ANTIOXIDANTS & REDOX SIGNALING
Volume 2, Number 2, 2000
Mary Ann Liebert, Inc.

Forum Original Research Communication

Coenzyme Q Protects Cells Against Serum Withdrawal-Induced Apoptosis by Inhibition of Ceramide Release and Caspase-3 Activation

DANIEL J.M. FERNÁNDEZ-AYALA,[1,3] SERGIO F. MARTÍN,[2,3] MARÍA P. BARROSO,[2] CONSUELO GÓMEZ-DÍAZ,[1] JOSÉ M. VILLALBA,[2] JUAN C. RODRÍGUEZ-AGUILERA,[1] GUILLERMO LÓPEZ-LLUCH,[1] and PLÁCIDO NAVAS[1]

ABSTRACT

Coenzyme Q_{10} (CoQ_{10}) is a component of the antioxidant machinery that protects cell membranes from oxidative damage and decreases apoptosis in leukemic cells cultured in serum-depleted media. Serum deprivation induced apoptosis in CEM-C7H2 (CEM) and to a lesser extent in CEM-9F3, a subline overexpressing Bcl-2. Addition of CoQ_{10} to serum-free media decreased apoptosis in both cell lines. Serum withdrawal induced an early increase of neutral-sphingomyelinase activity, release of ceramide, and activation of caspase-3 in both cell lines, but this effect was more pronounced in CEM cells. CoQ_{10} prevented activation of this cascade of events. Lipids extracted from serum-depleted cultures activated caspase-3 independently of the presence of mitochondria in cell-free *in vitro* assays. Activation of caspase-3 by lipid extracts or ceramide was prevented by okadaic acid, indicating the implication of a phosphatase in this process. Our results support the hypothesis that plasma membrane CoQ_{10} regulate the initiation phase of serum withdrawal-induced apoptosis by preventing oxidative damage and thus avoiding activation of downstream effectors as neutral-sphingomyelinase and subsequent ceramide release and caspase activation pathways. Antiox. Redox Signal. 2, 263–275.

INTRODUCTION

THE PLASMA MEMBRANE of eukaryotic cells contains diverse electron transport mechanisms involved in redox regulations related to growth control and development (Crane *et al.*, 1985). Among these systems, a basic one dependent on NADH integrates three antioxidants: ascorbate, α-tocopherol and coenzyme Q (CoQ), which are very effective in protection of the plasma membrane against oxidative stress (Beyer, 1994; Frei, 1994). In this system, CoQ acts as a key component because it is the only lipophilic antioxidant synthesized in humans and it maintains both α-tocopherol and ascorbate in their reduced state (Beyer, 1994; Villalba *et al.*, 1995). The reduced form of CoQ (ubiquinol) can be in turn regenerated by the NADH-cytochrome b_5-reductase flavoenzyme (Navarro *et al.*, 1995).

Serum withdrawal causes cell cycle arrest and apoptosis in several cell types (Ishizaki *et al.*, 1995). Serum provides survival signals for cells and its depletion activates an intrinsic cell

[1] Laboratorio Andaluz de Biología, Universidad Pablo de Olavide, 41013 Sevilla, Spain.
[2] Departamento de Biología Celular, Facultad de Ciencias, Universidad de Córdoba, 14004 Córdoba, Spain.
[3] These authors contributed equally to this work and should be considered as first authors.

death program (Raff, 1992; Raff *et al.*, 1993) whose machinery is constitutively expressed (Weil *et al.*, 1996). There is evidence for an indirect generation of oxidative stress during development of apoptosis caused by growth factors withdrawal (Slater *et al.*, 1996) because some endogenous antioxidant enzymes inhibit this process (Greelund *et al.*, 1995; Tilly and Tilly, 1995). Also, antioxidant molecules such as ascorbate, α-tocopherol, and CoQ can prevent apoptosis induced by deficiency of growth factors (Tilly and Tilly, 1995; Barroso *et al.*, 1997a,b). Furthermore, cell growth of immortalized cells cultured in absence of serum is stimulated by CoQ (Sun *et al.*, 1995), which also maintains the growth in mitochondrial respiratory chain-deficient cells (Martinus *et al.*, 1993; Barroso *et al.*, 1997b), indicating that these effects are independent of the mitochondrial function of the quinone. On the other hand, antagonists of CoQ trigger apoptosis upstream the antiapoptotic protein Bcl-2 (Wolvetang *et al.*, 1996; Macho *et al.*, 1998, 1999). Also, Bcl-2 itself has been reported to inhibit apoptosis, at least in part, by an antioxidant mechanism (Hockenbery *et al.*, 1993; Kane *et al.*, 1993; Bruce-Keller *et al.*, 1998).

Ceramide is released following stimulation of cells with several agents that induce differentiation and apoptosis such as 1α,25-dihydroxyvitamin D_3 (Okazaki *et al.*, 1989; 1990), tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) (Kim *et al.*, 1991), and interleukin-1β (IL-1β) (Ballou *et al.*, 1992). Ceramide acts as a mediator of stress responses in cells causing cell cycle arrest or apoptosis (Hannun, 1996), and modulating protein kinases, nuclear factors, enzymes of the eicosanoids pathway, and gene expression (Kim *et al.*, 1991; Ballou *et al.*, 1992; Raines *et al.*, 1993; Hannun *et al.*, 1994). Serum deprivation induces a progressive increase of ceramide levels in Molt-4 (Jayadev *et al.*, 1995) and HL-60 (Barroso *et al.*, 1997b) cells. Ceramide is then able to induce cell death after its intracellular accumulation (Obeid *et al.*, 1993; Jarvis *et al.*, 1994; Jayadev *et al.*, 1995; Barroso *et al.*, 1997b), by activating proteases of the interleukin-converting enzyme (ICE) family such as caspase-3 (Martin *et al.*, 1995b; Mizushima *et al.*, 1996; Smyth *et al.*, 1996). Thus, the increase of ceramide levels appears as an essential component in the stress response pathway triggered by serum withdrawal (Hannun, 1996).

We show here that CoQ_{10} prevents apoptosis induced by serum withdrawal, and decreases early neutral-sphingomyelinase (N-SMase) activity, transient short-term ceramide accumulation, and caspase-3 activation. This prevention was independent on the content of Bcl-2 and related to the concentration of CoQ at the plasma membrane (Barroso *et al.*, 1997a, b). We consider that CoQ, as central component of the plasma membrane antioxidant system, could break the lipid peroxidation chain reaction induced by serum withdrawal in this membrane as an initiation mechanism of apoptosis, acting upstream of intracellular regulators such as Bcl-2. In addition, our results support that ceramide activates caspase-3 via a cytosolic protein phosphatase, without participation of mitochondria.

MATERIALS AND METHODS

Cell lines and cultures

The human T-acute lymphatic leukemic cell line CEM-C7H2 (CEM), a subline of CCRF-CEM, and the cell line CEM-C7H2 expressing transgenic *bcl-2* (CEM-9F3) (Geley *et al.*, 1997; Susin *et al.*, 1997a), were provided by Dr. E. Muñoz (Dep. Inmunología, Facultad de Medicina, Córdoba, Spain). Cells were grown in 5% CO_2 atmosphere at 37°C in RPMI-1640 medium (Sigma, Spain) supplemented with 10% fetal calf serum (FCS) (PAA Labor, Austria), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 m*M* glutamine (Sigma, Spain). Transfectants were selected by supplementing the culture medium with 200 μg/ml hygromycin and 200 μg/ml G418 (Sigma, Spain). For serum withdrawal experiments, cells were previously washed twice in serum-free RPMI-1640 medium and then seeded in tissue culture flasks in serum-free RPMI-1640 medium. CoQ_{10} (Sigma, Spain) was dissolved in pure ethanol and added to serum-free culture media. Although no effect attributable to the vehicle was observed, 0.3% ethanol was added to control cultures.

Apoptosis determination

Cells were cultured in 10% FCS-supplemented or in serum-free media in the presence or absence of 30 μM CoQ_{10}. Cells were harvested at indicated times of incubation, and washed twice with ice-cold phosphate-buffered saline (PBS). Next, cells were fixed in 70% ethanol for at least 24 hr at 4°C, washed with Hank's balanced salt solution (HBSS) and then incubated for 30 min at 37°C with propidium iodide (PI) (Sigma, Spain) staining buffer [PBS, pH 7.5, 0.1 mM EDTA, 0.1% Triton X-100, 50 U/ml DNAse free-RNase A (Sigma, Spain), and 50 μg/ml PI]. Cells were collected in a FACScan (Becton-Dickinson, USA) and data analyzed using Lysis II software (Becton-Dickinson, USA).

