process believed to be associated with apoptosis and mediated by caspase-3 (Ghayur et al., 1996). Moreover, the importance of caspase activities for the generation of apoptosis by heat shock, and for its potentiation by catalase, could be confirmed using the pan-caspase inhibitor *N*-benzyloxycarbonyl-Val-Ala-Asp. In fact, it was observed that *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone almost totally prevented the heat-provoked death induction, in both the absence and the presence of the antioxidant (Fig. 2C).

It is known that the mitochondrial pathway of apoptosis is regulated by members of Bcl-2 protein family, such as Bcl-2 and Bcl-X_L (antiapoptotic) and Bax (proapoptotic) (Adrain and Martin, 2001). Hence, immunoblot assays were carried out to determine the expression of these proteins in heat shock-treated cells with and without catalase. As indicated in Fig. 3, the total cellular levels of Bcl-2 and Bax were unaffected by the treatments, whereas Bcl-X_L experienced a

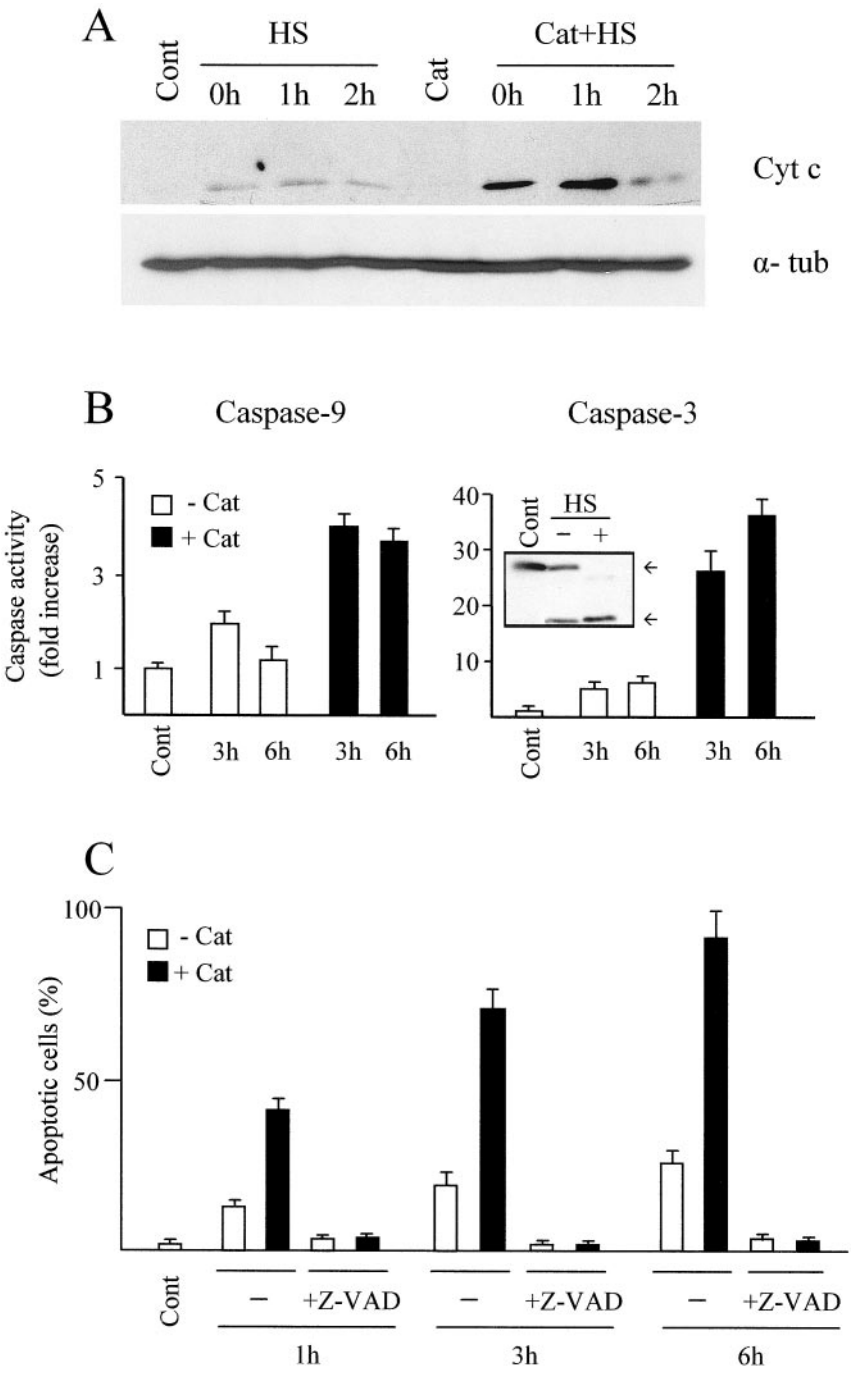

Fig. 2. Cytochrome *c* release, caspase activities, and effect of caspase inhibitors. U-937 cells were subjected to heat shock and allowed to recover for the indicated time periods in the absence (HS) or presence (Cat+HS) of catalase. A, cytosolic extracts (25 μg protein per lane) were used to measure the relative levels of cytochrome *c* by means of immunoblotting. The level of α-tubulin was also measured as an internal control. B, total cellular extracts (10 μg of protein per sample) were used to determine caspase 9- and -3 activities, using as substrates Leu-Glu-His-Asp-*p*-nitroanilide and Asp-Glu-Val-Asp-*p*-nitroanilide, respectively. The results (mean ± S.D. of three determinations) are represented in relation to untreated cells, which was given the arbitrary value of one. Inset, PKCδ cleavage, as determined by immunoblotting, at 6 h of recovery after heat shock in the absence (−) or presence (+) of catalase. The upper band corresponds to the whole protein (approximately 78 kDa) and the lower band to the main cleavage fragment (approximately 40 kDa). C, frequency of apoptotic cells, in the absence (−) or presence of 50 μM *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone. The caspase inhibitor was present during the entire treatment and recovery periods. All other conditions were as in Fig. 1.

