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# Ascorbate Stabilization Is Stimulated in $\rho^{\circ}$HL-60 Cells by $CoQ_{10}$ Increase at the Plasma Membrane

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**Long-term treatment with ethidium bromide of HL-60 cells induced a mitochondria-deficient $\rho^{\circ}$ cell line, where mitochondrial DNA can not be identified by PCR and cytochrome *c* oxidase activity was 80% decreased. These cells showed a progressive increase of ascorbate stabilization which was 52% higher in the established $\rho^{\circ}$HL-60 cells. Both $CoQ_{10}$ and NADH-ascorbate free radical reductase of the plasma membrane were increased in $\rho^{\circ}$ HL-60 cells compared to parental cells, while NADH-cytochrome *c* reductase was unchanged. $CoQ_{10}$ is a component of the ascorbate stabilization activity in the plasma membrane that would provide both a mechanism to deplete the excess of NADH produced in $\rho^{\circ}$ HL-60 cells and for resistance to oxidative stress.** 

Stabilization of ascorbate is an enzymatic mechanism that maintains the antioxidant property of this vitamin at the plasma membrane (1), resembling the properties of the ascorbyl radical quenching at the cell surface (2). This activity is modulated by growth factors (3), cAMP (4) and N-myc expression (5). $CoQ_{10}$ is a lipid-soluble antioxidant that mediates the electron transport in the plasma membrane (6), and is reduced by a phospholipid-dependent NADH-$CoQ_{10}$ reductase activity (7,8). This activity is also required for the reduction of the ascorbate free radical (AFR) in the plasma membrane and, as a consequence, external ascorbate is stabilized (9).

Long-term treatment of animal cells with ethidium bromide depletes cells of their mitochondrial DNA (mtDNA) (10), but surviving $\rho^{\circ}$ cells require uridine and pyruvate to maintain growth (11). The latter provides a mechanism for the reoxidation of excess NADH generated during glycolysis (12), and can be replaced by $CoQ_{10}$ for this purpose (13). Ethidium bromide-induced $\rho^{\circ}$ HL-60 cells showed decreased respiration parallel to an increase of the ascorbate stabilization, and also both $CoQ_{10}$ content and the $CoQ_{10}$-dependent NADH-AFR reductase were increased in the plasma membrane. The increased $CoQ_{10}$ and reductase in the plasma membrane can serve to oxidize internal NADH to provide $NAD^+$ for glycolysis as well as providing external resistance to oxidative stress (14).

## MATERIALS AND METHODS

*Cell lines and culture conditions.* Cells deficient in mitochondria ($\rho^{\circ}$) were generated by culturing HL-60 cells in the presence of 50 ng/ml ethidium bromide (Sigma, Spain) for 5-6 weeks as described (10). Both HL-60 and $\rho^{\circ}$ HL-60 cells were 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. $\rho^{\circ}$ HL-60 cells were also supplemented with 50 ng/ml uridine and 1 mM pyruvate. MtDNA was determined the PCR using $D_H$ and $E_L$ primers as described, using λ DNA as marker (15). Cells were concentrated from stock cultures by centrifugation at 1,000 xg for 5 min and washed twice in serum-free medium. Cell viability was determined by trypan blue exclusion.

*Plasma membrane preparation and $CoQ_{10}$ quantification.* Microsomes were obtained from HL-60 or $\rho^{\circ}$HL-60 cell homogenates. Plasma membrane vesicles were then isolated by the two-phase partition method (16). 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 (16).

*Enzyme activities.* Cytochrome *c* oxidase as a marker of respiratory chain was measured as described in microsomal fractions (17). Ascorbate stabilization was calculated from the difference between the rates of ascorbate oxidation (direct reading at 265 nm) with and without cells (4). NADH-AFR reductase was assayed in plasma membrane fractions by measuring NADH oxidation at 340 nm upon addition of 66 mU ascorbate oxidase to a reaction mixture containing 0.4 mM ascorbate (7). NADH-cytochrome *c* reductase was assayed in plasma membrane fractions as described (7).

## RESULTS AND DISCUSSION

Treatment of animal cells for a long period inhibits the replication of mitochondrial DNA by intercalating

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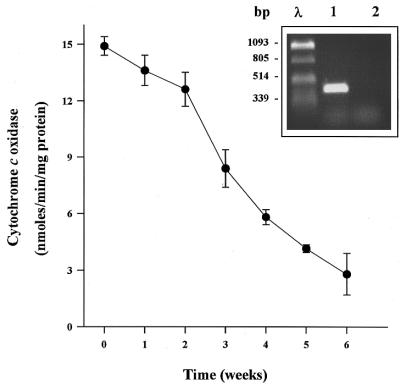


**FIG. 1.** Effect of ethidium bromide treatment on mitochondria of HL-60 cells. Cytochrome *c* oxidase activity. was assayed in microsomal fractions isolated from cells harvested once a week during the treatment. Specific activity: mean ± SD. n=4. Insert: PCR products of mtDNA in parental HL-60 cells (1) and HL-60 ($\rho^\circ$) cells treated for 6 weeks with ethidium bromide (2).

