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Plasma Membrane Ubiquinone Controls Ceramide Production and Prevents Cell Death Induced by Serum Withdrawal

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Serum provides cultured cells with survival factors required to maintain growth. Its withdrawal induces the development of programmed cell death. HL-60 cells were sensitive to serum removal, and an increase of lipid peroxidation and apoptosis was observed. Long-term treatment with ethidium bromide induced the mitochondria-deficient ρ°HL-60 cell line. These cells were surprisingly more resistant to serum removal, displaying fewer apoptotic cells and lower lipid peroxidation. HL-60 cells contained less ubiquinone at the plasma membrane than ρ°HL-60 cells. Both cell types increased plasma membrane ubiquinone in response to serum removal, although this increase was much higher in ρ° cells. Addition of ubiquinone to both cell cultures in the absence of serum improved cell survival with decreasing lipid peroxidation and apoptosis. Ceramide was accumulated after serum removal in HL-60 but not in ρ°HL-60 cells, and exogenous ubiquinone reduced this accumulation. These results demonstrate a relationship between ubiquinone levels in the plasma membrane and the induction of serum withdrawal-induced apoptosis, and ceramide accumulation. Thus, ubiquinone, which is a central component of the plasma membrane electron transport system, can represent a first level of protection against oxidative damage caused by serum withdrawal.

KEY WORDS: HL-60, ρ°HL-60; ubiquinone; plasma membrane; apoptosis; ceramide.

INTRODUCTION

Cells contain diverse mechanisms to respond to different forms of stress that usually leads to cell growth arrest, and under severe or irreparable damages they undergo programmed cell death (apoptosis). Withdrawal of serum is one of the conditions causing severe damage to and increased death of cells in culture (Ishizaki *et al.*, 1995). Serum contains those survival factors required to maintain growth by a social control (Raff, 1992). The machinery required to develop apoptosis is constitutively expressed (Weil *et, al.*, 1996), and a great variety of signal transduction pathways can be activated by stimuli which mediate signals from outside the cell, which converge upon the activation of this machinery (Vaux and Strasser, 1996).

Mild oxidative stress is one condition involved in the development of cell death (Slater *et al.*, 1996). Although it has been shown that various forms of induced apoptosis are independent of ambient oxygen tension (Schulze-Osthoff *et al.*, 1994; Jacobson and Raff, 1995; Muschel *et al.*, 1995; Shimizu *et al.*, 1995), there is evidence for an indirect generation of oxidative stress in developing apoptosis after growth factor withdrawal, which is inhibited by intracellular antioxidants (Greelund *et al.*, 1995; Tilly and Tilly, 1995). Oxidative damage is not synonymous with molecular oxygen, and changes in cellular reduction potential may still occur and coordinate important events in apoptosis, even in the absence of reactive oxygen species (ROS)

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(Slater *et al.*, 1996). Bcl-2 protein inhibits apoptosis by an antioxidant mechanism that suppresses lipid peroxidation developed after the apoptotic signal (Hockenbery *et al.*, 1993; Kane *et al.*, 1993), and lipid hydroperoxides trigger apoptosis in antioxidant-deficient cells (Sandstorm *et al.*, 1994).

Serum removal also induces the production of ceramide by the activation of a membrane-bound Mg^{2+}-dependent neutral sphingomyelinase (Jayadev *et al.*, 1995). Ceramide is able to induce cell death after its intracellular accumulation (Obeid *et al.*, 1993; Jarvis *et al.*, 1994) by activating proteases of the interleukin converting enzyme (ICE) family (Martin *et al.*, 1995; Smyth *et al.*, 1996; Mizushima *et al.*, 1996). Thus, ceramide accumulation appears as a key component in the stress response pathway triggered by serum withdrawal (Hannun, 1996).

Plasma membranes contain lipophilic antioxidants such as α-tocopherol and ubiquinone (coenzyme Q) that prevent lipid peroxidation caused by different agents (Beyer, 1994), and consequently can protect against free radical-mediated cell injury (Matsura *et al.*, 1995). Ubiquinone is a central molecule in the protection of the plasma membrane because it is directly reduced by cytochrome b_5 reductase (Villalba *et al.*, 1995) and then maintains other antioxidants such as α-tocopherol or ascorbate (Beyer, 1994; Villalba *et al.*, 1995). This paper indicates a role of plasma membrane ubiquinone in the prevention of apoptosis induced by serum withdrawal in HL-60 cells. The increase of ubiquinone at the plasma membrane can prevent both lipid peroxidation and apoptosis in these cells. Surprisingly, long-term ethidium bromide-induced mitochondria-deficient HL-60 (ρ°) cells: (i) were more resistant to serum removal, (ii) had more ubiquinone concentrated in the plasma membrane, and (iii) showed less lipid peroxidation than parental cells. Also, ceramide accumulation was significantly increased in HL-60 but not in ρ°HL-60 cells after serum removal, and its production was prevented by externally added ubiquinone. Thus, we show a clear participation of plasma membrane ubiquinone in control of lipid peroxidation and apoptosis induced by serum withdrawal, coincident with decrease of ceramide release into the cytosol as an apoptotic signal.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

HL-60, a human promyelocytic cell line, was cultured in RPMI-1640 medium (Sigma, Spain) supplemented with 10% fetal calf serum (FCS) (Flow, Scotland), 100 units/ml penicillin, 100 mg/ml streptomycin, and 2.5 mg/ml amphotericin B (Sigma, Spain), at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Mitochondria-deficient ρ° cells were generated by culturing HL-60 cells in the presence of 50 ng/ml ethidium bromide (Sigma, Spain) for 5–6 weeks as described elsewhere (Desjardins *et al.*, 1985). For these cells, culture media were supplemented with 50 ng/ml uridine (Sigma, Spain) and 1 mM pyruvate (Sigma, Spain). Cells were concentrated from stock cultures by centrifugation at 1,000 × *g* for 5 min and washed twice in serum-free medium. Cell viability was determined by trypan blue exclusion.