slight, progressive decrease in the case of heat shock plus catalase (Fig. 3).

Heat-Shock Protein Expression. Another group of proteins that may prevent cell death by interfering with the mitochondrial pathway of apoptosis are the HSPs, especially HSP70 and HSP27 (for review, see Garrido et al., 2001). It is known that heat shock and heavy metals are potent inducers of the stress response, as characterized by the stimulation of HSP expression (Vilaboa et al., 1995). Hence, we speculated about whether the potentiation by catalase of the heat shock lethality could be because of the capacity of the antioxidant to inhibit the stress response. To investigate this possibility, immunoblot assays were carried out to measure HSP expression under some of the experimental conditions used previously to analyze apoptosis (see Fig. 1). The results were as follows: 1) Heat shock induced the expression of HSP70 and HSP27, and the induction was greatly reduced by catalase (Fig. 4A). The antioxidant also inhibited the induction of HSP70 by cadmium, which, by difference with heat shock, did not modify HSP27 expression (Fig. 4A). 2) Catalase failed to inhibit the heat-provoked induction of HSP70 in RPMI 8866 (Fig. 4B), the only examined cell line in which the antioxidant did not increase the toxicity of heat shock. 3) Etoposide and cisplatin, the toxicity of which was not increased by catalase, failed to stimulate HSP70 and HSP27 expression (Fig. 4C). In addition, complementary experiments using antioxidants other than catalase revealed that SOD (specific for anion superoxide) and probucol (seldom used as a scavenger of hydroxyl radical; Lieberthal et al., 1996) failed to prevent the induction of HSP70 expression or to affect the generation of apoptosis by heat shock (Fig. 4D).

In all the above-described heat shock experiments, catalase was continuously present during both the treatment and recovery periods. The 2-h treatment period corresponds to the moment in which heat-shock factor 1 (HSF1, the transcription factor responsible for HSP70 expression under stress conditions) undergoes activation and DNA binding, whereas the recovery period corresponds to the moment in which HSP70 expression is executed (Vilaboa et al., 1995,