Ceramide quantification

Ceramide production was determined as previously described (Jayadev *et al.*, 1995). Briefly, cells were harvested at indicated times and washed twice with PBS. Lipids were then extracted by the method of Bligh and Dyer (1959). After drying under nitrogen, lipids were resuspended in 100 μl of chloroform. Twenty-five microliters were used for phosphate measurements and 25 μl for the diacylglycerol kinase assay (Preiss *et al.*, 1986). For ceramide determination, lipids were dried and dissolved in 20 μl of 1 mM diethylenetriamine pentaacetic acid, 7.5% octyl-β-D-glucopyranoside, and 5 mM cardiolipin (Sigma, Spain). After sonication for 15 sec, 50 μl of 2× reaction buffer (100 mM imidazole-HCl, pH 6.6, 100 mM NaCl, 25 mM $MgCl_2$, and 2 mM EGTA), DTT to a final concentration of 50 μM, 5 μg diacylglycerol-kinase (Sigma, Spain), and distilled water were added to a final volume of 90 μl. Reaction was started by the addition of a mixture of cold ATP and [γ-^{32}P]ATP (specific radiactivity 100.000–500.000 cpm/nmol) (Amersham Iberica, Spain). After incubation for 20 min at 25°C, lipids were extracted with chloroform as indicated (Okazaki *et al.*, 1989). Five hundred microliters of the chloroform phase were dried with N_2 gas and dissolved in 100 μl of 5% methanol in chloroform. Ceramide-1-phosphate generated (25 μl) was resolved by thin-layer chromatography (TLC) using chloroform/acetone/methanol/acetic acid/water (10:4:3:2:1) as solvent. Ceramide-phosphate spots were quantified using an automatic TLC-lineal analyzer (Berthold, Germany). Ceramide was quantified using external standards and normalised to phosphate as indicator of total phospholipids.

Assay for N-SMase

N-SMase was assayed by the method of Okazaki *et al.* (1994) with minor modifications. Cells were placed in the presence or absence of serum plus additives and maintained in culture. At indicated times, cells were harvested and washed twice in cold PBS. The cellular pellet was suspended in lysis buffer (25 mM Tris-HCl, pH 7.4, 5 mM EDTA, 0.05 % Triton X-100, 5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 2 μl/ml CLAP (5 mg/ml each, chymostatin, leupeptin, antipain, pepstatin A) (Sigma, Spain) and cells disrupted by vortex. Lysates were centrifuged at 3,000 × g for 5 min at 4°C and supernatants containing solubilized enzymes (50–100 μg of protein) were mixed with assay buffer for N-SMase (50 mM Tris-HCl, pH 7.4, 0.5% Triton X-100, 5 mM DTT, and 10 mM $MgCl_2$) plus 10 nmol of a mixture of cold sphingomyelin (SM) and [*methyl*-^{14}C]SM (specific radioactivity, 10,000 cpm/nmol) (Amersham Iberica, Spain). After incubation for 30–60 min at 37°C, the reaction was stopped by adding 1.5 ml of chloroform/methanol (2:1) and 200 μl of distilled water. Tubes were vortexed and then centrifuged at 1,500 × g for 5 min to separate two phases. [^{14}C]Phosphocholine present in the aqueous phase was quantified using a liquid scintillation counter (Beckman, USA). SMase activity was expressed as nmol SM hydrolyzed/mg of protein per hour.

Preparation of cell lysates and caspase activity determination

Cell lysates were prepared as described (Enari *et al.*, 1995). Briefly, cells were washed twice in cold PBS and resuspended in extraction buffer (50 mM PIPES, pH 7.4, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM DTT, 20 μM

cytochalasin B, 1 m*M* PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 50 μg/ml antipain, and 10 μg/ml chymopapain; Sigma, Spain). Cells were disrupted by five cycles of freezing and thawing. Crude fractions were obtained from cell homogenates by removal of cell debris, nuclei, and heavy membranes (as mitochondria) by centrifugation at 13,000 × *g* for 15 min at 4°C. In some cases, a further clarification of the crude fraction was performed by centrifugation at 50,000 × *g* for 30 min in a ultracentrifuge TLX-100 (Beckman, USA) to separate light membranes (pellet) and cytosolic fractions (supernatant).

Activities of ICE and caspase-3 caspases were assayed in cell extracts by a colorimetric method using Ac-YVAD-pNa and Ac-DEVD-pNa (Bachem, Switzerland), respectively, as substrates. Inhibitors for each caspase, Ac-YVAD-CHO and Ac-DEVD-CHO, (Bachem, Switzerland), respectively, were used to determine the specificity of the reaction (García-Calvo *et al.*, 1998). Fifty micrograms of protein were diluted in assay buffer (100 m*M* HEPES-KOH buffer, pH 7.5, 10% sucrose, 0.1% CHAPS, 0.1 mg/ml ovoalbumin, containing either, 5 m*M* DTT for ICE assay or 10 m*M* DTT per caspase-3 assay). Activity was developed in 96-well plates at 37°C for 30–60 min, and pNa release was determined spectrometricaly at 405 nm using a multiwell reader. Activity was expressed in units (1 unit = 1 pmol pNa released/30 min per mg of protein) as determined previously (Enari *et al.*, 1996).

In vitro *activation of caspase-3 by ceramide*

Lipids were extracted from 6 × 10^6 cells cultured in the absence or presence of serum plus or minus additives as described (Bligh and Dyer, 1959). Samples from these extracts were mixed with caspase reaction buffer and incubated for 30–60 min with crude or cytosolic fractions obtained from the same number of cells cultured in RPMI-1640 supplemented with 10% FCS. Caspase-3 activity was determined as described above.

Cytochrome c *oxidase activity*

The mitochondrial marker cytochrome *c* (cyt *c*) oxidase was assayed as described (Storrie and Madden, 1990) to control the presence of this organelle during subcellular fractionation. The same amount of protein from each sample was added to 40 m*M* potassium phosphate buffer, pH 6.2, containing 0.27 mg/ml reduced cyt *c* (Sigma, Spain) and 0.2% Lubrol PX (Sigma, Spain) to permeabilize mitochondria. The decrease in O.D. at 550 nm due to oxidation of the cyt *c* was followed for 3 min. Specific activity was expressed as nmol cyt *c* oxidized/mg of protein per min.

Statistical analysis

Presented data represent the mean ± SD from three or four different experiments. Significant differences were determined using Student's *t*-test.

RESULTS

Prevention of apoptosis in serum-deprived cultures by CoQ_{10}

CEM cells contain a moderate amount of Bcl-2, and CEM-9F3 cells, a transgenic *bcl-2* CEM-derived cell line, overexpress this protein (Geley *et al.*, 1997; Susin *et al.*, 1997a). Apoptotic cells accounted about 5% of total cells in 10% FCS cultures of both lines through all times of incubation (Fig. 1). The absence of serum induced apoptosis in both cell lines, with the number of apoptotic cells being increased in serum-depleted cells through time of incuba-

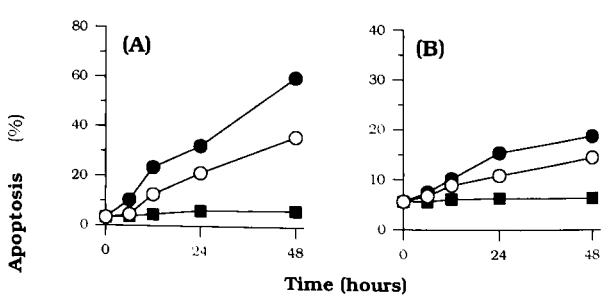

FIG. 1. Time course of apoptosis induction by serum withdrawal in CEM (A) and CEM-9F3 (B) cells. Cells were plated in tissue culture flasks at 5 × 10^5 cells/ml in 10% FCS-supplemented medium or serum-free medium in the absence or presence of 30 μ*M* CoQ_{10}. (●) 0% FCS; (○) 0% FCS plus 30 μ*M* CoQ_{10} (n = 5) (SD ≤ 8%).

