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Water-soluble formulation of Coenzyme Q_{10} inhibits Bax-induced destabilization of mitochondria in mammalian cells

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Abstract Oxidative stress leads to mitochondrial dysfunction, which triggers the opening of the permeability transition pores (PTP) and the release of pro-apoptotic factors causing apoptotic cell death. In a limited number of cell systems, anti-oxidants and free-radical scavengers have been shown to block this response. We have previously reported that coenzyme Q_{10} (CoQ_{10}), an electron carrier in the mitochondrial respiratory chain, is involved in the reactive oxygen species (ROS) removal and prevention of oxidative stress-induced apoptosis in neuronal cells. However, the mechanism of this protection has not been fully elucidated. In the present study we investigated the effects of CoQ_{10} on the mitochondrial events characteristic to apoptosis, especially on the function of pro-apoptotic protein Bax. Our results demonstrated that following a brief exposure of two human cell lines (fibroblasts and HEK293 cells) to H_2O_2 the intracellular levels of ROS and the association of Bax with the mitochondria significantly increased and the cells underwent apoptosis. Both of these events, as well as the release of cytochrome *c* from the mitochondria, were blocked by a 24 h pre-treatment with CoQ_{10}. It is therefore believed that CoQ_{10} prevented the collapse of the mitochondrial membrane potential in response to the H_2O_2 treatment. Recombinant Bax protein alone caused the ROS generation and release of cytochrome *c* from isolated mitochondria and, again, CoQ_{10} inhibited these Bax-induced mitochondrial dysfunctions.

Keywords Apoptosis · Bax · CoQ_{10} · Mitochondrial dysfunction · Oxidative stress · Permeability transition pores

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Introduction

Apoptosis or physiological programmed cell death is essential for the maintenance of tissue homeostasis as it is responsible for the removal of damaged and/or infected cells. Apoptosis can be initiated through two major pathways: the extrinsic also called plasma membrane death receptor-dependent pathway and the intrinsic or mitochondrion-dependent pathway. Mitochondrial depolarization and dysfunction play a major role in the intrinsic pathway [1]. Opening of the mitochondrial permeability transition pore (PTP) followed by a collapse of inner mitochondrial membrane potential and release of pro-apoptotic factors such as cytochrome *c* and/or apoptosis inducing factor (AIF) represent the initial steps in this pathway [2].

The Bcl-2 family, which consists of both pro-apoptotic and anti-apoptotic proteins, is a primary regulator of the intrinsic pathway and it has a profound impact on the mitochondria. Pro-apoptotic protein Bax, for example, upon induction of apoptosis translocates from the cytoplasm to the mitochondrial membrane and oligomerizes on the membrane. The insertion of Bax into the membranes disrupts the membrane integrity, results in a collapse of the membrane potential and release of cytochrome *c*, AIF, SMAC/DIABLO and various pro-caspases. Release of cytochrome *c*, in turn, triggers the assembly of apoptosome complex, which activates pro-caspase-9 [1]. The primary

target of apoptosome/caspase-9 complex is caspase-3, which is one of the most potent effecter caspases in the apoptotic process. It has been reported that pro-apoptotic factors such as Bax, calcium ions and ROS cause the opening of PTP, whereas Bcl-2, ATP and cyclosporine A inhibit PTP opening [3, 4]. Although the sequence of mitochondrial events related to apoptosis is relatively well characterized, the exact mechanism by which pro-apoptotic factors, such as Bax, participate in the opening of PTPs is still poorly understood. However certain structural components of PTPs like voltage-dependent anion channel (VDAC), adenine nucleotide translocator (ANT) and cyclophilin-D may offer clues to better understand this interaction [1, 5, 6].

Ubiquinone 50 (Coenzyme Q_{10}, CoQ_{10}), which is present in most aerobic microorganisms and in all animals, is an essential cofactor in the mitochondrial electron transfer chain (ETC). CoQ_{10} participates in the ETC by carrying electrons from complex I (NADH-Ubiquinone oxidoreductase) and complex II (succinate-ubiquinone oxidoreductase) to complex III (ubiquinone-cytochrome *c* oxidoreductase). CoQ_{10} is present in the body in both reduced and oxidized forms and a considerable body of evidence suggests that it functions as free radical scavenger [7]. CoQ_{10} has protective effects in disease processes such as Parkinson's disease [8], Alzheimer's disease [9], diabetes [10] as well as oxidative stress [11, 12] and aging [11]. However, the therapeutic applications of CoQ_{10} are greatly limited by its lack of solubility in aqueous media and poor bio availability. A water-soluble formulation of CoQ_{10}, which is readily applicable in any experimental conditions, has been developed at the National Research Council (NRC) of Canada (US patents # 6,045,826,6,191,172 B1 and 6,632,443). We have previously reported that CoQ_{10}, delivered as this water-soluble formulation, protects cells from apoptosis. For example, the pretreatment of differentiated human neuroblastoma SHSY-5Y cells with CoQ_{10} inhibits the ROS production, DNA fragmentation and significantly reduces the number of apoptotic cells in response to oxidative stress [13, 14]. This formulation has also been shown to prevent glutamate-induced neurotoxicity in human NT2-derived neurons and astrocytes [15]. Most significantly, it protects hippocampal pyramidal neurons from ischemic damage [16].

Despite the growing body of evidence showing protective effects of CoQ_{10} against damaging insults in variety of cell systems, the exact molecular mechanisms involved in this protection are not known. In the present study we have investigated the role of CoQ_{10} as a stabilizer of mitochondria. Using two human cell lines, normal fibroblasts and transformed HEK293 cells, which were challenged with apoptosis-inducing oxidative stress, we have established that CoQ_{10} offset the ROS generation and allowed the cells to maintain mitochondrial membrane potential under otherwise lethal doses of H_2O_2. Furthermore, we were able to show that CoQ_{10} could actually block the pro-apoptotic activity of Bax as it reduced the ROS generation and release of cytochrome *c* triggered by recombinant Bax protein from the isolated mitochondria. To our knowledge, other than the anti-apoptotic Bcl-2 protein, CoQ_{10} is the only compound shown thus far to interfere with the mitochondria permeabilasation function of Bax. Taken together, our results indicate that CoQ_{10} supplements could offset oxidative stress induced-apoptosis in neuronal and/or muscle cells and, therefore, it could play a preventative role in neurodegenerative and cardiovascular diseases.