Mitochondrial Markers of ρ°HL-60 Cells

Mitochondrial DNA (MtDNA) was determined by the polymerase chain reaction (PCR) as described previously (Larm *et al.*, 1994). Cell pellet was resuspended in 0.4 ml of 50 mM Tris/HCl, pH 7.4, 10 mM EDTA, and 100 mM NaCl. Nonspecific nuclear DNA sequences were eliminated incubating DNA samples (1 mg) overnight at 37°C with the restriction enzyme Bgl II and then digested for 90 min at 37°C with the exonuclease Bal 31 (Boehringer Mannheim, Germany). PCR was then carried out in a DNA Thermal Cycler 480 machine (Perkin Elmer, U.S.A.) for 30 cycles. Taq polymerase was purchased from Perkin Elmer (U.S.A.). The PCR primers D_H, complementary to the mitochondrial genome positions 11918–11942, and E_L, complementary to the positions 11580–11603, were used (Marzuki *et al.*, 1991). PCR products were analyzed by electrophoresis in 1% agarose gels containing 1 mg/ml ethidium bromide, using DNA size markers from λ DNA (Boehringer Mannheim, Germany). Cytochrome *c* oxidase as marker of respiratory chain was carried out as described (Storrie and Madden, 1990).

Plasma Membrane Preparation

Microsomes were obtained from HL-60 or ρ°HL-60 cell homogenates. Plasma membrane vesicles were then isolated by the two-phase partition method (Alcaín *et al.*, 1991). Membranes were resuspended in 50 mM Tris/HCl, pH 7.6, containing 10% glycerol, 1 mM PMSF, and 1 mM DTT, and stored either under liquid nitrogen or at −86°C. Purity of fractions was

checked by marker enzymes analysis (Alcaín *et al.*, 1991).

Ubiquinone Quantification

Plasma membranes (12 mg) were first disrupted with 1% SDS in a final volume of 1 ml, and 2 ml of 95% ethanol–5% isopropanol was added. Ubiquinone was then recovered from SDS-alcoholic solution by extraction with 5 ml of hexane. Extraction was repeated once and both hexane phases were combined. After evaporation under vacuum, extracts were resuspended in 100 μl ethanol. Ubiquinone determination was carried out by HPLC separation with UV monitoring at 275 nm. The procedure described here recovered more than 98% of ubiquinone.

Apoptosis Detection

Apoptosis was detected by labeling of 3′ OH ends of DNA breaks using the Apotag detection system as specified by the manufacturer (Oncor, USA). Briefly, cells were fixed in 4% neutral-buffered formalin, dried onto microscope slides, and washed with PBS. Cells were then incubated with terminal deoxynucleotidyl transferase in a reaction buffer containing digoxigenin dUTP for 1 h at 37°C. Once the reaction was terminated, slides were washed for 30 min and incubated with antidigoxigenin antibody coupled to fluorescein for 30 min at room temperature and then washed three times with PBS. Slides were then mounted for photomicrography under phase and epifluorescence illumination.

Lipid Peroxidation Measurements

Cells were preincubated with 45 μM *cis*-parinaric acid (molecular Probes, USA) in 0.1 M Tris/HCl, pH 7.5, for 5 min and then submitted to different treatments for 1 h at 37°C in the presence of *cis*-parinaric acid. Fluorescence measurements were carried out at room temperature, using a stirred cuvette in an SFM 25 spectrofluorometer (Kontron, Germany) with wavelengths of 334 nm for excitation and 375 nm for emission.

Ceramide Measurements

Ceramide content was determined as described (Jayadev *et al.*, 1995). Briefly, cells were harvested and the lipids extracted. Lipids were dried under nitrogen and then resuspended in 100 μl of chloroform, using 25 μl for phosphate measurements and 25 μl for the diacyl-glycerol kinase assay (Preiss *et al.*, 1986). The phosphorylated lipids were extracted and run on TLC using chloroform/acetone/methanol/acetic acid/water (10:4:3:2:1) as solvent. Ceramide-phosphate spots were quantitated using an automatic TLC-linear analyzer (Berthold, Germany). Ceramide was quantitated using external standards and was normalized to phosphate.