tion. CEM cells were more sensitive to serum withdrawal than CEM-9F3 cells, as determined by the presence of sub-$G_0/1$ cells by flow cytometry in such a way that about 60% of CEM cells were apoptotic after 48 hr of incubation whereas only 20% of CEM-9F3 population showed apoptotic nuclei (Fig. 1). In both cases, the presence of 30 μM CoQ_{10} in serum-free culture media decreased apoptosis (Fig. 1). This concentration was chosen as the more effective in the protection of HL-60 cells in previous experiments (Barroso *et al.*, 1997b). The number of cells showing apoptotic nuclei after 48 hr of incubation in CoQ_{10}-supplemented serum-free culture media decreased to 35% in CEM and to 11% in CEM-9F3 cells (Fig. 1). Thus, addition of CoQ_{10} resulted in a 40% protection for both cells through the time of experiments. These results were confirmed by the trypan blue dye exclusion test for cell viability (data not shown). In addition to the higher expression of Bcl-2 in CEM-9F3, plasma membranes from these cells also contained more CoQ_{10} under serum-withdrawal conditions without CoQ_{10} supplementation (about 100 pmol CoQ_{10}/mg protein in CEM-9F3 versus 70 pmoles CoQ_{10}/mg protein in CEM cells), which may also contribute to the different sensitivity of both cell lines to serum depletion.

Changes in ceramide levels in CEM and CEM-9F3 cells after serum deprivation

Short-term accumulation of ceramide was determined in both CEM and CEM-9F3 cells cultured in the absence of serum with or without 30 μM CoQ_{10}. In both cell lines, ceramide levels increased after 10 min in culture medium without serum, reaching a peak at about 30 min of incubation (Fig. 2). In CEM cells, a two-fold increase in ceramide was observed, and this was sustained for at least 60 min. In CEM-9F3 cells, however, the increase was only of about 50% and ceramide levels returned to initial levels after 60 min of incubation. When cells were cultured in serum-free media but supplemented with 30 μM CoQ_{10}, the behavior of both lines was quite similar in terms of ceramide accumulation. As shown in Fig. 2, a small increase in ceramide levels was found at 20 min both in CEM and CEM-9F3 cells cultured in serum-free, CoQ_{10}-supplemented media and then decreased to initial levels at 40 min of incubation. The maximal inhibition in ceramide accumulation due to the presence of CoQ_{10} was observed at about 30 min of incubation, and estimated to about 80% in both cell lines. Cultures with 10% FCS did not show any change in ceramide content through incubation (data not shown).

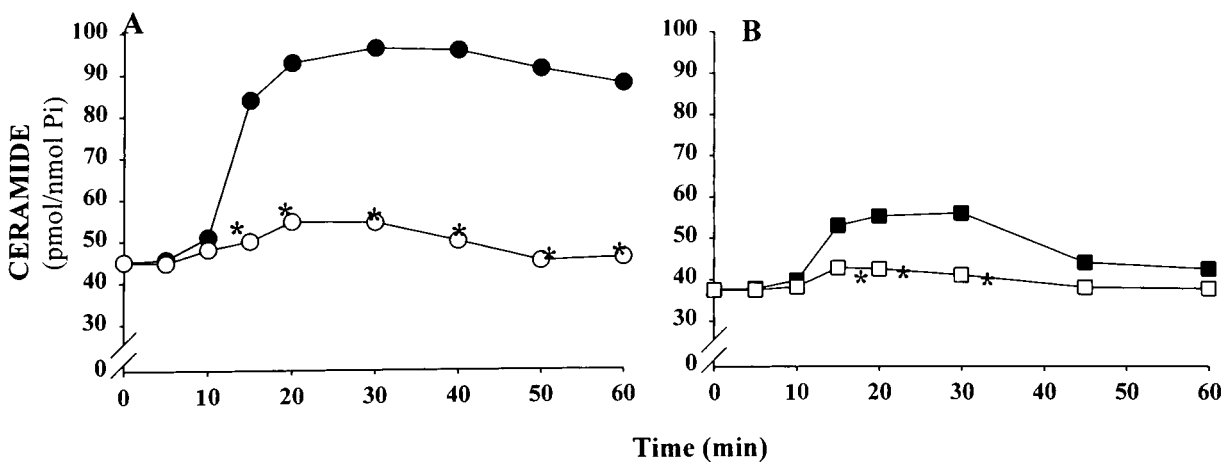

FIG. 2. Ceramide levels in serum-free cultures. Cells were cultured in serum-free media in the absence (●) or presence (○) of 30 μM CoQ_{10} and plated as described in Fig. 1. After the indicated times of incubation, cells were harvested and ceramide levels were determined by TLC. Ceramide levels in CEM cells (**A**) and CEM-9F3 cells (**B**). Data represent the mean from three different experiments performed in duplicate. (*) Significant differences versus serum-free cultures in the absence of CoQ_{10}, $p \leq]0.05$. SD was less than 10% in each point.

SMase activity in serum-deprived cells

Two enzymatic activities have been related to ceramide release in apoptotic events, N-SMase and acidic SMase (A-SMase) (Jayadev *et al.*, 1995; Monney *et al.*, 1998). The time course of these activities was followed in CEM and CEM-9F3 lines after serum withdrawal in the absence or presence of 30 μM CoQ_{10}, but no significant modification of A-SMase activity was found along our experiments (data not shown). N-SMase in 10% FCS-supplemented media did not show significant modifications along incubation times, although basal activity of N-SMase was about 10-fold higher in CEM-9F3 cells than in CEM cells (Fig. 3A). When cells were placed in serum-free medium, N-SMase was activated, and a three- to four-fold increase was observed in CEM cells after 3 hr of culturing without serum, whereas less increase was found in CEM-9F3 cells during the same period of time (Fig. 3B, C). As stated above for ceramide, behavior for both cell lines in terms of N-SMase activation during time was quite similar when 30 μM CoQ_{10} was added to serum-free medium. In this case, CoQ_{10} prevented very efficiently the increase in N-SMase of CEM cells after 1 hr of culture (Fig. 3B), but no significant differences between CoQ_{10}-supplemented and nonsupplemented media were obtained for CEM-9F3 at all times tested (Fig. 3C).

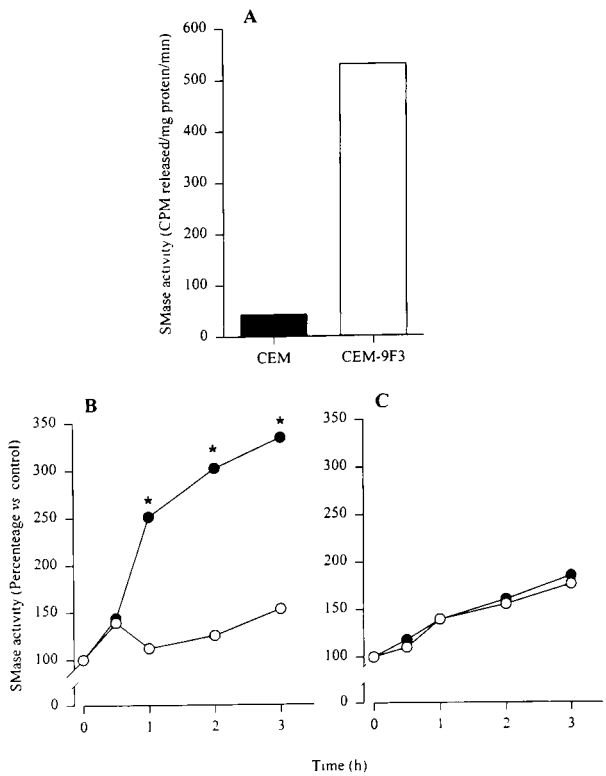

FIG. 3. Neutral SMase activity in serum-depleted cell cultures. Cells were harvested at indicated times and SMase activities determined. Basal activities of CEM and CEM-9F3 cells cultures in 10% FCS supplemented media **(A)**; CEM **(B)** and CEM-9F3 **(C)** activities in cells grown serum-free media in the absence (closed symbols) or the presence (open symbols) of 30 μM CoQ_{10}. Activity of 10% FCS-supplemented cultures did not change significantly along incubation. Data represent the activity of SMase after indicated times of incubation referred to 10% FCS cultured cells taken as control (100%). Data represent the mean from three different experiments performed in duplicate. (*) Significant differences versus serum-free cultures in the absence of CoQ_{10}, $p \leq 0.05$. SD was less than 10% in each point.

