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Ceramide-dependent Caspase 3 Activation is Prevented by Coenzyme Q from Plasma Membrane in Serum-deprived Cells

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Coenzyme Q (CoQ) is the key factor for the activity of the eukaryotic plasma membrane electron transport chain. Consequently, CoQ is essential in the cellular response against redox changes affecting this membrane. Serum withdrawal induces a mild oxidative stress, which produces lipid peroxidation in membranes. In fact, apoptosis induced by serum withdrawal can be prevented by several antioxidants including CoQ. Also, CoQ can maintain cell growth in serum-limiting conditions, whereas plasma membrane redox system (PMRS) inhibitors such as capsaicin, which compete with CoQ, inhibit cell growth and induce apoptosis. To understand how plasma membrane CoQ prevents oxidative stress-induced apoptosis we have studied the induction of apoptosis by serum withdrawal in CEM cells and its modulation by CoQ. Serum-withdrawal activates neutral sphingomyelinase (N-SMase), ceramide release and caspase-3-related proteases. CoQ addition to serum-free cultures inhibited a 60% N-SMase activation, an 80% ceramide release, and a 50% caspase-3 activity induced by serum deprivation. Caspase activation dependent on ceramide release since C_2-ceramide was only able to mimic this effect in 10% foetal calf serum cultured cells but not in serum-free cultures. Also, *in vitro* experiments demonstrated that C_2-ceramide and ceramide-rich lipid extracts directly activated caspase-3. Taken together, our results indicate that CoQ protects plasma membrane components and controls stress-mediated lipid signals by its participation in the PMRS.

Keywords: Coenzyme Q; Caspase; Plasma Membrane; Ceramide

INTRODUCTION

Human plasma membrane contains significant amounts of CoQ_{10}, which plays an essential role as antioxidant and electron carrier mediating the regeneration of other important antioxidants such as ascorbate and α-tocopherol. These antioxidants are components of a plasma membrane-associated electron transport system (PMRS) which protects the membrane against damage caused by oxidative stress, where CoQ_{10} is the central component.[1] Serum provides survival signals for cells and its depletion activates an intrinsic cell death program,[2,3] whose machinery is constitutively expressed.[4] There are evidences for an indirect generation of oxidative stress during apoptosis caused by growth factors withdrawal,[5] since this apoptotic program is inhibited by endogenous antioxidant enzymes[6,7] and other antioxidant molecules such as ascorbate, α-tocopherol and CoQ_{10}.[7–9] Furthermore, CoQ_{10} is able to stimulate cell growth in the absence of serum,[10] and maintain growth in mitochondrial respiratory chain deficient cells.[9,11] In addition, CoQ_{10} seems to be the key factor in preventing apoptosis induced by oxidative stress since antagonists of CoQ_{10} trigger the apoptotic program.[12–14] The role of antioxidants in the prevention of apoptosis is keen in several

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systems, including regulation of antioxidant response mechanism by Bcl-2.[15–17]

Ceramide acts as a mediator of stress responses modulating enzymes of the eicosanoid pathway, protein kinases, nuclear factors, and gene expression.[18] Ceramide is also able to induce cell death after its intracellular accumulation,[9,19–22] by activating proteases of the caspase family.[23] Serum deprivation also induces a progressive increase of ceramide levels in Molt-4 and HL-60 cells.[9,20] Thus, the increase of ceramide levels appears to be an essential component in the stress response pathway triggered by serum withdrawal.[19]

We show here that CoQ_{10} acts at the initiation phase of apoptosis by preventing the activation of neutral sphingomyelinase (N-SMase), consequently, decreasing the short-term ceramide accumulation and caspase-3 activation. Our results support the idea that CoQ_{10} prevents apoptosis-induced by serum withdrawal through the inhibition of plasma membrane N-SMase and ceramide release.