RESULTS

Cells require growth and survival factors that are provided by serum to maintain cell growth in culture. Its withdrawal induces cell growth arrest and some cells develop the death program (Ishizaki *et al.*, 1995). Figure 1A shows how removal of serum from a culture of HL-60 cells induced death of a significant number of cells by apoptosis. In parallel, lipid peroxidation rates in HL-60 cells cultured in the absence of serum were significantly increased (Fig. 1B). HL-60 cells contained about 70 pmol of ubiquinone per mg of protein in the plasma membrane (Fig. 2). After 48 h growth without serum, ubiquinone content in this membrane of surviving cells was increased 40%. Plasma membrane fractions were prepared by two-phase partitioning optimized to cells in culture, and the marker enzymes analysis guaranteed the significant absence of mitochondrial inner membranes and other endomembranes contamination (Alcaín *et al.*, 1991). Ubiquinone determined here is thus primarily derived only from the plasma membrane.

Treatment of HL-60 cells with ethidium bromide for a long period inhibits the replication of mitochondrial DNA by intercalating between adjacent DNA base pairs (Leibowitz, 1971). Long-term treatment of these cells in the presence of ethidium bromide depleted cells of their mitochondrial DNA (Desjardins *et al.*, 1985), but surviving cells (ρ° cells) can maintain their growth if supplemented with pyruvate and uridine. After 6 weeks, DNA extracted from these cells did not contain any mitochondrial DNA detectable by PCR using the D_H and E_L primers as described (Marzuki *et al.*, 1991), and these cells were then used for

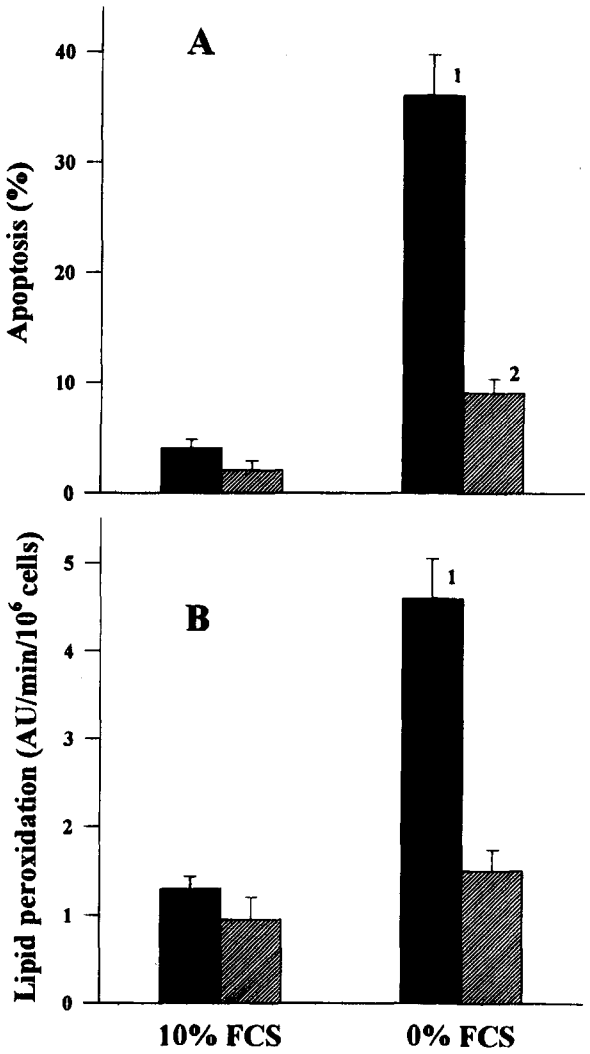

Fig. 1. Serum withdrawal effect on HL-60 and ρ°HL-60 cells. Cells used were grown in the presence or in the absence of 10% FCS for 48 h and both the percentage of apoptotic cells (A) and lipid peroxidation rates (B) were determined. $n = 4$. $^1p \leq 0.01$ vs 10% FCS, $^2p \leq 0.05$ vs 10% FCS. Closed bars: HL-60 cells. Dotted bars: ρ°HL-60 cells.

further experiments. Also, cytochrome *c* oxidase, a marker for the respiratory chain, was decreased from 14 ± 1.5 nmol/min/mg protein in parental HL-60 cells to 2 ± 0.5 nmol/min/mg protein in ρ°HL-60 cells. As shown in Fig. 1A, ρ°HL-60 cells were much less sensitive to serum removal and only about 9% apoptotic cells were identified after 48 h. Also, the lipid peroxidation rate was not significantly changed in comparison to ρ°HL-60 cells growing in 10% serum (Fig. 1B). Plasma membrane ubiquinone was increased 40% in ρ°HL-60 cells compared to parental cells and, when submitted to serum withdrawal, ubiquinone concentration was triplicated in these cells (Fig. 2).

Externally addition of ubiquinone to serum-free cultures improved survival of cells (Fig. 3). HL-60 cells were more sensitive to serum removal than ρ°HL-60 cells, but showed a significant ability to survive in the presence of ubiquinone at concentrations of 30 μM or higher. We analyzed then apoptosis and lipid peroxidation in both HL-60 and ρ°HL-60 cells growing for 48 h without serum but in the presence of 40 μM ubiquinone or ubiquinol (Fig. 4). The number of apoptopic cells was significantly decreased by ubiquinone in both cell cultures (Fig. 4A), although reduction was more evident in HL-60 cells that naturally contained less ubiquinone in the plasma membrane (see above). It is also important to observe that the ubiquinol was more effective than the oxidized form. Further, lipid peroxidation rates were also affected by the presence of ubiquinone in culture media (Fig. 4B). They were clearly decreased in HL-60 cells with both ubiquinone and ubiquinol although the latter was more effective. The protection provided by ubiquinone to ρ°HL-60 cells was also significant although less evident than in parental cells. No differences were detected between ubiquinone and ubiquinol in ρ°HL-60 cells.