Materials and methods

Cell culture

Transformed human embryonic kidney cells (HEK 293 cell line) (ATCC, USA) and Normal Human Fibroblasts (NHF) were grown using DMEM/F-12 media and Minimum Essential Medium Eagle respectively (Sigma, Mississauga, Ontario). The media were supplemented with 15% (v/v) Fetal Bovine Serum (FBS) in tissue culture flasks and/or Petri dishes and incubated in 5% CO_2 at 37°C and 95% humidity.

Experimental treatments

All treatments were performed on 50–70% confluent cells. Oxidative stress was induced by an addition of H_2O_2 (Sigma-Aldrich, Mississauga, Ontario) into the growth media at a final concentration of 100 μM for HEK 293 cells and 250 μM for NHF. The cells were incubated with H_2O_2 for 1 h at 37°C, the media were then replaced and the cells were incubated in fresh complete media at 37°C for additional periods of time (i.e., 3 and 6 h). In some experiments cells were pretreated for 24 h with a 30 μg/ml CoQ_{10} formulation added directly to the media. Subsequently, the cells were exposed for 1 h to H_2O_2 as described above and incubated further for 3 and 6 h in fresh media containing CoQ_{10}.

Preparation of water-soluble formulation of CoQ_{10}

Polyoxyethanyl α-tocopheryl sebacate (PTS) was synthesized by conjugating polyethylene glycol 600 to α-tocopherol via bi-functional sebacic acid (Sigma-Aldrich, St. Louis, MO) as previously described [16, 17]. Water-soluble formulation of CoQ_{10} was prepared from PTS and CoQ_{10} (Kyowa Hakko, New York, NY) by directly combining both components in a molar ratio 2:1 and heating them to a temperature higher than their respective melting points to form a clear melt, which was water-soluble and could be diluted

with aqueous solutions (e.g., water, saline, phosphate buffer) to a desired concentration [16, 17]. Typically, a stock solution of 50 mg/ml of PTS was made and used in all the experimental treatments.

Cellular staining and microscopy

Following the experimental treatments cells were stained with 10 μM of 33342 Hoechst Dye (Molecular Probes, Eugene, Oregon). After 5 min incubation at 37°C, the cells were examined under a fluorescence microscope (Leica DM IRB, Germany) and phase contrast and fluorescence pictures were taken. The images were processed using Improvision OpenLab v3.1.2 and Adobe Photoshop v7.0.

Annexin V staining

The cells were grown and treated as previously described. After an allotted time period, an Annexin-V binding assay was conducted using a purchased kit and the manufacturer's protocol. Cells were harvested via trypsinization, washed twice in PBS, and then re-suspended in Annexin-V binding buffer (10 mM HEPES and NaOH pH 7.5, 140 mM NaCl, 2.5 mM $CaCl_2$) at a concentration of $\sim 10^6$ cells/mL. 100 μL of each solution ($\sim 10^5$ cells) was transferred to micro centrifuge tubes, 5 μL of annexin-V-FITC conjugate was added, and the mixture was incubated for 15 min at room temperature. After the incubation period, the cells were re-suspended in 100 μL fresh binding buffer, cells were stained with Hoechst 33342, and 10 μL of each suspension was transferred to a glass slide and covered with a cover-slip. Slides were examined under a fluorescent microscope and pictures were taken (*Leica DM IRB, Germany*).

Isolation of mitochondria

Mitochondria were isolated from HEK 293 cells using previously published protocol [18]. Approximately 70% confluent cells were harvested using a rubber policeman and pelleted at 2500 rpm for 8 min at 22°C. The supernatant was discarded and the cell pellet was washed with ice-cold PBS and centrifuged at 2500 rpm for 8 min at 22°C. The pellet was washed with a hypotonic buffer (1 mM EDTA, 10 mM HEPES, pH 7.4, 250 mM Sucrose) and spun down at 3000 rpm for 5 min at 22°C. Finally, the cell pellet was re-suspended in the hypotonic buffer, incubated in ice for 10 min and homogenized in a glass dounce homogenizer using approximately 20 strokes. The homogenate was centrifuged at 3000 rpm for 5 min at 4°C to remove unbroken cells and nuclei and, subsequently, at 10,000 rpm for 10 min at 4°C to pellet the mitochondria.

Measurement of ROS in HEK 293 cells

Cells were cultured and treated with H_2O_2 as described above. ROS production was measured using membrane permeable dye 2′7′-dichlorodihydrofluoroscein di-acetate (H_2DCFDA) (Molecular Probes, Eugene, Oregon). The dye was added to the culture plates to a final concentration of 1 μM and the plates were incubated at 37°C for 15 min. The cells were then harvested with a rubber policeman and a 100 μL cell suspension per well was used in a 96 well reading plate. The fluorescence activity was measured (excitation at 513 nm and emission at 530 nm) using a Spectra Max Gemini XS multi-well plate fluorescence reader and Softmax Pro (Molecular Devices, Sunnyvale, California). Statistical analysis and representation of data was done using Microsoft Excel 2002.

Measurement of ROS in isolated mitochondria

ROS generation in isolated mitochondria was measured using oxidation-sensitive parahydroxyphenylacetic acid (PHPA) and horseradish protease (HRP) (Sigma) as described by Li et al. [18]. The mitochondrial pellet, isolated as described above, was re-suspended in a reaction buffer consisting of 0.25 M Sucrose, 1 mM $MgCl_2$, 10 mM HEPES, 100 mM Succinate and 4 mg/mL PHPA (the final concentration of PHPA was approximately 0.2 μg/μL). Mitochondria, either control untreated or pretreated for 30 min with 0.5 mg/mL CoQ_{10} formulation, were incubated with 5 μg/mL of recombinant Bax for 5 min at room temperature. The mitochondrial suspensions (100 μL per well) were placed in a 96 well plate and the wells were subsequently treated with 0.4 units of HRP and fluorescence was measured starting immediately after the addition of HRP for 10 min in 30-s intervals. The fluorescence readings were performed using the Spectra Max Gemini XS at an excitation wavelength of 320 nm and an emission wavelength of 400 nm.

