Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 6458–6463, June 1996
Cell Biology

Mitochondria are selective targets for the protective effects of heat shock against oxidative injury

(heat shock proteins/hydrogen peroxide/mitochondrial membrane potential/antioxidant/*bcl-2*)

B. S. POLLA*†‡, S. KANTENGWA†, D. FRANÇOIS§, S. SALVIOLI¶, C. FRANCESCHI¶, C. MARSAC§, AND A. COSSARIZZA¶

*Laboratoire de Physiologie Respiratoire, Unité de Formation et de Recherche Cochin Port-Royal, 24 Rue du Faubourg St. Jacques, 75014 Paris, France; †Allergy Unit, University Hospital, 1211 Geneva 14, Switzerland; §Biochimie Pharmacologique et Métabolique, Institut National de la Santé et de la Recherche Médicale, Unité Associée 75, Faculté de Médecine Necker Enfants Malades, 75730 Paris, France; and ¶Department of Biomedical Sciences, Section of General Pathology, University of Modena, 41100 Modena, Italy

Communicated by Ewald R. Weibel, Universität Bern, Bern, Switzerland, February 20, 1996 (received for review May 17, 1995)

ABSTRACT **Heat shock (HS) proteins (HSPs) induce protection against a number of stresses distinct from HS, including reactive oxygen species. In the human premonocytic line U937, we investigated in whole cells the effects of preexposure to HS and exposure to hydrogen peroxide (H_2O_2) on mitochondrial membrane potential, mass, and ultrastructure. HS prevented H_2O_2-induced alterations in mitochondrial membrane potential and cristae formation while increasing expression of HSPs and the protein product of *bcl-2*. Protection correlated best with the expression of the 70-kDa HSP, hsp70. We propose that mitochondria represent a selective target for HS-mediated protection against oxidative injury.**

Heat shock (HS) proteins (HSPs) are a set of conserved proteins induced in prokaryotes and eukaryotes by elevated temperatures and a number of other types of cellular stresses, including oxidative stress. HSPs are classified according to their apparent molecular weight, their intracellular location, their main inducer(s), and their functions. Members of the hsp70 family locate themselves to the cytoplasm (hsp72–73), to the endoplasmic reticulum (grp78), or to the mitochondria (mthsp70, grp75), whereas hsp65 is essentially a mitochondrial protein (for reviews, see refs. 1 and 2). These HSPs act as molecular chaperones and contribute to protein folding, refolding, transport, and translocation (1). HSPs induce thermotolerance and protection against a number of stresses distinct from HS, including reactive oxygen species (ROS), tumor necrosis factor α, and, *in vivo*, ischemia and reperfusion, sepsis, and acute inflammation (3–7), whereas all of these conditions are characterized by an increased oxidative burden (8). We have shown in the premonocytic line U937 that the induction of a HS response prevents subsequent H_2O_2-induced cell death (3, 9). However, the precise molecular targets for HSP protection from oxidative stress have remained elusive; cell membranes, DNA, and proteins have all been suggested to be protected by HSP (10). It has also been suggested that HS may increase the expression or the activity of endogenous scavengers of ROS, such as catalase or superoxide dismutase (11, 12).

We hypothesize that mitochondria are a selective target for protection from oxidative injury by HSP. A number of observations favor such a possibility. First, these organelles have been suggested from the earliest HS studies to be directly involved in the HS response (13). Second, whereas oxidative stress induces many alterations within the cell, mitochondria may be the first to be hit by ROS (14), which may be exemplified by the cytotoxicity of tumor necrosis factor α. Indeed, on the one hand, the earliest postreceptor event to be observed after addition of tumor necrosis factor α to cells is the generation of ROS within mitochondria (15), whereas on the other hand, the HS-induced protection from the cytotoxicity of the cytokine is clearly attributable to HSP (4, 5). Another example is injury after postischemic reperfusion, which is associated with mitochondrial disruption and which is prevented by HS *in vivo* (6, 16). Third, Patriarca and Maresca (17) have shown in *Saccharomyces cerevisiae* that, along with the induction of HSP, acquired thermotolerance prevents impairment in mitochondrial ATPase activity at elevated temperatures.

To test the hypothesis that HS protects cells from oxidative injury by preventing alterations in mitochondrial function, we preexposed U937 cells to HS, then exposed them to hydrogen peroxide (H_2O_2) and analyzed the mitochondrial membrane potential ($\Delta\varphi$), mass, and ultrastructure. Our data indicate that mitochondria may be a major target for the protective effects of HSP against oxidative stress. We then analyzed the correlations between the kinetics of the HS-induced protection against H_2O_2-mediated alterations in $\Delta\varphi$ and the levels of expression of hsp70 and hsp65, respectively. In addition, the possibility that a modulation in the expression of *bcl-2* protein [the product of a gene that is essential for controlling cell death by apoptosis and protecting cells via antioxidant mechanisms (18, 19)] contributes to the antioxidant effects of HS was also examined.

MATERIALS AND METHODS

Reagents and Media. 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) and nonyl acridine orange (NAO) were purchased from Molecular Probes. 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] (kindly provided by U. Fisher, Hoffmann–La Roche, Switzerland) was dissolved in ethanol and used at 100 ng/ml.