Removal of serum induces the accumulation of ceramide in different cells (Jayadev *et al.*, 1995), which appears as a signal for triggering cell growth arrest and death (Hannum, 1996). In order to study the relationship between mild oxidate damages produced by serum removal and the plasma membrane antioxidant ubiquinone, we studied the production of ceramide in HL-60 and ρ°HL-60 cells, and the effect of externally added ubiquinone (Fig. 5). Both HL-60 and ρ°HL-60 cells contained similar basal levels of ceramide in 10% FCS-supplemented cultures. Removal of serum for 48 h induced a significant accumulation of ceramide in HL-60 cells (Fig. 5A), which was totally prevented if supplemented with 40 μM ubiquinone. However, when ρ°HL-60 cells were submitted to serum withdrawal, there was no new production of ceramide and the addition of ubiquinone decreased the content of ceramide in ρ°HL-60 cells after 48 h without serum (Fig. 5A). Total phosholipids in both HL-60 and ρ°HL-60 cells did not change significantly in all the conditions used in this work (Fig. 5B).

DISCUSSION

Diverse agents and environmental conditions produce different levels of damage to cells and when it

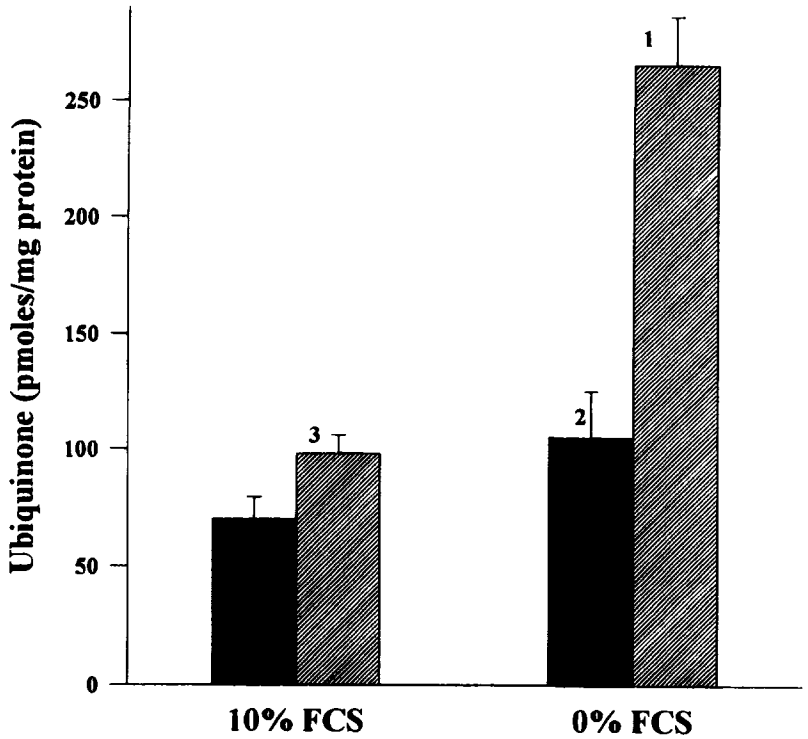

Fig. 2. Contents of ubiquinone at the plasma membrane. HL-60 (closed bars) and ρ°HL-60 (dotted bars) cells were grown for 48 h in the presence of 10% FCS or in its absence. Cells were then harvested and plasma membrane vesicles were separated by two-phase partition. Ubiquinone was extracted from these plasma membrane samples and quantitated by HPLC. $^1p \leq 0.01$ vs 10% FCS. $^2p \leq 0.05$ vs 10% FCS. $^3p \leq 0.05$ vs HL-60 cells.

is irreparable, cells develop the program of cell death. These stimuli activate the constitutive machinery of apoptosis through many signaling pathways (Vaux and Strasser, 1996). Serum provides to cells in culture those survival signals from other cells for survival and proliferation (Raff, 1992), and its removal is one stimulus acting at the plasma membrane that triggers cell death by default (Ishizaki *et al.*, 1995). Removal of serum from HL-60 cultures induced a significant amount of apoptotic cells and, also, higher rates of lipid peroxidation were observed under these conditions. However, ethidium bromide-induced mitochondria-deficient ρ°HL-60 cells were more resistant to death caused by serum withdrawal and no significant changes in lipid peroxidation were observed. Removal of serum or growth factors induces oxidative stress in various cell types leading to death, and this type of apoptosis can be prevented by antioxidants such as cytosolic superoxide dismutase, catalase, or glutathione peroxidase (Greenlund *et al.*, 1995; Tilly and Tilly, 1995; Hockenbery *et al.*, 1993). Also, the action of externally added ascorbate can prevent growth factor withdrawal-induced apoptosis in rat ovarian follicles (Tilly and Tilly, 1995). We have found that the plasma membrane of HL-60 cells contains a lower concentration of ubiquinone than that of ρ°HL-60 cells, and these cells overexpress ubiquinone at the plasma membrane as a stress response caused by the removal of serum. However, this response was very low in parental HL-60 cells. Ubiquinone is an antioxidant that can prevent some degree of oxidative stress, either by a direct interaction with lipid peroxyl radicals or in cooperation with α-tocopherol (Beyer 1994), and can then protect against radical-mediated cell injury (Matsura *et al.*, 1995). The ubiquinone-deficient yeast strain *coq*3Δ is very sensitive to autoxidation products of polyunsaturated fatty acids and the resistance can be regained by the introduction of the COQ_3 gene, indicating that the sensitive phenotype results solely from the inability to produce ubiquinone (Do *et al.*, 1996). Ubiquinone also supports growth of different cell lines in serum-limiting media (Sun *et al.*, 1995). Thus, the up-regulation of ubiquinone in ρ°HL-60 cells under the stress caused by serum removal, or the external supplementation

