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Original Research Communication

Dual Involvement of Coenzyme Q_{10} in Redox Signaling and Inhibition of Death Signaling in the Rat Heart Mitochondria

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ABSTRACT

Coenzyme Q_{10} (CoQ) has long been utilized as a cardioprotective agent in various heart diseases. One of the most important mechanisms by which CoQ exerts cardioprotection is aerobic ATP production as a mobile electron carrier in the mitochondrial electron transfer chain. The ability of CoQ to afford myocardial protection is also attributed to its antioxidant property. However, CoQ may also act as a pro-oxidant through the generation of reactive oxygen species. Although excess oxidative stress is known to induce death signaling via cytochrome *c* release from mitochondria, it is now apparent that a brief exposure to oxidative stress stimulates redox signaling for acquisition of tolerance to oxidative stress. Therefore, we have investigated dual involvement of CoQ in redox signaling generation through enhanced production of reactive oxygen species and death signaling inhibition through antioxidation. Mitochondria were isolated from the rat heart and incubated with CoQ (10 or 100 μM) or its vehicle HCO 60 for 1 h. H_2O_2 and cytochrome *c* release from respiring mitochondria were increased by antimycin A (2 μM), an inhibitor of complex III respiratory chain, or by high Ca^{2+} (10 μM). This enhanced release of H_2O_2 was associated with an increase in lipid peroxidation as measured with 4-hydroxy-2-nonenal-modified proteins and with large amplitude swelling of mitochondria. CoQ potentiated H_2O_2 release from antimycin A- or high Ca^{2+}-treated mitochondria, but was capable of inhibiting lipid peroxidation and large amplitude swelling, and attenuated cytochrome *c* release from the mitochondria. In addition, CoQ increased ATP synthesis by mitochondria. These results suggest that CoQ plays dual roles in mitochondrial generation of intracellular signaling. CoQ acts as a pro-oxidant that participates in redox signaling. CoQ also acts as an antioxidant that inhibits permeability transition and cytochrome *c* release, and increases ATP synthesis, thereby attenuating death signaling toward apoptosis and necrosis. Antioxid. Redox Signal. 3, 103–112.

INTRODUCTION

Coenzyme Q_{10} (CoQ) has long been known as a cardioprotective agent that has been utilized in treatment for ischemic heart disease, heart failure, and cardiotoxic chemical intoxication such as adriamycin (14). One of the important mechanisms by which CoQ exerts cardioprotection has been attributed to its role as a mobile electron carrier in the mitochondrial electron-transport process of respiration and coupled phosphorylation (19). CoQ represents "substrate-like molecules" linking successive enzymes as in a metabolic pathway, and in this respect it may represent controlling devices of the overall rate of electron transfer. Thus, un-

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der a normal aerobic environment, abundance of CoQ in mitochondria is an important determinant for ATP synthesis. Synthesized ATP can be utilized for maintaining intracellular ionic homeostasis by activating ATP-requiring ion pumps, thereby alleviating myocardial injury induced by various noxious stimuli.

The ability of CoQ to afford myocardial protection is also attributed to its antioxidant property. It is apparent that reactive oxygen species (ROS) are a common mediator of cytotoxic stress. Biochemical mechanisms underlying the toxicity of ROS are their ability to peroxidize membrane phospholipids with unsaturated free fatty acid and interaction with certain sulfhydryl proteins. The net result of ROS-induced damage appears to be altered membrane function and structure. Eventually, the altered handling of ionic gradient results in intracellular Ca^{2+} overload, the major pathogenesis of myocardial reperfusion injury (25). Mitochondria are especially vulnerable to increased Ca^{2+}. Intracellular Ca^{2+} overload facilitates energy-linked uptake of Ca^{2+} by mitochondria that dissipates mitochondrial membrane potential and inhibits ATP synthesis. Elevation of intracellular Ca^{2+} is a prerequisite for increased ROS generation by mitochondria (13). Furthermore, accumulation of Ca^{2+} in the mitochondrial matrix above the critical level is known to result in the activation of cell death cascade by provoking permeability transition (PT) and releasing cytochrome *c* from the intermembrane space (18, 26). Cytochrome *c* in the presence of Apaf (apoptotic protease activating factors) and dATP in the cytosol activates caspases leading to the degradation phase of apoptosis (2, 30).

In addition to an antioxidative role, CoQ may also act as a pro-oxidant. Redox cycling of CoQ in the mitochondrial electron transfer chain has been shown to be involved in O_2^- generation (5). ROS released into cytosol from mitochondria can trigger intracellular signal transduction pathways that may mediate cytoprotection (8, 28) and gene expression through activation of redox-sensitive transcriptional factors such as nuclear transcription factor κB and activating protein-1 (16). It is, therefore, anticipated that increased ROS generation in mitochondria with abundance of CoQ could represent a novel mechanism of cardioprotection through the potentiation of redox signaling. Thus, the objective of the present study was to test the hypothesis that CoQ increases ROS generation, but prevents oxidative damage and dysfunction of mitochondria under excess ROS-generating conditions.