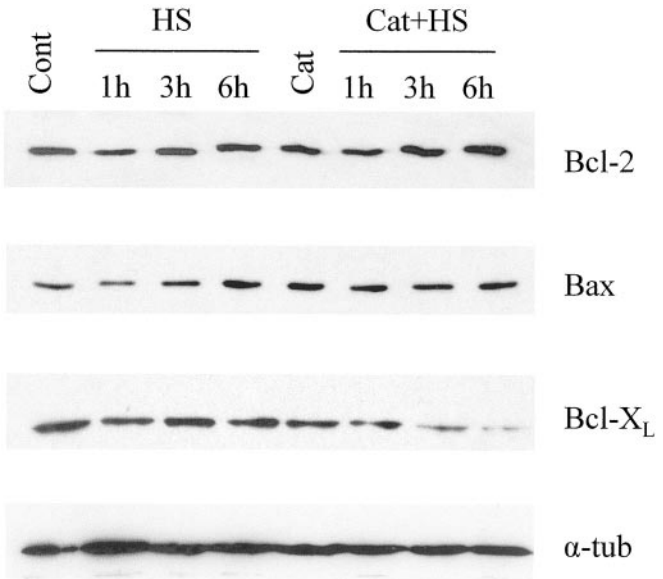

Fig. 3. Expression of Bcl-2 protein family members. Total cellular extracts (10 μg protein per lane) obtained from U-937 untreated cells (Cont), from cells treated with catalase alone (Cat), and from cells subjected to heat shock and allowed to recover for the indicated time periods in the absence (HS) or the presence (Cat+HS) of catalase were used to determine the relative Bcl-2, Bax, and Bcl-X_L levels. The level of α-tubulin was also measured as an internal control. All determinations were repeated at least twice, with essentially similar results. All other conditions were as in Fig. 1.