Cells and Exposure to Stresses. Mycoplasma-free U937 (a human premonocytic cell line), HL-60 (a human promyelocytic cell line), and CCRF-CEM (CEM) (a lymphoblastoid human T cell line) were maintained in stationary suspension in RPMI 1640 medium (GIBCO) containing 10% fetal calf serum (FCS) (GIBCO) and 1% glutamine (GIBCO) (complete medium). In some experiments (e.g., electron microscopy), U937 cells were differentiated, or not, by incubation with 1,25-$(OH)_2D_3$ (10 ng/ml) for 72 hr before exposure to HS or to H_2O_2. Exposure to HS was performed as described (20) in the presence of 25 mM Hepes; recovery after HS ranged from 0 to 24 h. Exposure to H_2O_2 (1 mM) was for 1 hr at 37°C.

Determination of Mitochondrial Membrane Potential ($\Delta\varphi$). $\Delta\varphi$ was analyzed by an original method that we recently described, which allows the direct measurement of $\Delta\varphi$ either

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: HS, heat shock; HSP, HS protein; ROS, reactive oxygen species; NAO, nonyl acridine orange; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3.

‡To whom reprint requests should be addressed.

in intact cells (21) or in isolated mitochondria (22). Briefly, JC-1 is a lipophilic cation that is able to selectively enter into mitochondria (23). JC-1 exists in a monomeric form emitting at 527 nm after excitation at 490 nm; however, depending on $\Delta\varphi$, JC-1 is able to form J-aggregates that are associated with a large shift in emission (590 nm) (24, 25). Thus, the color of the dye changes reversibly from green to greenish orange as the mitochondrial membrane becomes more polarized (23). Both colors can be detected using filters commonly mounted in flow cytometers, so that the green emission can be analyzed in fluorescence channel 1 (FL1) and the greenish orange emission in channel 2 (FL2).

The cell staining was performed as follows: cell suspensions were adjusted to a density of 0.5×10^6 cells per ml and incubated in complete medium with JC-1 (10 μg/ml) for 10 min at room temperature in the dark. At the end of the incubation period, the cells were washed twice in cold phosphate-buffered saline (PBS) (GIBCO), resuspended in a total volume of 400 μl, and analyzed. Flow cytometry was performed using a FACScan flow cytometer (Becton Dickinson) equipped with a single 488 nm argon laser. A minimum of 10,000 cells per sample were acquired in list mode and analyzed using LYSYS II software as reported (26).

Determination of Mitochondrial Mass: NAO Staining of the Phospholipid Cardiolipin. Mitochondrial mass was measured by flow cytometry as described (26, 27). We took advantage of NAO, a fluorescent dye capable of binding the mitochondrial phospholipid cardiolipin in an energy-independent manner and that subsequently can provide an indication of mitochondrial mass (28). Briefly, cell suspension was adjusted at 0.5×10^6 cells per ml and incubated with 10 mM NAO for 10 min at room temperature in the dark, washed twice with cold PBS, and immediately analyzed. A minimum of 10,000 cells per sample were acquired in list mode using a log scale for the photomultiplier which detected the green fluorescence emission of the dye. Median NAO fluorescence values were then calculated as reported elsewhere (29).

Analysis of HSP Expression. U937 cells were exposed to HS and then allowed to recover for the indicated times. Aliquots corresponding to equal cell numbers were resolved by SDS/PAGE (with 10% polyacrylamide) and revealed by autoradiography as described (30). hsp70 and hsp65 expression was revealed by Western blot analyses with mouse monoclonal antibodies against, respectively, the human inducible hsp70 (SPA810, StressGen Biotechnologies, Sidney, Canada) or the human hsp65 4B9-89 (a kind gift of G. Rook, London) (31). Bound antibodies were revealed with anti-mouse IgG-peroxidase conjugated (Sigma) in the presence of H_2O_2 and 4-chloro-1-naphthol (Sigma).

Determination of *bcl-2* Levels. The amount of *bcl-2* present in different cell lines was quantified by flow cytometry. U937 cells, HL-60 and CCRF-CEM cells were heat shocked as described above, kept for 18 hr at 37°C, and stained with anti-*bcl-2* mAb, as described elsewhere (32). Briefly, a pellet of 2×10^6 cells was resuspended in 1 ml of 2% paraformaldehyde in PBS and kept for 10 min at 4°C; 100 μl of 0.5 Triton X-100 (Sigma) in PBS was then added and 1 min later the cells were washed twice in cold PBS with 1% bovine serum albumin. For labeling, cells were incubated for 30 min at 4°C with saturating doses of fluorescein-conjugated anti-*bcl-2* mAb (Dako), washed twice with cold PBS, and analyzed. Negative controls were represented by cells treated in the same manner except that the labeling was performed with fluorescein isothiocyanate-conjugated isotype-matched mAbs (Dako). A minimum of 10,000 cells per sample was analyzed. To better describe the variations in the fluorescence emissions of untreated or heat-shocked cells after staining them with the anti-*bcl-2* mAb, for each sample the median linear fluorescence channel was calculated using the LYSYS II software according to procedures established by Schmid *et al.* (29). Then, from the median fluorescence value of each stained sample, the value of its negative control was subtracted. The resulting number indicated the net value of fluorescence of cells stained with anti-*bcl-2* mAb and was directly proportional to the amount of protein present inside the cell.

Electron Microscopy. After the respective treatments, U937 cells were fixed in suspension ($\approx 10^6$ cells per specimen) with 2.5% glutaraldehyde in 0.1 M PBS, pH 7.4, for 10 min at 4°C and as a pellet for 2 hr. After washing in the same buffer to which 0.2 M sucrose had been added, the pellets were postfixed using 2% osmic acid in 0.1 M sodium cacodylate, pH 7.3, for 30 min at room temperature, then washed again in distilled water, stained *en bloc* with aqueous uranyl acetate, washed in distilled water, dehydrated in a graded series of ethanol, and embedded in Spurr resin (33). Ultra-thin sections were cut using a Reichert-Young ultramicrotome, equipped with a diamond knife, and counterstained with lead citrate. They were examined in a Philips EM-300 electron microscope operating at 60 kV.