MATERIALS AND METHODS

Isolation of mitochondria

Male Sprague–Dawley rats weighing 250–300 g fed standard diet were used for experiments. These animals received humane care according to the animal-welfare regulations of the Kansai Medical University. The rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (100 mg/kg), and the hearts were quickly removed. The hearts were mounted on the nonrecirculating Langendorff's perfusion apparatus and were perfused with Krebs–Henseleit bicarbonate buffer solution equilibrated with a 95% O_2/5% CO_2 gas mixture at 37°C. After washout of blood from the coronary circulation, the hearts were removed from the apparatus and immersed in ice-cold isolation buffer (buffer A) with the following composition: 150 m*M* KCl, 5 m*M* MOPS, 2 m*M* EGTA, and 0.1% bovine serum albumin, pH 7.25. After atria and major blood vessels were discarded, ventricles were finely minced and gently homogenized with a Teflon homogenizer in the isolation buffer containing 10 mg of Nagarse (Wako Pure Chemical Industries Co., Osaka, Japan). The homogenate was centrifuged at 800 *g* for 5 min. The supernatant was retrieved and centrifuged at 7,000 *g* for 10 min. The resulting pellet designated as the mitochondrial function was resuspended in the buffer above lacking Nagarse to a protein concentration of 30–50 mg/ml and kept on ice. Mitochondria were incubated with CoQ or its vehicle HCO 60 for 1 h before experiments. CoQ and HCO 60 were gifts from Eisai Pharmaceutical Co. (Tokyo, Japan). Protein concentration was determined by Bradford's method using bovine serum albumin as a standard. Ca^{2+} concentrations in incubation buffer were calculated according to the formula (11).

Measurement of hydrogen peroxide (H_2O_2) release from mitochondria

H_2O_2 release from isolated mitochondria was assessed by the oxidation of scopoletin by horseradish peroxidase in the presence of H_2O_2 as described previously (15). In brief, mitochondria were resuspended in the reaction buffer containing 124 m*M* sucrose, 65 m*M* KCl, 10 m*M* HEPES buffer, pH 7.2. Mitochondrial suspension was transferred into a fluorometer (Hitachi F-2000 Fluorescence Spectrophotometer, Hitachi Ltd., Instruments, Tokyo, Japan). Mitochondria were then incubated with 1 m*M* scopoletin and 0.5 m*M* horseradish peroxidase, and the change in the fluorescence intensity at 450 nm was continuously measured for 15 min after incubation with 2 μM antimycin A or 10 μM Ca^{2+}.

Measurement of large amplitude swelling of mitochondria

Large amplitude swelling of mitochondria was measured according to the methods described by Marchetti et al. (17). Isolated mitochondria were resuspended in buffer A containing 5 m*M* succinate. Large amplitude swelling of mitochondria was induced by adding 2 μM antimycin A or 10 μM Ca^{2+}. The change of OD_{540} was monitored continuously for 15 min at 23°C by a spectrophotometer (Hitachi).

Release of cytochrome c from isolated mitochondria

Isolated mitochondria were resuspended in buffer A containing 5 m*M* succinate and incubated for 15 min at 23°C after addition of 2 μM antimycin A or 10 μM Ca^{2+}. The reaction medium was centrifuged at 3,000 *g* for 15 min at 4°C, and the supernatant was assayed for cytochrome *c* by western-blot analysis as described below.

Western-blot analysis

Mitochondrial suspension or the supernatant was solubilized in 1 ml of RIPA buffer containing 10 m*M* sodium phosphate, 150 m*M* NaCl, 1 m*M* EDTA, 1 m*M* EGTA, 1% (wt/vol) sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 100 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 m*M* sodium orthovanadate, pH 7.4. Equal amounts of the mixture protein (60 μg) were loaded on reducing SDS/10–20% gradient polyacrylamide gels (buffer composition: 4% SDS, 20% glycerol, 4% β-mercaptoethanol, 0.2 *M* Tris-HCl, pH 6.8, and 0.02% bromophenol blue). Protein was transferred onto nitrocellulose membranes and blocked for 1 h at room temperature. The membrane was then incubated with either polyclonal anti-cytochrome *c* antibodies (1:1,000 dilution) or polyclonal anti-4-hydroxy-2-nonenal (HNE)-modified protein antibodies (1:1,000 dilution) for 1 h at room temperature. Rabbit polyclonal anti-cytochrome *c* antibodies and rabbit polyclonal anti-HNE-modified protein antibodies were generous gifts from Dr. Kanji Tomioka (Process Technology and Research Laboratories, Research Institute, Kaneka, Hyogo, Japan) and Dr. Koji Uchida (Laboratory of Food and Biomedics, Nagoya University School of Agricultural Science, Nagoya, Japan), respectively. After membrane washing, horseradish peroxidase-conjugated secondary antibodies (1:2,000 dilution; Dako Japan Co., Kyoto, Japan) were used. Bound antibodies were detected using enhanced chemiluminescence with a kit from Amersham (Arlington Heights, IL, U.S.A.). Intensity of the signal was analyzed and quantified by the NIH Image program (NIH-Image ver. 1.59).