Isolation and purification of recombinant Bax

E. coli expressing His tag-conjugated Bax was grown in LB media containing 20 μg/mL Chloramphenicol. The expression of Bax was induced at 0.5 OD_{600} by adding arabinose (Sigma-Aldrich, Mississauga, Ontario) to a final concentration of 1 g/L, followed by overnight incubation at room temperature. The samples were then centrifuged at 10,000 rpm at 4°C for 10 min, and the pellet was re-suspended in hypotonic buffer (0.02 M Sodium Phosphate, pH 7.4) containing 0.2% SDS and sonicated 4 times in 10 sec spurts. Isolation of Bax from this sample was performed using the HiTrap metal Chelating HP column (Amersham Biosciences) as per manufacturer's instructions.

Protein extraction

Cells, treated and untreated, were harvested with a rubber policeman, washed twice with 1X PBS and centrifuged for 5 min at 3000 rpm. The supernatants were discarded and the pellets were re-suspended in an extraction buffer (10 mM Tris-HCl pH 7.2, 1 mM EDTA, 10 mM HEPES, 50 mM Sucrose and 1% (v/v) Triton X-100) containing protease inhibitors (1 μg/mL leupeptin and 1 μg/mL pepstatin and 0.5 mM PMSF). The cell suspension was transferred to a glass homogenizer (Kontes Glass Company, Vineland, New Jersey) and the cell membranes were mechanically disrupted by approximately 20 strokes. The homogenized suspension was centrifuged at 3000 rpm for 5 min. The pellet was discarded and the supernatant was again centrifuged for 10,000 rpm for 10 min. The pellet contained the mitochondrial proteins, while the supernatant consisted of the cytoplasmic proteins. Protein concentration in these cell lysates was measured using BioRad protein assay dye reagent (Bio-Rad Laboratories, California).

Western blot analysis

Total cellular and mitochondrial proteins (25 μg/lane) were resolved on 12% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked for 1 h with a 5% non-fat milk solution in TBST (0.02 M Tris HCl, 0.2 M NaCl, and 0.001% TWEEN (v/v), pH 7.2) and, subsequently, were incubated for 1 h with primary antibodies in a 2% non-fat milk solution in TBST. Both anti-Bax and anti-cytochrome *c* sera were from (Santa Cruz Biotechnology Inc, California) and were diluted as per manufacturer's instructions. The nitrocellulose membranes were washed with TBST three times for 15, 5 and 5 min. The membranes were incubated for another hour with anti-mouse secondary antibody, diluted 1:5000 in a 2% non-fat milk solution and were finally washed with TBST. Chemiglow kit (Alpha Innotech Corporation, CA) was used to detect and Chemilmager v5.5 (Alpha Innotech Corporation) was used to visualize the antigens.

Comet assay

The comet assay was performed using a slight modification of previously described method [19]. Briefly, 10,000 cells in 10 μl in volume were mixed with 80 μl of warm Low-melting point agarose (LMP) (0.75%, 37°C) in a microfuge tube and spread on a glass slide pre-coated with 200 μl of 0.1% agarose in such a way that half of the gel was on the frosted surface and other half on the smooth and transparent surface (agarose gel tends to slide away from smooth surface during processing). A cover slip was laid over the gel mixture and slides were kept at 4°C. After gelling at 4°C, the cover slip was removed. Slides were immersed immediately in a jar containing a freshly prepared cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10, 1% Triton X-100 and 10% DMSO). Lysis was done at 4°C for 1 h in the dark. Slides were washed in a freshly prepared alkaline electrophoresis buffer (0.3 M NaOH, 1 mM Na_2 EDTA, pH >13). The DNA was electrophoresed at 300 mA (0.8 V/cm) and washed twice in a neutralizing buffer (0.4 M Tris, pH 7.5) and stained with Hoechst 33342 (1 μM). The fluorescence signals of the stained 'comets' were examined using Fluorescent microscope (Zeiss, Axiovert 200) and images were processed using Northern Eclipse 6.0 software.

Results

CoQ_{10} decreased number of apoptotic cells in NHF and 293 cells upon induction of oxidative stress

In order to investigate the protective effect of CoQ_{10} against apoptosis induced by oxidative stress we challenged NHF and transformed human kidney cells (293 cells) with H_2O_2 treatment in the presence or absence of water-soluble CoQ_{10} as described in material and method section. Various morphological and biochemical parameters including nuclear chromatin condensation, DNA fragmentation and intracellular ROS production were measured. Figure 1 shows the nuclear morphology of NHF and 293 cells stained with Hoechst in normal conditions (1a, 1d) and following oxidative stress (1b, 1e). As shown, apoptosis was initiated in the cells that were not pre-treated with CoQ_{10}. However, in the cells with CoQ_{10} pre-treatment nuclear morphology remained similar to control cells both in NHF and 293 cells (1c, 1f). The apoptotic bright nuclei were counted and the percentage of apoptotic cells in NHF after treated with H_2O_2 is shown in Fig. 2. As indicated in the cells treated with H_2O_2 following pre-treatment with CoQ_{10}, the number of apoptotic cells decreased significantly in comparison to the cells without pre-treatment of CoQ_{10}, or pre-treated with the carrier PTS alone which shows the effect of CoQ_{10} in preventing apoptosis. DNA fragmentation is a hallmark of apoptotic cell death. We applied comet assay to demonstrate DNA fragmentation in cells undergoing apoptosis by oxidative stress. Figure 3 indicates apoptosis and DNA fragmentation in NHF cells under oxidative stress in the absence of CoQ_{10}, whereas CoQ_{10} pretreated cells were protected from oxidative stress, and CoQ_{10} inhibited apoptosis and DNA fragmentation.

Furthermore, evaluation of cells with Annexin V binding assay indicated that CoQ_{10} protected 293 cells from apoptosis upon induction of oxidative stress (Fig. 4). 293 cells with CoQ_{10} pre-treatment resisted from undergoing apoptosis (Fig. 4C). However, cells with no CoQ_{10} pre-treatment