RESULTS

HS Protected Mitochondria from H_2O_2-Induced Functional and Morphological Alterations. The first aim of this investigation was to ascertain whether HSPs were able to protect cells from oxidative stress at the mitochondrial level. Thus, a cytofluorimetric approach was used to study, at the single cell level, the modifications of $\Delta\varphi$ induced by the oxidative damage. The main results are depicted in Fig. 1 (one representative experiment) and in Table 1 (results obtained from three disctinct experiments). In Fig. 1, in each panel, the number indicates the percentage of cells with depolarized mitochondria. A small percentage of control U937 cells had a low membrane potential (Fig. 1, *Upper Left*). HS *per se* was able to induce a small increase in such a percentage (data not shown); however, cells returned to normal after 18 hr (Fig. 1, *Upper*

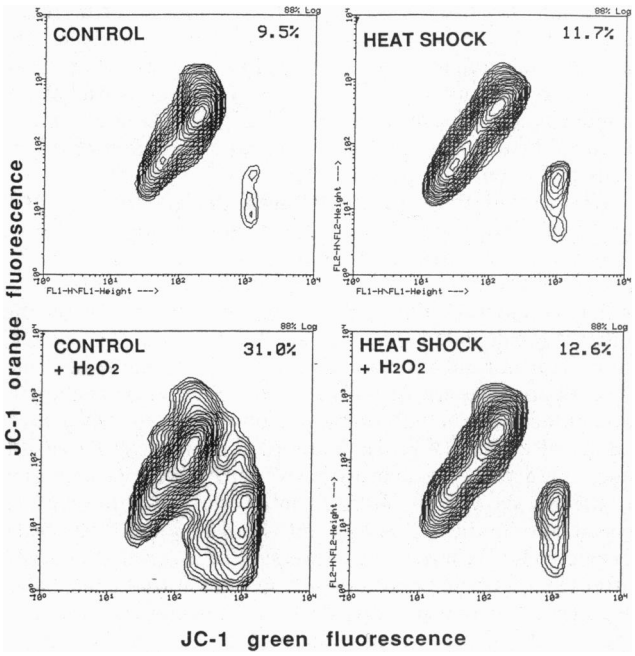

FIG. 1. HS protects mitochondria from H_2O_2-mediated damage. Cytofluorimetric analysis of mitochondrial membrane potential as assessed by JC-1 staining showing changes in control and heat-shocked U937 cells subsequently exposed to H_2O_2. All cells were incubated for 18 hr. One representative experiment out of three is shown. HS completely prevented the H_2O_2-induced alteration in $\Delta\varphi$ (compare the two lower panels). Numbers (in the upper right corner of each panel) indicate the percentage of cells with depolarized mitochondria.

Table 1. HS prevents H_2O_2-induced mitochondrial membrane depolarization

Treatment	Control	HS 18 hr recovery
None	10.6 ± 0.9[a]	11.7 ± 2.0[b]
H_2O_2	29.7 ± 2.5[c]	12.8 ± 1.4[d]

Numbers refer to % cells with depolarized mitochondria (mean ± SEM, $n = 3$). Statistical analysis, performed by two-tailed paired Student's t test, indicates that: a vs. b, P = NS; a vs. c, $P < 0.01$; a vs. d, P = NS; b vs. d, P = NS; c vs. d, $P < 0.01$. NS, not significant.

Right). When cells were treated with H_2O_2, significant modifications of the $\Delta\varphi$ took place (Fig. 1, *Lower Left*). Preexposure to HS (followed by 18 hr recovery) completely prevented the H_2O_2-induced mitochondrial depolarization (Fig. 1, *Lower Right*).

To rule out the possibility that changes in $\Delta\varphi$ were due to a loss in mitochondrial content, we studied the staining of cardiolipin by NAO, a recognized indicator of mitochondrial mass (28). Fig. 2 shows that exposure of cells to H_2O_2 did not provoke any loss in their mitochondrial mass.

The morphological data confirmed the functional data (Fig. 3). HS induced a transient perinuclear relocalization of mitochondria, which returned to normal by 18 hr (unpublished data). We have shown before that 1,25-$(OH)_2D_3$ increases both intracellular Ca^{2+} and the sensitivity of U937 cells to H_2O_2 (3). In agreement with these results, the morphological alterations in mitochondria of U937 cells preincubated with 1,25-$(OH)_2D_3$ appeared more important (compare Fig. 3 *e* and *f* to *a* and *b*). This is likely to be the case because the previous accumulation of Ca^{2+} induced by the hormone within these organelles rendered them unable to cope with an increased oxidative burden, resulting in an increase of cytosolic Ca^{2+}. It is noteworthy that the morphological changes observed (in particular, the alterations in mitochondrial volume) occurred in the absence of alterations in mitochondrial mass.

In both undifferentiated and 1,25-$(OH)_2D_3$-differentiated cells, HS prevented, at least in part, the morphological alterations induced in mitochondria by H_2O_2 (compare Fig. 3 *c* and *d* to *a* and *b* and *g* and *h* to *e* and *f*). In contrast, nuclear condensation (absent in control U937 cells; data not shown) induced by exposure to H_2O_2 was similar whether or not the cells had been preexposed to HS.