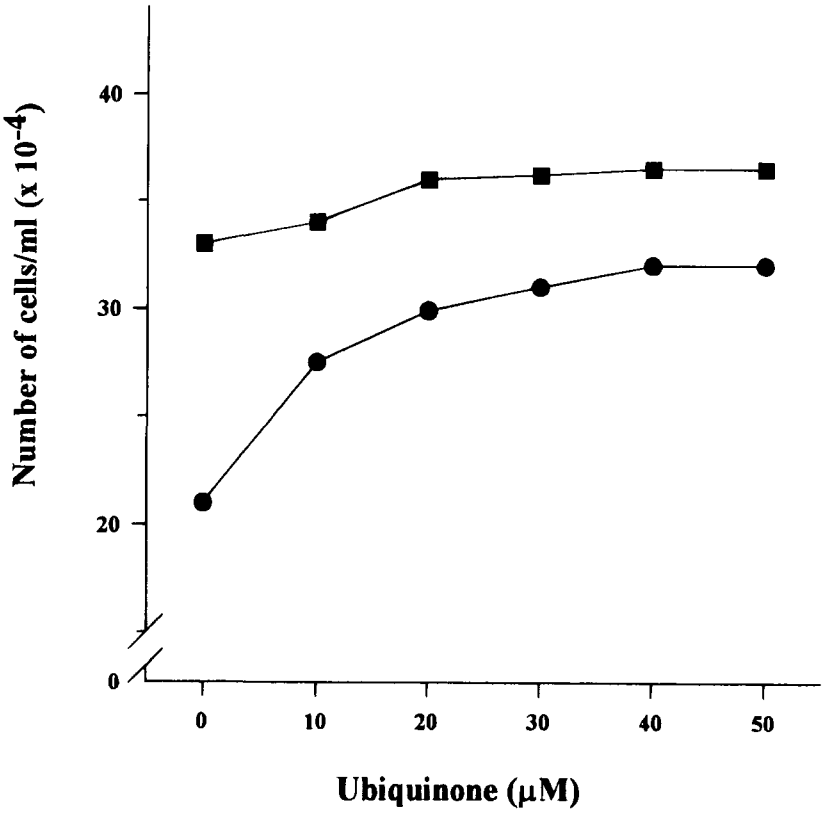

Fig. 3. Effect of externally added ubiquinone on the survival of HL-60 (circles) and ρ°HL-60 (squares) cells. Cells were seeded at a concentration of $37 \cdot 10^4$ cells/ml and grown for 48 h in the absence of FCS and different concentrations of ubiquinone. The numbers of surviving cells were plotted. $n = 3$. SD ≤ 12%.

with ubiquinone in the HL-60 cells, could explain the resistance to programmed cell death due to a decrease in the level of damage at the plasma membrane.

Our results also show that supplementation of HL-60 cells with ubiquinone prevented both apoptosis and lipid peroxidation caused by serum removal. Also, survival of these cells was clearly increased by the external addition of ubiquinone. These effects are more evident in HL-60 than the ρ°HL-60 cells, because the latter overexpressed ubiquinone at the plasma membrane under serum deficient conditions. The fibroblast-derived ρ°701.2a cell line is much more sensitive to serum withdrawal than the parental human fibroblast line GM701, and both cells are protected from apoptosis by the overexpression of Bcl-2 (Jacobson *et al.*, 1993). Bcl-2 can prevent apoptosis caused by multiple agents including withdrawal of growth factors, membrane peroxidation, and free radical-induced damage (Zhong *et al.*, 1993) by an antioxidant mechanism (Hockenbery *et al.*, 1993), acting downstream of apoptosis signaling during the effector phase of the cell death program (Vaux and Strasser, 1996). In fact, Bcl-2 could act either by blocking the activation of the ICE family of proteases or by preventing them from reaching their substrates (Vaux and Strasser, 1996). However, it is very interesting that Bcl-2 also blocks the accumulation of lipid peroxides in those membranes where this protein is located intracellularly as in mitochondria, endoplasmic reticulum, and nuclear envelope (Hockenbery *et al.*, 1993; Jacobson *et al.*, 1993; Monaghan *et al.*, 1992; Krajewski *et al.*, 1993; Lithgow *et al.*, 1994; de Jong *et al.*, 1994), but not in the plasma membrane. If the blockade of lipid peroxides is essential to prevent the development of the apoptotic program, and this is a basic function for the Bcl-2 family of proteins (Reed, 1994), a similar function could be assigned to ubiquinone and the other associated antioxidants at the plasma membrane to prevent lipid peroxide formation under stress conditions such as serum withdrawal (Beyer, 1994; Villalba *et al.*, 1996).