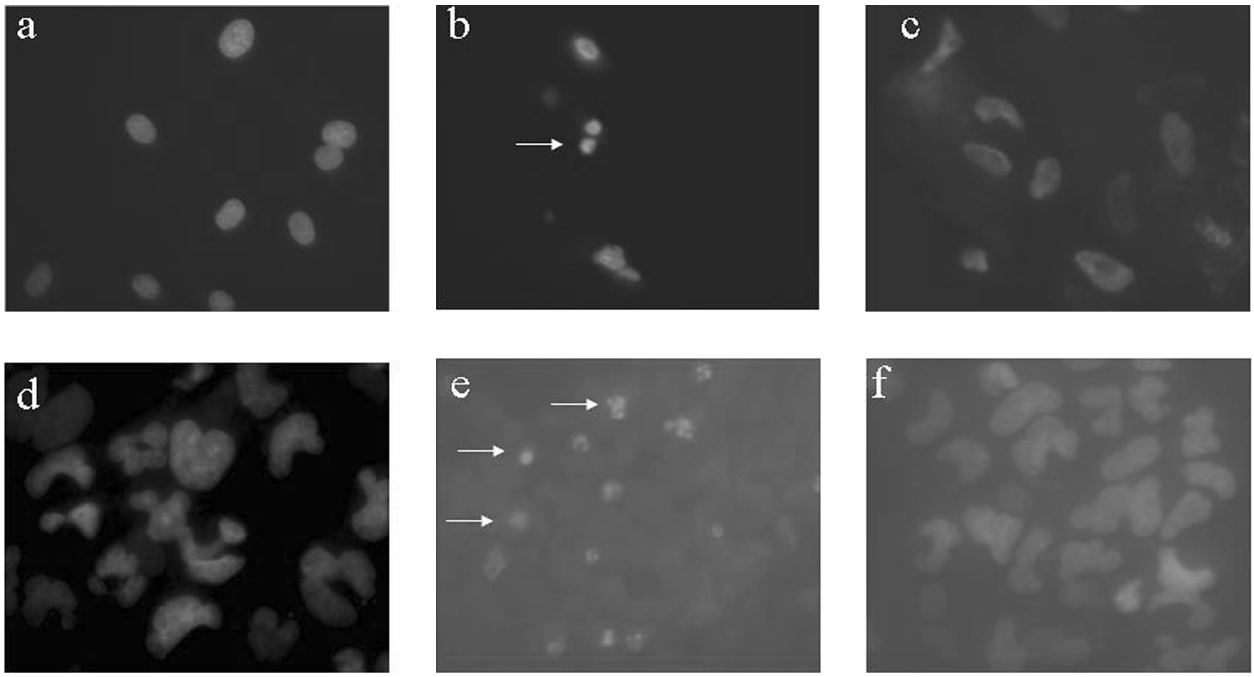

Fig. 1 Evaluation of nuclear morphology of NHF and 293 cells after application of H_2O_2, treated with CoQ_{10}: NHF and 293 cells were treated with 250 μM and 100 μM H_2O_2 respectively in the presence and absence of CoQ_{10} as described in Materials and Methods. Panels (a) and (d) depict control untreated NHF and 293 cells respectively. Treatment with H_2O_2 resulted in cell death in both NHF and 293 cells characterized by nuclear condensation, as pointed out in (b) and (e) respectively. 24 h pretreatment with CoQ_{10} led to protection of both cell lines from cell death induced by oxidative stress, as shown in (c) and (f) respectively

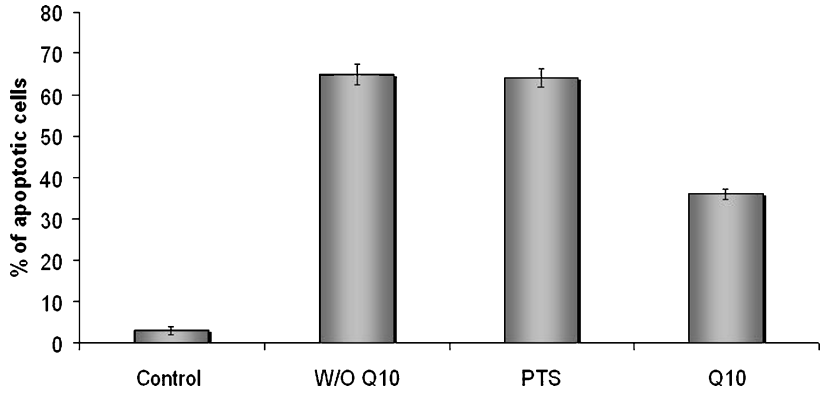

Fig. 2 CoQ_{10} significantly decreased cell death in NHF cells: Untreated control cells were compared to NHF cells treated with 200 μM H_2O_2 for 1 h in three sets, which had been subjected to: (i) no CoQ_{10} pretreatment, (ii) PTS carrier (placebo) pretreatment for 24 h and (iii) PTS carrier containing CoQ_{10} pretreatment for 24 h. Microsoft Excel Version 6.0 was used to represent data and calculate standard deviation. Percentages of apoptotic cells were determined by counting, and the protective effects of CoQ_{10} against oxidative stress were clearly demonstrated

or treated with PTS (Fig. 4(b) and (d)) underwent apoptosis after being subjected to H_2O_2.

ROS generation is decreased in the presence of CoQ_{10}

Mitochondrial dysfunction has been associated with the increased production of reactive oxygen species. ROS levels were quantified in 293 cells in the presence and absence of CoQ_{10} using a redox-sensitive dye. Apoptosis was induced by a brief exposure of cells to 100 μM H_2O_2 followed by incubation in normal growth media. Results shown in Fig. 5 indicated that intracellular ROS generation was increased in the cells exposed to H_2O_2 compared to control untreated cells. However, CoQ_{10} significantly decreased ROS levels in the cells. Interestingly, this effect is more prominent after 24 h post-treatment, in which ROS levels are almost 6 fold less than corresponding cells without pre-treatment with CoQ_{10}. Thus, the results presented in the previous sections indicated that mitochondrial membrane collapse and increased ROS generation are involved in apoptosis induced by oxidative