Measurement of mitochondrial ATP generation

Isolated mitochondria were resuspended in buffer A and incubated for 15 min at 37°C after addition of 5 m*M* KH_2PO_4, 5 m*M* succinate, and 5 m*M* ADP. The reaction was terminated by adding an equal volume of 12% trichloroacetic acid. The reaction medium was centrifuged at 3,000 *g* for 15 min at 4°C, and the supernatant was neutralized with 6 *M* KOH. After centrifugation at 3,000 *g* for 15 min at 4°C, the supernatant was assayed for ATP by an enzymatic method using a kit from Sigma Aldrich Co. (Tokyo, Japan).

Statistical analysis

All numerical data are presented as means ± SEM. Statistical comparisons were done by an

analysis of variance and Scheffé's multiple comparison test.

RESULTS

CoQ enhances H_2O_2 release from mitochondria

There was no appreciable H_2O_2 release from mitochondria in the absence of antimycin A or Ca^{2+}. Incubation of mitochondria with the vehicle in the presence of antimycin A or Ca^{2+} only slightly increased H_2O_2 release (Fig. 1). CoQ at a concentration of 100 μM enhanced H_2O_2 release from mitochondria incubated with 2 μM antimycin A and to a lesser extent with 10 μM Ca^{2+}.

CoQ inhibits HNE-modified protein generation in mitochondria

HNE-modified proteins are formed in biological tissues during oxidative stress. It is produced as a result of α-cleavage of alkoxy or peroxy radicals. Alkoxy and peroxy radicals are formed from the decomposition of lipid hydroperoxides, which are produced when membrane β-6 polyunsaturated fatty acids (e.g., linoleic acid, arachidonic acid, and docosahexaenoic acid) undergo free radical-mediated peroxidation (10, 29). HNE-modified proteins have a considerably longer half-life than ROS and are capable of inhibiting numerous protein functions by reacting with the amino acids cysteine, histidine, and lysine. Therefore, we used HNE-modified proteins as an index for lipid peroxidation and peroxidative damage of mitochondria.

The western blot analysis for HNE-modified proteins shown in Fig. 2A demonstrates a number of discrete bands that are discernible in mitochondria incubated for 15 min with 2 μM antimycin A or 10 μM Ca^{2+} under state 4 respiration. Quantitative analysis demonstrated