Stress caused by serum withdrawal induces sphingomyelin breakdown by a Mg^{2+}-dependent membrane neutral sphingomyelinase (Jayadev *et al.*, 1995), and

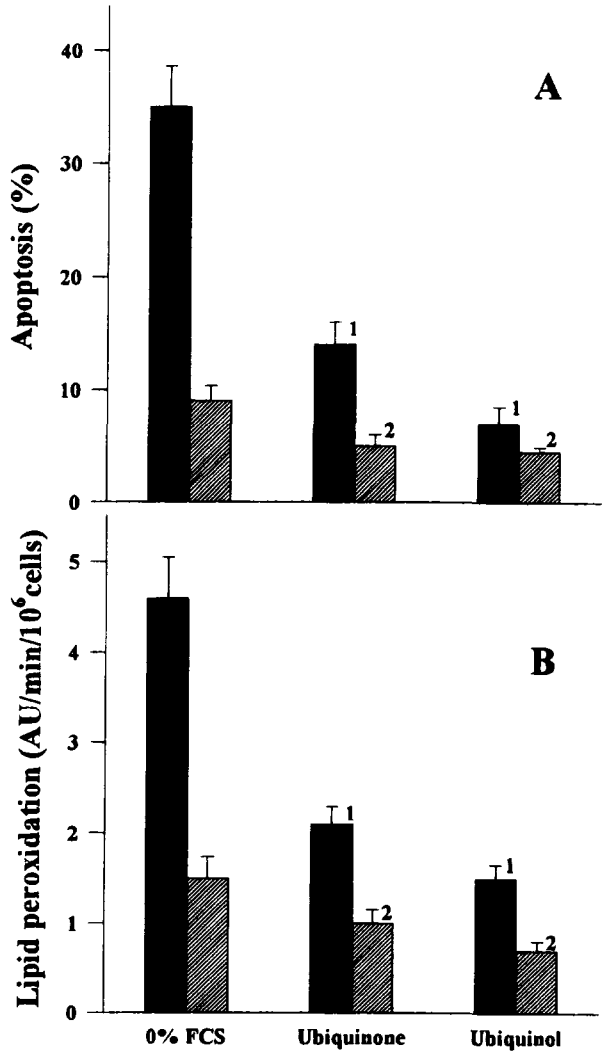

Fig. 4. Role of externally added ubiquinone on apoptosis and lipid peroxidation caused by serum withdrawal. HL-60 (closed bars) and ρ°HL-60 (dotted bars) cells were grown for 48 h in the absence of FCS or supplemented with 40 μM of ubiquinone or ubiquinol. Ubiquinone was freshly reduced to ubiquinol by adding a crystal of borohydride till the yellow color of ubiquinone solution disappears, and then the excess of borohydride is descarded. Cells were harvested and the number of apoptotic cells (A) and lipid peroxidation rates (B) were determined. $n = 4$. $^1p \leq 0.01$ vs 0% FCS, $^2p \leq 0.05$ vs 0% FCS.

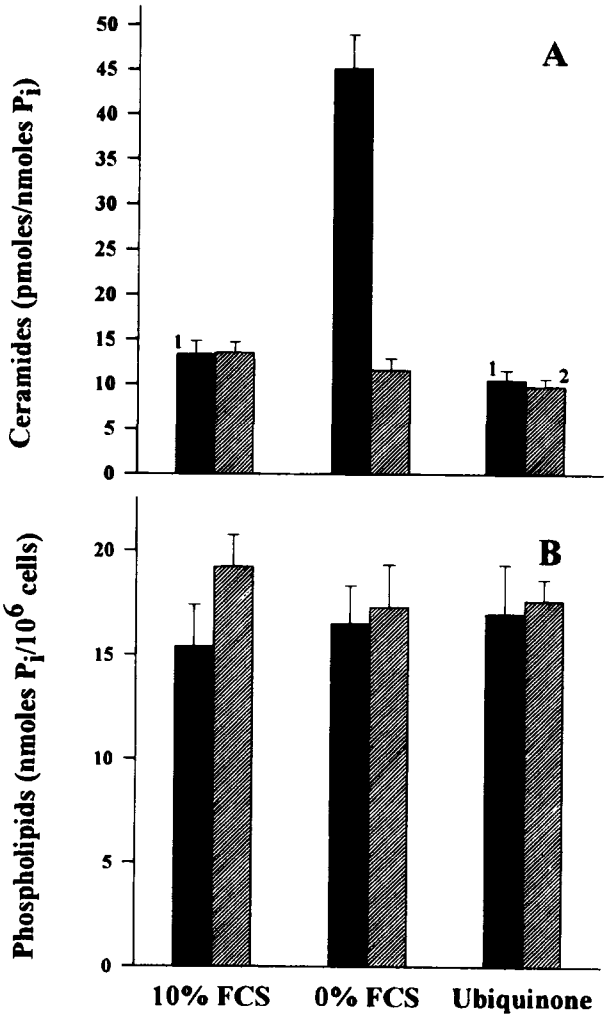

Fig. 5. Ceramide contents in HL-60 and ρ°HL-60 cells after serum withdrawal. Cells were grown for 48 h in the presence or absence of FCS. Some cultures growing without serum were also supplemented with 40 μM ubiquinone. The accumulation of ceramide was analyzed (A) and the total content of phospholipids was determined (B). $n = 3$. $^1p \leq 0.01$ vs 0% FCS, $^2p \leq 0.05$ vs 10% FCS.