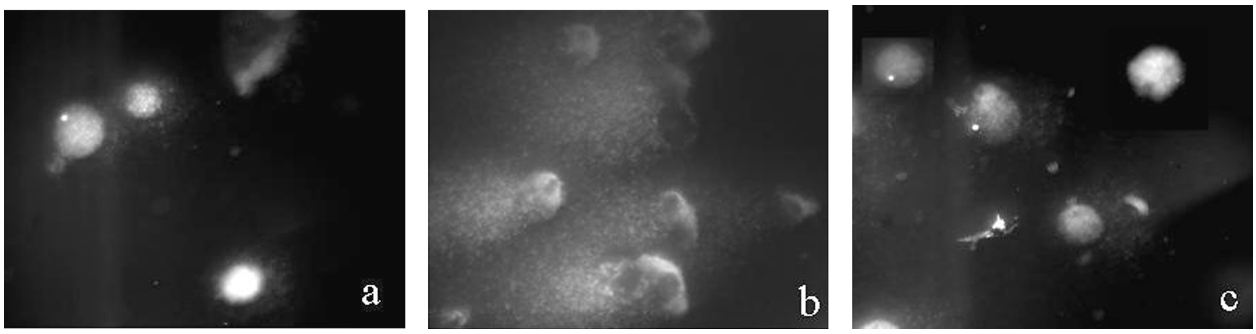

Fig. 3 Estimation of DNA fragmentation by comet assay: NHF cells were grown and pre-treated with CoQ_{10} or placebo for 24 h before 1 h exposure to H_2O_2 as described previously. Cells were harvested by trypsinization 24 h after the treatment and comet assay was performed as described in Materials and Methods. As shown in (a) there was no apparent DNA fragmentation in untreated control cells. However, significant DNA fragmentation resulted in H_2O_2-treated cells w/o CoQ_{10} pretreatment, as depicted in (b), but no significant DNA fragmentation in H_2O_2-treated cells in the presence of CoQ_{10} (c)

stress and interestingly, water soluble CoQ_{10} was capable of significantly inhibiting these parameters. Therefore, it appears CoQ_{10} is functioning at the mitochondrial level in inhibiting apoptosis in these cells. The following experiments were designed to investigate this possibility.

CoQ_{10} prevents mitochondrial depolarization

Several researches have indicated that external oxidative stress initiates apoptosis mainly through the intrinsic pathway in which mitochondria has a major role. To this end, we

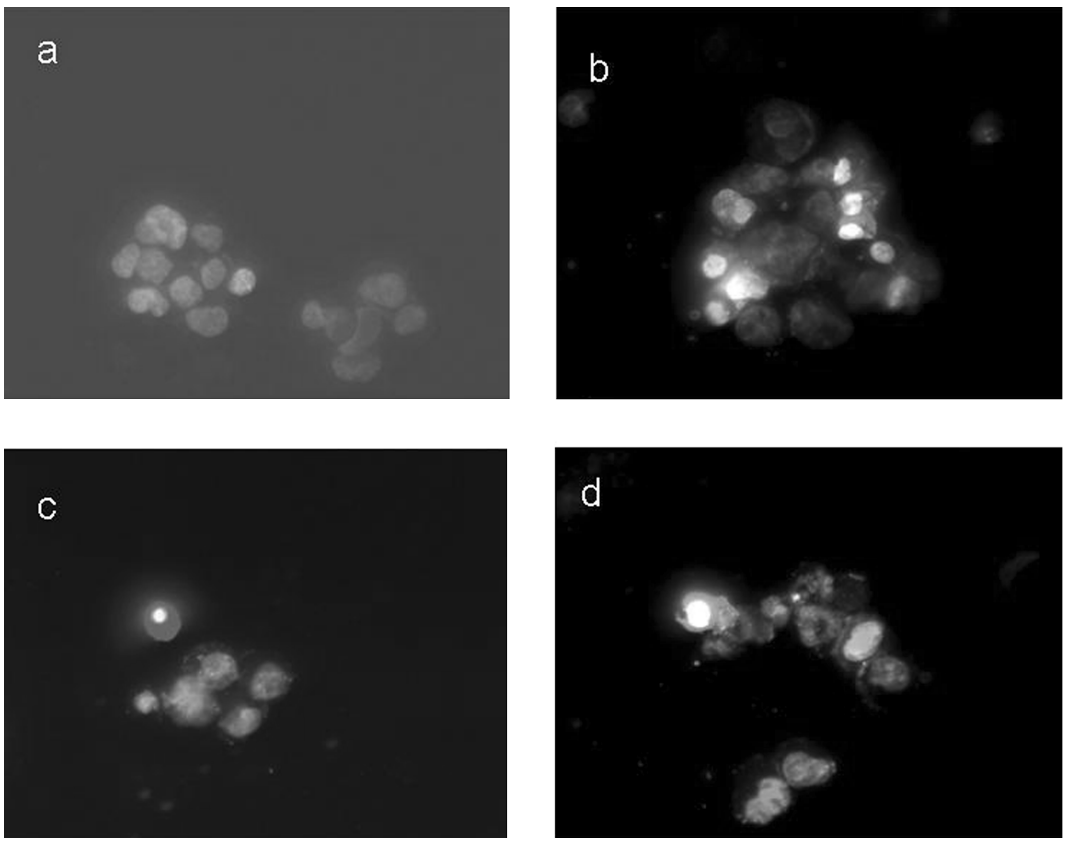

Fig. 4 Evaluation of protective effect of CoQ_{10} on 293 cells using Annexin V assay. Oxidative stress was induced to 293 cells and Annexin V assay was performed as described in the Materials and methods. (a) Control cells. (b) Cells treated with 100 μM H_2O_2 without CoQ_{10} (c) and cell with 30 μM CoQ_{10} pre-treatment. Cells with pre-treatment of PTS underwent apoptosis after induction of oxidative stress (d)

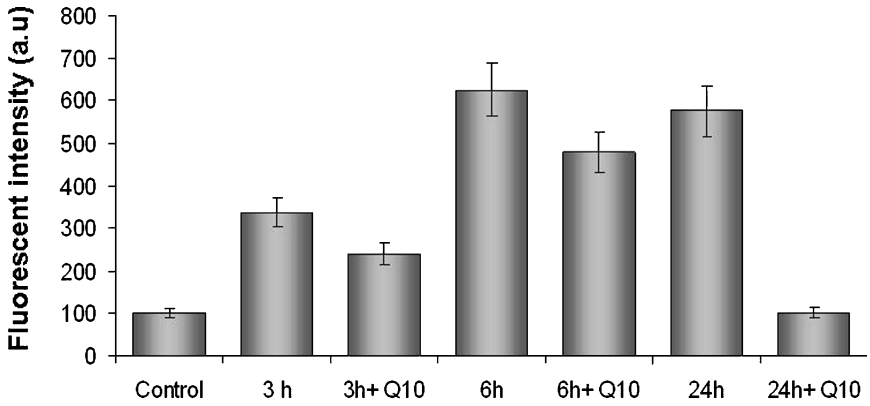

Fig. 5 CoQ_{10} inhibited ROS generation in 293 cells following induction of oxidative stress. 293 cells were treated with H_2O_2 and ROS levels were measured using $DCFDAH_2$ as described in Materials and methods. Microsoft Excel Version 6.0 was used to represent data and calculate standard deviation. ROS levels were higher in cells both pretreated and untreated with CoQ_{10} when compared to control cells following application of H_2O_2. However, the increment of ROS levels was significantly higher in CoQ_{10} untreated cells after 3 and 6 h compared to CoQ_{10} pretreated cells. Furthermore, ROS levels in CoQ_{10} untreated cells remained high after 24 h but declined drastically in CoQ_{10} pretreated 293 cells

