BioFactors 9 (1999) 171–177
IOS Press

Role of plasma membrane coenzyme Q on the regulation of apoptosis

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Abstract. Serum withdrawal is a model to study the mechanisms involved in the induction of apoptosis caused by mild oxidative stress. Apoptosis induced by growth factors removal was prevented by the external addition of antioxidants such as ascorbate, α-tocopherol and coenzyme Q (CoQ). CoQ is a lipophilic antioxidant which prevents oxidative stress and participates in the regeneration of α-tocopherol and ascorbate in the plasma membrane. We have found an inverse relationship between CoQ content in plasma membrane and lipid peroxidation rates in leukaemic cells. CoQ_{10} addition to serum-free culture media prevented both lipid peroxidation and cell death. Also, CoQ_{10} addition decreased ceramide release after serum withdrawal by inhibition of magnesium-dependent plasma membrane neutral-sphingomyelinase. Moreover, CoQ_{10} addition partially blocked activation of CPP32/caspase-3. These results suggest CoQ of the plasma membrane as a regulator of initiation phase of oxidative stress-mediated serum withdrawal-induced apoptosis.

1. Introduction

Plasma membrane contains an electron transport chain involved in the maintenance of an antioxidant system (see Navarro et al. this volume), involving ascorbate, α-tocopherol and CoQ. Ascorbate is a first order antioxidant which scavenges lipid peroxidation-initiating radicals [10]. In addition, ascorbate reduces membrane bound α-tocopheroxyl radicals produced by the oxidation of α-tocopherol after scavenging of lipid-peroxyl radicals thus restoring the antioxidant capacity of this compound [10,12]. CoQ is a lipid-soluble antioxidant which can be considered as the central molecule in plasma membrane protection against lipid peroxidation because it is directly reduced by cytochrome b_5 reductase [31,36], and maintains both ascorbate and α-tocopherol in their reduced state [10,12,25].

Serum withdrawal causes a mild oxidative stress to cells in culture leading to membrane damage and cell death [17]. Serum removal induces ceramide release to the cytosol by the activation of Mg^{2+}-dependent neutral sphingomyelinase (N-SMase) [21]. Ceramides are able to induce cell death after its intracellular accumulation by activating proteases of the caspase family [18,27]. Thus, ceramide accumulation appears as a key component in the stress response pathway triggered by serum removal [14]. Further, cellular accumulation of antioxidants can prevent apoptosis induced by growth factors deprivation [3,13,32].

We show here the protection caused by antioxidants such as ascorbate, α-tocopherol and CoQ against apoptosis induced by serum removal. Also, we demonstrate a correlation between the content of CoQ at the plasma membrane and the extinction of lipid peroxidation. Further, plasma membrane CoQ inhibits

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neutral sphingomyelinase and, as a consequence, prevents ceramide release and caspase-3 activation. CoQ, as central part of the antioxidant system of the plasma membrane, regulates the initiation phase of apoptosis induced by growth factor withdrawal.

2. Material and methods

2.1. Cell lines and cultures

HL-60 (myelocytic), K562 (erithroblastic), and CEM (T-lymphoid) cells, were cultured 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 mM glutamine (Sigma, Spain). Daudi (B-lymphoid) cells were cultured in the same medium supplemented with 20% FCS. Mitochondrial deficient ρ^o 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 [8] and cultured in the same medium as HL-60 cells but with the addition of 1 mM pyruvate and 50 ng/ml uridine (Sigma, Spain). Prior to serum withdrawal experiments, cells were harvested and washed twice in serum-free medium. Viability was assessed by trypan blue dye exclusion method.

2.2. Plasma membrane isolation and CoQ quantification

Microsomes were obtained from cell homogenates and plasma membrane vesicles were then isolated by the two-phase partition method [1]. Membranes were resuspended in 50 mM Tris/HCl, pH 7.6 containing 10% glycerol, 1 mM PMSF and 1 mM DTT, and stored under liquid nitrogen or at -86°C until needed. Purity was checked by marker enzyme analysis [1]. CoQ quantification was performed as described previously [2]. CoQ determination was carried out by HPLC separation with UV monitoring at 275 nm. CoQ was quantified by integration of peak areas and comparison with external standards, and referred to membrane protein. The procedure recovered more than 98% CoQ.