Correlation Between HS-Induced Protection from H_2O_2 and hsp70 Expression. Kinetic experiments were performed and cells were exposed to H_2O_2, either immediately or in increments of 2, 4, 12, 18, and 24 hr after HS. Control cells were not heat shocked. For each increment of time, a protection index was calculated (i.e., the ratio between the percentage of cells with normal mitochondria in heat-shocked cells and a control population). As a result, 100% relative protection was attributed to the highest protection observed, which was always 18 hr after HS (Fig. 4). Kinetics for protection was then correlated with the expression of hsp70 (Fig. 4) and hsp65 (data not shown), as obtained by Western blotting with the specific antibodies (SPA-801 and 4B9-89 for hsp70 and hsp65, respectively). Whereas the expression of hsp65 doubled 12 hr after HS, then remained constant up to 24 hr (data not shown), the expression of hsp70 varied with time in close correlation to the degree of relative protection (Fig. 4), and the time for maximal protection (18 hr) correlated with maximal expression of hsp70. In the presence of the transcriptional inhibitor Actinomycin D, for which we have previously published in detail the conditions under which it selectively prevents HSP induction without altering overall protein synthesis (34, 35), hsp70 expression was completely inhibited (data not shown). This result also paralleled the inhibition of any protective effect of HS on subsequent H_2O_2 exposure (Fig. 4).

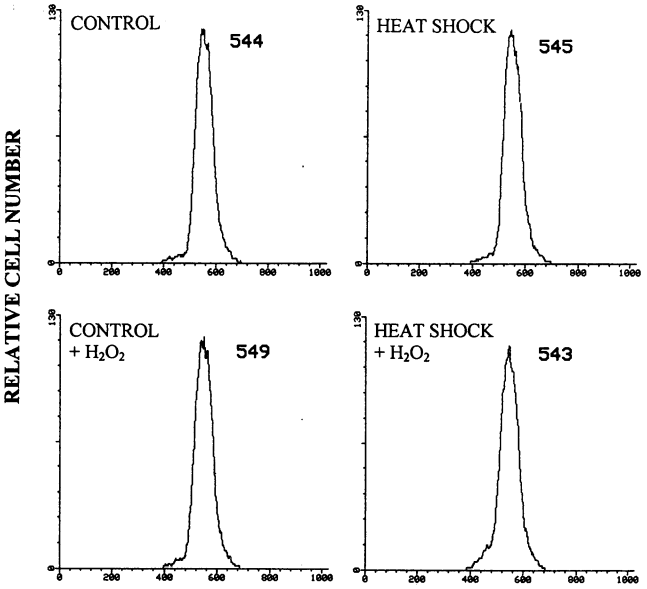

FIG. 2. H_2O_2 induces no alteration in mitochondrial mass. Exposure of U937 cells to H_2O_2 did not alter mitochondrial cardiolipin staining by NAO as revealed by cytofluorimetric analysis. Numbers in each panel indicate the linear median fluorescence channel, in proportion to the cardiolipin content; this parameter is related to mitochondrial mass. One representative experiment out of five is shown; all cells were incubated for 18 hr.

HS Increased the Expression of *bcl-2* in Different Human Cell Lines. To determine whether *bcl-2* was involved in the protective effect of HS on mitochondria, we studied using flow cytometry the amount of the protein present in three untreated or heat-shocked human cell lines. Fig. 5 shows the fluorescence emission of cells stained with anti-*bcl-2* mAb, the height of each column being directly proportional to the intracellular quantity of *bcl-2*. The data indicate that (*i*) different cell lines expressed different basal levels of *bcl-2* protein, and (*ii*) HS (after 18 hr of recovery) induced an increase in *bcl-2* protein expression in all three cell lines tested.

DISCUSSION

Here we report that the induction of a HS response prevented alterations in $\Delta\varphi$, which were induced by a subsequent exposure to H_2O_2 in the human premonocytic line U937.

Previously we reported that the induction of a HS response protects U937 cells from H_2O_2-induced cell death (3). We have been investigating several possible targets for this protective effect. Protection in terms of membrane alteration (measuring lipid peroxidation) ranges from 20 to 25% (36), whereas protection at the level of DNA (measuring ethidium bromide fluorescence) varies according to *in vitro* cell aging (ref. 37; M. Perin-Minisini, unpublished work). The HS-induced protection against H_2O_2-mediated mitochondrial alterations consistently ranged from 60 to 90%. Protection was thus higher for mitochondria than for DNA or membranes, suggesting that mitochondria might represent a primary target for protection by HS.

The morphological analysis also confirmed the functional studies. Preexposure to HS prevented, at least in part, the alterations in mitochondrial ultrastructure. The morphological mitochondrial alterations that we observed were very similar to those observed by Currie *et al.* (16), i.e., swelling and cristae disruption, and the protective effects of HS were similar, whether HS was administered *in vivo* (16) or *in vitro* (our study). As reported, differentiation of U937 cells with

