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Coenzyme Q_{10} Prevents Apoptosis by Inhibiting Mitochondrial Depolarization Independently of Its Free Radical Scavenging Property*

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Laura Papucci, Nicola Schiavone, Ewa Witort, Martino Donnini, Andrea Lapucci‡, Alessio Tempestini, Lucia Formigli§, Sandra Zecchi-Orlandini§, Giovanni Orlandini§, Giuseppe Carella¶, Rosario Brancato¶, and Sergio Capaccioli‖

From the Department of Experimental Pathology and Oncology and §Department of Anatomy, Histology, and Forensic Medicine, University of Florence, 50134-Florence, and the ¶Department of Ophthalmology and Visual Sciences, University-Hospital San Raffaele of Milan, 20132 Milan, Italy

The permeability transition pore (PTP) is a mitochondrial channel whose opening causes the mitochondrial membrane potential ($\Delta\psi$) collapse that leads to apoptosis. Some ubiquinone analogues have been demonstrated previously to modulate the PTP open-closed transition in isolated mitochondria and thought to act through a common PTP-binding site rather than through oxidation-reduction reactions. We have demonstrated recently both *in vitro* and *in vivo* that the ubiquitous free radical scavenger and respiratory chain coenzyme Q_{10} (CoQ_{10}) prevents keratocyte apoptosis induced by excimer laser irradiation more efficiently than other antioxidants. On this basis, we hypothesized that the antiapoptotic property of CoQ_{10} could be independent of its free radical scavenging ability and related to direct inhibition of PTP opening. In this study, we have verified this hypothesis by evaluating the antiapoptotic effects of CoQ_{10} in response to apoptotic stimuli, serum starvation, antimycin A, and ceramide, which do not generate free radicals, in comparison to control, free radical-generating UVC irradiation. As hypothesized, CoQ_{10} dramatically reduced apoptotic cell death, attenuated ATP decrease, and hindered DNA fragmentation elicited by all apoptotic stimuli. This was accompanied by inhibition of mitochondrial depolarization, cytochrome *c* release, and caspase 9 activation. Because these events are consequent to mitochondrial PTP opening, we suggest that the antiapoptotic activity of CoQ_{10} could be related to its ability to prevent this phenomenon.

Ubiquinone Q_{10} (coenzyme Q_{10}, CoQ_{10})[1] is both a well known electron transporter in complexes I (NADH-ubiquinone oxidoreductase), II (succinate-ubiquinone oxidoreductase), and III (ubiquinone-cytochrome *c* oxidoreductase) of mitochondrial respiratory chain and a ubiquitous free radical scavenger (1, 2). Our preliminary observations that CoQ_{10} is endowed with antiapoptotic activity as a free radical scavenger (3) prompted us to challenge the ability of CoQ_{10} to prevent keratocyte apoptosis induced by excimer laser photorefractive keratectomy. Apoptotic death of keratocytes is the earliest pathogenetic event of haze formation, the main drawback of photorefractive keratectomy (4, 5). We demonstrated that a 2-h pretreatment with CoQ_{10} lowered the number of apoptotic keratocytes in response to excimer laser irradiation to a much higher extent than other free radical scavengers both *in vitro* (6) and *in vivo* (5). The antiapoptotic effects of CoQ_{10} as free radical scavenger have been demonstrated consistently by others (2, 7–11) in parallel or in successive works.

The mitochondrial permeability transition pore (PTP) is a complex, large conductance channel that plays a pivotal role in triggering apoptosis (12). The opening of PTP is responsible for disruption of the mitochondrial transmembrane electrochemical gradient ($\Delta\psi$ from −180 to 0 mV). The oligomerization of apoptotic members of the *bcl-2* family follows, thus forming mitochondrial transmembrane channels through which a number of apoptotic factors are released into the cytoplasm to trigger the mitochondrion-dependent (intrinsic) pathway of apoptosis execution (13–15). This is orchestrated by caspase cascade activation, which leads to cleavage of cellular substrates. Mitochondrion-dependent (intrinsic) and membrane death receptor-dependent (extrinsic) apoptotic pathways are known to share the same caspases with two exceptions: caspase 9 for the former (16) and caspase 8 for the latter (17). Factors and mechanisms involved in PTP formation are still unclear. Nevertheless, three proteins, the adenine nucleotide translocator, the voltage-dependent anion channel, and cyclophilin-D, appear to play the main structural role (13–15, 18). Besides a number of ancillary structural/regulative proteins, such as benzodiazepine peripheral receptor, creatine kinase, hexokinase, and Bax, that are recruited by the voltage-dependent anion channel-adenine nucleotide translocator-cyclophilin-D complex, several molecules are positive (Bax, Ca^{2+}, ROS, and atractyloside) or negative (Bcl-2, ATP, bongkrekic acid, and cyclosporin A) determinants of PTP opening (15, 18).

Some authors (19–24) demonstrated in isolated mitochondria that the PTP harbors a ubiquinone-binding site and is regulated by complex I of the mitochondrial respiratory chain. Irrespective of the method used to induce permeability transition, the opening of PTP was strongly inhibited by some ubiqui-

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‖ To whom correspondence should be addressed: Dept. of Experimental Pathology and Oncology, University of Florence, Viale G.B. Morgagni 50, 50134-Florence, Italy. Tel.: 39-55-4282308; Fax: 39- 55-4282309; E-mail: sergio@unifi.it.

[1] The abbreviations used are: CoQ_{10}, coenzyme Q_{10}; PTP, permeability transition pore; ROS, reactive oxygen species; FBS, fetal bovine serum; MDA, malondialdehyde; 4-HNE, 4-hydroxy-2-nonenal; SOD, superoxide dismutase; Ub, ubiquinone; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; $\Delta\psi$, mitochondrial membrane potential.

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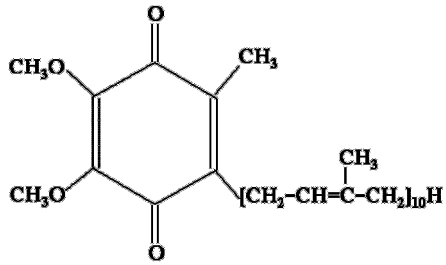
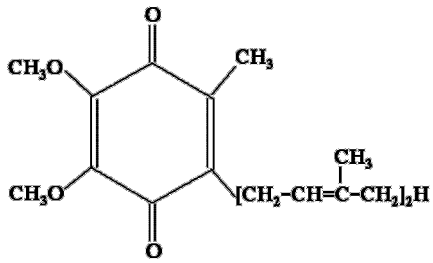


Q_{10}



Q_2

FIG. 1. **The chemical structures of the physiological coenzyme Q_{10} and its synthetic analog CoQ_2 (Ub_{10}, see Fig. 1 of Ref. 23).** Their difference is restricted to the number of isoprenoid units in the side chain; the 10 units (50 carbon atoms) in the CoQ_{10} are substituted by 2 units (10 carbon atoms) in the synthetic analog CoQ_2 (Ub_{10}).