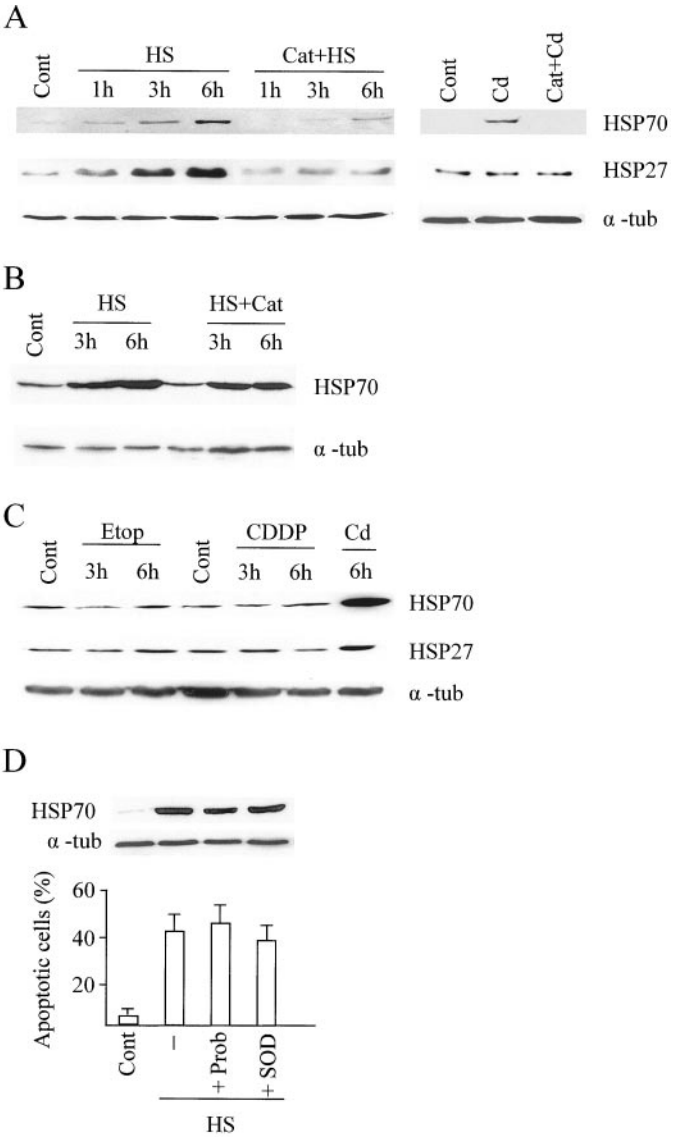

Fig. 4. Modulation of HSP expression. A, relative levels of HSP70, HSP27, and α-tubulin (used as a control) proteins in U-937 untreated cells (cont); in cells subjected to heat shock and allowed to recover for the indicated time periods in the absence (HS) or presence (Cat+HS) of catalase; and in cells treated with cadmium chloride and allowed to recover for 6 h in the absence (Cd) or presence (Cat+Cd) of catalase. B, relative levels of HSP70 and α-tubulin proteins in RPMI 8866 cells subjected to the same treatments as in A. C, relative levels of HSP70, HSP27, and α-tubulin proteins in untreated U-937 cells and in cells treated for the indicated time periods with etoposide and cisplatin. In these experiments, cells treated with cadmium chloride and allowed to recover for 6 h were used as a positive control. D, relative HSP70 and α-tubulin protein levels and frequency of apoptotic cells in U-937 cell cultures subjected to heat shock and allowed to recover for 6 h, either in the absence (–) or presence of probucol (Prob, 100 μM) or SOD (400 U/ml). The antioxidants were added 30 min before heat shock and maintained during the treatment and recovery periods. The immunoblots were carried out using 5 μg of total protein extracts per lane in A, 10 μg in B and D, and 25 μg in C (to clearly detect the basal HSP70 protein level). All other conditions were as in Fig. 1.

1997). For this reason, new experiments were carried out in which the antioxidant was separately applied during each period. The obtained results are represented in Fig. 5. The administration of catalase only during the 2-h period of heating sufficed to prevent the subsequent induction HSP70 expression (Fig. 5A) and to potentiate cell death (Fig. 5B). Correspondingly, under these conditions, catalase attenuated the heat-provoked stimulation of HSF1 binding (Fig. 5C). Of note was that heat shock provoked a slight decrease in Sp1 binding (here examined as a control) that was not affected by catalase (Fig. 5C). In contrast, the application of catalase alone during recovery after the heating period did not prevent the induction HSP70 expression nor affect apoptosis (Fig. 5, A and B). The differential action of catalase on cell death, depending on the period of administration, was corroborated by measuring