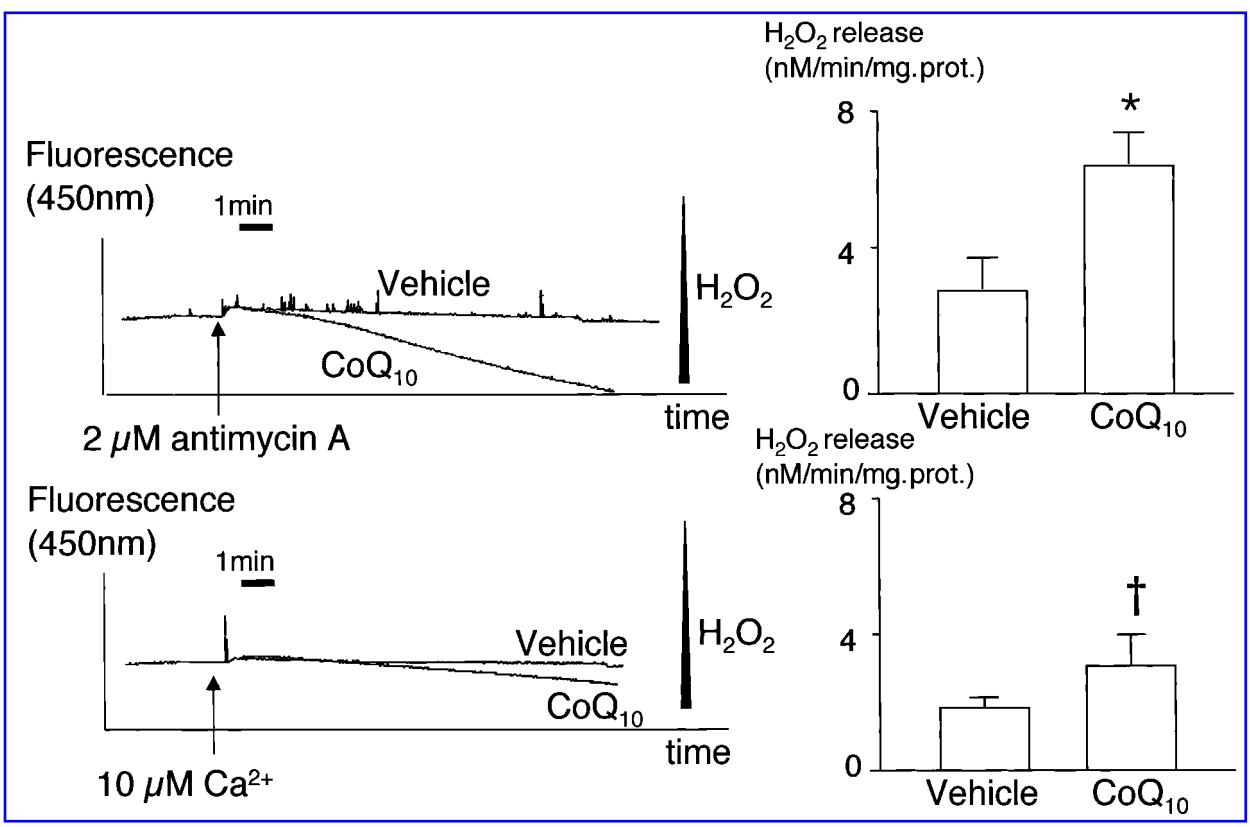

FIG. 1. Effect of CoQ on H_2O_2 release from isolated mitochondria. Mitochondria were resuspended in the reaction buffer as described in Materials and Methods. Mitochondrial suspension was transferred into a fluorometer. Mitochondria were then incubated with 1 μM scopoletin and 0.5 μM horseradish peroxidase, and the change in the fluorescence intensity at 450 nm was continuously measured after incubation with 2 μM antimycin A or 10 μM Ca^{2+} (**left panel**). Quantitative analysis corresponding to the left panel is shown in the **right**. Each column expresses the mean ± SEM of five experiments. *$p < 0.05$, †$p < 0.1$ compared with vehicle.