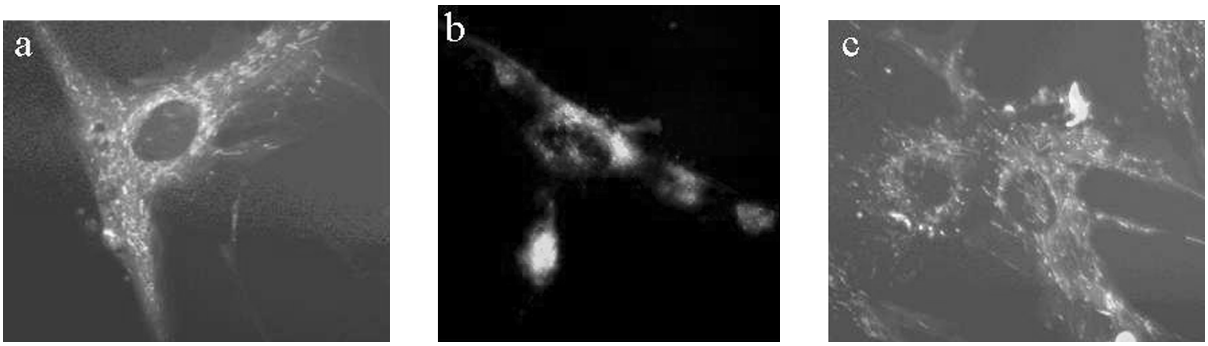

Fig. 6 Mitochondria in NHF cells remain intact after oxidative stress when pretreated with CoQ_{10}: NHF cells were stained with JC1 as described in Materials and methods. Mitochondrial membrane potential was measured using JC1 staining and evaluated by fluorescent microscopy. In (a), control untreated NHF cells, the mitochondria stained red, indicating they were intact and healthy. As shown in (b), mitochondrial membrane potential (MMP) collapsed following treatment with H_2O_2, as indicated by the green staining. However, as is evident due to the red staining in panel (c), MMP remains hyperpolarized when cells were treated with CoQ_{10} prior to inducing oxidative stress

evaluated the mitochondrial membrane potential by uptake of a membrane potential-sensitive fluorescent dye JC-1. Disappearance of red-stained mitochondria (hyperpolarized membrane) and an increase in green-stained mitochondria (loss of $\Delta\psi$) was observed following H_2O_2 treatment, however, in the cells pre-treated with CoQ_{10}, mitochondria largely remained hyperpolarized after induction of oxidative stress (Fig. 6).

CoQ_{10} stabilizes mitochondrial membrane and inhibits cytochrome *c* release into the cytoplasm

Rising ROS levels in the cells indicated mitochondria were affected by oxidative stress. Since mitochondria are in the center of the intrinsic pathway and any effect on these potentially begins apoptosis in the cells, we examined the expression of Bax, a pro-apoptotic protein of Bcl-2 family in the cells by western blot analysis. Figure 7(a) shows levels of Bax expression were increased 6 h after induction of oxidative stress. However, no increase in the levels of Bax was observed after the same period in the cells pretreated with CoQ_{10}. Since Bax is able to release cytochrome *c* from mitochondria, the analysis of cytochrome *c* levels in the mitochondrial fraction of cell lysate was conducted. cytochrome *c* levels were higher in the cells with pre-treatment of CoQ_{10} than cells with no pre-treatment (Fig. 7(b) and (c)), which means CoQ_{10} prevented release of cytochrome *c* from mitochondria into the cytoplasm.

Recombinant Bax increased ROS generation in isolated mitochondria from 293 cells; CoQ_{10} inhibited the effect of Bax

To evaluate the effect of Bax directly on isolated mitochondria, we used recombinant Bax protein expressed in *E.coli* [20]. Bax was isolated and purified as discussed in