2.3. 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 as described [2]. Fluorescence measurements were carried out at 37°C with a stirred cuvette in a SFM 25 spectrofluorometer (Kontron, Germany). Excitation wavelength was 334 nm and emission wavelength, 375 nm.

2.4. Apoptosis determination

Apoptosis was detected by the labeling of 3′OH ends of DNA breaks (Apotag detection system, Oncor, USA) as specified by the manufacturer, or by cell cycle analysis using flow cytometry after fixation of cells in 70% ethanol and labeling with PI staining buffer (PBS pH 7.5, 0.1 mM EDTA, 50 U/ml DNAse free-RNAse A and 50 μg/ml PI (Sigma, Spain). Cell cycle analysis was performed in a FACScan and data analyzed using a Lysis II software (Becton–Dickinson, USA). Apoptotic cells were determined by the presence of subG_0/1 population.

2.5. Ceramide and SMase activity determination

Ceramide production was determined as previously described [2,21]. Cells were harvested at indicated times of incubation and lipid extracted as described by Bligh and Dyer [5]. After drying under nitrogen and resuspension in chloroform, lipids were used for phosphate measurements and diacylglycerol kinase assay as described [29]. Ceramide-1-phosphate generated in the reaction was resolved by TLC using chloroform/acetone/methanol/acetic acid/water (10 : 4 : 3 : 2 : 1) as solvent. Ceramide-1-phosphate spots were quantified with an automatic TLC-lineal analyzer (Berthold, Germany), using external standards, and normalized to phosphate as indicator of total phospholipids.

N-SMase was measured by the method of Okazaki et al. [28]. Thirty nM final concentration of a mixture of cold and [*methyl*-^{14}C]-sphingomyelin (Amersham Iberica, Spain) was used as substrate for the SMase. Enzymatic hydrolysis of [*methyl*-^{14}C]-sphingomyelin by SMase was quantified using a liquid scintillation counter (Beckman, USA). SMase activity was expressed as nmol sphingomyelin hydrolyzed/mg protein/hour.

2.6. Caspase activity determination

Cell lysates were prepared by five cycles of freezing and thawing and vortex in extraction buffer (50 mM PIPES, pH 7.4; 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM DTT; 20 mM cytochalasin B; 1 mM PMSF; 1 μg/ml leupeptin, 1 μg/ml pepstatin, 50 μg/ml antipain and 10 μg/ml chymopapain). After centrifugation at 12,000 $\times$ *g* for 15 min at 4°C, 50 μg of protein from extract was 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) and CPP32 specific substrate, Ac-DEVD-pNa (Bachem, Switzerland) was added. Activity was developed in 96 well plates at 37°C and *p*-nitroanilide (pNa) release was determined spectrometrically by using a multiwell plate reader at 405 nm. Activity is expressed in units (1 unit = 1 pmol pNa released/30 min/mg protein) as was determined by Enari et al. [9].

3. Results and discussion

The reduced form of CoQ has been proposed to act as a chain-breaking antioxidant in the plasma membrane [11]. This role depends on the existence of a recycling system to reduce the oxidized CoQ [25], and also on its capacity to regenerate α-tocopherol [22]. Two enzymes, the cytochrome b_5 reductase and DT-diaphorase have been shown to act as CoQ-reductases [4,24,36] which are important in the maintenance of appropriate levels of antioxidants in the plasma membrane [7,37].

Cells in culture undergo controlled rates of lipid peroxidation involved in their surviving equilibrium. As is show in Fig. 1, we found a clear inverse correlation between the content of CoQ in the plasma membrane and the rates of lipid peroxidation in different leukemic cell lines when submitted to oxidative stress. The higher rate of peroxidation showed by K562 cells corresponded to the lower content of CoQ. Also differences were found between ρ^oHL-60 cells and parental HL-60 cells, the former showing lower rates of lipid peroxidation and a higher content of CoQ. Accordingly, serum deprivation induces lower rates of both apoptosis and lipid peroxidation in ρ^oHL-60 than in HL-60 cells [2]. Moreover, after serum deprivation the content of CoQ in plasma membrane was increased in ρ^oHL-60 cells than in parental HL-60 cells [2]. Lipid peroxidation induced *in vitro* in egg phosphatidylcholine liposomes enriched with CoQ was prevented by the addition of purified cytochrome b_5 reductase from plasma membrane [35].