Activation of caspase-3 in serum-deprived cells

Caspases such as ICE and caspase-3 are cysteinyl-proteases directly related to apoptosis. We did not find any significant modification on ICE activity along treatments, using the chromogenic substrate Ac-YVAD-pNa (data not shown). However, using the substrate Ac-DEVD-pNa, we found a transient increase of caspase-3 caspase activity in both CEM and in CEM-9F3 cells (Fig. 4). Activity of caspase-3 was always higher in CEM than in CEM-9F3 cells and these cell lines showed different patterns of activation during time. In CEM cells, the activity of caspase-3 increased gradually to reach a peak at about 120 min of culture without serum. At this moment, a four-fold increase over initial levels was observed (Fig. 4A). In CEM-9F3 cells, however, a two-fold increase of caspase-3 was observed at 30–60 min and then activity returned to initial values after 120 min (Fig. 4B). In both cell lines, the presence of CoQ_{10} in serum-free cultures induced a 50% significant inhibition of caspase-3 activity (at 120 min in CEM cells, and at 30–60 min in CEM-9F3 cells).

The specificity of the reaction was verified by using the inhibitor Ac-DEVD-CHO, which completely abolished the activity of caspase-3 in all cases (Fig. 4A, B).

Effect of C_2-ceramide in caspase-3 activity in serum-deprived cells

To determine if ceramide was involved in caspase-3 activation, we studied the effect of

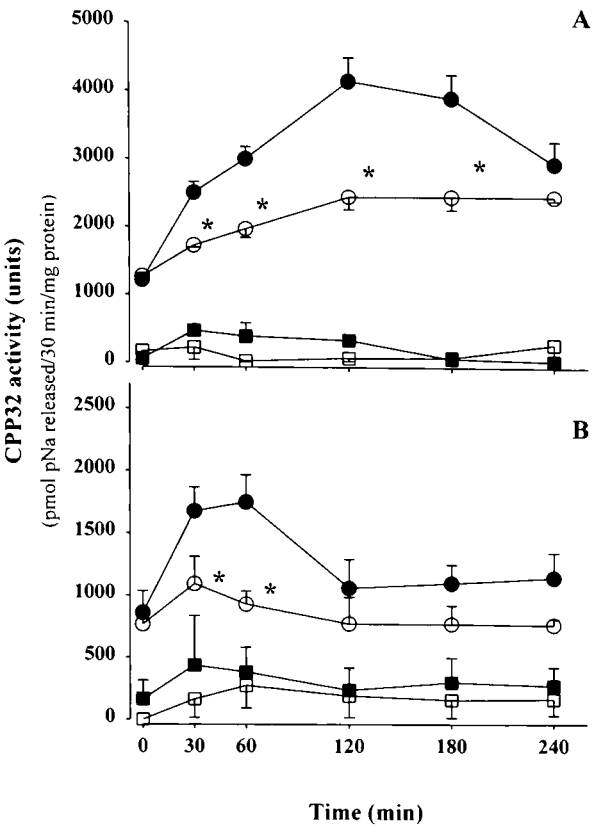

FIG. 4. Caspase-3 activity in serum-deprived cells. Caspase-3 activity (circles) was determined in cell extracts from CEM (**A**) and CEM-9F3 (**B**) cells grown in serum-free media in the absence (closed symbols) or the presence (open symbols) of 30 μM CoQ_{10}. The specific inhibitor of caspase-3, Ac-DEVD-CHO, was added at 1 μM final concentration (squares) in the assay. Data represent the mean ± SD from three different experiments performed in triplicate. (*) Significant differences versus serum-free cultures in the absence of CoQ_{10}, $p \leq 0.05$.

the cell-permeable analogue C_2-ceramide in CEM and CEM-9F3 cells cultured in 10% FCS-supplemented, or in serum-free media either in the presence or absence of 30 μM CoQ_{10}. C_2-ceramide (10 μM) increased about threefold the activity of caspase-3 in both cell lines growing in 10% FCS (Fig. 5A). When cultures were carried out with serum-free medium, caspase activity was not further increased by the addition of C_2-ceramide to CEM cells, whereas in CEM-9F3 cells, which showed less activation of caspase-3 induced by serum withdrawal, a 50% increase was observed after the addition of C_2-ceramide (Fig. 5B). The addition of C_2-ceramide to cells growing in serum-deprived cultures but supplemented with 30 μM CoQ_{10} induced a significant increase of caspase-3 activity in both cell lines, following a pattern similar to that observed in cells grown in medium containing 10% FCS (Fig. 5C).

Caspase-3 is activated in vitro *by lipid extracts from serum-free cultures*

If ceramide released in cells after culturing in serum-free medium was involved in caspase-3 activation, then a lipid fraction obtained from these cells should mimic the effect of C_2-ceramide as a caspase activator. For these experiments, we obtained a lipid extract from equal amounts of cells grown in the presence or absence of serum, or in serum-free cultures supplemented with 30 μM CoQ_{10}. Then, we tested whether or not any of these extracts was able to activate caspase-3 *in vitro* in crude fractions obtained from CEM cells grown in 10% FCS. We also included 50 μM C_2-ceramide as a positive control. As shown in Table 1, lipid extracts from cells grown either in 10% FCS or in serum-deficient CoQ_{10}-supplemented medium did not activate caspase-3 significantly. However, lipid extracts obtained from CEM cells cultured for 30 min in the absence of serum induced a significant activation of the caspase caspase-3 in crude extracts of CEM cells, as it was also found for C_2-ceramide. To check if added CoQ had a direct *in vitro* effect on the caspase-3 activity, CoQ was added to the control assay and no significant differences were observed (Table 1). Assays were specific for caspase-3, as including the inhibitor Ac-DEVD-CHO into the assay prevented all activations.

Mitochondria have been related to ceramide effect on caspase-3 activation (Susin *et al.*, 1997b) and could be responsible for the activation observed in this work. Because the presence of mitochondria in our crude extracts (13,000 × *g* supernatants) was not expected, we also studied the activation of caspase-3 in cytosolic fractions (50,000 × *g* supernatants) from 10% FCS-grown CEM cells, which contained only 5% of the mitochondrial marker cytochrome *c* oxidase (0.13 ± 0.01 nmol cytochrome *c* oxidized/min per mg protein) relative to cell homogenates (2.5 ± 0.8 nmoles/min per mg protein). As found with crude extracts (Table 1), both 0% FCS-derived lipid extracts and C_2-ceramide activated the caspase in these frac-

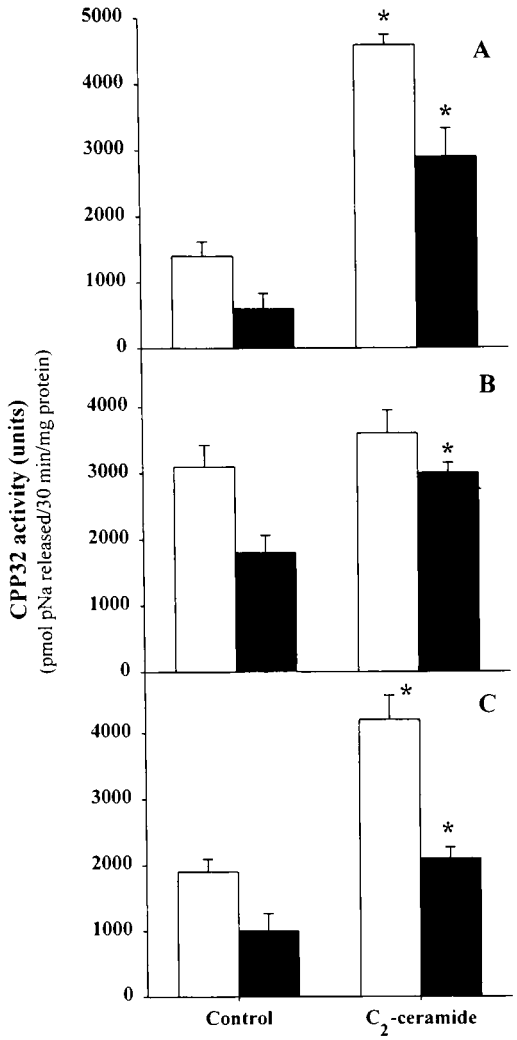

FIG. 5. C_2-ceramide effect on caspase-3 activity in CEM and CEM-9F3 cells. CEM cells (white bars) and CEM-9F3 cells (black bars) were cultured in 10% FCS medium or serum-free media with or without 30 μM CoQ_{10}, in the presence or absence of 10 μM C_2-ceramide for 2 hr, homogenized and caspase-3 activity determined. (**A**) Caspase-3 activity in 10% FCS supplemented cultures. (**B**) Caspase-3 activity in serum-free cultures without CoQ_{10} addition. (**C**) Caspase-3 activity in serum-free cultures supplemented with 30 μM CoQ_{10}. Data represent the mean ± SD from three different experiments performed in duplicate. (*) Significant differences versus respective control levels, $p \leq 0.05$.

tions. Specificity of the activation was also verified with the Ac-DEVD-CHO inhibitor (data not shown).