none analogues, including Ub_0, decyl-Ub, and Ub_{10}, whereas other tested quinones were ineffective or had antithetic effects (23). Specific structural features have been demonstrated to be necessary for regulation of PTP by ubiquinone analogues, independently of their ability to decrease mitochondrial ROS production to the same extent. These results supported the idea that PTP regulation by ubiquinones is exerted through binding to a common site rather than through redox reactions (23). Interestingly, the natural CoQ_{10} has a very strict structural analogy with the synthetic Ub_{10} (CoQ_2); as shown in Fig. 1, their difference is restricted to the number of side isoprenoid units within the side chain, which has been reported to not influence substantially the folding of the molecule (25). These considerations prompted us to hypothesize that what was observed with ubiquinone analogues applied to isolated mitochondria (19–24) could underlie a natural phenomenon operating in the whole cell. In line with this possibility, the respiratory chain complex I has been suggested to be a constituent of mitochondrial PTP (26). Armstrong and co-workers (27, 28) have designed a new model for mitochondrial PTP, in which the ROS producer respiratory chain complex III is functionally linked to the permeability transition. The ability of complex III to generate ROS and to open mitochondrial PTP could override the antiapoptotic function of Bcl-2. Noteworthy, both respiratory chain complexes I and III share CoQ_{10} as electron acceptor or electron donor, respectively. These observations led us to explore the possibility that, besides its free radical scavenging property, CoQ_{10} may be endowed with antiapoptotic activity as a modulator of PTP opening.

In this study we demonstrate that CoQ_{10} provides effective protection against apoptosis of rabbit keratocytes not only in response to the free radicals generated by UVC irradiation but also in response to three apoptotic stimuli generally known to act independently of free radical generation such as chemical hypoxic drug antimycin A (29), the C_2-ceramide, a cell-permeable analogue of natural lipidic apoptotic messenger ceramide (30), and survival factor withdrawal (serum starvation) (18). This evidence emerged from examination of two classes of different parameters: 1) ROS, MDA, and superoxide dismutase (SOD) activity levels for assessment of the effect of our treatments on free radical generation; and 2) cellular morphology, number of living and apoptotic cells, ATP levels, and DNA status for assessment of the effect of our treatments on cell life and death. In contrast, another well known free radical scavenger, vitamin E (α-tocopherol) (31–32), whose effect in association with CoQ_{10} has been described in our previous work (5), provides effective protection against apoptosis only in response to UVC irradiation.

The antiapoptotic activity of CoQ_{10} was mediated by hindering mitochondrial depolarization, cytochrome *c* release to cytoplasm, and procaspase 9 activation. This suggests that the mechanism by which CoQ_{10} prevents apoptosis may involve inhibition of mitochondrial PTP opening that triggers the intrinsic execution pathway of apoptosis. This possibility and the hypothesis that this novel functional activity may underlie physical localization of CoQ_{10} in the PTP, possibly in association with other components of the complex, is now under study.

EXPERIMENTAL PROCEDURES

Cell Cultures—Rabbit corneal keratocytes (RCE cell line) were maintained in Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12 1:1, supplemented with 15% fetal bovine serum (FBS), 2 mM glutamine, 5 μg/ml insulin, 10 ng/ml epidermal growth factor, and 50 IU/ml penicillin, in humidified atmosphere of 5% CO_2 at 37 °C. Cells were plated at the density of 3×10^5 cells/plate.

Treatments—Each of the four damaging agents was applied at doses experimentally established to induce apoptosis: UVC irradiation (254 nm) at 15 mJ/cm^2, the respiratory chain blocker antimycin A at 200 μM concentration, the apoptotic signaling lipid C_2-ceramide (a synthetic cell-permeable analogue of endogenous ceramides) at 20 μM concentration, and FBS restriction to 0.5%. Treatments with 10 μM CoQ_{10} or with 10 μM vitamin E (α-tocopherol), both dissolved in 0.04% Lutrol F127 used as vehicle to ensure cellular uptake of this hydrophobic molecule (5, 6), were commenced 2 h prior to application of apoptotic stimuli. Vehicle alone-treated cells were used as controls. In some experiments vitamin C (ascorbic acid) was also used.

Identification of Apoptosis on the Basis of Cellular Morphology and DNA End Labeling by Klenow-FragEL™ Assay—Apoptotic RCE cells were identified by light microscopy following end labeling of DNA fragments by Klenow-FragEL™ kit (Oncogene Research Products, Boston). Briefly, following apoptotic treatments, the keratocytes were detached from substrate and fixed in 4% formaldehyde and 80% ethanol at a concentration of 10^6 cells/ml. Prior to detection, the cells (3×10^5) were affixed to glass slides by cytocentrifugation, air-dried, and processed according to the manufacturer's instructions. In this assay Klenow binds to exposed ends of DNA fragments and catalyzes the template-dependent addition of biotin-labeled deoxyribonucleotides, which are then detected using streptavidin-horseradish peroxidase conjugate. Diaminobenzidine reacts with the labeled fragmented DNA of apoptotic cell nuclei, generating a dark brown insoluble chromogen that contrasts with counterstained methyl green cytoplasms. In contrast, viable cells appear uniformly green or even unstained.

Measurement of ROS and MDA-4-HNE Levels and of SOD Activity—The extent of free radical generation by cultured cells exposed to UVC, antimycin A, C_2-ceramide, or FBS restriction to 0.5% was measured by means of three independent indexes: reactive oxygen species (ROS), lipid peroxidation level, and superoxide dismutase activity. Generation of ROS was assessed immediately after treatments, using 2′,7′-dichlorofluorescein diacetate (Sigma) (33), by a confocal laser scanning microscope (Bio-Rad MRC 1024 ES scanning microscope) equipped with a krypton/argon laser source. The emitted fluorescence was monitored at 488 and 568 nm wavelengths with a Nikon plan Apo × 60 oil-immersion objective. To this purpose, series of optical sections (512 × 512 pixels) were taken through the depth of cells with a thickness of 1 μm at intervals of 0.8 μm. Twenty optical sections for each samples examined were projected as a single composite image by superimposition. To monitor the rate of lipid peroxidation, the levels of typical end products