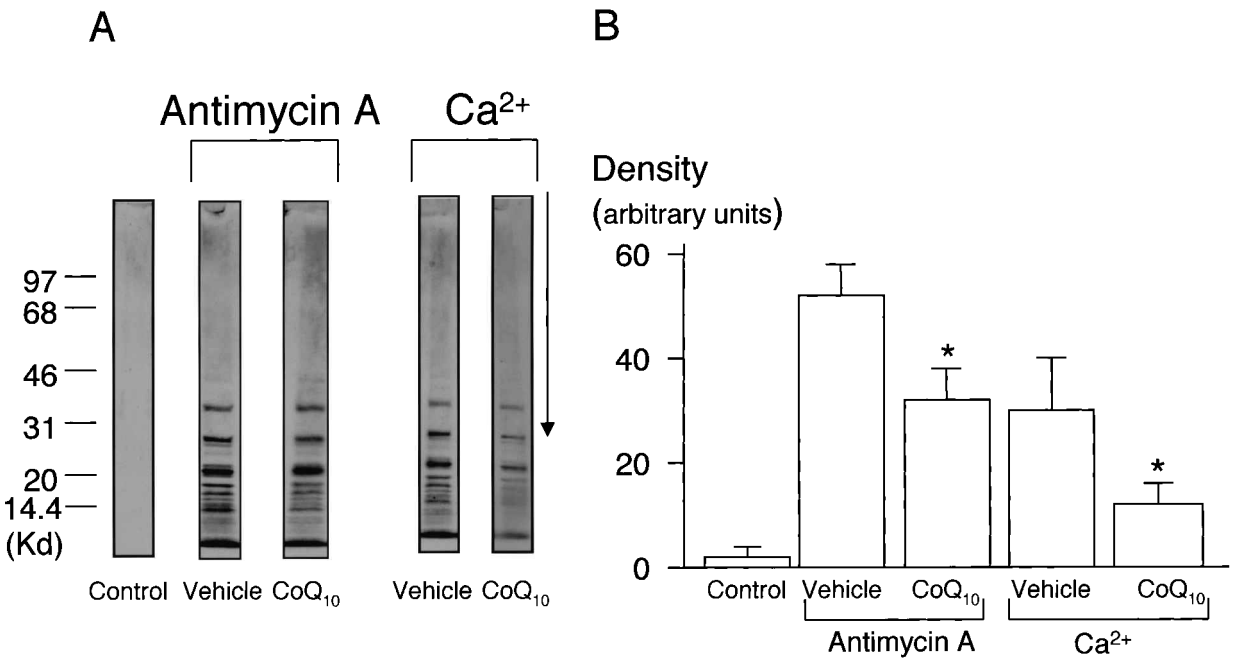

FIG. 2. Effect of CoQ on HNE modification of mitochondrial protein. Mitochondria were exposed with 2 μM antimycin A or 10 μM Ca^{2+} under state 4 respiration. (**A**) Western-blot analysis for HNE-modified proteins. (**B**) Densitometric analysis of HNE-modified protein western blot. Each column expresses the mean ± SEM of five experiments. Control samples were obtained after incubation of mitochondria for 15 min with 5 mM succinate in the absence of antimycin A or Ca^{2+}. *$p < 0.05$ compared with vehicle.

that this enhanced HNE-modified protein signal was significantly attenuated by preincubation with 100 μM CoQ (Fig. 2B).

CoQ inhibits large amplitude swelling in mitochondria

The decline of OD_{540}, which is an index of large amplitude swelling of mitochondria, during a 15-min incubation with succinate was potentiated by 2 μM antimycin A or by 10 μM Ca^{2+} (Fig. 3). The large amplitude swelling was significantly inhibited by preincubation with 10 or 100 μM CoQ.

CoQ inhibits cytochrome c release from mitochondria

There was a trivial amount of cytochrome *c* released from mitochondria when incubated without succinate for 15 min or in the absence of antimycin A or Ca^{2+} (data not shown). Cytochrome *c* release from mitochondria was markedly increased by addition with antimycin A and to a lesser extent with Ca^{2+} under state 4 respiration (Fig. 4A). This increase in cytochrome *c* release was attenuated by preincubation with CoQ. Quantitative analysis demonstrated dose-dependent inhibition of cytochrome *c* release from mitochondria by CoQ (Fig. 4B).

CoQ increases ATP synthesis in mitochondria

Mitochondria contained only a small amount of ATP before incubation with inorganic phosphate, succinate, and ADP (Fig. 5). Preincubation of mitochondria with 100 μM CoQ significantly enhanced ATP synthesis after an addition of inorganic phosphate, succinate, and ADP. However, addition of 2 μM antimycin A or 10 μM Ca^{2+} completely abrogated mitochondrial ATP synthesis in both vehicle- and CoQ-treated mitochondria (data not shown).

DISCUSSION

The results of the present study demonstrated that H_2O_2 release from mitochondria by antimycin A treatment or by Ca^{2+} loading was