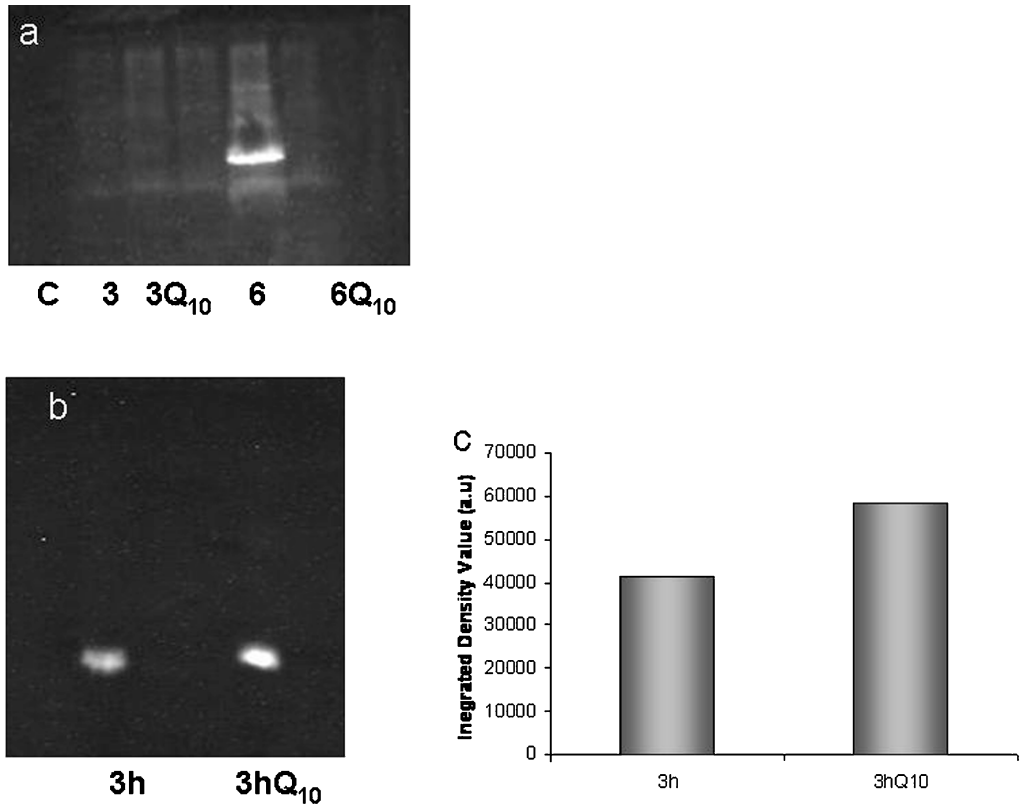

Fig. 7 CoQ_{10} inhibited expression of Bax in 293 cells and aided in retention of cytochrome *c* in the mitochondria following oxidative stress. (a) Western blot analysis was performed to evaluate the expression of Bax as described in Material and Methods. Following oxidative stress in 293 cells induced by 1 h H_2O_2 treatment, CoQ_{10} prevented the expression of Bax after 6 h. (b) Cytochrome *C* levels were measured in isolated mitochondria from 293 cells after 3 h H_2O_2 treatment as described in Materials and Methods. cytochrome *c* levels were higher in mitochondrial pellets with Q_{10} pre-treatment (M + Q) in comparison to the cells without Q_{10} treatment (M). (c) The intensity of the bands in (b) is depicted in a graph in arbitrary units

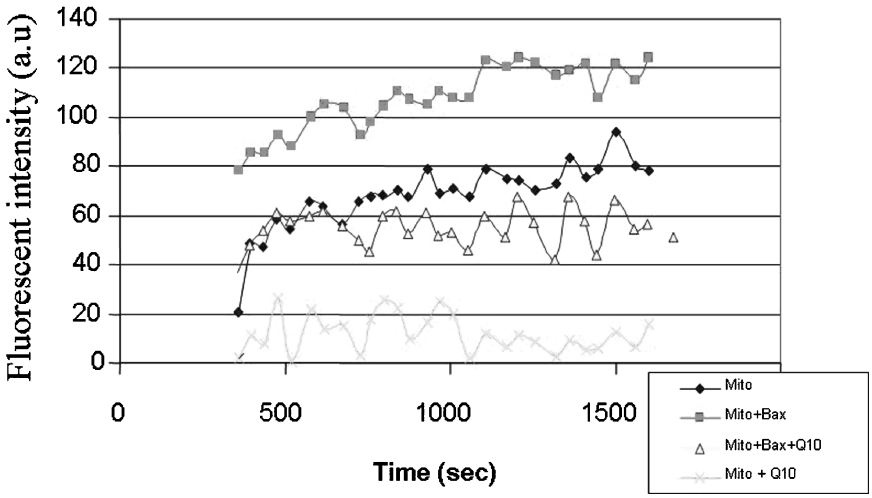

Fig. 8 CoQ_{10} inhibits ROS generation in isolated mitochondria from 293 cells in the presence of Bax: Isolated mitochondria from 293 cells were incubated with Bax in the presence and absence of CoQ_{10} and ROS production was measured using PHPA as described in the Materials and Methods. Bax increased ROS production in isolated mitochondria; however, mitochondria pretreated with CoQ_{10} displayed significantly lower levels of ROS. Furthermore, mitochondria treated with CoQ_{10} only showed remarkably low ROS levels

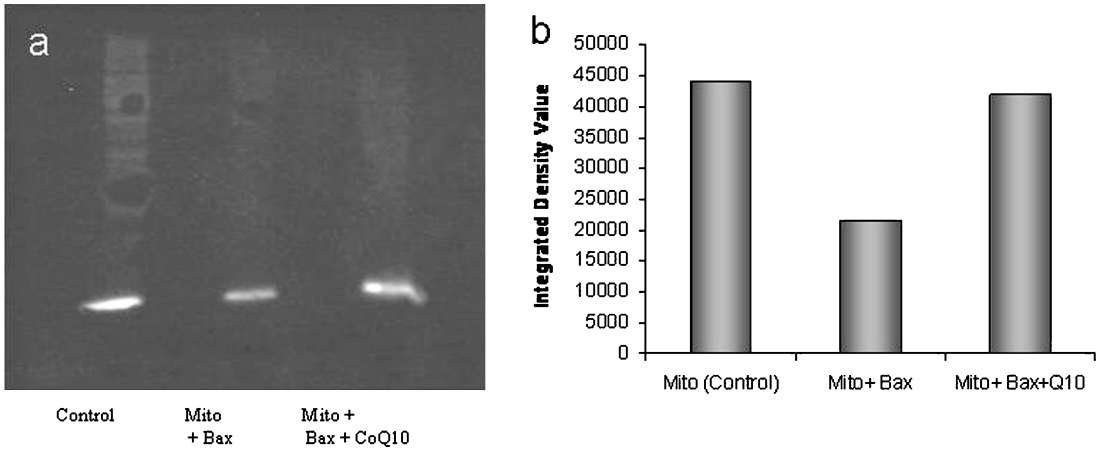

Fig. 9 CoQ_{10} stabilizes mitochondrial membrane and prevents release of cytochrome *c*. (a) Mitochondria were isolated from 293 cells and incubated with Bax as described in Materials and Methods. Lane 1 shows cytochrome *c* levels in untreated control mitochondria, lane 2 contains mitochondria treated with Bax and lane 3 contains mitochondria pretreated with CoQ_{10} prior to Bax treatment (a). cytochrome *c* levels were higher in the mitochondria which were pretreated with CoQ_{10} when compared to Bax treatment only, implying increased retention of cytochrome *c* within the mitochondrial membrane due to CoQ_{10} pretreatment. (b) Intensities of the bands detected in western blot analysis in Fig. 9(a) were measured using ChemiImager software from Alpha Innotech Corporation (Mississauga, Ontario)

Materials and Methods using His-Tag affinity column. Purified Bax was resolved in SDS-PAGE. Purity of the isolated Bax protein was checked by Coomassie blue staining and immunoblot analysis using a monoclonal anti-Bax antibody. Mitochondria were isolated from healthy 293 cells as described in Materials and Methods. Equal amounts of isolated mitochondria were treated with Bax protein. In order to compare the effect of CoQ_{10} with Bax, the mitochondria were pre-treated with CoQ_{10} for 20 min. Mitochondria incubated with Bax in the absence of CoQ_{10} were and mitochondria incubated with CoQ_{10} alone were used as controls. Results (Fig. 8) indicate that ROS production from isolated mitochondria was increased in the presence of Bax, implying that Bax causes mitochondrial dysfunction. However, in CoQ_{10} pre-treated mitochondria ROS production was inhibited significantly. These results indicated that water soluble CoQ_{10} is capable of directly inhibiting Bax–induced mitochondrial dysfunction in the assay involving isolated mitochondria.