Ceramide-dependent activation of caspase-3 is inhibited by okadaic acid

Our results indicated that ceramide activate caspase-3 caspase without the participation of mitochondria. Some authors have demonstrated that okadaic acid (OA) interferes in the ceramide-dependent apoptotic pathway inhibiting the ceramide-activated protein phosphatase (CAPP) (Dobrowsky and Hannun, 1992). Thus, we studied the effect of OA in our *in vitro* experiments and found that activation of caspases achieved by treatment of crude fractions of CEM cells were activated by 50 μM C_2-ceramide or 0% FCS-derived cellular lipid extracts in as described above, and this activation was completely prevented by 1 nM okadaic acid (Table 2).

DISCUSSION

The aim of the present work was to determine how CoQ prevents apoptosis in serum-deprived cultures of CEM and CEM-9F3 cells. CoQ inhibited serum withdrawal-induced SMase activation and ceramide release and subsequent caspase-3 activation in both cells. The two cell lines showed a different apoptotic response to serum withdrawal, which may be related to their different Bcl-2 expression levels, and CoQ reduced apoptosis in both. Thus, protection by CoQ added to the medium was independent of Bcl-2, in agreement with our previous published results (Barroso *et al.*, 1997a).

Data from the literature suggest that increased SMase activity and ceramide production are important for the induction of apoptosis by several agents, including oxidative injury (Okazaki *et al.*, 1989, 1990; Kim *et al.*, 1991; Jayadev *et al.*, 1995; Hannun, 1996). Ceramide accumulation induces cell death by activation of proteases such as caspase-3 or related caspases (Martin *et al.*, 1995b; Mizushima *et al.*, 1996; Smyth *et al.*, 1996). In our study, caspase-3 (or a related caspase) was activated in both cell lines at the time ceramide was accumulating. The addition of C_2-ceramide to cells increased caspase-3 activity to the same level in both cultures grown with 10% FCS and in CoQ-supplemented serum-free cultures, but it was unable to increase caspase activity further in those cells that already showed the highest levels of activation, such as those grown in serum-free media without CoQ. These results suggest

TABLE 1. EFFECT OF CELL LIPID EXTRACTS AND C_2-CERAMIDE ON CASPASE-3 ACTIVATION *IN VITRO*[a]

Additions	*Caspase-3 activity (pmol pNa/mg protein per 30 min)*		
	− Inhibitor	*+ Inhibitor*	*Activation (%)*
None (ethanol)	1,064 ± 370	254 ± 160	100
+ CoQ_{10} (30 μM)	982 ± 110	275 ± 142	97
10% FCS extract	1,310 ± 264	306 ± 195	123
0% FCS extract	2,431 ± 470[b]	306 ± 87	228[b]
0% FCS + CoQ_{10} extract	1,460 ± 214	330 ± 57	137
None (DMSO)	947 ± 163	333 ± 109	100
C_2-ceramide (50 μM)	3,500 ± 622[b]	115 ± 100	370[b]

[a]Data represent the mean ± SD of the caspase-3 activity in the presence or absence of the especific inhibitor Ac-DEVD-CHO from four different experiments performed in triplicate.
[b]Significant differences versus respective control, $p \leq 0.05$.

that the CoQ-dependent inhibition of ceramide release prevents caspase-3 activation, whereas exogenously added C_2-ceramide restores ceramide levels for maximal activation of the caspase.

CEM-9F3 cells, which overexpress Bcl-2, showed a higher basal levels of N-SMase activity. However the levels of ceramide were similar to those in CEM cells. Thus, increased conversion of ceramide to sphingosine-1-phosphate may take place in Bcl-2-overexpressing cells. Ceramide conversion has in fact been correlated with increased cell proliferation and survival after withdrawal of trophic factors (Mandala *et al.*, 1998; Van Brocklyn *et al.*, 1998). Bcl-2 has complex effects on ceramide levels. It has been reported that it inhibits ceramide accumulation induced by several apoptotic stimuli (Yoshimura *et al.*, 1998; Tepper *et al.*, 1999), although it cannot prevent the ceramide accumulation induced by TNF-α (Monney *et al.*, 1998). Thus, it seems that Bcl-2 acts both upstream and downstream of the ceramide pathway depending on the apoptotic stimulus.

TABLE 2. OKADAIC ACID EFFECT ON CASPASE-3 ACTIVATION *IN VITRO*[a]

Additions	*Caspase-3 activity (pmol pNa/mg protein per 30 min)*
None (ethanol)	1,091 ± 100
0% FCS extract	2,592 ± 457[b]
+ 1 n*M* OA	1,070 ± 121[c]
None (DMSO)	874 ± 91
C_2-ceramide (50 μM)	4,694 ± 772[b]
+ 1 n*M* OA	921 ± 76[c]

[a]Data represent the mean ± SD from three experiments performed in triplicate.
[b]Significant differences versus respective controls, $p \leq 0.05$.
[c]Significant differences versus respective additions without okadaic acid, $p \leq 0.05$.

It is clear that a redox modulation of cellular components is taking place after growth factors or serum withdrawal (Slater *et al.*, 1996). Different antioxidants prevent both apoptosis and lipid hydroperoxide accumulation in growth factor withdrawal conditions (Hockenbery *et al.*, 1993; Tilly and Tilly, 1995; Barroso *et al.*, 1997a, b). Then, prevention of lipid peroxidation seems to be a critical event in oxidative damage-dependent cell death. Related to this apoptotic mechanism, phospholipase A_2 (PLA_2) is activated by hydroperoxides in plasma membrane (Hashizume *et al.*, 1991; Salgo *et al.*, 1993), and activation of N-SMase by PLA_2 has been reported (Jayadev *et al.*, 1994, 1997). Moreover, inhibitors of PLA_2 delay cell death (Neale *et al.*, 1988). Thus, a putative relationship between PLA_2 and SMase activation is suggested. However, a direct activation of N-SMase by hydroperoxides cannot be discarded because an *in vitro* inhibition of this lipase by glutathione has been also reported (Liu and Hannun, 1997). In our model, the antiapoptotic activity of CoQ resides in the prevention of the lipid peroxidation chain reaction by itself or together with

other antioxidants (Barroso *et al.*, 1997a) both in plasma membrane (Navarro *et al.*, 1998) and whole cells (Barroso *et al.*, 1997b). According to this model, in serum-deprived cells, the accumulation of hydroperoxides that activates N-SMase by a direct or an indirect mechanism is prevented by CoQ, avoiding further steps in the ceramide-dependent pathway.

On the other hand, CoQ is an essential factor in the mitochondrial electron chain and ceramide shows a direct inhibition of this electron chain (García-Ruiz *et al.*, 1997; Gudz *et al.*, 1997). Thus, a further protective role of exogenously added CoQ on mitochondria cannot be ruled out. However, in our system, the inhibition of ceramide release induced by CoQ would mask this putative direct effect of CoQ on mitochondria.