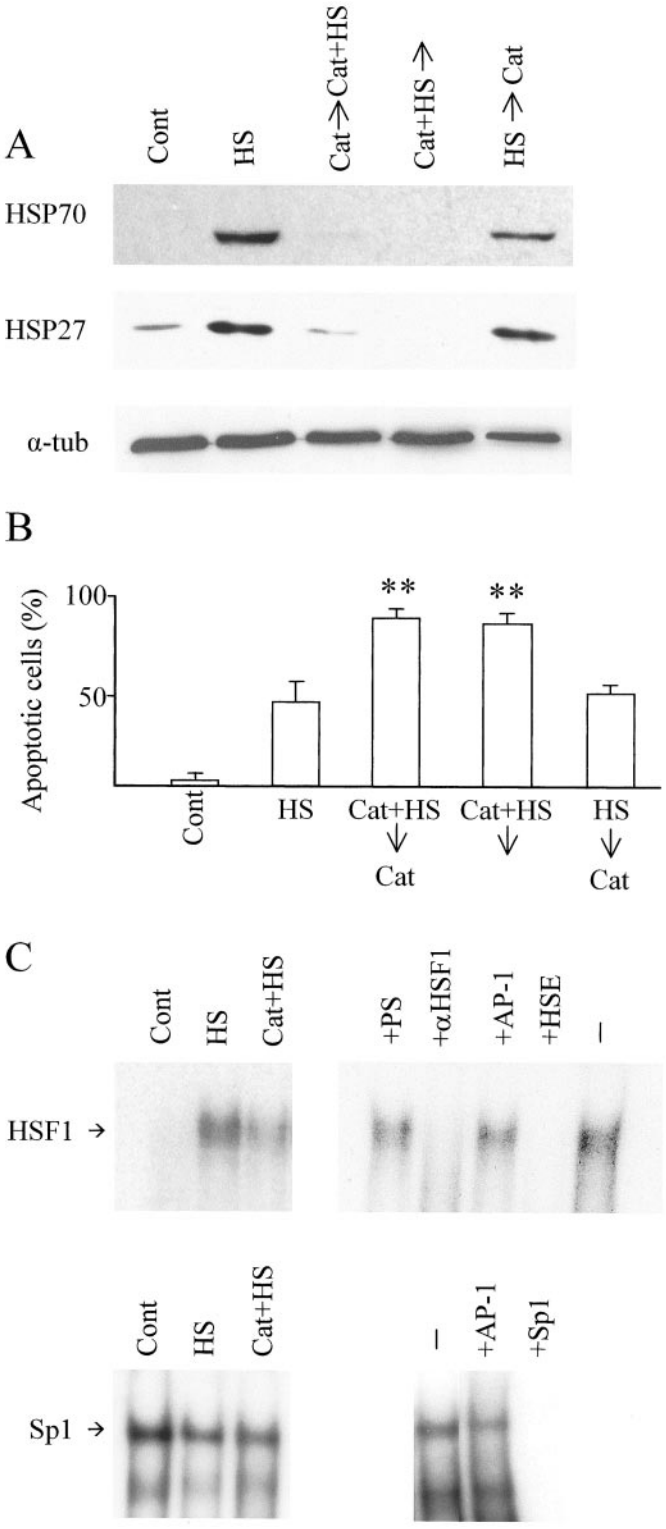

Fig. 5. Effects of sequential treatments with catalase on cell death, HSP70 and HSP27 expression, and HSF1 binding activity. Relative levels of HSP70, HSP27, and α-tubulin proteins (A) and frequency of apoptotic cells (B) in U-937 untreated cell cultures (Cont); in cultures subjected to heat shock and allowed to recover, always without catalase (HS); in cultures subjected to heat shock and allowed to recover, always in the presence of catalase (Cat+HS→Cat); in cultures subjected to heat shock in the presence of catalase and then allowed to recover without the antioxidant (Cat+HS→); and in cultures subjected to heat shock without catalase and then allowed to recover in the presence of the antioxidant (HS→Cat). All determinations were carried out at 6 h of recovery. The values in B represent the mean ± S.D. of three determinations. **, $P < 0.01$ (Student's t test) versus HS treatment. C, HSF1 binding activity to an oligoprobe containing a HSE consensus sequence, using nuclear extracts from U-937 untreated cells (Cont), and from cells subjected to heat shock for 2 h either in the absence (HS) or the presence (Cat+HS) of catalase, as determined by gel shift assay. To check the specificity of binding, reactions were carried out using the extract from heat-shocked cells in the absence of competitor (−), in the presence of 50-fold excess unlabeled HSE oligoprobe (+HSE), in the presence of 50-fold excess heterologous (AP-1 recognizing) oligoprobe (+AP-1), and in the presence of anti-HSF1 antibody (+αHSF1) or preimmune serum (+PS). As a control, the same extracts were assayed with an oligoprobe containing a Sp1-consensus binding sequence. The specificity of binding was corroborated using 50-fold excess unlabeled homologous (+Sp1) and heterologous (+AP-1) oligoprobes. All other conditions were as in Figs. 1 and 4.

the fraction of cells with sub-G_1 DNA content (results not shown).

The preceding results strongly suggested that the increase of the heat shock toxicity by catalase is caused, at least in part, by the capacity of the antioxidant to abrogate the stress response. To corroborate this hypothesis, experiments were carried out in which cells were preconditioned by a soft heating (1 h at 42°C), before being subjected to the normal treatment with heat shock (2 h at 42.5°C), with and without catalase (Fig. 6A, experimental scheme). Such a soft preconditioning is not lethal but suffices to cause a transient elevation of HSP70 levels (Vilaboa et al., 1995, and results not shown). The results in Fig. 6, B and C, reveal that heat preconditioning caused a significant decrease in the frequency of apoptotic cells in cultures subjected to heat shock alone and heat shock plus catalase. This confirms the inverse relationship between HSP70 expression and apoptosis induction in the promonocytic cell model.