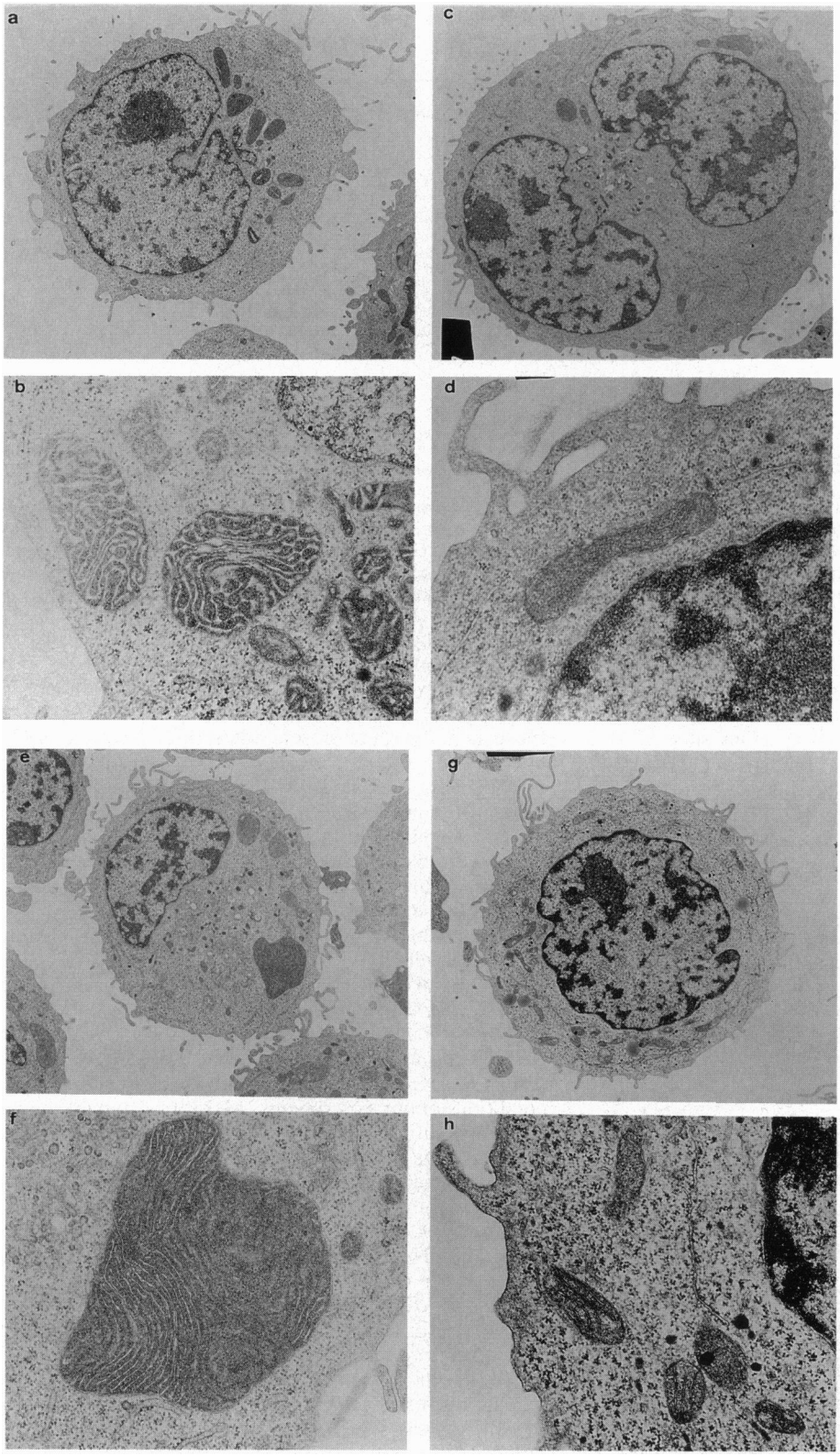

FIG. 3. HS prevents structural mitochondrial alterations in both control and differentiated U937 cells. Undifferentiated U937 cells (*a–d*) and cells differentiated with 1,25-$(OH)_2D_3$ (*e–h*) were either exposed to HS (42°C, 20 min) (*c*, *d*, *g*, and *h*) or were maintained at 37°C (*a*, *b*, *e*, and *f*). Eighteen hours after HS, all cells were exposed to H_2O_2 and prepared for electron microscopy as described. Mitochondria of cells that were exposed to HS (*c*, *d*, *g*, and *h*) retained a normal morphology, as compared with non-heat-shocked H_2O_2-exposed cells (*a*, *b*, *e*, and *f*), both in undifferentiated (*c* and *d*) and in differentiated (*g* and *h*) cells. In contrast, intranuclear chromatin condensation was similar under all the conditions tested. The pictures shown are representative of four distinct experiments, in each of which a mean of 20 pictures was examined. (*a*, *c*, *e*, and *g*, ×4560; *b*, *d*, *f*, and *h*, ×26,500.)

$1,25(OH)_2D_3$ potentiated oxidative damage, an effect related to an accumulation of intracellular Ca^{2+} (3). Interestingly, $1,25(OH)_2D_3$ up-regulates HSP expression (38, 39), as well as superoxide dismutase (unpublished work), both of which may represent a self-protecting mechanism aiming at counteracting the increase in oxidative stress.

The kinetics for protection against H_2O_2-induced alterations in $\Delta\varphi$ correlated with those of hsp70 accumulation within the heat-shocked cells, whereas protection was abolished when HS was performed in the presence of the transcriptional inhibitor Actinomycin D. These results indicate that the expression of HSP, and not solely HS, is required for protection. Such correlations between HSP expression and protection are usually considered to be a valuable argument for implying a direct role for HSP in protection both *in vitro* and *in vivo* (3, 34, 35). Our results are consistent with the fact that hsp70 likely represents the major protective stress protein, as also proposed by a number of investigators (4, 6, 34, 36, 40–42).