Our data suggest that ceramide is the factor responsible for caspase-3 activation after serum deprivation. However, how ceramide activates caspase-3 remains controversial. It has been suggested that ceramide induces apoptosis through a mitochondrial factor released once the mitochondrial transmembrane potential ($\Delta\Psi_m$) decreases and the mitochondrial pore complex is opened (Susin *et al.*, 1997a, b). However, C_2-ceramide is also able to induce nuclear apoptosis in the isolated nuclei of CEM cells (Martin *et al.*, 1995a) and can activate caspase-3 without the participation of mitochondria (Newmeyer *et al.*, 1994; Martin *et al.*, 1995a). In agreement with the above results, our data show caspase-3 activation in mitochondria-free fractions by either C_2-ceramide or ceramide-containing lipid extracts from serum-free grown cells. Thus, it seems that mitochondria are not required for ceramide activation of caspase-3 in our system.

Ceramide-dependent activation of caspase-3 seems to need the participation of phosphatases. In fact, phosphorylation and dephosphorylation cycles have been related to apoptotic processes (Wolf *et al.*, 1997; Cardone *et al.*, 1998). Furthermore, CAPP modulates the ceramide-dependent signal transduction (Dobrowsky and Hannun, 1992; Wolff *et al.*, 1994). Moreover, ceramide activates a mitochondrial protein phosphatase involved in Bcl-2 dephosphorylation and subsequently inactivation (Ruvolo *et al.*, 1999), and the protein phosphatase inhibitor OA inhibits the ceramide-induced PKCα inactivation by dephosphorylation (Lee *et al.*, 1996). OA inhibited ceramide-dependent caspase-3 activation in CEM cell extracts. Thus, it is suggested that CAPP or related phosphatases participate in the cytosolic activation of caspase-3.

In summary, in the model of oxidative injury induced by serum withdrawal, CoQ may participate in the initiation phase of apoptosis preventing N-SMase activation. Consequently, plasma membrane CoQ inhibits ceramide accumulation and ceramide-mediated activation of caspase-3 independent of mitochondria. This model suggests that plasma membrane CoQ constitutes a first defensive barrier in the protection of cells against apoptosis induced by oxidative damage.

ACKNOWLEDGMENTS

This work has been partially supported by the Spanish Dirección General de Educación Superior grant number PB98-0329-C02-01. Critical reading and comments from Dr. Lodewijk V. Dekker are kindly acknowledged. Daniel J. M. Fernández-Ayala is recipient of a fellowship FPD from the Junta de Andalucia. Sergio F. Martín is recipient of a fellowship from the Spanish Government Ministerio de Educación y Cultura.

ABBREVIATIONS

A-SMase, Acidic sphingomyelinase; CAPP, ceramide-activated protein phosphatase; CoQ, coenzyme Q, ubiquinone; cyt *c*, cytochrome *c*; DTT, dithiothreitol; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; ICE, interleukin-converting enzyme; IFN-γ, interferon-γ; IL-1-β, interleukin-1β; $\Delta\Psi$m, mitochondrial transmembrane potential; N-SMase, magnesium-dependent neutral sphyngomyelinase; OA, okadaic acid; PI, propidium iodide; PLA_2, phospholipase A_2; PLC, phospholipase C; PMSF, phenylmethylsulfonyl fluoride; pNa, paranitroaniline; ROS, reactive oxygen species;

SM, sphingomyelin; TLC, thin-layer chromatography; TNF-α, tumor necrosis factor-α.

REFERENCES

BALLOU, L.R., CHAO, C.P., HOLNESS, M.A., BRKER, S.C., and RAGHOW, R. (1992). Interleukin-1–mediated PGE_2 production and sphingomyelin metabolism. J. Biol. Chem. **267,** 20044–20050.

BARROSO, M.P., GÓMEZ-DÍAZ, C., LÓPEZ-LLUCH, G., MALAGÓN, M.M., CRANE, F.L., and NAVAS, P. (1997a). Ascorbate and α-tocopherol prevent apoptosis induced by serum removal independent of Bcl-2. Arch. Biochem. Biophys. **343,** 243–248.

BARROSO, M.P., GÓMEZ-DÍAZ, C., VILLALBA, J.M., BURÓN, M.I., LÓPEZ-LLUCH, G., and NAVAS, P. (1997b). Plasma membrane ubiquinone controls ceramide production and prevents cell death induced by serum withdrawal. J. Bioenerg. Biomemb. **29,** 259–267.

BEYER, R.E. (1994). The role of ascorbate in antioxidant protection of biomembranes: interaction with vitamin E and coenzyme Q. J. Bioenerg. Biomembr. **26,** 349–358.

BLIGH, E., and DYER, W. (1959). A rapid method for total lipid extraction and purification. Can. J. Biochem. Physiol. **37,** 911–917.

BRUCE-KELLER, A.J., BEGLEY, J.G., FU, W., BUTTERFIELD, D.A., BREDESEN, D.E., HUTCHINS, J.B., HENSLEY, K., and MATTSON, M.P. (1998). Bcl-2 protects isolated plasma and mitochondrial membranes against lipid peroxidation induced by hydrogen peroxide and amyloid β-peptide. J. Neurochem. **70,** 31–39.

CARDONE, M.H., ROY, N., STENNICKE, H.R., SALVESEN, G.S., FRANKE, T.F., STANBRIDGE, E., FRISCH, S., and REED, J.C. (1998). Regulation of death protease caspase-9 by phosphotylation. Science **282,** 1318–1321.

CRANE, F.L., SUN, I.L., CLARK, M.G., GREBING, C., and LÖW, H. (1985). Transplasma-membrane redox systems in growth and development. Biochim. Biophys. Acta **811,** 233–264.

DOBROWSKY, R.F., and HANNUN, Y.A. (1992). Ceramide stimulates a cytosolic protein phosphatase. J. Biol. Chem. **267,** 5048–5051.

ENARI, M., HASE, A., and NAGATA, S. (1995). Apoptosis by a cytosolic extract from Fas-activated cells. EMBO J. **14,** 5201–5208.

ENARI, M., TALANIAN, R.V., WONG, W.W., and NAGATA, S. (1996). Sequential activation of ICE-like and CPP32–like proteases during Fas-mediated apoptosis. Nature **380,** 723–726.

FREI, B. (1994). Reactive oxygen species and antioxidant vitamins: mechanisms of action. Am. J. Med. **97,** 5S-13S.

GARCÍA-CALVO, M., PETERSON, E.P., LEITING, B., RUEL, R., NICHOLSON, D.W., and THORNBERRY, N.A. (1998). Inhibition of human caspases by peptide-base and macromolecular inhibitors. J. Biol. Chem. **273,** 32608–32613.

GARCÍA-RUÍZ, C., COLLEL, A., MARÍ, M., MORALES, A., and FERNÁNDEZ-CHECA, J.C. (1997). Direct effect of ceramide on the mitochondrial electron transport chain leads to generation of reactive oxygen species. Role of mitochondrial glutathione. J. Biol. Chem. **272,** 11369–11377.

GELEY, S., HARTMANN, B.L., and KOFLER, R. (1997). Ceramides induce a form of apoptosis in human acute lymphoblastic leukemia cells that is inhibited by Bcl-2, but not by CrmA. FEBS Lett. **400,** 15–18.

GREELUND, L.J.S., BECKWERT, T.L., and JOHNSON, E.M.J. (1995). Superoxide dismutase delays neuronal apoptosis: a role for reactive oxygen species in programmed neuronal death. Neuron **14,** 303–314.

GUDZ, T.I., TSERNG, K.-I., and HOPPEL, C.L. (1997) Direct inhibition of mitochondrial respiratory chain complex III by cell-permeable ceramide. J. Biol. Chem. **272,** 24154–24158.

HANNUN, Y.A. (1994). The sphingomyelin cycle and the second messenger function of ceramide. J. Biol. Chem. **269,** 3125–3128.

HANNUN, Y.A. (1996). Functions of ceramide in coordinating cellular responses to stress. Science **274,** 1855–1859.

HASHIZUME, T., YAMAGUCHI, H., KAWAMOTO, A., TAMURA, A., SATO, T., and FUJII, T. (1991). Lipid peroxide makes rabbit platelet hyperaggregable to agonists through phospholipase A_2 activation. Arch. Biochem. Biophys. **289,** 47–52.

HOCKENBERY, D.M., OLTVAI, Z.N., YIN, X-M., MILLIMAN, C.L., and KORSMEYER, S.J. (1993). Bcl-2 functions in an antioxidant pathway to prevent apoptosis. Cell **75,** 241–251.