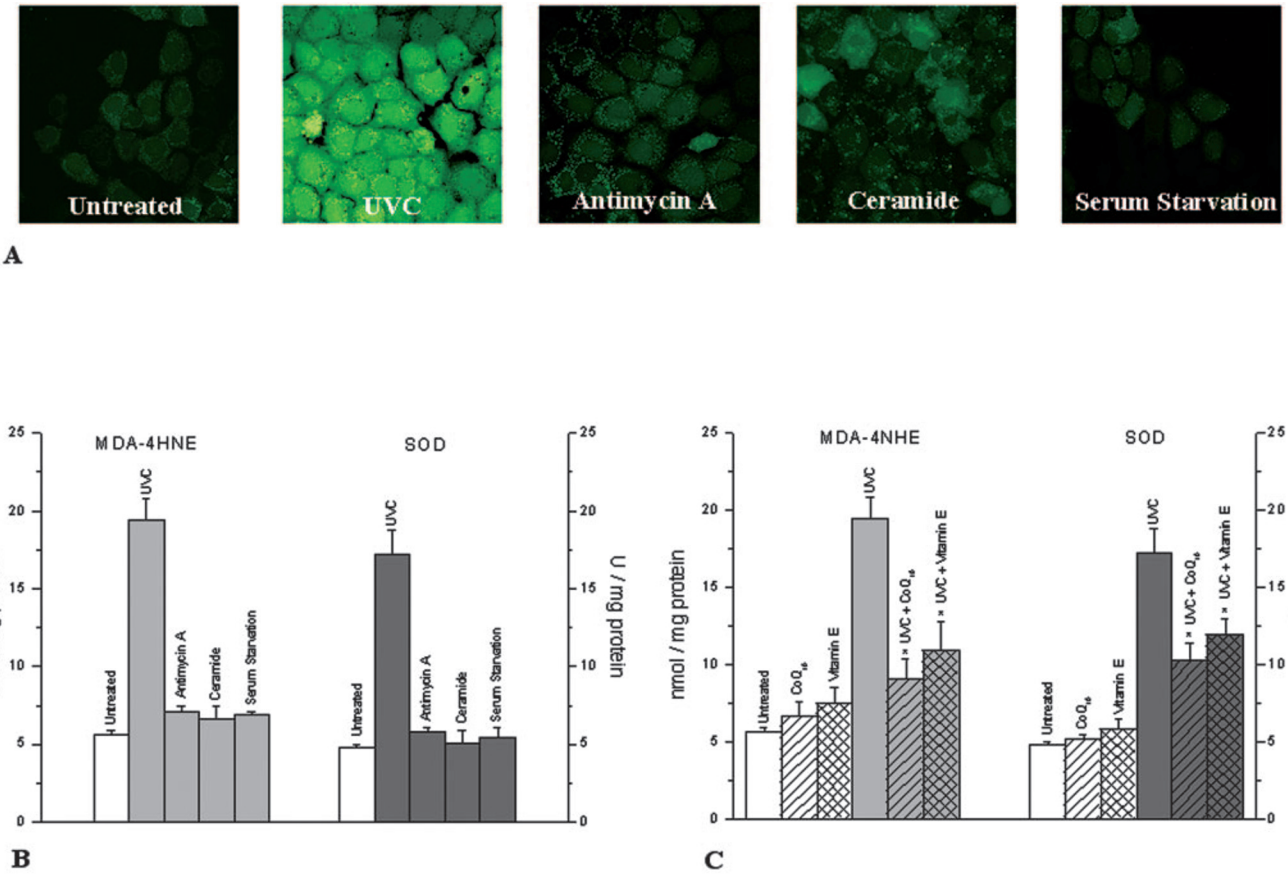


FIG. 2. **Quantification of ROS, MDA-4-HNE, and SOD activity levels in RCE cells after application of UVC at 254 nm (15 mJ/cm^2), antimycin A (200 μM), ceramide (20 μM), or serum starvation (0.5% fetal calf serum).** *A*, ROS cellular levels were evaluated by confocal microscopy following labeling with the ROS-specific fluorescent indicator immediately after administration of apoptotic stimuli, as described previously (33, 36). *B*, MDA-4-HNE cellular levels (*left panel*) and SOD activity (*right panel*) evaluated at the 2nd h after administration of apoptotic stimuli. Both parameters increase only in response to UVC irradiation. *C*, inhibitory effect of a 2-h pretreatment with 10 μM CoQ_{10} or vitamin E against the increase of MDA-4-HNE cellular levels (*left panel*) and SOD activity (*right panel*) evaluated at the 2nd h after UVC irradiation. Each point was the mean ± S.E. of three experiments; *, $p \leq 0.001$ compared with UVC alone.

of the process, which are MDA plus 4-hydroxy-2-alkenals, exemplified by 4-hydroxy-2-nonenal (4-HNE), were determined in the cellular extracts prepared and analyzed at 2 h following treatments. Measurements were made using a colorimetric method at 586 nm, according to the reaction of a chromogenic reagent, *N*-methyl-2-phenylindole, with MDA and 4-HNE in the presence of methanesulfonic acid at 45 °C (34). SOD activity was determined at 2 h following treatments using a spectrophotometric assay (Bioxytech, Portland, OR). For this purpose, RCE cells were lysed in 4 volumes of water added to 1 volume of cells, and the cellular lysates were extracted with ethanol/chloroform 62.5: 37.5 (v/v) and vortexed for 30 s. Cellular membranes were separated by centrifugation at 3000 × *g* at 4 °C for 10 min. The upper phase was collected to determine SOD activity. The absorbance of samples was measured spectrophotometrically at 525 nm.

Quantification of Living and Apoptotic Cells—The number of living cells was evaluated by the MTT (*C,N*-diphenyl-*N*′-4,5-dimethyl thiazol-2-yl tetrazolium bromide) colorimetric assay (reduction of tetrazolium salt to formazan as described previously (35)). The cumulative apoptotic events were scored by the time-lapse videomicroscopy using a Zeiss inverted phase contrast microscope equipped with a 10× objective, Panasonic CCD cameras, and JVC BR9030 time-lapse video recorders. After cell detachment from the substrate, an apoptotic event was counted the moment the cell had shrunk completely and blebbing started (36).

Analysis of ATP Levels—Cellular levels of ATP were evaluated 24 h following apoptotic stimuli, using untreated cells as control as reported previously (36). The cells (2–3 × 10^5) were pelletted, resuspended in distilled water, and boiled for 3–5 min. Samples were then cooled to room temperature and stored frozen at −20 °C for later measurements. ATP in the extracts was quantified by a bioluminescence assay with an ATP determination kit (Molecular Probes), using a liquid scintillation analyzer (Camberra Packard) for bioluminescence analysis, according to the manufacturer's instructions.

Detection of Change in Mitochondrial Transmembrane Potential ($\Delta\psi$)—The change in $\Delta\psi$ occurring during apoptosis was detected by fluorescence-based assay. The RCE cells were cultured on coverslips in Dulbecco's modified Eagle's medium containing the lipophilic cationic probe 5,5′,6,6′-tetrachloro-1,1′3,3′-tetraethylbenzimidazol-carbocyanine iodide (JC-1, 5 mg/ml, Molecular Probes, Eugene, OR) for 15 min at 37 °C. This dye has a unique feature: at hyperpolarized membrane potentials (to −140 mV) it forms a red fluorescent J-aggregate, whereas at depolarized membrane potentials (to −100 mV) it remains in the green fluorescent monomeric form. Prior to detection, cells were washed in phosphate-buffered saline and placed in an open slide-flow loading chamber that was mounted on the stage of a confocal scanning microscope (Bio-Rad) equipped with a krypton/argon laser source. The emitted fluorescence was monitored at 488 and 568 nm wavelengths with a Nikon plan Apo ×60 oil-immersion objective (37). Imaging acquisition of the optical sections through the cell was performed as described above.