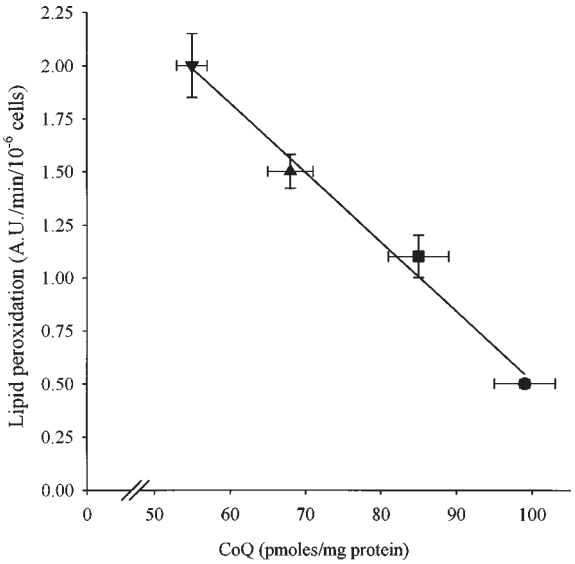

Fig. 1. Relationship between CoQ and lipid peroxidation in leukemic cells in culture. HL-60 (▼), ρ^oHL-60 (■), Daudi (●), and K562 (▲) cells were cultured in 10% FCS-supplemented culture media. Cells were harvested and plasma membrane obtained as described in "Materials and methods". Samples were divided in two fractions and both CoQ and lipid peroxidation rates were determined as described in "Material and methods". Data represent the mean ± SD from four separate experiments.

Thus, CoQ seems to play a central role in antioxidant protection against lipid peroxidation in plasma membrane.

Bcl-2 regulates apoptosis induced by several factors including serum withdrawal [26]. Bcl-2 has been proposed to function as antioxidant protecting cells from oxidative injury induced by most apoptotic factors [16] although this could be due to an indirect effect through the inhibition of cytochrome *c* release from mitochondria and the subsequent production of reactive oxygen species from the mitochondrial electronic chain. In order to test the role of Bcl-2 in the protective effect of antioxidants such as CoQ, ascorbate and α-tocopherol in apoptosis, we studied their effect in HL-60 cells, expressing Bcl-2, and Daudi cells, which do not express this protein. Externally added antioxidants prevented apoptosis induced by serum withdrawal in both cell lines (Fig. 2). In both cases, ascorbate, α-tocopherol and reduced CoQ_{10} prevented apoptosis induced after 48 h of serum withdrawal. Addition of oxidized CoQ_{10} resulted in a less but significant protective effect in both cell lines. Bcl-2 is located in different endomembranes but not in the plasma membrane [16], where the antioxidant system integrated by ascorbate, α-tocopherol and CoQ maintains its protective functions [12,22,24,36,37]. Plasma membrane performs then as the first barrier for the protection against oxidative stress generated from the outer side of cells. Prevention of apoptosis by antioxidants was then independent of the presence of Bcl-2 in cells, suggesting they act upstream Bcl-2 in apoptosis pathway.

To determine how CoQ_{10} prevents apoptosis induced by serum withdrawal in leukemic cells, we studied the release of ceramide, a known factor in apoptosis induced by several agents including serum deprivation [21]. By using CEM cells, a lymphoblastic cell line with moderate expression of Bcl-2, we determined the activity of N-SMase, ceramide release and activation of caspase-3/CPP32, one of the main executioners of apoptosis (Table 1). After 48 h of culture in serum free medium, CEM cells showed a high rate of apoptosis, which was 25% prevented by CoQ_{10} addition. N-SMase activity increased 20 fold in serum-free cultures after 1 h of incubation respect to 10% FCS-cultures. CoQ_{10} addition partially