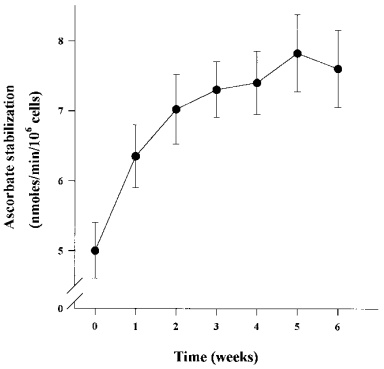


**FIG. 2.** Time course of ascorbate stabilization activity during the treatment of HL-60 cells with ethidium bromide. The stabilization of ascorbate was calculated as the difference between the rates of ascorbate oxidation with and without cells. Specific activity: mean ± SD. n=5.

between adjacent DNA base pairs (18), and as a consequence mtDNA is depleted in these cells (10) although surviving $\rho^\circ$ cells can grow if supplemented with uridine and pyruvate (11). During the treatment of HL-60 cells with 50 $\mu$M ethidium bromide, after an initial period of rapid cell death, surviving cells progressively lost cytochrome *c* oxidase activity as a marker for the respiratory chain (Fig. 1). After 6 weeks of treatment, the clone of $\rho^\circ$ HL-60 cells selected showed about 80% of loss of this activity. Similar levels of inhibition of the mitochondrial electron chain were observed in ethidium bromide-treated L929 cells (19). PCR using the primers $D_H$, complementary to the mitochondrial genome positions 11918-11942, and $E_L$, complementary to the positions 11580-11603 (15) established that no mtDNA was detected after the fourth week of treatment (Fig. 1, insert) Depletion of both the mitochondrial respiratory chain and mtDNA is characteristic of other $\rho^\circ$ cells induced by ethidium bromide, that also show a decline of oxygen uptake and an increase of aerobic glycolysis (19,20).

During the establishment of the $\rho^\circ$ HL-60 cell line, ascorbate stabilization through a transplasma membrane electron transport system (1) was increased in parallel to respiratory chain depletion (Fig. 2). Maximum activity about 50% higher than control was reached after the fifth week and $\rho^\circ$ HL-60 cells from older cultures were used for the analysis of plasma membrane as presented in Table I. Plasma membrane fractions isolated by two-phase partition have almost no mitochondrial contamination and thus are appropriate for the study of $CoQ_{10}$ content and redox reactions (16,21). We have shown that $CoQ_{10}$ is an electron carrier involved in both the ascorbate stabilization and the NADH-AFR reductase in the plasma membrane (7,9). $CoQ_{10}$ content was increased about 40% in plasma membranes from $\rho^\circ$ HL-60 cells compared to those from parental cells, and similar levels of increase were observed for both NADH-AFR reductase (37%) and ascorbate stabilization (52%). However, NADH-cytochrome *c* reductase. that is independent of $CoQ_{10}$ (7) was unchanged (Table I). Up-regulation of the ferricyanide reduction during the ethidium bromide treatment of human Namalwa cells was also observed (20), but ferricyanide reduction measures redox enzymes in the plasma membrane (22) and is not increased by addition of $CoQ_{10}$ to liver plasma membrane (6).

$CoQ_{10}$ can replace pyruvate to maintain growth of $\rho^\circ$ Namalwa cells (13), and maintains growth of different cells in serum-limiting media (23). Also, $CoQ_{10}$ protects HL-60 cells from both cell death and lipid peroxidation caused by serum removal (14). $CoQ_{10}$ is a component of the transplasma membrane electron transport system that is maintained reduced by the cytochrome b5 reduc-

**TABLE I**

Redox Characteristics of Plasma Membranes from HL-60 and $\rho^\circ$HL-6o Cells

| Parameter | HL-60 cells | $\rho^\circ$HL-60 cells | Ratio |
|---|---|---|---|
| Ascorbate stabilization[a] | 5.0 ± 0.4 | 7.2 ± 0.2 | 1.52 |
| NADH-AFR reductase | 4.8 ± 0.1 | 6.5 ± 0.3 | 1.35 |
| Coenzyme $Q_{10}$ | 70 ± 6 | 99 ± 5 | 1.40 |
| NADH cytochrome *c* reductase | 31 ± 4 | 29 ± 2 | 0.93 |

*Note.* Plasma membrane fractions were purified by the two-phase partition method and analyzed by biochemical markers to confirm that mitochondrial vesicles were absent (21,29). Specific activities are expressed as nmoles/min/$10^6$ cells for ascorbate stabilization, nmoles/min/mg protein for NADH-AFR reductase and NADH-cytochrome *c* reductase, and $CoQ_{10}$ content as pmoles/mg protein. Values were determined from four experiments ± SD. Ratio determined between $\rho^\circ$HL-6o and parental cells.

[a] Carried out in whole cells.

tase (7,8), and can then reduce both $\alpha$-tocopherol and ascorbate (9, 24). This system protects from membrane damage caused by mild oxidative stress, where $CoQ_{10}$ appears to be the central regulator (6). Also, the inhibition of the plasma membrane electron transport system increases cell death (20,25). Thus, the overexpression of $CoQ_{10}$ at the plasma membrane, and the corresponding increase of the $CoQ_{10}$-dependent electron transport, would explain the resistence of $\rho^{\circ}$ cells to redox mediated cell injury (14,19).

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