the activation of this enzyme coincides with the long-term phase of ceramide accumulation, suggesting its participation in the control of the apoptotic pathway (Hannun, 1996). Ceramide activates proteins of the interleukin converting enzyme (ICE) family (Martin *et al.*, 1995; Smyth *et al.*, 1996; Mizushima *et al.*, 1996), but this activation and induction of apoptosis is inhibited by Bcl-2 (Zhang *et al.*, 1996), indicating that ceramide functions upstream of both proteases and Bcl-2. Thus, ceramide may relay a stress signal that is modulated downstream to develop apoptosis (Hannum, 1996). Removal of serum from culture media induced the accumulation of ceramide in HL-60 but not in ρ°HL-60 cells, without a significant change of total phospholipids. Similarly, serum-starved Molt-4 cells accumulated ceramide and also were induced to death (Jayadev *et al.*, 1995). The supplementation of culture media with ubiquinone prevented the accumulation of ceramide in HL-60. This effect was not apparent in ρ°HL-60 cells, which already displayed an overexpression of ubiquinone at the plasma membrane. Thus, it appears that the increase of antioxidant capacity of the plasma membrane by

ubiquinone can be responsible for the blockade of sphingomyelin breakdown caused by stress-induced damage.

Ubiquinone is a component of the antioxidant system of the plasma membrane that can be maintained in its reduced form by the cytochrome b_5 reductase (Villalba *et al.*, 1995; Navarro *et al.*, 1995), and can then maintain the reduced state of both α-tocopherol and ascorbate (Beyer, 1994, Gómez-Díaz *et al.*, 1997). This constitutes a plasma membrane-associated electron transport system able to protect from membrane damage caused by mild oxidative stress, where ubiquinone appears as the regulable central component (Sun *et al.*, 1992). The inhibition of this electron transport chain triggers apoptosis that is downstream prevented by Bcl-2 (Wolvetang *et al.*, 1996). Ubiquinone, as a central component of the electron transport at the plasma membrane, is an antioxidant agent that in parallel controls lipid peroxidation and ceramide accumulation caused by serum withdrawal and, as a consequence, prevents apoptosis. Plasma membrane would be then a site for the initiation of cell death signaling by ceramide. When the antioxidant system dependent on ubiquinone is exceeded, a downstream protection system such as the Bcl-2 family of proteins would be required.