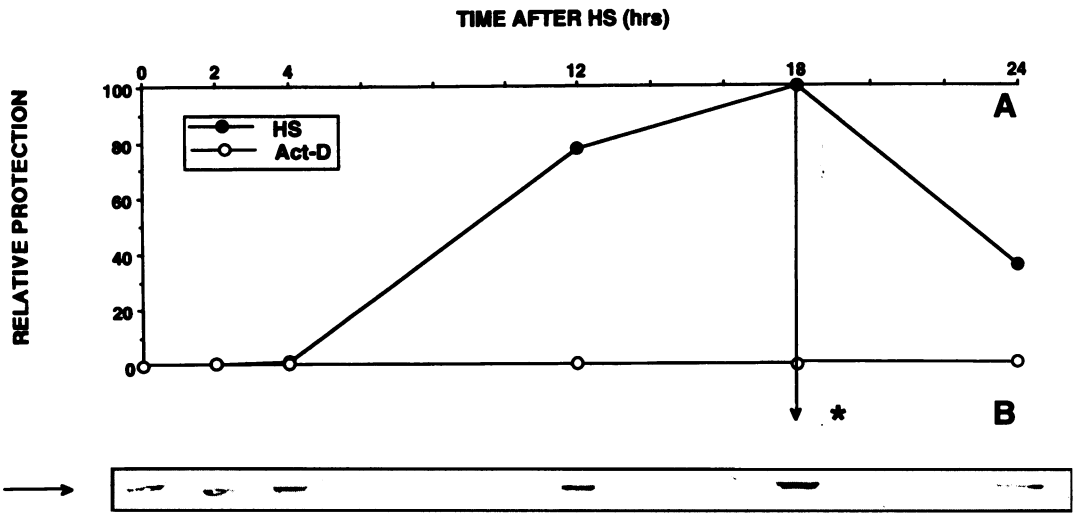

FIG. 4. Time course of hsp70 expression and HS-induced prevention of mitochondrial membrane depolarization in cells exposed to H_2O_2. The time for mitochondrial maximal protection (18 hr) (*A*) correlated with maximal expression of hsp70 (*B*, arrow). *, 100% relative protection, attributed to the highest protection observed, was always at 18 hr (one representative experiment out of six is shown). (*B*) Aliquots corresponding to equal cell numbers were loaded on each lane of the Western blot. The presence of the transcriptional inhibitor Actinomycin D completely inhibited hsp70 expression (data not shown), which paralleled the inhibition of any protective effect of HS on subsequent H_2O_2 exposure (open circles).

hsp70-mediated chaperoning and protection may be exerted at the mitochondrial membrane and/or within the mitochondria as well as within the cytoplasm, because among the members of the hsp70 family, there are members locating themselves specifically to these as well as other subcellular compartments (2). Interestingly, the correlation between protection and HSP expression was stronger for hsp70 than for the mitochondria-specific hsp65, which probably relates to the fact that the heat inducibility of the latter is lower than it is for hsp70. The specific role in mitochondrial protection of other HSPs remains to be established.

Although the precise mitochondrial function that is protected remains to be determined, several arguments favor some component of the respiratory chain:

(*i*) ATPase activity undergoes thermotolerance in *S. cerevisiae* (17), and HS prevents inhibition of oxidative phosphorylation by the ATPase inhibitor oligomycin in cells strictly dependent on oxidative metabolism (43).

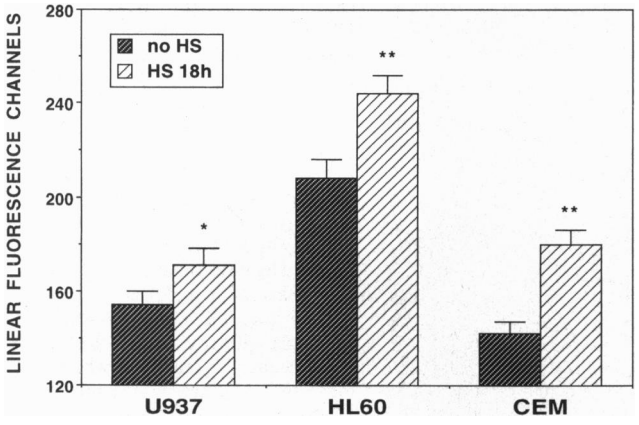

FIG. 5. HS increases *bcl-2* expression in different human cell lines (U937, HL-60, and CCRF-CEM). The graphs show the cytofluorimetric analysis of the expression of *bcl-2* in untreated or heat-shocked cells (after 18 hr of recovery). The data represent the difference between the median fluorescence channels (that have been converted from logarithmic to linear scale) of cells stained with an unrelevant, fluorescein isothiocyanate-labeled mAb (i.e., the negative control) and cells stained with the anti-*bcl-2* mAb. The height of the columns is directly proportional to the quantity of protein present inside the cell (mean ± SEM, $n = 3$; * = $P < 0.03$ and ** = $P < 0.01$ versus respective controls by two-tailed paired Student's *t* test). Basal levels of *bcl-2* were characteristic for each cell line and increased 18 hr after HS.

(*ii*) We have found that induction of a HS response *in vivo* in rats prevents alterations in mitochondrial respiration on subsequent exposure of isolated perfused hearts to H_2O_2 and, more specifically, in state III respiration (44). These data are in agreement with those of Borkan *et al.* (45), who demonstrated thermotolerance in stage III mitochondrial respiration in inner-medullary collecting duct cells from rat kidneys. Both of these studies (44, 45) were performed on isolated mitochondria, whereas the cytofluorimetric study of $\Delta\varphi$ in the intact cells we used here offered an advantage: the mitochondria were studied in their physiological environment, i.e., within the cell.