ISHIZAKI, Y., CHENG, L., MUDGE, A.W., and RAFF, M.C. (1995). Programmed cell death by default in embryonic cells, fibroblasts, and cancer cells. Mol. Biol. Cell. **6,** 1443–1458.

JARVIS, W.D., KOLESNICK, R.N., FORNARI, F.A., TRAYLOR, R.S., GEWIRTZ, D.A., and GRANT, S. (1994). Induction of apoptotic DNA damage and cell death by activation of the sphingomyelin pathway. Proc. Natl. Acad. Sci. USA **91,** 73–77.

JAYADEV, S., LINARDIC, C.M., and HANNUN, Y.A. (1994). Identification of arachidonic acid as a mediator of sphingomyelin hydrolysis in response to tumor necrosis factor alpha. J. Biol. Chem. **269,** 5757–5763.

JAYADEV, S., LIU, B., BIELAWSKA, A.E., LEE, J.Y., NAZAIRE, F., PUSHKAREVA, M.Y., OBEID, L.M., and HANNUN, Y.A. (1995). Role for ceramide in cell cycle arrest. J. Biol. Chem. **270,** 2047–2052.

JAYADEV, S., HAYTER, H.L., ANDRIEU, N., GAMARD, C.J., LIU, B., BALU, R., HAYAKAWA, M., ITO, F., and HANNUN, Y.A. (1997). Phospholipase A_2 is necessary for tumor necrosis factor alpha-induced ceramide generation in L929 cells. J. Biol. Chem. **272,** 17196–17203.

KANE, D.J., SARAFIN, T.A., ANTON, R., HAHN, H., GRALLA, E.B., VALENTINE, J.S., ÖRD, T., and BREDENSEN, D.E. (1993). Bcl-2 inhibition of neuronal death: decreased generation of reactive oxygen species. Science **262,** 1274–1277.

KIM, M-Y., LINARDIC, C., OBEID, L., and HANNUN,

Y.A. (1991). Identification of sphingomyelin turnover as an effector for the action of tumor necrosis factor alpha and gamma-interferon. Specific role in cell differentiation. J. Biol. Chem. **266,** 484–489.

LEE, J.Y., HANNUN, Y.A., and OBEID, L.M. (1996). Ceramide inactivates cellular protein kinase Cα. J. Biol. Chem. **271,** 13169–13174.

LIU, B., and HANNUN, Y.A. (1997). Inhibition of the neutral magnesium-dependent sphingomyelinase by glutathione. J. Biol. Chem. **272,** 16281–16287.

MACHO, A., BLÁZQUEZ, M-V., NAVAS, P., and MUÑOZ, E. (1998). Induction of apoptosis by vanilloid compounds does not require de novo gene transcription and activator protein 1 activity. Cell Growth Differ. **9,** 277–286.

MACHO, A., CALZADO, M.A., MUÑOZ-BLANCO, J., GÓMEZ-DÍAZ, C., GAJATE, C., MOLLINEDO, F., NAVAS, P., and MUÑOZ, E. (1999). Selective induction of apoptosis by capsaicin in transformed cells: the role of reactive oxygen species and calcium. Cell Death Differ. **6,** 155–165.

MANDALA, S.M., THORNTON, R., TU, Z., KURTZ, M.B., NICKELS, J., BROACH, J., MENZELEEV, R., and SPIEGEL, S. (1998). Sphingoid base 1–phosphate phosphatase: a key regulator of sphingolipid metabolism and stress response. Proc. Natl. Acad. Sci. USA **95,** 150–155.

MARTIN, J., NEWMEYER, D.D., MATHIAS, S., FARSCHON, D.M., WANG, H-G., REED, J.C., KOLESNICK, R.N., and GREEN, D.R. (1995a). Cell-free reconstitution of Fas-, UV radiation- and ceramide-induced apoptosis. EMBO J. **14,** 5191–5200.

MARTIN, S.J., O'BRIEN, G.A., NISHIOKA, W.K., McGAHON, A.J., MAHBOUBI, A., SAIDO, T.C., and GREEN, D.R. (1995b). Proteolisis of fodrin (non-erythroid spectrin) during apoptosis. J. Biol. Chem. **270,** 6425–6428.

MARTINUS, R.D., LINNANE, A.W., and NAGLEY, P. (1993). Growth of human namalwa cells lacking oxidative phosphorylation can be sustained by redox compounds ferricyanide or coenzyme Q putatively acting through the plasma membrane oxidase. Biochem. Molec. Biol. Int. **31,** 997–100510.

MIZUSHIMA, N., KOIKE, R., KOHSAKA, H., KUSHI, Y., HANDA, S., YAGITA, H., and MIYASAKA, N. (1996). Ceramide induces apoptosis via CPP32 activation. FEBS Lett. **395,** 267–271.

MONNEY, L., OLIVIER, R., OTTER, I., JANSEN, B., POIRIER, G.G., and BORNER, C. (1998). Role of an acidic compartment in tumor-necrosis-factor-α-induced production of ceramide, activation of caspase-3 and apoptosis. Eur. J. Biochem. **251,** 295–303.

NAVARRO, F., VILLALBA, J.M., CRANE, F.L., MACKELLAR, W.C., and NAVAS, P. (1995). A phospholipid dependent NADH coenzyme Q reductase from liver plasma membrane. Biochem. Biophys. Res. Commun. **212,** 138–143.

NAVARRO, F., NAVAS, P., BURGESS, J.R., BELLO, R.I., DE CABO, R., ARROYO, A., and VILLALBA, J.M. (1998). Vitamin E and selenium deficiency induces expression of the ubiquinone-dependent antioxidant system at the plasma membrane. FASEB J. **12,** 1665–1673.

NEALE, M.L., FIERA, A., and MATTHEWS, N. (1988). Involvement of phospholipase A_2 activation in tumor cell killing by tumour necrosis factor. Immunology **64,** 81–85.

NEWMEYER, D.D., FARSCHON, D.M., and REED, J.C. (1994). Cell-free apoptosis in Xenopus eggs extracts: inhibition by Bcl-2 and requirement for an organelle fraction enriched in mitochondria. Cell **79,** 353–364.

OBEID, L.M., LINARDIC, C.M., KAROLAK, L.A., and HANNUN, Y.A. (1993). Programmed cell death induced by ceramide. Science **259,** 1769–1771.

OKAZAKI, T., BELL, R.M., and HANNUN, Y.A. (1989). Sphingomyelin turnover induced by vitamin D_3 in HL-60 cells. Role in cell differentiation. J. Biol. Chem. **264,** 19076–19080.

OKAZAKI, T., BIELAWSKA, A., BELL, R.M., and HANNUN, Y.A. (1990). Role of ceramide as a lipid mediator of 1α,25–dihydroxyvitamin D_3-induced HL-60 cell differentiation. J. Biol. Chem. **265,** 15832–15831.

OKAZAKI, T., BIELAWSKA, A., DOMAE, N., BELL, R., and HANNUN, Y.A. (1994). Characteristics and partial purification of a novel cytosolic, magnesium-independent, neutral sphingomyelinase activated in the early signal transduction of 1α,25–dihydroxyvitamin D_3-induced HL-60 cell differentiation. J. Biol. Chem. **269,** 4070–4077.

PREISS, J., LOOMIS, C.R., BISHOP, W.R., STEIN, R., NIEDEL, J.E., and BELL, R.M. (1986). Quantitative measurement of sn-1,2–diacylglycerols present in platelets, hepatocytes, and ras- and sis-transformed normal rat kidney cells. J. Biol. Chem. **261,** 8597–8600.

RAFF, M.C. (1992). Social controls on cell survival and cell death. Nature **356,** 397–400.

RAFF, M.C., BARRES, B.A., BURNE, J.F., COLES, H.S., ISHIZAKI, Y., and JACOBSON, M.D. (1993). Programmed cell death and the control of the survival: lessons from the nervous system. Science **262,** 695–700.

RAINES, M.A., KOLESNICK, R.N., and GOLDE, D.W. (1993). Sphingomyelinase and ceramide activate mitogen-activated protein kinase in myeloid HL-60 cells. J. Biol. Chem. **268,** 14572–14575.

RUVOLO, P.P., DENG, X., ITO, T., CARR, B.K., and MAY, S. (1999). Ceramide induces Bcl2 dephosphorylation via a mechanism involving mitochondrial PP2A. J. Biol. Chem. **274,** 20296–20300.