Western Blot Analysis of Cytoplasmic Cytochrome c—RCE cells were evaluated 24 h following application of apoptotic stimuli. Cytosolic fractions were prepared as reported previously (38). Proteins in the cytosolic extracts were quantified by the BCA Protein Assay Reagent (Pierce). Proteins (25 μg/lane) were electrophoresed through SDS-polyacrylamide 12.5% gel and electroblotted onto nitrocellulose membrane (Schleicher & Schuell) using a transblotter (Bio-Rad). The nonspecific signals were blocked with blocking buffer (5% w/v instant nonfat milk powder in phosphate-buffered saline) and incubated overnight at 4 °C with 1 μg/ml of anti-cytochrome *c* monoclonal antibody (Pharmingen). The membrane was washed and subsequently incubated with goat anti-mouse IgG horseradish peroxidase conjugate (Sigma). Detection was carried out using a commercial chemiluminescence procedure (Amersham Biosciences).

Analysis of Caspase 9 Activity—Caspase 9 activity was determined by the Caspase-9 Colorimetric Protease Assay (BioSource Europe, S.A.,

Nivelles, Belgium). Cytosolic extracts, prepared by lysing cells with Cell Lysis Buffer provided in the kit, were incubated with the colorimetric substrate Leu-Glu-His-Asp (LEHD) conjugated to the chromophore *p*-nitroanilide, in 50 μl of 2× Reaction Buffer containing 10 mM dithiothreitol. After a 2-h incubation at 37 °C, the absorbance of samples was measured at $A^{405\ nm}$ in Bio-Rad enzyme-linked immunosorbent assay reader.

Analysis of DNA (Nucleosomal Laddering)—Apoptotic internucleosomal DNA fragmentation was evaluated by classical assay, detecting electrophoretically a separated ladder of fragmented DNA. The genomic DNA was extracted from RCE cells as described by Blankenberg *et al.* (39). The fragments were separated by gel electrophoresis in 0.8% agarose containing ethidium bromide (0.2 μg/ml), UV-visualized, and photographed.

Statistical Analysis—The statistical evaluation of the data was performed with the two-tailed Student's *t* test for unpaired values. Differences were considered statistically significant when $p < 0.05$. The data are reported as percentage of the maximal value.

RESULTS

UVC Irradiation, Antimycin A, C_2-ceramide, and Serum Starvation Committed Cultured Keratocytes to Apoptosis—We have previously optimized by time-lapse videomicroscopy the doses of stimuli necessary to commit a substantial number of RCE keratocytes to apoptosis, but not to necrosis, within 24 h after treatments. The following doses were chosen: 15 mJ/cm^2 UVC irradiation (254 nm), 200 μM antimycin A, 20 μM C_2-ceramide, and serum starvation (0.5% FBS).

Free Radical Generation Was Restricted to UVC-Irradiation—We have then analyzed the effects of the apoptotic doses of UVC irradiation, antimycin A, C_2-ceramide and serum starvation on free radical generation. For this purpose, three different parameters, which are cellular levels of ROS, MDA-4-HNE levels, and SOD activity, were analyzed after application of apoptotic stimuli (Fig. 2). As shown in Fig. 2*A*, UVC irradiation was the only apoptotic stimulus able to generate ROS as visualized, immediately after treatment, by confocal microscopy following ROS labeling with the fluorescent indicator (2′,7′-dichlorofluorescein diacetate) as described by Formigli *et al.* (36). In keeping with this observation, Fig. 2*B* shows that only UVC irradiation markedly enhanced MDA-4-HNE levels (*left panel*) and SOD activity (*right panel*), evaluated at the 2nd h after treatment, compared with untreated cells (from 5.6 nmol/mg of protein to 19.4 nmol/mg of protein, and from 4.8 units/mg of protein to 17.2 units/mg of protein, respectively). Increase of ROS, MDA-4-HNE, and SOD activity induced by UVC irradiation was substantially lowered by a 2-h pretreatment with either 10 μM CoQ_{10} or vitamin E (Fig. 2*C*).

CoQ_{10} but Not Vitamin E Increased Cell Survival in Response to Free Radical Independent Apoptotic Stimuli—Once ascertained that apoptosis by antimycin A, C_2-ceramide and serum starvation was not consequent to free radical generation, the effects of a 2-h pretreatment with 10 μM CoQ_{10} or vitamin E on cell survival at the 24th h following application of apoptotic stimuli was evaluated by the MTT assay. As shown in Fig. 3, UVC irradiation, antimycin A, C_2-ceramide, and serum starvation induced a dramatic decrease in the number of living cells compared with untreated controls, which was significantly attenuated by pretreatment with CoQ_{10} (*upper panel*: from 82 to 49%, from 56 to 29%, from 61 to 24%, and from 51 to 80%, respectively). On the contrary, pretreatment with vitamin E was able to attenuate significantly this decrease only in response to UVC irradiation (*lower panel*: from 82 to 58%).

The effects of the 2-h pretreatment with 10 μM CoQ_{10} or vitamin E on cell apoptosis induced by UVC irradiation, antimycin A, C_2-ceramide, and serum starvation were then evaluated using light microscopy and ultramicroscopy (not shown) as reported previously (3, 6, 36). Identification of apoptotic cells with fragmented DNA was also carried out by end labeling with