(*iii*) We can exclude the fact that the target for mitochondrial oxidative damage in our experiments is mitochondrial DNA. Indeed, although the concentrations of H_2O_2 we used induce damage to genomic DNA in the U937 cells (46), we were unable to detect any damage to mitochondrial DNA under the conditions of our experiments (M. Morris, unpublished data). The possibility that immortal cells benefit from an increased ability to efficiently repair mitochondrial DNA may be considered, in view of recent results indicating that mitochondrial DNA damage induced by ROS may indeed be repaired (47).

(*iv*) Finally, we can exclude loss in mitochondrial mass, as assessed by NAO binding to cardiolipin.

A number of similarities between the HS and the *bcl-2*-induced antioxidant effects suggested to us the possibility that overexpression of *bcl-2* may contribute to the mechanisms by which HS prevents mitochondrial oxidative damage (18, 19). Indeed, both HS and/or HSP and *bcl-2* protect cells from oxidative stress (3, 19); both decrease ROS production (19, 35). In both cases, protection is distal to the Ca^{2+} entry that is induced by oxidative stress (3, 19). Furthermore, HS prevents apoptosis, as does *bcl-2* (48, 49). We found that HS increased the expression of *bcl-2*, as detected by cytofluorimetric analysis of a number of human cell lines (Fig. 5). The promoter region of *bcl-2* contains a number of sequences resembling the HS element (including n-AGAA-n), as well as a classical HS element (n-GAA-nn-TTC-n) at position −622/−630, which may explain the increased expression of *bcl-2* protein after HS (50, 51). However, the lack of prevention of nuclear condensation by HS suggests that the contribution of *bcl-2* to the protective effects of HS was a minor one, because one would

expect *bcl-2* to prevent essentially those alterations associated with apoptosis.

In conclusion, our data suggest that mitochondria might represent the primary intracellular target for HS-induced protection against oxidative injury. Although protection correlated best with hsp70 expression, the respective role of other stress proteins remains to be determined. Furthermore, the use of different agents acting at specific stages of mitochondrial respiration (52) should help to define the targets for HS-induced protection in mitochondrial respiration; current knowledge suggests that state III respiration and F1-ATPase are such preferential targets.

Protection from oxidative injury has wide medical implications, including inflammation, reperfusion injury, cancer, and AIDS (8, 53). Therapeutic applications of HSP are being considered in these clinical conditions (54). A better understanding of the molecular mechanisms for HSP-mediated protection is a prerequisite for such applications.

We are grateful to Dr. M. Morris for the analysis of mitochondrial DNA, to Drs. D. Barbieri and G. Kalachnikova for their contribution to the cytofluorimetric analysis, to Y. R. A. Donati and E. Mariéthoz for technical help, to Dr. L. Bornman for many stimulating discussions, and to J. Rian for critical review. This work was supported by Institut National de la Santé et de la Recherche Médicale (B.S.P.) and grants from the CISC (Communauté de l'Industrie Suisse de la Cigarette) and the Fonds National de la Recherche Scientifique (Grant Nb.32.028645) to B.S.P.; by grants from Associazone Italiana per la Ricerca sul Cancro, VII and VIII Progetto di Ricerche sull'AIDS (Grants 9204/55 and 9304/64) to C.F. The financial support of Telethon (Italy) is also gratefully acknowledged.