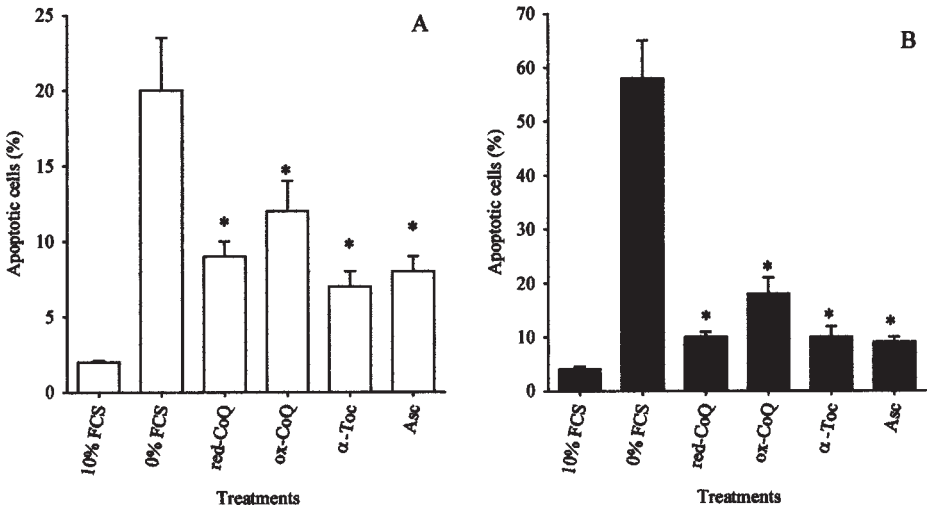

Fig. 2. Effect of antioxidants on apoptosis induced by serum withdrawal. Cells were grown in the absence of serum or supplemented with 0.2 mM ascorbate (Asc), 40 μM α-tocopherol (α-Toc), 40 μM oxidized CoQ_{10} (ox-CoQ) or 40 μM reduced CoQ_{10} (red-CoQ). Control cells were cultured in the presence of 10% FCS. (A) HL-60 cells. (B) Daudi cells. Apoptosis was detected by using Apotag detection kit. *Significant differences vs. serum-free cultures without antioxidant supplement from four different experiments, $p \leqslant 0.05$.

Table 1
Role of CoQ_{10} on the activation of apoptosis in serum-deprived CEM cells

Culture conditions	Apoptosis (%)	N-SMase (nmol SM /mg protein/h)	Ceramide (pmol/nmol Pi)	CPP32 (units)
10% FCS	5	2.0	45	1200
0% FCS	80	40.5	90	4100
+ CoQ_{10}	60*	27.3*	46*	2450*

Apoptosis was determined by flow cytometry after 48 h of incubation. N-SMase and ceramide were determined after 1 h of incubation. CPP32 activity was determined after 2 h of incubation. Data represent the mean ($n = 3$), and *significant differences *vs.* culture in absence of serum, $p \leqslant 0.05$. SD was less than 10% in each case.

inhibited N-SMase activation. Ceramide release was also inhibited by CoQ_{10} addition to serum-free cultures. The role of ceramide in apoptotic program has been related to the activation of CPP32 [14]. CPP32 activity was increased in serum-free cultured cells and the addition of CoQ_{10} inhibited about 40% of this increase. Thus, the presence of CoQ_{10} in serum-free cultures partially avoided the activation of apoptotic machinery through inhibition of SMase pathway. Ceramide quantification must be taken as qualitative instead of quantitative since the diacylglycerol kinase assay used is currently under discussion [33].

To date, the mechanism of SMase activation after serum withdrawal is not known, but must be related to the oxidative stress and lipid peroxidation produced in cultured cells by serum withdrawal [2]. Phospholipase A_2 (PLA_2) is activated by hydroperoxides in plasma membrane [15,30]. Also, a dramatic increase in Ca^{2+}-independent PLA_2 activity is induced by oxidative damage *in vivo* [23], possibly to increase metabolism of fatty acid hydroperoxides [34]. Moreover, PLA_2 has been related to activation of N-SMase in HL-60 cells and murine fibroblasts [19,20]. Thus, CoQ_{10} and other antioxidants added

to serum-free cultures and its incorporation to plasma membrane could avoid lipid peroxidation and subsequently impair activation of both PLA_2 and N-SMase, preventing the apoptotic machinery.

4. Conclusion

Antioxidants prevent apoptosis induced by mild oxidative stress caused by serum removal acting as chain-breaking factors in lipid peroxidation. CoQ is directly related to the lipid peroxidation rate in plasma membrane. Its presence in serum-deprived cultures inhibits N-SMase activation and ceramide release in cytosol, blocking the progression through the apoptosis program, including activation of caspases. So, the maintenance of the reduced state of CoQ_{10} in plasma membrane by enzymatic recycling and the other components of antioxidant system, ascorbate and α-tocopherol, is essential in the survival of cells affected by oxidative damage.

Acknowledgements

Supported by the Spanish D.G.E.S. Grant nr PB95-0560.

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