MATERIALS AND METHODS

Cell Lines and Cultures

The human T-acute lymphatic leukaemia cell line CEM-C7H2 (CEM), a sub-line of CCRF-CEM, was grown in 5% CO_2 atmosphere at 37°C in RPMI-1640 medium (Sigma, Spain). Cultures were supplemented with 10% foetal calf serum (FCS) (PAA Labor, Austria), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B (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 grown in 10% FCS supplemented RPMI medium. For treatments, cells were previously harvested, washed twice in serum-free medium and resuspended in 10% FCS-supplemented medium or in serum-free media supplemented or not with 30 μM CoQ_{10}. After indicated times of incubation, cells were harvested, washed twice with ice cold PBS and fixed in 70% ethanol for at least 24 h at 4°C. After fixation, cells were washed with Hank's balanced salt solution (HBSS) and 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 analysed using a Lysis II software (Becton-Dickinson, USA).

Ceramide Quantification

Ceramide production was determined as previously described.[20] Briefly, cells were harvested at indicated times and washed twice with PBS. Lipids were then extracted by the method of Bligh and Dyer.[24] Lipids were dried and dissolved in 20 μl 1 mM diethylenetriamine pentaacetic acid, 7.5% octyl-β-D-glucopyranoside, and 5 mM cardiolipin (Sigma, Spain). After sonication for 15 s, 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 radioactivity 100,000–500,000 cpm/nmol) (Amersham Iberica, Spain). After incubation for 20 min at 25°C, lipids were extracted with chloroform. Chloroform phase (500 μl) 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 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 analyser (Berthold, Germany). Ceramide was quantified using external standards and normalised to phosphate as indicator of total phospholipids.

Assays for N-SMase and Caspase-3

Proteins (50–100 μg) from cell lysates, plasma membrane or purified N-SMase 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 $MgCl_2$). Then, 10 nmol of a mixture of cold sphingomyelin (SM) and [methyl-^{14}C]-SM (specific radioactivity 10,000 cpm/nmol) (Amersham Iberica, Spain) were added. After incubation for 30–60 min at 37°C, the reaction was stopped by adding 1.5 ml chloroform/methanol (2:1) and 200 μl distilled water. Tubes were vortexed and then centrifuged at 1500*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 hydrolysed/mg protein/hour.

Caspase-3 activity was assayed by a colorimetric method using Ac-DEVD-pNa (Bachem, Switzerland) a specific substrate for caspase-3 and other related executioner caspases. The specific inhibitor Ac-DEVD-CHO (Bachem, Switzerland) was used to

TABLE I Effect of serum withdrawal on the ceramide-dependent pathway components of apoptosis and its inhibition by CoQ in CEM cells

	Growth conditions		
Component	+Serum	−Serum	−Serum + CoQ
Mg-dependent nSMase	60 ± 8	250 ± 21	105 ± 9*
Ceramide concentration	45 ± 6	89 ± 5	48 ± 5*
Caspase-3 activity	1100 ± 185	4200 ± 300	2000 ± 180*

N-SMase, ceramide levels and caspase-3 activity were determined in cells cultured in 10% FCS, serum-free and CoQ supplemented serum-free cultures. Cells were harvested after 1 h of incubation for both N-SMase and ceramide assays and after 2 h for caspase activity determination. Neutral SMase activity is expressed in cpm released/min/mg protein, ceramide concentration in pmol/nmol Pi, and caspase-3 activity in units (1 pmol pNa released/30 min/mg protein). *Significant differences vs. serum-free conditions media, $p \leq 0.05$ ($n = 4$).

determine the specificity of the reaction. Protein (50 μg) were diluted in assay buffer (100 mM HEPES–KOH buffer, pH 7.5; 10% sucrose, 0.1% CHAPS, 0.1 mg/ml ovoalbumin, and 10 mM DTT). Activity was developed in 96 well plates at 37°C for 30–60 min, and paranitroaniline (pNa) released was determined spectrophotometricaly at 405 nm using a multiwell reader. Activity was expressed in units (1 unit = 1 pmol pNa released/30 min/mg protein).