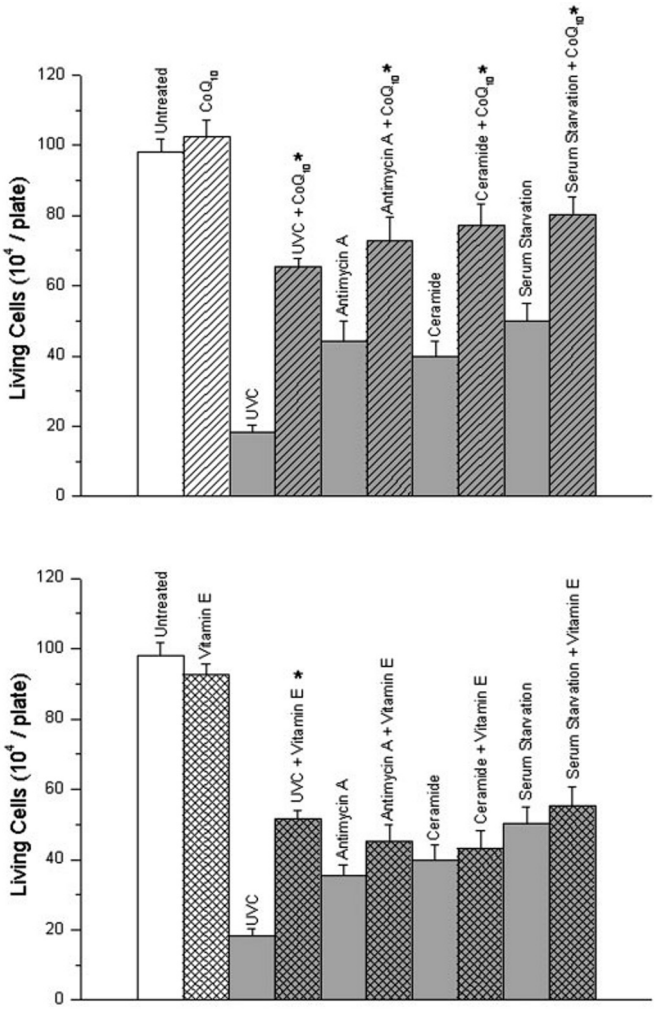


FIG. 3. **RCE survival expressed as number of living cells/plate determined by MTT analysis at the 24th h after UVC irradiation or treatment with 200 μM antimycin A, 20 μM ceramide, or serum starvation (FBS 0.5%) preceded or not by pretreatment with 10 μM CoQ_{10} (*upper panel*) or 10 μM vitamin E (*lower panel*).** Cells were initially plated at 3×10^5 cells/plate. Each point is the mean ± S.E. of three experiments. CoQ_{10} increased cell survival in response to all treatments, whereas vitamin E was effective only in response to UVC irradiation. *, $p \leq 0.005$ compared with cells subjected only to pretreatment with either CoQ_{10} or vitamin E.

the Klenow-FragEL™, by which apoptotic cells are easily recognized by the presence of a dark brown stain in contrast to viable cells that appear green or even unstained. At the 24th h following treatment with UVC irradiation, antimycin A, C_2-ceramide, and serum starvation, a significant number (about 30–60% depending on type of apoptotic stimulus) of RCE cells contained brown-stained fragmented DNA (apoptotic cells). Treatment with 10 μM CoQ_{10} 2 h before application of apoptotic stimuli dramatically reduced the number of brown-stained cells, so that the number of cells stained green (viable cells) overcame 90% of all cells. Treatment with 10 μM vitamin E significantly reduced brown-stained (apoptotic) cells in response to UVC irradiation but not in response to the other, free radical unrelated, apoptotic stimuli. Results obtained with UVC and serum starvation, either pretreated or not with CoQ_{10} or vitamin E, are shown in Fig. 4. Quite similar results have been obtained using 10 μM vitamin C (ascorbic acid) as antioxidant (not shown).

CoQ_{10} Decreased the Number of Apoptotic Events in Response to Free Radical Independent Apoptotic Stimuli—The possibility that CoQ_{10} increased cell survival by inhibiting apoptosis was

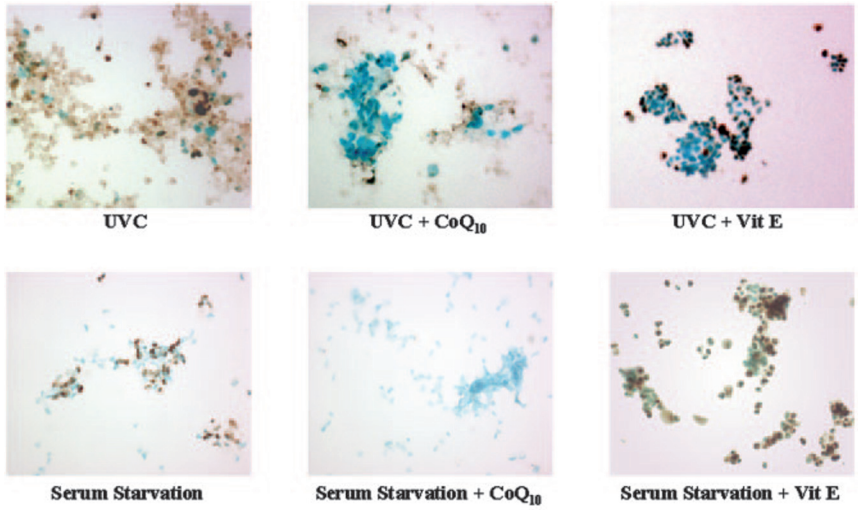


FIG. 4. **Detection of DNA fragmentation in apoptotic nuclei by the Klenow fragment end labeling (FragEL, Oncogene Research Products) at the 24th h following UVC irradiation or serum starvation preceded or not by pretreatment with 10 μM CoQ_{10} or vitamin E.** CoQ_{10} prevented DNA fragmentation in response to both stimuli, whereas vitamin E (*Vit E*) was effective only in response to UVC irradiation.

then evaluated by time-lapse videomicroscopy at 24 h following UVC irradiation or treatment with antimycin A, C_2-ceramide, or serum starvation. Following all treatments, a high number of apoptotic RCE cells was observed in cultured plates, which dramatically decreased if cells were pretreated with CoQ_{10} (not shown). Corresponding quantitative data are reported in Fig. 5. When compared with untreated or CoQ_{10}-pretreated controls, the number of cumulative apoptotic events scored by time-lapse videomicroscopy markedly increased, following application of all apoptotic stimuli, but to a significantly lower extent if the cells were pretreated with CoQ_{10} for 2 h before the induction of apoptosis. These results clearly indicated that CoQ_{10} was able to increase cell survival by preventing apoptosis even in response to stimuli that do not generate free radicals.

CoQ_{10} Attenuated Lowering of ATP Levels in Response to All Apoptotic Stimuli—Execution of the apoptotic death program requires massive consumption of ATP and, consequently, is accompanied by a dramatic reduction in ATP cellular levels. As shown in Fig. 6, UVC irradiation, antimycin A, C_2-ceramide, and serum starvation markedly lowered ATP cellular levels as compared with untreated controls. Nevertheless, this lowering was significantly attenuated by a 2-h pretreatment with CoQ_{10} (from 65 to 28%, from 76 to 41%, from 81 to 51%, and from 60 to 27%, respectively, as compared with respective controls). In further experiments the effects of CoQ_{10} in response to UVC irradiation were omitted.

CoQ_{10} Counteracted Mitochondrial Depolarization, Cytochrome c Release, Caspase 9 Activation, and DNA Fragmentation in Response to All Apoptotic Stimuli—The effects of antimycin A, C_2-ceramide, and serum starvation on mitochondrial membrane charge was quantified by the uptake of JC-1 (Fig. 7). The shift in membrane charge was observed as disappearance of fluorescent red-orange-stained mitochondria (large negative $\Delta\psi$) and an increase in fluorescent green-stained mitochondria (loss of $\Delta\psi$). Following application of apoptotic stimuli most cells underwent mitochondrial membrane depolarization; indeed, cells with red-orange-stained mitochondria dropped to ~20% of total cells. Treatment with CoQ_{10} before application of apoptotic stimuli prevented significantly mitochondrial membrane depolarization, because more than 60% of cells examined maintained red-orange-stained mitochondria. CoQ_{10} was most efficient in serum-starved cells and had less effect in response to antimycin A or C_2-ceramide.