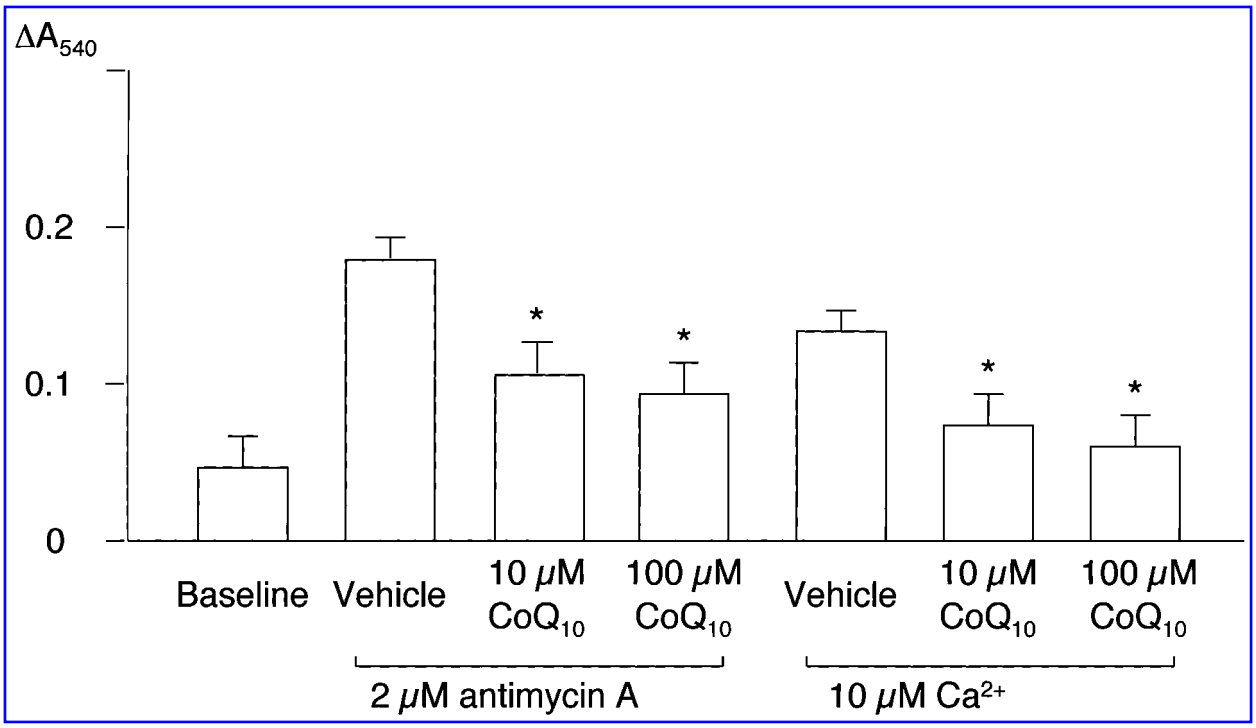

FIG. 3. Effect of CoQ on large amplitude swelling of mitochondria. Isolated mitochondria were resuspended in buffer A containing 5 m*M* succinate as described in Materials and Methods. Large amplitude swelling of mitochondria was induced by adding 2 μM antimycin A or 10 μM Ca^{2+}. The change of OD_{540} (ΔA_{540}) was monitored by a spectrophotometer for 15 min. Each column expresses the mean ± SEM of five experiments. $^*p < 0.05$ compared with vehicle.

enhanced by pretreatment with CoQ. We used 10 and 100 μM CoQ, which are several orders of magnitude higher than the reported concentration of total CoQ in the rat heart mitochondria (21). These concentrations were chosen because it has been shown that CoQ at concentrations up to 4.6 μM was not sufficient to increase H_2O_2 production in the beef heart mitochondria (5). We presume that only a small amount of exogenous CoQ was incorporated into mitochondria and functioned as an electron carrier. Lipid peroxidation and peroxidative modification of mitochondrial proteins were inhibited by CoQ treatment as evidenced by attenuation of HNE-modified protein generation in mitochondria. These findings suggest that exogenously added CoQ can amplify ROS generation, but simultaneously act as an antioxidant within mitochondria. Pro-oxidative action of CoQ has been a matter of debate. O_2^- can stem from a divergent single electron transfer from redox-cycling ubisemiquinone. It has been demonstrated that exogenously added CoQ enhances O_2^- generation in isolated complex I (NADH-CoQ reductase) and III (CoQ-cytochrome *c* reductase) (5). Other lines of evidence that also support redox cycling of CoQ as an alternative site of direct oxygen interaction during respiration were derived from the experiments showing that H_2O_2 release from decomposing O_2^- was inhibited after removal of CoQ from mitochondria, but was reestablished after reincorporation of CoQ (27). In addition, myxothiazol, which prevents the existence of ubisemiquinone at its outer binding center to the bc_1 complex, inhibited mitochondrial O_2^- formation (22). On the other hand, argument against the role of CoQ in the source of O_2^- has also been provided by Nohl and Stolze (23), who reported that O_2^- formation did not occur through redox cycling of CoQ in a water-free nonpolar reaction system that resembles the lipophilic character of the inner mitochondrial membrane, but became significant when the membrane was permeable to protons by toluene pretreatment. This observation suggests that CoQ does not play a major role in O_2^- generation in intact mitochon-