Statistical Analysis

Presented data represent the mean ± SD from three or four different experiments. Significant differences were determined using the one way ANOVA test.

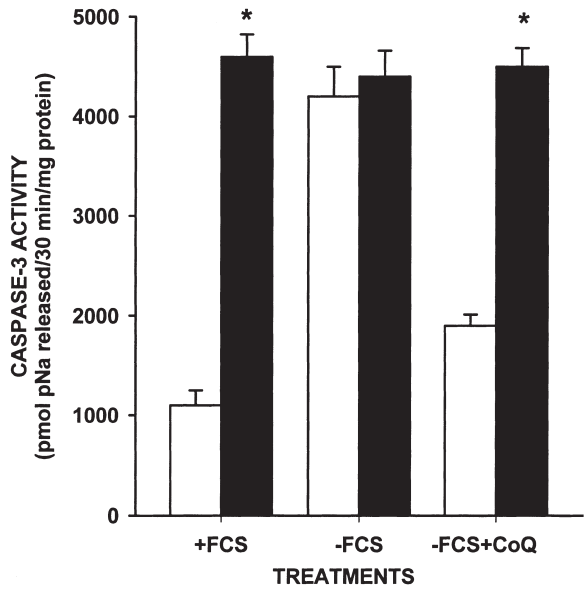

FIGURE 1 Role of growth conditions on the activation of caspase-3 in CEM cells. Cells were incubated in the presence of serum or in serum free or 30 μM CoQ-supplemented serum-free media. C_2-ceramide (50 μM) was also added to all the above-indicated conditions. After 2 h of incubation, cells were harvested and caspase activity determined. White bars: activity in the absence of C_2-ceramide. Black bars: plus C_2-ceramide. *Significant differences vs. C_2-ceramide free conditions ($p \leq 0.01$).

RESULTS

CEM cells growing in a complete medium showed about 5% of apoptotic population at 48 h of culture but the culture in serum-free medium induced an increase of apoptotic cells reaching about 60% of the population at the same time. Addition of 30 μM CoQ_{10} to serum-free cultures decreased apoptosis up to 35% of the population at the same time.

Different components of the ceramide-dependent apoptotic pathway were analysed in CEM cells and data are included in Table I. Two enzyme activities have been related to ceramide release in apoptotic events, N-SMase and acidic SMase (A-SMase). Latter activity was not affected in CEM cells after serum removal, but N-SMase activity increased four folds after the first hour of incubation in serum-free media. The presence of CoQ_{10} in serum-free media clearly prevented N-SMase activation. Ceramide increased up to two folds in these cells after 1 h of culture in the absence of serum, but no differences in ceramide accumulation were observed when CoQ_{10} was added to serum-free cultures. No changes in ceramide concentration occurred in 10% serum growing CEM cells at any time of incubation (data not shown). Further, caspase-3 proteases, able to recognise the aminoacidic sequence DEVD, were activated about four folds after 2 h of culture in CEM cells in the absence of serum, whereas the presence of CoQ decreased this activation by 50%.

TABLE II *In vitro* activation of caspase-3 by lipid extracts from CEM cells and C_2-ceramide

Additions	Caspase-3 activity	Activity (%)
None	1164 ± 170	100
+CoQ 30 μM	1082 ± 110	93
10% FCS extract	1310 ± 264	113
0% FCS extract	2520 ± 350*	216*
0% FCS +CoQ extract	1480 ± 197	127
C_2-ceramide 50 μM	3820 ± 540*	327*

Cytosol extracts from 10% FCS-cultured CEM cells were incubated *in vitro* with lipid extracts from 10% FCS, serum-free and 30 μM CoQ-supplemented serum-free cultured CEM cells. Controls were performed by incubation of cytosol extracts with ethanol, 30 μM CoQ and C_2-ceramide (50 μM). Caspase-3 activity is expressed in units (1 pmol pNa released/30 min/mg protein). *Significant differences vs. no additions, $p \leq 0.05$ ($n = 3$).