Cytochrome *c*, caspase 9 activity, and DNA status were analyzed as indexes of apoptosis execution by means of the intrinsic (mitochondrion-dependent) pathway (14) (Fig. 8). Fig. 8*A* shows the release of cytochrome *c* to cytoplasm, evaluated by Western blotting analysis; at 24 h after application of all free radical unrelated apoptotic stimuli, cytoplasmic levels of cytochrome *c* markedly increased but remained substantially unaffected if treatments were preceded by CoQ_{10} administration. Similarly, Fig. 8*B* shows that caspase 9 activity undergoes a 6–8-fold enhancement at 24 h after application of apoptotic stimuli. This enhancement was dramatically lower when apoptotic stimuli application was preceded by CoQ_{10} administration. Fig. 8*C* shows that pretreatment with CoQ_{10} also prevented DNA internucleosomal fragmentation elicited by all free radical unrelated apoptotic stimuli (40).

The ability of CoQ_{10} to prevent cytochrome *c* release and caspase 9 activation in response to free radical unrelated apoptotic stimuli, two events that are triggered by PTP opening, suggests that, independently of its free radical scavenging property, CoQ_{10} inhibits apoptosis by directly maintaining mitochondrial PTP in the closed conformation. Finally, prevention by CoQ_{10} of DNA internucleosomal fragmentation indicates that blocking of intrinsic apoptotic pathway by pretreatment with CoQ_{10} suffices to prevent the "ignition" of the entire apoptotic machinery triggered by apoptotic stimuli.

DISCUSSION

We have shown previously (5, 6) that apoptosis of rabbit keratocytes induced by excimer laser irradiation, a free radical-generating treatment, was prevented both *in vitro* and *in vivo* by the free radical scavenger CoQ_{10} dissolved in Lutrol F127 and administered 2 h before treatment.

By using the same keratocyte cell line (RCE), we show here that CoQ_{10} prevents apoptosis also in response to apoptotic stimuli that do not generate free radicals, which are antimycin A, C_2-ceramide, and serum starvation. UVC irradiation, a well known free radical-generating damaging agent, has been used as control. We also demonstrate that the mechanism underlying the free radical scavenging independent antiapoptotic properties of CoQ_{10} is inhibition of mitochondrial depolarization.

Multiple and often interrelated mechanisms have been described to account for the effects of a single apoptotic stimulus. The damaging effect of UVC irradiation is consequent to direct alteration of molecular substrates, such as proteins and nucleic

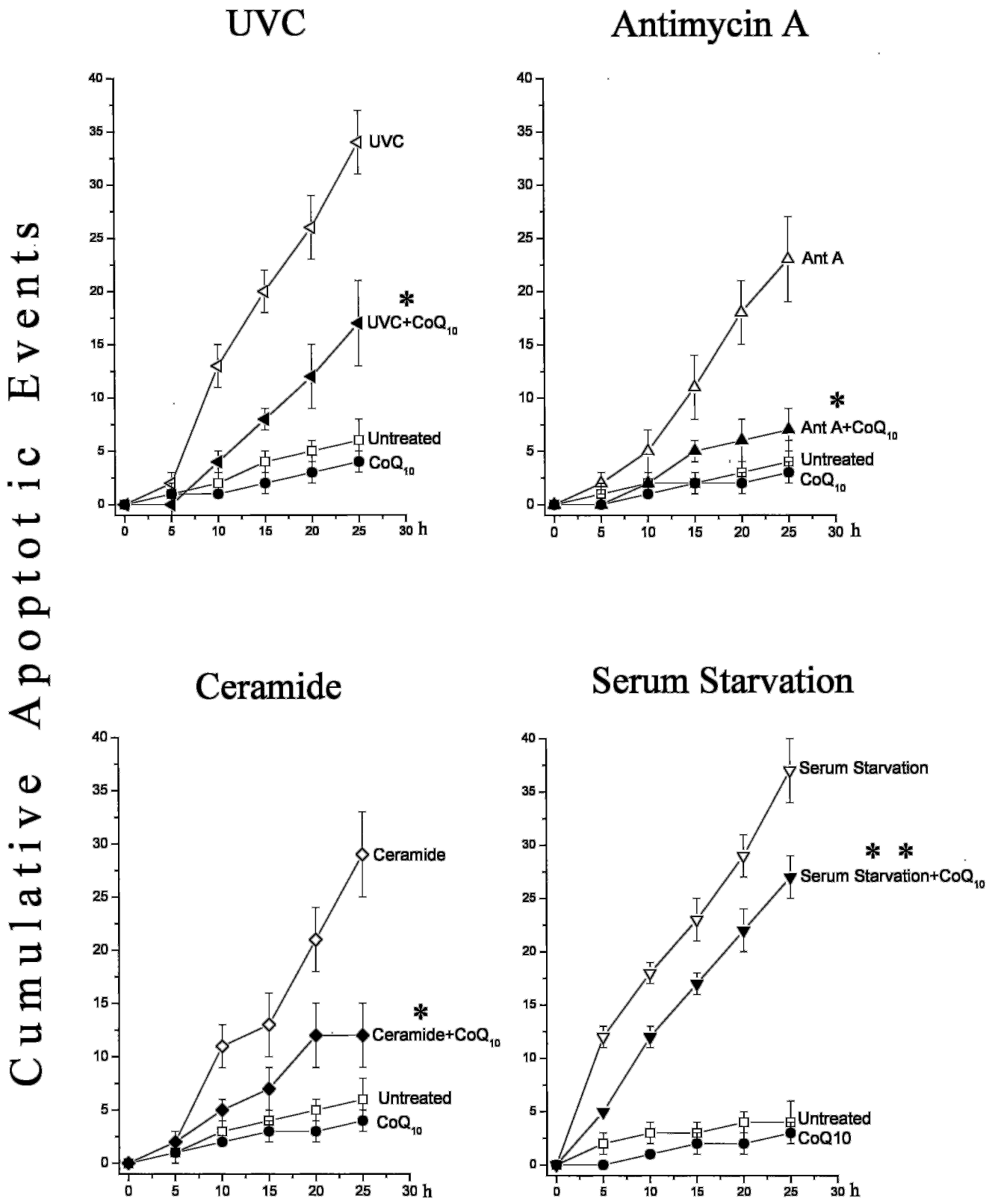


FIG. 5. **Reduction of cumulative apoptotic events by pretreatment with CoQ_{10} in RCE cells.** Apoptotic events were detected and registered progressively by time-lapse videomicroscopy during 24 h after application of apoptotic stimuli. An apoptotic event was scored the moment the cell detached from the substrate, shrank, and blebbed. In comparison to untreated cells, UVC irradiation, antimycin A, ceramide, and serum starvation induced a marked increase in cumulative apoptotic events that was significantly reduced by treatment with the CoQ_{10}. Each point is the mean ± S.E. of three experiments. *, $p \leq 0.005$ compared with CoQ_{10} untreated cells; **, $p \leq 0.05$ compared with CoQ_{10} untreated cells.