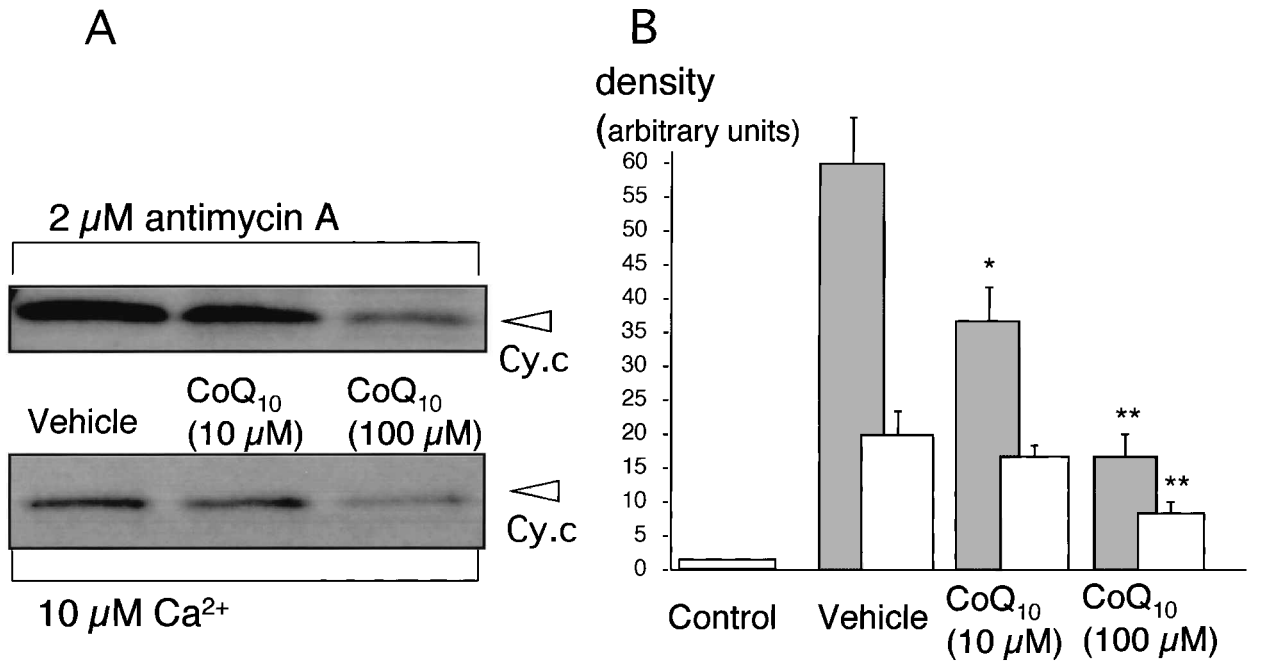

FIG. 4. Cytochrome *c* (Cy.c) release from isolated mitochondria. Isolated mitochondria were resuspended in buffer A containing 5 m*M* succinate and incubated for 15 min after addition of 2 μM antimycin A or 10 μM Ca^{2+}. (**A**) Western-blot analysis for Cy.c (**B**) Densitometric analysis of Cy.c western blot. Each column expresses the mean ± SEM of five experiments. Control samples were obtained after incubation of mitochondria for 15 min with 5 m*M* succinate in the absence of antimycin A or Ca^{2+}. Shaded columns, antimycin A; open columns, Ca^{2+}. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with vehicle.

dria, but may become an important source of O_2^- under certain pathological conditions in which the inner mitochondrial membrane is protonated (24). Antimycin A treatment or Ca^{2+} loading to mitochondria used in the present study may produce such pathological conditions that are capable of inducing mitochondrial inner membrane leaky to proton and allowing interaction with CoQ, to potentiate ROS generation.

Despite enhanced H_2O_2 release, mitochondria pretreated with CoQ appear to be less affected by peroxidative damage as evidenced by reduced generation of HNE-modified proteins. Such a discrepancy can be explained by an antioxidant role of CoQ. The ability of reduced CoQ to interfere with the formation of ROS-induced chemical changes in liposomes, lipid emulsions, and other purely chemical systems has been confirmed, extended, and reported in a significant number of publications. Above all, the main function of CoQ as an antioxidant appears to inhibit lipid peroxidation and not to scavenge ROS, because localization of CoQ in the hydrophobic region of the membrane phospholipid bilayer is a particularly favorable position to inhibit lipid peroxidation and oxidative damage of proteins associated with the membranes.

The inhibition of lipid peroxidation and peroxidative protein damage of mitochondria pretreated with CoQ was associated with attenuation of PT and cytochrome *c* release by antimycin A treatment or Ca^{2+} loading. Although the exact mechanism of pro-oxidant- or Ca^{2+}-induced PT and cytochrome *c* release remains elusive, recent studies (6, 12, 15) have suggested that mitochondrial generation of ROS promotes the oxidation and cross-linkage of mitochondrial membrane protein thiol groups, leading to PT. Ca^{2+} loading to mitochondria, on the other hand, induces PT not only by promoting vicious Ca^{2+} cycling (26), but also by increasing ROS generation (13). It is, thus, likely that an antioxidant property of CoQ plays a role in inhibition of PT and cytochrome *c* release under ROS-generating and Ca^{2+}-loading conditions.