1. Feige, U. & Polla, B. S. (1994) *Experientia* **50,** 979–986.
2. Stuart, R. A., Cyr, D. M. & Neupert, W. (1994) *Experientia* **50,** 1002–1011.
3. Polla, B. S., Bonventre, J. V. & Krane, S. M. (1988) *J. Cell Biol.* **107,** 373–380.
4. Jäättelä, M. (1993) *J. Immunol.* **151,** 4286–4294.
5. Mehlen, P., Preville, X., Chareyron, P., Briolay, J., Klemenz, R. & Arrigo, P. (1995) *J. Immunol.* **154,** 363–374.
6. Plumier, J.-C. L., Ross, M. B., Currie, R. W., Angelidis, C. E., Kazlaris, H., Kollias, G. & Pagoulatos, G. N. (1995) *J. Clin. Invest.* **95,** 1854–1860.
7. Villar, J., Ribeiro, S. P., Mullen, J. B., Kuliszewski, M., Post, M. & Slutsky, A. S. (1995) *Crit. Care Med.* **22,** 914–921.
8. Halliwell, B. (1987) *FASEB J.* **1,** 358–364.
9. Donati, Y. R. A., Slosman, D. O. & Polla, B. S. (1990) *Biochem. Pharmacol.* **40,** 2571–2577.
10. Polla, B. S., Mili, N. & Kantengwa, S. (1991) in *Heat Shock and Oxidative Injury in Human Cells*, eds. Maresca, B. & Lindquist, S. (Springer, Berlin), pp. 279–290.
11. Karmazyn, M. (1991) *Can. J. Physiol. Pharmacol.* **69,** 719–730.
12. Polla, B. S., Stubbe, H., Kantengwa, S., Maridonneau-Parini, I. & Jacquier-Sarlin, M. R. (1995) *Inflammation* **3,** 363–378.
13. Ritossa, F. (1962) *Experientia* **18,** 571–573.
14. Richter, C. & Kass, G. E. N. (1991) *Chem. Biol. Interact.* **77,** 1–23.
15. Hennet, T., Richter, C. & Peterhans, E. (1993) *Biochem. J.* **289,** 587–592.
16. Currie, R. W., Karmazyn, M., Kloc, M. & Mailer, K. (1988) *Circ. Res.* **63,** 543–549.
17. Patriarca, E. J. & Maresca, B. (1990) *Exp. Cell. Res.* **190,** 57–64.
18. Kane, D. J., Sarafian, T. A., Anton, R., Hahn, H., Gralla, E. B., Valentine, J. S., Ord, T. & Bredesen, D. E. (1993) *Science* **262,** 1274–1277.
19. Hockenbery, D. M., Oltvai, Z. N., Yin, X.-M., Milliman, C. L. & Korsmeyer, S. J. (1993) *Cell* **75,** 241–251.
20. Clerget, M. & Polla, B. S. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 1081–1085.
21. Cossarizza, A., Baccarani-Contri, M., Kalachnikova, G. & Franceschi, C. (1993) *Biochem. Biophys. Res. Commun.* **197,** 40–45.
22. Cossarizza, A., Ceccarelli, D. & Masini, A. (1996) *Exp. Cell Res.* **222,** 84–94.
23. Smiley, S. T., Reers, M., Mottola-Hartshorn, C., Lin, M., Chen, A., Smith, T. W., Steele, G. D. & Chen, L. B. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 3671–3675.
24. Hada, H., Honda, C. & Tanemura, H. (1977) *Photogr. Sci. Eng.* **21,** 83–91.
25. Reers, M., Smith, T. W. & Chen, L. B. (1991) *Biochemistry* **30,** 4480–4486.
26. Cossarizza, A., Kalashnikova, G., Grassilli, E., Chiappelli, F., Salvioli, S., Capri, M., Barbieri, D., Troiano, L., Monti, D. & Franceschi, C. (1994) *Exp. Cell Res.* **214,** 323–330.
27. Cossarizza, A., Cooper, E. L., Quaglino, D., Salvioli, S., Kalachnikova, G. & Franceschi, C. (1995) *Biochem. Biophys. Res. Commun.* **214,** 503–510.
28. Petit, J. M., Maftah, A., Ratinaud, M. H. & Julien, R. (1992) *Eur. J. Biochem.* **209,** 267–273.
29. Schmid, I., Schmid, P. & Giorgi, J. V. (1988) *Cytometry* **9,** 533–538.
30. Jacquier-Sarlin, M. R., Jornot, L. & Polla, B. S. (1995) *J. Biol. Chem.* **270,** 14094–14099.
31. Sharif, M., Worral, J., Singh, B., Gupta, R. S., Lydyard, P. M., Lambert, C., McCulloch, J. & Rook, G. A. W. (1992) *Arthritis Rheum.* **35,** 1427–1433.
32. Delia, D., Aiello, A., Soligo, D., Fontanella, A., Melani, C., Pezzella, F., Pierotti, M. A. & Della Porta, G. (1992) *Blood* **79,** 1291–1298.
33. Spurr, A. R. (1969) *J. Ultrastruct. Res.* **26,** 31–43.
34. Mariéthoz, E., Tacchini-Cottier, F., Jacquier-Sarlin, M., Sinclair, F. & Polla, B. S. (1994) *Int. Immunol.* **6,** 925–930.
35. Maridonneau-Parini, I., Malawista, S. E., Stubbe, H., Russo-Marie, F. & Polla, B. S. (1993) *J. Cell Physiol.* **156,** 204–211.
36. Jacquier-Sarlin, M. R., Fuller, K., Dinh-Xuan, A. T., Richard, M.-J. & Polla, B. S. (1994) *Experientia* **50,** 1031–1038.
37. Perin, M. & Polla, B. S. (1992) *Int. J. Radiat. Biol.* **62,** 114 (abstr.).
38. Polla, B. S., Healy, A. M., Amento, E. P. & Krane, S. M. (1986) *J. Clin. Invest.* **77,** 1332–1339.
39. Kantengwa, S., Capponi, A. M., Bonventre, J. V. & Polla, B. S. (1990) *Am. J. Physiol.* **259,** C77–C83.
40. Barbe, M. F., Tytell, M., Gower, D. J. & Welch, W. J. (1988) *Science* **241,** 1817–1820.
41. Riabowol, K. T., Mizzen, L. A. & Welch, W. J. (1988) *Science* **242,** 433–436.
42. Johnston, R. N. & Kucey, B. L. (1988) *Science* **242,** 1551–1554.
43. Polla, B. S. & Bonventre, J. V. (1987) *Clin. Res.* **35,** 555A (abstr.).
44. Bornman, L., Gericke, G. S., Steinmann, C. M. L. & Polla, B. S. (1994) *J. Free Rad. Biol. Med.* **2,** R6 (abstr.).
45. Borkan, S. C., Emami, A. & Schwartz, J. H. (1993) *Am. J. Physiol.* **265,** F333–F341.
46. Perin Minisini, M., Kantengwa, S. & Polla, B. S. (1994) *Mutat. Res.* **315,** 169–179.
47. Driggers, W. J., LeDoux, S. P. & Wilson, G. L. (1993) *J. Biol. Chem.* **268,** 22042–22045.
48. Mailhos, C., Howard, M. K. & Latchman, D. S. (1993) *Neuroscience* **55,** 621–627.
49. Mosser, D. D. & Martin, L. H. (1992) *J. Cell. Physiol.* **151,** 561–570.
50. Cleary, M. L. & Sklar, J. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 7439–7443.
51. Cleary, M. L., Smith, SD, & Sklar, J. (1986) *Cell* **47,** 19–28.
52. Gabai, V. L. & Kabakov, A. E. (1993) *FEBS Lett.* **327,** 247–250.
53. Pace, G. W. & Leaf, C. D. (1995) *Free Rad. Biol. Med.* **4,** 523–528.
54. van Eden, W. & Young, D. B., eds. (1996) *Stress Proteins in Medicine* (Dekker, New York).