CoQ_{10} inhibits release of cytochrome *c* from isolated mitochondria in the presence of recombinant Bax

Although the increased ROS production from isolated mitochondria in response to Bax treatment is a good indication of mitochondrial dysfunction, it did not indicate if Bax caused PTP opening or mitochondrial permeabilization. In order to investigate the mitochondrial permeabilization we examined the release of cytochrome *c* from isolated mitochondria following Bax treatment either in the absence or presence of CoQ_{10}. The incubation and reactions were carried out as described above and following the incubation time reaction mixtures were centrifuged to separate supernatant and mitochondrial pellet. Since the amount of cytochrome *c* released in the supernatant of the reaction mixture was very small and diluted, mitochondrial pellet was used to estimate the amount of cytochrome-c remaining within the mitochondria. The mitochondrial proteins were resolved on SDS-PAGE, transferred to a nitrocellulose membrane and western blot analysis using anti-cytochrome *c* antibody was performed as described in Materials and Methods. As shown in Fig. 9, cytochrome *c* levels were lower in Bax treated mitochondrial pellet compared to control indicating the release of cytochrome *c* in the supernatant. However, in the presence of CoQ_{10}, cytochrome *c* mainly remained inside of mitochondria, indicating mitochondrial membranes were largely intact.

Discussion

In our previous studies we have shown that the water soluble coenzyme Q_{10} is capable of inhibiting apoptosis of differentiated human neuronal cells challenged with glutamate excitotoxicity or hydrogen peroxide or indirect oxidative stress by paraquat [13–15]. Since increase in the intracellular ROS level was a universal feature in many of these studies, it is plausible to hypothesize that CoQ_{10} might be inhibiting apoptosis by simply scavenging the free radicals. Indeed, several researchers have demonstrated that free radical scavenger activity of CoQ_{10} is responsible for its anti-apoptotic activity [21–24].

In the present study we have shown the ability of water-soluble CoQ_{10} to prevent apoptosis in fibroblasts and transformed embryonic kidney cells closely related to its ability in

stabilizing the mitochondrial membrane and decrease ROS generation. Our results clearly indicated that mitochondrial membrane potential ($\Delta\psi$) collapsed upon inducing oxidative stress. However, if cells were pre-treated with CoQ_{10} prior to induction of oxidative stress, membrane potential remained intact. It could be argued that CoQ_{10} inhibits the mitochondrial collapse and apoptosis simply by scavenging the intracellular ROS. Since induction of expression of Bax and its role in mitochondrial destabilization has been implicated in apoptosis in the intrinsic pathway of apoptosis [1], we wanted to investigate if CoQ_{10} is acting by interfering with the Bax activity.

We have demonstrated that recombinant Bax protein causes mitochondrial dysfunction in isolated mitochondria as indicated by increased ROS generation and leakage of cytochrome *c*. Furthermore, our results indicated direct inhibition of Bax activity on isolated mitochondria. These results suggest that the protective effect of CoQ_{10} could be attributed to its ability to stabilize the mitochondrial membrane potential and blocking the Bax activity.

We have used a water-soluble CoQ_{10} formulation [25] for our studies firstly because the treatment of cells occurs in an aqueous culture media at any concentration and secondly the uptake of CoQ_{10} using this formulation has been shown to be better in both *in vitro* as well as *in vivo* systems [25].

The CoQ_{10} formulation contains equimolar ratio of reduced and oxidized form of ubiquinone. Thus, it does not favor the redox status as such. We have measured the H_2O_2 amount in the media in the absence and presence of 30 μg/ml water-soluble CoQ_{10} and we did not see any significant change in the amount of H_2O_2 (data not shown). Furthermore, other anti-oxidants such as Vitamin E are not able to prevent apoptosis in the cells in the similar situation (data not shown). The dose and duration of water-soluble CoQ_{10} for pre-treatment was decided based on previous results where the best dose and time for neuro-protection were optimized [13–15]. We have observed that amount of total mitochondrial CoQ_{10} is increased significantly after 24 h incubation with this formulae (Sikorska et al. unpublished results and Borowski et al. US patents # 6,045,826,6,191,172 B1and 6,632,443). We observed that in the cells, which were pre-treated with CoQ_{10}, mitochondrial membranes were more stable (maintained membrane potential), ROS levels were lower and in these cells cytochrome *c* mostly remained inside the mitochondria following oxidative stress.

As described before, Bax is a pro-apoptotic protein that moves from the cytoplasm to the mitochondrial membrane upon apoptotic stimulation. It has been postulated that Bax is recruited by VDAC-ANT-cyclophilin complex, a structural component of PTP leading to the formation and opening of the PTP [3, 6]. Our finding that recombinant Bax can destabilize isolated mitochondria, causing increased ROS generation and cytochrome *c* release is a direct demonstration of Bax activity *in-vitro*. More importantly, we found that CoQ_{10} was able to block the Bax activity in isolated mitochondria. These results suggest that CoQ_{10} may interfere with the association of Bax with PTP. Indeed it has been demonstrated that there might be ubiquinone-binding sites on PTP that are regulated by complex I of the electron transfer chain [4, 26]. Interestingly, recent reports suggest that CoQ_{10} prevents apoptosis by inhibiting mitochondrial depolarization independently of its free radical scavenging property [27]. Our result that CoQ_{10} directly inhibited Bax-induced dysfunction of isolated mitochondria further confirms these results. We have previously reported that CoQ_{10} pre-treatment leads to significant increase in overall ATP generation in mammalian cells [14, 15].

The mechanism of protective effects of CoQ_{10} is still elusive and needs more investigation. The results obtained in this study have clearly demonstrated that water-soluble CoQ_{10} is capable of preventing apoptosis by inhibiting mitochondrial dysfunction caused by pro-apoptotic activities of Bax protein. These findings further extend the scientific basis for the development of water-soluble CoQ_{10} as a neuro-protective therapy for neurodegenerative diseases.

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