acids, or mediated by generation of free radicals, which are mainly reactive oxygen species (41). Antimycin A is a chemical hypoxic drug that has been reported to prevent free radical generation (27, 42–47). Antimycin A induces apoptosis by poisoning the mitochondrial respiratory chain complex III (29) but also by mimicking a cell death-inducing Bcl-2 homology domain (48). C_2-ceramide is a cell-permeable analogue of physiological ceramides, a small group of membrane sphingolipids involved

in cell growth, differentiation, and apoptosis (30, 49, 50). Ceramide-induced apoptosis is mainly consequent to its ability to collapse mitochondrial $\Delta\psi$ (51), either by direct inhibition of mitochondrial respiratory chain complex III (52) or by formation of large transmembrane channels that raise mitochondrial permeability (53, 54). Withdrawal of survival factors, achieved in cultured cells by serum starvation, commit cells to apoptosis by different mechanisms, such as mitogen-activated protein kinase induction (55), ceramide release (56), and COX-2 activation (57), whose common target is triggering the mitochondrion-dependent apoptotic pathway (18). On the other hand, some authors have reported that treatments with antimycin A (58, 59), C_2-ceramide (60), and serum starvation (11) can also lead to ROS generation. Because apoptosis execution is accompanied by ROS generation (27, 28, 41, 61, 62), whether a ROS increase during apoptosis is a cause or an effect of this phenomenon is not easy to establish. Because this point is very crucial for our study, in which it was critical to exclude antimycin A, C_2-ceramide, and serum starvation as directly generating free radicals, we quantified three different free radical generation indexes: ROS, MDA, and SOD activity levels. Quantification was performed early after application of apoptotic stimuli, which is prior to commencement of apoptosis execution as monitored by time-lapse videomicroscopy. A marked increase in ROS, MDA, and SOD activity levels occurred in response to UVC irradiation but not to treatment with antimycin A or C_2-ceramide or serum starvation, which indicated that, at least in our experimental system, the three latter apoptotic stimuli do not generate free radicals.

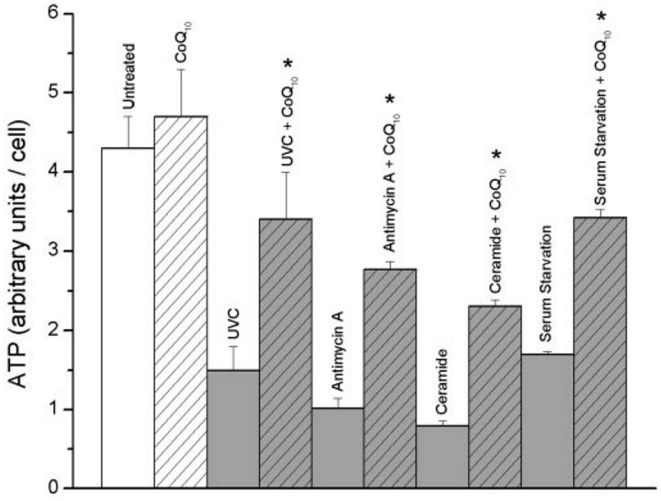


FIG. 6. **Evaluation of ATP cellular levels at 24 h after application of apoptotic stimuli.** Preparation of cell lysates is described under "Experimental Procedures." Pretreatment with CoQ_{10} significantly reduced the drop in ATP cellular levels induced by all apoptotic stimuli. Each point is the mean ± S.E. of three experiments. *, $p \leq 0.005$ compared with CoQ_{10} non-pretreated cells.

The administration of CoQ_{10} 2 h prior to apoptotic stimuli prevents apoptosis not only in response to UVC irradiation but also to antimycin A, C_2-ceramide, or serum starvation, *i.e.* independently of the ability of apoptotic stimuli to trigger or not trigger free radical generation. This protective effect was clearly demonstrated by several evidences, including changes of cell morphology detected by light microscopy and ultramicroscopy, quantification of living and apoptotic cells, and analysis of ATP cellular levels. Indeed, CoQ_{10} significantly enhances the number of living cells evaluated by the MTT analysis and lowers the number of cumulative apoptotic events scored by time-lapse videomicroscopy in response to any apoptotic stimulus, whereas the free radical scavenger vitamin E was effective only against the free radical-generating UVC irradiation. In line with this assertion, pretreatment with CoQ_{10} was able to prevent the massive reduction in ATP levels induced by all apoptotic stimuli, a phenomenon that is typically associated with the energy-consuming apoptosis execution and in particular with $\Delta\psi$ collapse consequent to PTP opening.

We suggest that the mechanism by which CoQ_{10} exerts its antiapoptotic activity is associated with inhibition of PTP open-