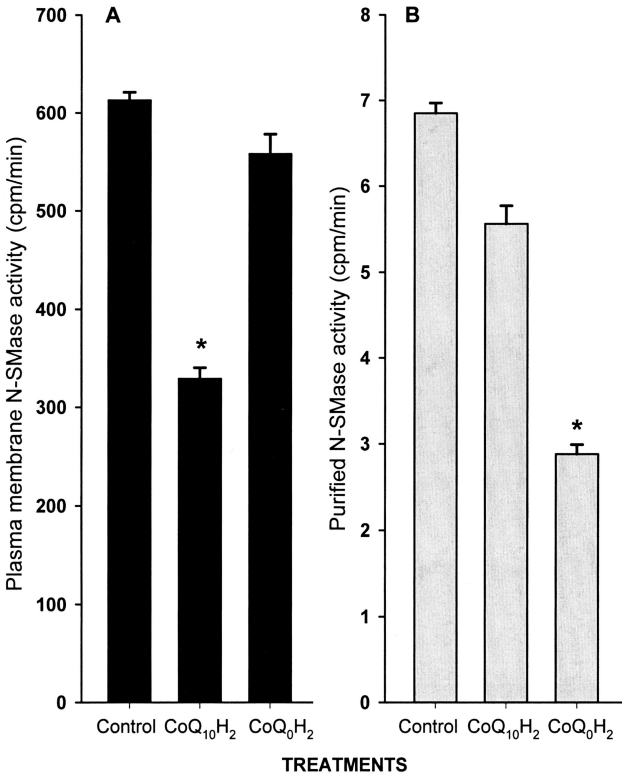

FIGURE 2 Inhibition of plasma membrane and purified N-SMase by reduced forms of CoQ_0 and CoQ_{10}. (A) Plasma membrane from pig liver cells (60 μg) was incubated with vehicle, or 100 μM of $CoQ_{10}H_2$ or CoQ_0H_2 and N-SMase activity determined as indicated in "Material and methods" section. (B) Purified N-SMase (2 ng in 20 μl) from plasma membrane from pig liver cells was incubated in the presence or absence of 100 μM of $CoQ_{10}H_2$ or CoQ_0H_2. Data represent the mean ± SD from three independent experiments. *Significant differences vs. control, ($p \leq 0.05$).

To determine if caspase-3 was present in the cytosol and ceramide was involved in its activation, C_2-ceramide was added to CEM cells (Fig. 1). Clearly, caspase-3 was activated after the addition of C_2-ceramide to cells growing in both the presence of serum and in the absence of serum plus CoQ_{10}. However, C_2-ceramide was unable to increase caspase-3 activity in cells growing in the absence of serum since these cells already reached the peak of activation of this caspase by incubation in serum-free media.

To determine if ceramide directly affected caspase-3 activity we performed an *in vitro* experiment by incubation of the cytosolic fraction of 10% FCS cultured CEM cells with C_2-ceramide or lipid extracts from cells cultures in 10, 0% FCS or 0% FCS plus CoQ_{10} supplemented-media (Table II). *In vitro* activation of cytosolic caspase-3 was significantly induced by lipid extracts from 0% FCS growing cells and by C_2-ceramide but not by lipid extracts from both CEM cells growing in the presence of serum or in the absence of serum plus CoQ_{10}. The effect of the lipid extracts from cells grown in serum-free media was probably due to their higher concentration in ceramide. As control, CoQ_{10} itself did not show any effect on *in vitro* cytosolic caspase-3 activation.

In order to study the mechanism of inhibition by CoQ_{10} of ceramide-dependent apoptosis pathway, we analysed the effect of CoQ_{10} on purified N-SMase from plasma membrane of pig liver hepatocytes (Fig. 2).[25] Plasma membranes purified from pig liver contain a N-SMase activity that was inhibited about 50% by $CoQ_{10}H_2$, but was not affected by the soluble form CoQ_0H_2. However, purified N-SMase was inhibited by CoQ_0H_2 in a 60% but was not significantly affected by $CoQ_{10}H_2$. These results indicate lipid environment is important for the effective ubiquinone inhibition of N-SMase in plasma membrane.