SALGO, M.G., CORONGIU, F.P., and SEVANIAN, A. (1993). Enhanced interfacial catalysis and hydrolytic specifity of phospholipase A_2 toward peroxidized phosphatidylcholine vesicles. Arch. Biochem. Biophys. **304,** 123–132.

SLATER, A.F.G., STEFAN, C., NOBEL, I., VAN DEN DOBBELSTEEN, D.J., and ORRENIUS, S. (1996). Intracellular redox changes during apoptosis. Cell Death Differ. **3,** 57–62.

SMYTH, M.J., PERRY, D.K., ZHANG, J.D., POIRIER, G.G., HANNUN, Y.A., and OBEID, L.M. (1996). prICE:

a downstream target for ceramide-induced apoptosis and for the inhibitory action of Bcl-2. Biochem J. **316,** 25–28.

STORRIE, B., and MADDEN, E.A. (1990). Isolation of subcellular organelles. Methods Enzymol. **182,** 203–225.

SUN, I.L., SUN, E.E., and CRANE, F.L. (1995). Comparison of growth stimulation of Hela cells, HL-60 cells, and mouse fibroblasts by coenzyme Q_{10}. Protoplasma **184,** 214–219.

SUSIN, S.A., ZAMZAMI, N., CASTEDO, M., DAUGAS, E., WANG, H-G., GELEI, S., FASSY, F., REED, J.C., and KROEMER, G. (1997a). The central executioner of apoptosis: multiple connections between protease activation and mitochondria in Fas/APO-1/CD95- and ceramide-induced apoptosis. J. Exp. Med. **186,** 25–37.

SUSIN, S.A., ZAMZAMI, N., LAROCHETTE, N., DALLAPORTA, B., MARZO, Y., BRENNER, C., HIRSCH, T., PETIT, P.X., GEUSKENS, M., and KROEMER, G. (1997b). A cytofluorometric assay of nuclear apoptosis induced in a cell-free system: application to ceramide-induced apoptosis. Exp. Cell Res. **236,** 397–403.

TEPPER, A., DE VRIES, E., VAN BLITTERSWIJT, W.J., and BORST, J. (1999) Ordering of ceramide formation, caspase activation, and mitochondrial changes during CD95- and DNA damage-induced apoptosis. J. Clin. Invest. **103,** 971–978.

TILLY, J.L., and TILLY, K.I. (1995). Inhibitors of oxidative stress mimic the ability of follicle-stimulating hormone to suppress apoptosis in cultured rat ovarian follicles. Endocrinology **136,** 242–252.

VAN BROCKLYN, J.R., LEE, M-J., MENZELEEV, R., OLIVERA, A., EDSALL, L., CUVILIER, O., THOMAS, D.M., COOPMAN, P.J.P., THANGADA, S., LIU, X.M., HLA, T., and SPIEGEL, S. (1998). Dual actions of sphingosine-1–phosphate: extracellular through the Gi-coupled receptor Edg-1 and intracellular to regulate proliferation and survival. J. Cell Biol. **142,** 229–240.

VILLALBA, J.M., NAVARRO, F., CÓRDOBA, F., SERRANO, A., ARROYO, A., CRANE, F.L., and NAVAS, P. (1995). Coenzyme Q reductase from liver plasma membrane: purification and role in transplasma membrane electron transport. Proc. Natl. Acad. Sci. USA **92,** 4887–4891.

WEIL, M., JACOBSON, M.D., COLES, H.S.R., DAVIES, T.J., GARDNER, R.L., RAFF, K.D., and RAFF, M.C. (1996). Constitutive expression of the machinery for programmed cell death. J. Cell Biol. **133,** 1053–1059.

WOLF, C.M., REYNOLDS, J.E., MORANA, S.J., and EASTMAN, A. (1997). The temporal relationship between protein phosphatase, ICE/CED-3 protease, intracellular acidification, and DNA fragmentation in apoptosis. Exp. Cell Res. **230,** 22–27.

WOLFF, R.A., DOBROWSKY, R.T., BIELAWSKA, A., OBEID, L.M., and HANNUN, Y.A. (1994). Role of ceramide-activated protein phosphatase in ceramide mediated signal transduction. J. Biol. Chem. **269,** 19605–19609.

WOLVETANG, E.J., LARM, J.A., MOUTSOULAS, P., and LAWEN, A. (1996) Effectors of the mammalian plasma membrane NADH-oxidoreductase system. Short-chain ubiquinone analogues as potent stimulators. Cell Growth Differ. **7,** 1315–1325.

YOSHIMURA, S., BANNO, Y., NAKASHIMA, S., TAKENAKA, K., SAKAI, H., NISHIMURA, Y., SAKAI, N., SHIMIZY, S., EGUCHI, Y., TSUJIMOTO, Y., and NOZAWA, Y. (1998). Ceramide formation leads to caspase-3 activation during hypoxic PC12 death. J Biol. Chem. **273,** 6921–6927.

Address reprint requests to:
Dr. Plácido Navas
Laboratorio Andaluz de Biología
Universidad Pablo de Olavide
Carretera de Utrera, Km 1.0
41013 Sevilla, Spain.

E-mail: pnavas@gob.upo.es

Received for publication December 21, 1999; accepted February 27, 2000.

This article has been cited by:

1. Beatrice A. Golomb, Edwin K. Kwon, Sabrina Koperski, Marcella A. Evans. 2009. Amyotrophic Lateral Sclerosis-Like Conditions in Possible Association with Cholesterol-Lowering Drugs. *Drug Safety* **32**:8, 649-661. [CrossRef]
2. Jose M. Munoz-Olaya, Xavier Matabosch, Carmen Bedia, Meritxell Egido-Gabás, Josefina Casas, Amadeu Llebaria, Antonio Delgado, Gemma Fabriàs. 2008. Synthesis and Biological Activity of a Novel Inhibitor of Dihydroceramide Desaturase. *ChemMedChem* **3**:6, 946-953. [CrossRef]
3. G. López-Lluch, M. Rios, M. A. Lane, P. Navas, R. Cabo. 2005. Mouse liver plasma membrane redox system activity is altered by aging and modulated by calorie restriction. *AGE* **27**:2, 153-160. [CrossRef]
4. David A. Groneberg, Birgit Kindermann, Martin Althammer, Maja Klapper, Jürgen Vormann, Gian P. Littarru, Frank Döring. 2005. Coenzyme Q10 affects expression of genes involved in cell signalling, metabolism and transport in human CaCo-2 cells. *The International Journal of Biochemistry & Cell Biology* **37**:6, 1208-1218. [CrossRef]
5. Beatrice Alexandra Golomb. 2005. Implications of statin adverse effects in the elderly. *Expert Opinion on Drug Safety* **4**:3, 389-397. [CrossRef]
6. John SmythiesSection I. The Cholinergic System **64**, 1-122. [CrossRef]
7. Norma Marchesini, Yusuf A Hannun. 2004. Acid and neutral sphingomyelinases: roles and mechanisms of regulation. *Biochemistry and Cell Biology* **82**:1, 27-44. [CrossRef]
8. Rangaprasad Sarangarajan, Shireesh P. Apte. 2004. Ocular Melanogenesis: The Role of Antioxidants. *Ophthalmic Research* **36**:6, 303-311. [CrossRef]
9. Steven K Baker, Mark A Tarnopolsky. 2003. Targeting cellular energy production in neurological disorders. *Expert Opinion on Investigational Drugs* **12**:10, 1655-1679. [CrossRef]
10. E Casalino. 2002. Antioxidant effect of hydroxytyrosol (DPE) and Mn2+ in liver of cadmium-intoxicated rats. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* **133**:4, 625-632. [CrossRef]
11. Christopher C Lindsey, Consuelo Gómez-D##az, José M Villalba, Thomas R.R Pettus. 2002. Synthesis of the F11334's from o-prenylated phenols: µM inhibitors of neutral sphingomyelinase (N-SMase). *Tetrahedron* **58**:22, 4559-4565. [CrossRef]
12. Nathalie Andrieu-Abadie, Valérie Gouazé, Robert Salvayre, Thierry Levade. 2001. Ceramide in apoptosis signaling: relationship with oxidative stress. *Free Radical Biology and Medicine* **31**:6, 717-728. [CrossRef]