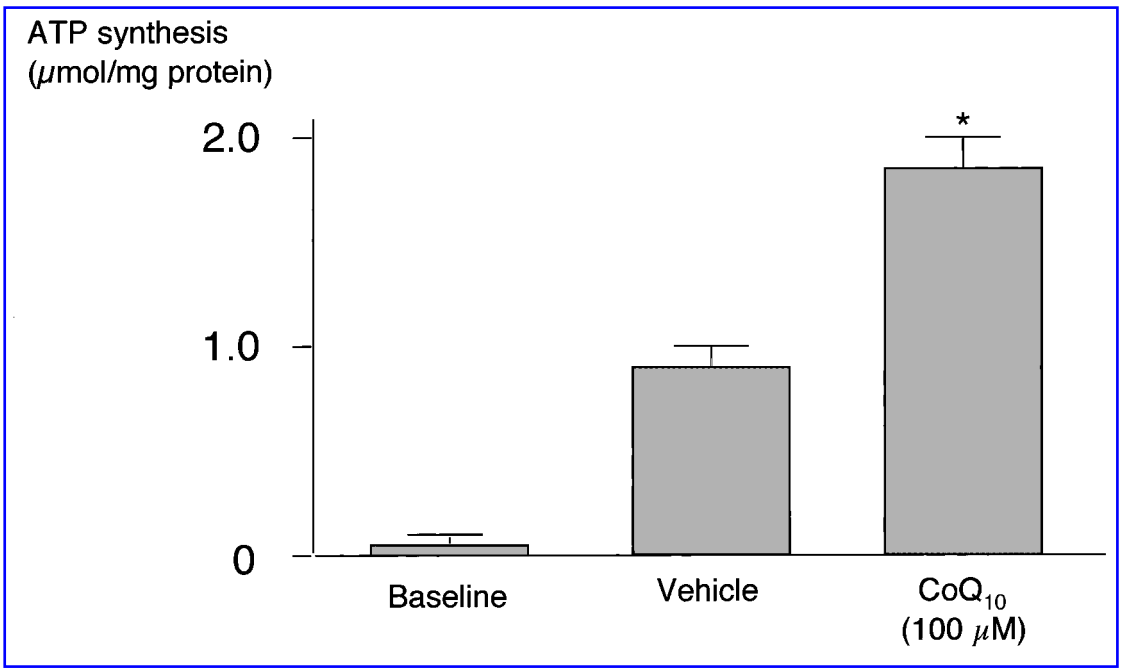

FIG. 5. ATP synthesis of isolated mitochondria. Isolated mitochondria were resuspended in buffer A and incubated for 15 min at 37°C after addition of 5 m*M* KH_2PO_4; 5 m*M* succinate, and 5 m*M* ADP. Each column expresses the mean ± SEM of five experiments. Baseline samples were obtained before incubation of mitochondria with the above-mentioned ATP-generating buffer. *$p < 0.05$ compared with vehicle.

PT and cytochrome *c* release from mitochondria have been considered to be involved in an execution phase of cell death. PT pore opening causes dissipation of the H^+ gradient across the membrane. The loss of electrochemical gradient results in uncoupling of the respiratory chain and subsequent abrogation of ATP synthesis via F_0–F_1 ATPase. Depriving the cell of ATP is a primary step for necrosis. On the other hand, cytochrome *c* released into the cytosol can activate a cysteine protease (caspase) cascade involved in apoptotic processes in the presence of Apaf and dATP (2, 30). Therefore, replenishment of mitochondria with CoQ may have a therapeutic advantage against ROS- and Ca^{2+}-loading-mediated myocardial injury by preventing the pathways for cardiomyocyte necrosis and apoptosis.

Finally, CoQ-induced potentiation of ROS generation deserves discussion from the viewpoint of myocardial acquisition of tolerance to ischemia and reperfusion injury. It is increasingly clear that redox signaling plays a crucial role in promoting cell survival and tolerance under various noxious conditions. Aerobic organisms are known to take advantage of a small amount of ROS as a sensor to stimulate signal transduction pathways that feed back to protect against lethal hypoxia and oxidative stress (4). Ischemic preconditioning is a typical paradigm of such an adaptive mechanism in that a preceding brief period of ischemia can alleviate cellular injury induced by a more prolonged period of ischemia (20). Recent studies have raised the hypothesis that sublethal oxidant stress generated during a brief period of ischemia and reperfusion could be a trigger of ischemic preconditioning (1, 7, 28). These studies also suggest that mitochondria are the source of ROS generation during the preconditioning challenge. Thus, pro-oxidant function of CoQ may be involved in the generation of redox signaling and the activation of cellular defense systems against lethal oxidative damage.

In conclusion, CoQ may play dual roles in myocardial protection against oxidative stress by directly acting as an antioxidant that inhibits death signaling, i.e., PT, cytochrome *c* release, and ATP depletion, and by potentiating ROS release from mitochondria that may be participating in generation of the redox signaling for

cellular acquisition of tolerance to oxidant stress.

ABBREVIATIONS

Apaf, apoptotic protease activating factors; CoQ, coenzyme Q_{10}; HNE, 4-hydroxy-2-nonenal; H_2O_2, hydrogen peroxide; PT, permeability transition; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate.

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