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REFERENCES

Alcaín, F. J., Burón, M. I., Villalba, J. M., and Navas, P. (1991). *Biochim. Biophys. Acta* **1073**, 380–385.
Beyer, R. E. (1994). *J. Bioenerg. Biomembr.* **26**, 349–358.
de Jong, D., Prins, F. A., Maason, D. Y., Reed, J. C., van Ommen, G. B., and Kluin, P. M. (1994). *Cancer Res.* **54**, 256–260.
Desjardins, P., Frost, E., and Morais, R. (1985). *Mol. Cell. Biol.* **5**, 1163–1169.
Do, T. Q., Schultz, J. R., and Clarke, C. F. (1996). *Proc. Natl. Acad. Sci. USA* **93**, 7534–7539.
Gómez-Díaz, C., Rodríguez-Aguilera, J. C., Barroso, M. P., Villalba, J. M., Navarro, F., Crane, F. L., and Navas, P. (1997). *J. Bioenerg. Biomembr.* **29**, 251–257.
Greenlund, L. J. S., Beckwerth, T. L., and Johnson, E. M. J. (1995). *Neuron* **14**, 303–314.
Hannun, Y. A. (1996). *Science* **274**, 1855–1859.
Hockenbery, D. M., Oltvai, Z. N., Yin, X.-M., Milliman, C. L., and Korsmeyer, S. J. (1993). *Cell* **75**, 241–251.
Ishizaki, Y., Cheng, L., Mudge, A. W., and Raff, M. C. (1995). *Mol. Biol. Cell.* **6**, 1443–1458.
Jacobson, M. D., and Raff, M. C. (1995). *Nature* **374**, 814–816.
Jacobson, M. D., Burne, J. F., King, M. P., Miyashita, T., Reed, J. C., and Raff, M. C. (1993). *Nature* **361**, 365–369.
Jarvis, W. D., Kolesnick, R. N., Fornari, F. A., Traylor, R. S., Gewirtz, D. A., and Grant, S. (1994). *Proc. Natl. Acad. Sci. USA* **91**, 73–77.
Jayadev, S., Lin, B., Bielawska, A. E., Lee, J. Y., Nazaire, F., Pushkareva, M. Y., Obeid, L. M., and Hannun, Y. A. (1995). *J. Biol. Chem.* **270**, 2047–2052.
Kane, D. J., Sarafian, T. A., Anton, R., Hahn, H., Gralla, E. B., Valentine, J. S., Örd, T., and Bredesen, D. E. (1993). *Science* **262**, 1274–1277.
Krajewski, S., Tanaka, S., Takayama, S., Schibler, M. J., Fenton, W., Reed, J. C. (1993). *Cancer Res.* **53**, 4701–4714.
Larm, J. A., Vaillant, F., Linnane, A. W., and Lawen, A. (1994). *J. Biol. Chem.* **269**, 30097–30100.
Leibowitz, R. D. (1971). *J. Cell Biol.* **51**, 116–122.
Lithgow, T., van Driel, R., Bertram, J. F., and Strasser, A. (1994). *Cell Growth Diff.* **5**, 411–417.
Martin, S. J., Obrein, G. A., Nishioka, W. K. Mcgahon, A. J., Mahboubi, A., Saido, T. C., and Green, D. R. (1995). *J. Biol. Chem.* **270**, 6425–6428.
Marzuki, S., Noer, A. S., Lertrit, P., Uttanaphol, P., Thyagarajan, D., Kapsa, R., Sudoyo, H., and Byrne, E. (1991). In *Progress in Neuropathology* (Sato, T., and DiMauro, S., eds.), Raven Press, New York, Vol. 7, pp. 181–193.
Matsura, T., Yamada, K., and Kawasaki, T. (1995). *Redox Rep.* **1**, 343–347.
Mizushima, N., Koike, R., Kohsaka, H., Kushi, Y., Handa, S., Yagita, H., and Miyasaka, N. (1996). *FEBS Lett.* **395**, 267–271.
Monaghan, P., Robertson, D., Amos, A. S., Dyer, M. J. S., Mason, D. Y., and Greaves, M. F. (1992). *J. Histochem. Cytochem.* **40**, 1819–1825.
Muschel, R. J., Bernhard, E. J., Garza, L., Mackenna, W. C., and Koch, C. J. (1995). *Cancer Res.* **55**, 995–998.
Navarro, F., Villalba, J. M., Crane, F. L., Mackellar, W. C., and Navas, P. (1995). *Biochem. Biophys. Res. Commun.* **212**, 138–143.
Obeid, L. M., Linardic, C.-M., Karolak, L. A., and Hannun, Y. A. (1993). *Science* **259**, 1769–1771.
Preiss, J., Loomis, C. R., Bishop, W. R., Stein, R., Niedel, J. E., and Bell, R. M. (1986). *J. Biol. Chem.* **261**, 8597–8600.
Raff, M. C. (1992). *Nature.* **356**, 397–400.
Reed, J. C. (1994). *J. Cell Biol.* **124**, 1–6.
Sandstrom, P. A., Tebbey, P. W., Van Cleave, S., and Buttke, T. M. (1994). *J. Biol. Chem.* **269**, 798–801.
Schulze-Osthoff, K., Kramer, P. H., and Döge, W. (1994). *EMBO J.* **13**, 4587–4596.
Shimizu, S., Eguchi, Y., Kosaka, H., Kamiike, W., Matsuda, H., and Tsujimoto, Y. (1995). *Nature* **374**, 811–813.
Slater, A. F. G., Stefan, C., Nobel, I., van den Dobbelsteen, D. J., and Orrenius, S. (1996). *Cell. Death Diff.* **3**, 57–62.
Smyth, M. J., Perry, D. K., Zhang, J. D., Poirier, G. G., Hannun, Y. A., and Obeid, L. M. (1996). *Biochem. J.* **316**, 25–28.
Storrie, B., and Madden, E. A. (1990). *Methods Enzymol.* **182**, 203–225.
Sun, I. L., Sun, E. E., Crane, F. L., Morré, D. J., Lindgren, A., and Löw, H. (1992). *Proc. Natl. Acad. Sci. USA* **89**, 11126–111130.
Sun, J. L., Sun, E. E., and Crane, F. L. (1995). *Protoplasma.* **184**, 214–219.

Tilly, J. L. and Tilly, K. I. (1995). *Endocrinology* **136**, 242–252.
Vaux, D. L., and Strasser, A. (1996). *Proc. Natl. Acad. Sci. USA* **92**, 8443–8447.
Villalba, J. M., Navarro, F., Córdoba, F., Serrano, A., Arroyo, A., Crane, F. L., and Navas, P. (1995). *Proc. Natl. Acad, Sci. USA.* **92**, 4887–4891.
Villalba, J. M., Córdoba, F., and Navas, P. (1996). In *Subcellular Biochemistry* (Harris, J. R., ed.), Plenum Press, New York, Vol. 25, pp. 57–81.
Weil, M., Jacobson, M. D., Coles, H. S. R., Davies, T. J., Gardner, R. L., Raff, K. D., and Raff, M. C. (1996). *J. Cell. Biol.* **133**, 1053–1059.
Wolvetang, E. J., Larm, J. A., Montsoulas, P., and Lawen, A. (1996). *Cell Growth Diff.* **7**, 1315–1325.
Zhang, J., Alter, N., Reed, J. C., Borner, C., Obeid, L. M., and Hanum, Y. A. (1996). *Proc. Natl. Acad. Sci. USA* **93**, 5325–5328.
Zhong, L. -T., Sarafian, T., Kane, D. J., Charles, A. C., Mah, S. P., Edwards, R. H., and Bredesen, D. E. (1993). *Proc. Natl. Acad. Sci. USA* **90**, 4533–4537.