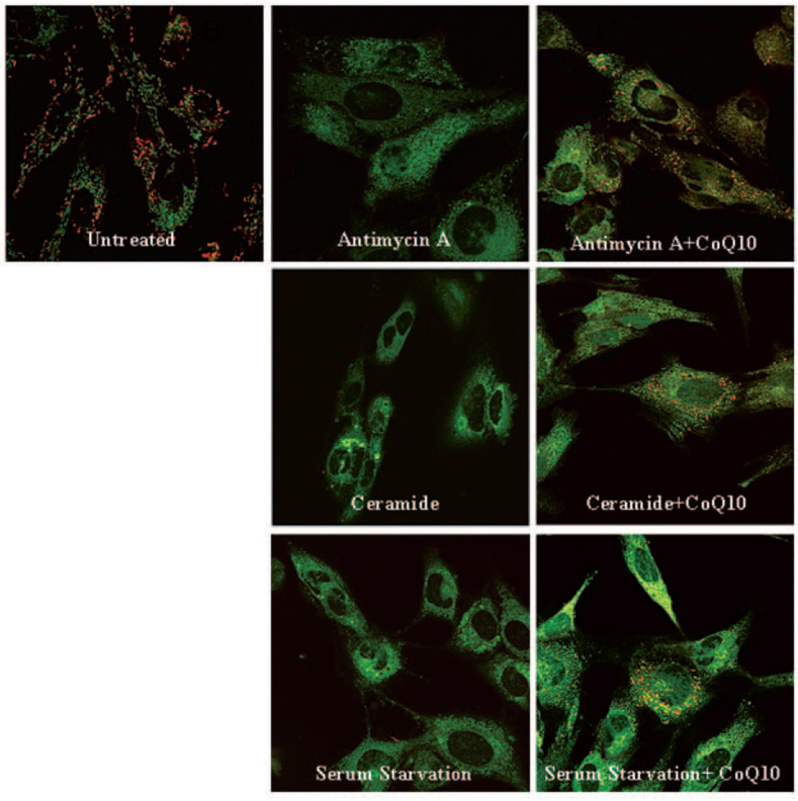


FIG. 7. **Detection of the $\Delta\psi$ by double fluorescence JC-1 assay at the 24th h following treatment with 200 μM antimycin A or 20 μM ceramide or serum starvation (0.5% FBS) preceded or not by pretreatment with CoQ_{10}.** Dual emission images (525 and 590 nm) represent the signal from monomeric (*green*) and J-aggregate (*red*) JC-1 fluorescence in RCE cells. Untreated RCE cells show *red*-stained mitochondria (large negative $\Delta\psi$). Most cells treated with apoptotic stimuli are uniformly *green*-stained (loss of $\Delta\psi$). Pretreatment with CoQ_{10} significantly protected cells against loss of $\Delta\psi$ as demonstrated by the maintenance of the red-stained mitochondria in more than 60% of cells. Each image is representative of at least three independently performed experiments with similar results. The total number of cells analyzed is as follows: 40 (untreated); 38 (antimycin A); 35 (antimycin A + CoQ_{10}); 35 (ceramide); 40 (ceramide + CoQ_{10}); 32 (serum starvation); 34 (serum starvation + CoQ_{10}).

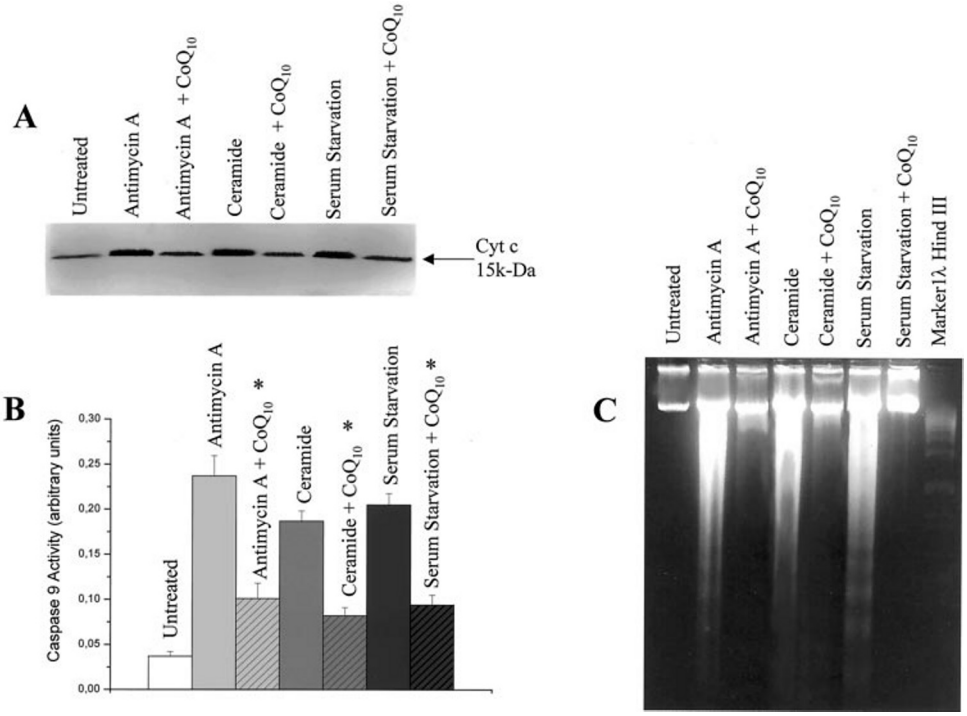


FIG. 8. **Analysis of events involved in mitochondrial permeability transition at 24th h after application of antimycin A, ceramide, or serum starvation (0.5% FBS).** All parameters were evaluated in three independent experiments. *A*, typical Western blot analysis of cytosolic extracts from RCE cells with 1 μg/ml anti-cytochrome *c* monoclonal antibody. The 15-kDa cytochrome *c* (*Cyt c*) (*arrow*) band density markedly increased at the 24th h after application of apoptotic treatments as compared with untreated controls. Pretreatment with CoQ_{10} resulted in a significant reduction of cytochrome *c* band density. *B*, activity of caspase 9 increased 6–8-fold at the 24th h following apoptotic stimulus. Pretreatment with CoQ_{10} largely reduced activity of caspase 9. Each point is the mean ± S.E. of three experiments. *, $p \leq 0.005$ compared with CoQ_{10} non-pretreated cells. *C*, a typical electrophoretic analysis of DNA fragmentation. Nucleosomal laddering of DNA was detected in RCE cells induced to apoptosis by all three stimuli but was largely reduced when treatment with CoQ_{10} preceded apoptotic treatments.

ing. This possibility is supported by a body of literature. First, Fontaine *et al.* (20–22) have demonstrated that Ca^{2+}-dependent opening of PTP in isolated mitochondria can be prevented by two synthetic quinone analogues, ubiquinone 0 and the decyl-ubiquinone, whereas the other quinones tested are ineffective; on this basis, they have proposed a model of mitochondrial PTP that harbors a ubiquinone-binding site. Second, Walter *et al.* (23) have identified the structural features required for regulation of the mitochondrial PTP by ubiquinone analogues and defined three functional classes of ubiquinone analogues, PTP inhibitors, PTP inducers, and PTP-inactive quinones, on the basis of their ability to keep the mitochondrial PTP open or closed or to counteract the effects of both inhibitors and inducers. Because all ubiquinones used decrease mitochondrial ROS production to the same extent, they claim that their different regulative effect on PTP, when present, is mediated by their binding to a common site rather than by their free radical scavenging activity, possibly through secondary changes in the PTP Ca^{2+} binding affinity. Our result indicating that CoQ_{10}, but not vitamin E, prevents mitochondrial depolarization, cytochrome *c* release, and caspase 9 activation induced by apoptotic stimuli strongly support this possibility. Indeed, these three sequential events are specifically associated with opening of PTP and lead to mitochondrion-dependent apoptosis execution. The PTP opening-dependent mitochondrial permeability transition collapses the mitochondrial $\Delta\psi$ and induces a massive ATP hydrolysis, an early event in the apoptotic pathway. The consequent release of cytochrome *c* into cytoplasm and caspase 9 activation triggers caspase cascade that culminates in DNA laddering and apoptosis (12). All these apoptosis execution-related events occurred in our experimental model and were substantially prevented by treatment with CoQ_{10}. On the basis of these considerations, CoQ_{10} may be a candidate as a functional analogue of the Bcl-2 protein (63). Moreover, association of complexes I and III of the mitochondrial respiratory chain with mitochondrial PTP (19, 20, 23–28) and the integration of CoQ_{10} in both complexes suggest the role of CoQ_{10} as a structural element and modulator of mitochondrial PTP. Experiments are now in progress to obtain direct evidence of this possibility and to "pin-point" localization of CoQ_{10} in the PTP complex.

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