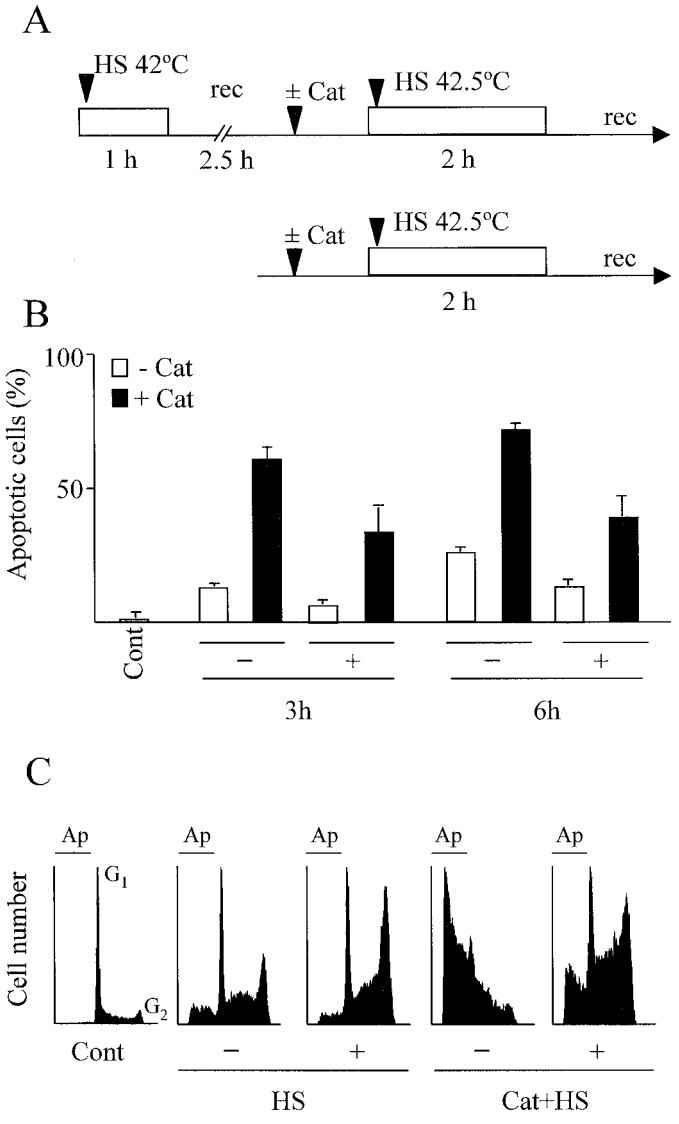

Fig. 6. Effect of heat preconditioning on heat shock-induced apoptosis. A, scheme of the experimental design used. U-937 cells were preconditioned with a soft heating (1 h at 42°C) followed by a 2.5-h recovery, before being subjected to the normal heat shock (2 h at 42.5°) with or without catalase. B, frequency of apoptotic cells, as determined by chromatin fragmentation, at the indicated times of recovery, with (+) and without (−) preconditioning. The values represent the mean ± S.D. of five determinations. Significant differences ($p < 0.05$, Student's t test) were always detected in each pair of values (preconditioned (+) versus non preconditioned (−) cells). C, frequency of apoptotic cells (Ap), as revealed by sub-G_1 DNA content, at 6 h of recovery after heat shock alone (HS) or catalase plus heat shock (Cat+HS), with (+) or without (−) preconditioning. All other conditions were as in Fig. 1.

Discussion

Catalase and glutathione peroxidase are the major intracellular enzymes responsible for H_2O_2 catabolism. Although it is generally accepted that the catalase applied to the culture medium is not able to penetrate the cell membrane (with some possible exceptions; see Sundaresen et al., 1995), the enzyme may still provide antioxidant protection, probably by scavenging the H_2O_2 that diffuses outside the cells. Actually, we have recently demonstrated that exogenous catalase attenuated the cisplatin-provoked increase in dichlorodihydrofluorescein diacetate-derived fluorescence in promonocytic cells, indicating that the enzyme effectively scavenged intracellular peroxides (Troyano et al., 2001). Whatever the case, our present results indicate that catalase differentially modulates apoptosis induction in U-937 human promonocytic cells, depending on the inducer used. On the one hand, it attenuated the toxicity of antitumor drugs, a result that is in full agreement with earlier reports (Gorman et al., 1997; Ikeda et al., 1999) and that corroborates the importance of H_2O_2 as a mediator of apoptosis in myeloid cells. However, other works indicate that H_2O_2 also mediates the generation of apoptosis induction by heat shock and cadmium (Katschinski et al., 2000; Szuster-Ciesielka et al., 2000). Hence, the observation that catalase did not attenuate and even potentiated the toxicity of the stress inducers was unexpected; to our knowledge, it represents the first demonstration of a proapoptotic action of this antioxidant agent.