DISCUSSION

Data from the literature suggest that increased SMase activity and ceramide production are important factors in apoptosis induced by several agents including oxidative injury.[19,20,26] Ceramide accumulation induces cell death by activation of caspases.[23,27,28] In our study, caspase-3 was activated in CEM cells after ceramide release. The addition of C_2-ceramide to cells increased caspase-3 activity to the same level in cultures grown with 10% FCS and in CoQ_{10}-supplemented serum-free media. However, C_2-ceramide was unable to further increase caspase activity in cells grown in serum-free media without CoQ_{10}. These results suggest that CoQ_{10}-dependent inhibition of ceramide release prevents caspase-3 activation. Exogenously added C_2-ceramide restores ceramide levels for maximal activation of the caspases.

It is clear that growth factors or serum withdrawal induce a redox modulation of cellular activities.[5] Different antioxidants prevent both lipid hydroperoxides accumulation and apoptosis in serum-free conditions.[7–9,15] Then, lipid peroxidation seems to be a critical event in oxidative damage-dependent cell death and its prevention is one of the main functions of physiological antioxidants such as ascorbate, α-tocopherol and CoQ. Related to this apoptotic mechanism triggered by oxidative damage, phospholipase A_2 (PLA_2) is activated by hydroperoxides in plasma membrane.[29] PLA_2 activation can be the responsible of subsequent activation of N-SMase initiating the ceramide-dependent apoptotic pathway.[30] In fact, inhibitors of PLA_2 delay cell death in other apoptotic systems linked to oxidative processes.[31] Thus, a putative relationship between PLA_2 and N-SMase activation is suggested. However, a direct activation of N-SMase by hydroperoxides can not be discarded

since intracellular antioxidants like glutathione are able to inhibit this lipase.[32] In our model, the antiapoptotic activity of CoQ_{10} resides in the prevention of the lipid peroxidation chain reaction by itself or together with other antioxidants.[1,8,9]

Our results indicate that N-SMase localised in plasma membrane was inhibited by CoQ_{10} but not by CoQ_0, whereas the isolated enzyme was not affected by CoQ_{10} but was inhibited by the soluble form CoQ_0. These results clearly indicate a direct inhibition of N-SMase by ubiquinone and the importance of the lipid environment for the access of CoQ analogs to the enzyme.

Our data also suggest that ceramide is the factor responsible for the activation of caspase-3 related proteases after serum-deprivation. However, how ceramide activate these caspases remains controversial. It has been suggested that ceramide induce apoptosis through a mitochondrial factor, which is released once the mitochondrial transmembrane potential decreases and the mitochondrial pore complex is opened.[33] However, C_2-ceramide is also able to induce nuclear apoptosis in isolated nuclei of CEM cells, and activate caspase-3 without the participation of mitochondria.[27,34] Our *in vitro* results confirm the caspase-3 activation in mitochondria-free fractions by C_2-ceramide or ceramide-containing lipid extracts from serum-free grown cells. Thus, it seems that mitochondria are not absolutely required for ceramide activation of executioner caspases in our system.

In summary, in this model of oxidative injury induced by serum-withdrawal, CoQ may participate in the initiation phase of apoptosis preventing N-SMase activation, apparently by its direct inhibition, although an indirect effect through inhibition of hydroperoxide-induced PLA_2 activation can not be disregarded. In any case, plasma membrane CoQ_{10} inhibits N-SMase activation and ceramide accumulation and thus, ceramide-mediated activation of executioner caspases, like caspase-3, independently of mitochondria. This model suggests that plasma membrane CoQ_{10} is the first defensive level for the protection of cells against apoptosis induced by oxidative damage.

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