The chaperon-like property of HSPs enables them to interact with and modify the function of many other proteins. In particular, HSP70 and HSP27 may bind and repress several components of the mitochondrial pathway of apoptosis (for review, see Garrido et al., 2001). Hence, one may imagine that the induction of HSP expression restrains the concomitant execution of apoptosis that would otherwise be facilitated if HSP expression were prevented. In this regard, our present results strongly indicate that the potentiation of the heat shock and cadmium toxicity by catalase is the consequence, at least in part, of its capacity to inhibit HSPs induction. The facts are: 1) There was a clear inverse relationship between the down-regulation of HSP70 expression and the up-regulation of apoptosis in heat-treated U-937 cell cultures incubated with catalase. Of note was that catalase potentiated apoptosis only when added at the time of the activation of the stress response (i.e., HSF1 activation and binding), not when added later, when the expression of HSPs was no longer prevented. 2) Catalase failed to potentiate the lethality of treatments that do not induce HSP expression in U-937 cells, such as the antitumor drugs, and failed also to potentiate the lethality of heat shock in a cell line (RPMI 8866) in which catalase did not prevent HSP expression. 3) Antioxidants such as SOD and probucol did not affect HSP expression and also failed to potentiate the heat shock-provoked apoptosis. 4) A soft heat preconditioning, which suffices to elevate HSP70 expression before catalase administration,

attenuated the potentiation of apoptosis by the antioxidant. Hence, the modulation by catalase of the stress-provoked apoptosis may be contemplated as the result of the interplay of two conflicting signals: the direct antiapoptotic action of catalase as an antioxidant and its indirect proapoptotic action as a suppressor of protective proteins (HSP70 and HSP27). This second mechanism seems to be predominant, at least in promonocytic cells, hence favoring cell death, but the result could be different in other myeloid cell types or when a different experimental procedure is used. For instance, Katschinski et al. (2000) reported that the over-expression of endogenous catalase slightly attenuated the heat shock-provoked apoptosis in HL-60 human promyelocytic cells, but in this case, the antioxidant caused only a minor decrease in HSP27 expression. Our own experiments indicated that exogenous catalase increased the generation of apoptosis by heat shock in HL-60 cells, but the increase was much lower than that observed in the promonocytic cell lines (U-937, THP-1). Concerning the exact mechanism responsible for the potentiation of apoptosis, our results indicate that catalase up-regulates the mitochondrial pathway at an early stage, namely the release of cytochrome *c* to the cytosol. This is consistent with recent publications indicating that HSP70 and HSP27 may inhibit cytochrome *c* release (Samali et al., 2001; Klein and Brune, 2002). Nonetheless, this does not exclude additional regulatory roles of the HSPs at later stages (e.g., by direct binding and inactivation of released cytochrome *c*, of Apaf-1, or of the caspases themselves) (Garrido et al., 2001).

It has been reported that the stress response is an oxidation-regulated process. On the one hand, cell treatment with H_2O_2 causes HSF1 activation; on the other hand, H_2O_2 may inhibit in vitro the HSF1-DNA binding reaction (Jacquier-Sarlin and Polla, 1996; Jornot et al., 1997). Our results indicating that catalase inhibits HSF1-HSE binding and, consequently, HSP synthesis are in agreement with earlier results in other cell models (Nishizawa et al., 1999; Ozaki et al., 2000) and corroborate the importance of H_2O_2 as a regulator of the stress response. Nevertheless, HSF1 activation is a complex process that sequentially involves transcription factor homotrimerization, nuclear translocation, and DNA binding (Baler et al., 1993); hence, the exact catalase-sensitive steps remain to be determined. In addition, the fact that catalase did not affect the induction by heat shock of HSP70 expression in RPMI 8866 lymphoblastic cells indicates that the oxidation-mediated regulation of the stress response is a cell type-specific phenomenon. These aspects are now under investigation in our laboratory.

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