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A novel mechanism of coenzyme Q10 protects against human endothelial cells from oxidative stress-induced injury by modulating NO-related pathways☆,☆☆

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Abstract

Background: Atherosclerosis is a chronic inflammatory disease of the vessel wall associated with oxidized low-density lipoprotein (oxLDL)-induced apoptosis of endothelial cells. Coenzyme Q10 (CoQ10), a potent antioxidant and a critical intermediate of the electron transport chain, has been reported to inhibit LDL oxidation and thus the progression of atherosclerosis. However, its molecular mechanisms on endothelial cells remain still unclarified.
Methods: In this study, primary human umbilical vein endothelial cell cultures treated with oxLDL were used to explore the protective effects of CoQ10.
Results: Our results showed that CoQ10 attenuated the oxLDL-induced generation of reactive oxygen species and improved the antioxidant capacity. CoQ10 also attenuated the oxLDL-mediated down-regulation of endothelial nitric oxide synthase (eNOS) and up-regulation of inducible nitric oxide synthase (iNOS). In addition, CoQ10 suppressed oxLDL-activated NF-κB and downstream inflammatory mediators, including expression of adhesion molecules, release of proinflammatory cytokines and the adherence of monocytic THP-1 cells. Moreover, CoQ10 attenuated oxLDL-altered proapoptotic responses. The inhibitor of eNOS (L-NIO 10 μM) and iNOS (1400W 10 μM) as well as NO enhancer (SNP 10 μM) were used to clean up the mechanism.
Conclusion: These results provide new insight into the possible molecular mechanisms by which CoQ10 protects against atherogenesis by NO-related pathways.

Keywords: oxLDL; CoQ10; NO; ROS; Apoptosis; Inflammation

1. Introduction

Atherosclerotic cardiovascular disease is associated with oxidative stress [1]. There is an increasing body of evidence showing that oxidized low-density lipoprotein (oxLDL)-induced apoptosis of vascular endothelial cells participates in the pathogenesis of atherosclerosis. The early stages of the atherosclerotic process are initiated by accumulation of oxLDL and activation of endothelial cells with subsequent expression of adhesion molecules and increased binding of monocytes to the vascular endothelium. Proinflammatory cytokines, such as interleukin 8 (IL-8) and tumor necrosis factor α (TNF-α), which are released when endothelial cells are exposed to oxLDL, up-regulate the expression of cell adhesion molecules. This series of adverse changes is also associated with a decrease in the bioavailability of nitric oxide (NO); NO is produced in endothelial cells by the constitutively expressed enzyme endothelial nitric oxide

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synthase (eNOS). Considerable evidence indicates that oxLDL-induced endothelial dysfunction is associated with down-regulation of eNOS and up-regulation of inducible nitric oxide synthase (iNOS). Reactive oxygen species (ROS), especially superoxide, generated by oxLDL directly reacts with NO to form peroxynitrite, a stable molecule that is toxic to endothelial cells. As a superoxide scavenger, NO inhibits the generation of hydrogen peroxide and impedes the activation of NF-κB and the subsequent expression of inflammatory mediators that promote leukocyte adhesion [2] and macrophage recruitment [3].

Moreover, several pathways involved in the cytotoxicity of oxLDL are dependent on the generation of ROS [4]. At high concentrations, ROS can induce cell injury and death by oxidatively modifying proteins, carbohydrates, nucleic acids and lipids. In addition, the proapoptotic effects of oxLDL-induced ROS generation in endothelial cells involve the disturbance of mitochondrial membrane permeability followed by cytochrome *c* release and subsequently the activation of executioner caspases [5]. Therefore, therapeutic interventions involving inhibitors of oxLDL-induced endothelial apoptosis may prevent the development of atherogenic diseases as well as reduce the morbidity and increase the survival rate of patients with cardiovascular diseases.

Coenzyme Q10 (CoQ10) is a key component of mitochondrial oxidative phosphorylation and adenosine triphosphate production [6]. It has also been shown that CoQ10 located in the mitochondria, lysozomes, Golgi and plasma membranes has antioxidant activity either by directly reacting with free radicals or by regenerating tocopherol and ascorbate from their oxidized state [6]. In humans, CoQ10 content is highest at 20 years old in the lung, heart, spleen, liver and kidney and then gradually decreases upon further aging [7]. In addition, patients with cardiovascular disease show decreased CoQ10 levels in the myocardium [8]. CoQ10 has been reported to have numerous biologic effects. For example, it has been shown *in vitro* that CoQ10 promotes cellular membrane repair *via* patch formation [9], affects the expression of genes involved in G-protein-coupled receptor-mediated JAK/STAT signaling pathways and mediates inflammatory-related response by inhibiting NF-κB activation [10]. In addition, it has been shown *in vivo* that CoQ10 reduces exercise-induced muscular injury and leads to a reduction in plasma concentrations of the oxidative stress marker malondialdehyde [11], attenuates hypertension-mediated oxidative damage [12], increases the antioxidant capacity of glutathione reductase and superoxide dismutase (SOD) [13] and attenuates obesity-induced inflammation and oxidative stress damage [14]. Hamilton et al. [15] showed that CoQ10 improved endothelial dysfunction in statin-treated type II diabetic patients. Clinically, CoQ10 has potential for use in prevention and treatment of cardiovascular diseases such as myocardial infarction, congestive heart failure and other drug-induced/disease-induced cardiomyopathies [8,16]. Daily supplementation of CoQ10 decreases plasma LDL concentration as well as increase HDL concentration in humans with hypercholesterolemia [17]. Chapidze et al. [18] reported that treatment with CoQ10 provide roles in positive modification of oxidative stress, anti-atherogenic fraction of lipid profile, atherogenic ratio and platelet aggregability; moreover, CoQ10 revealed anti-aggregatory ability in patients with coronary atherosclerosis [19].

However, the direct effects of CoQ10 against oxidative stress-induced endothelial injury have not been well clarified. In this study, we explored whether CoQ10 attenuates oxLDL-induced damage by modulating the NO-related pathways.

2. Materials and methods

2.1. Reagents

Fetal bovine serum, M199 and trypsin–EDTA were obtained from Gibco (Grand Island, NY); low serum growth supplement was obtained from Cascade (Portland, OR); CoQ10 (purity ≥98% by HPLC), 2′,7′-bis-2-carboxyethyl-5 (and -6)-carboxyfluorescein-acetoxymethyl ester (BCECF-AM), 1400 W, (L-N^5-(1-iminoethyl)ornithine hydrochloride; L-NIO), sodium nitroprusside (SNP),4,6-diamidino-2-phenylindole (DAPI), EDTA, penicillin, and streptomycin were obtained from Sigma (St. Louis, MO); the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining kit was obtained from Boehringer Mannheim (Mannheim, Germany); the SOD activity assay kit and the catalase activity assay kit were obtained from Calbiochem (San Diego, CA); 2′,7′-dichlorofluorescein acetoxymethyl ester (DCF-AM), Fura-2 AM and the EnzChek caspase 3 assay kit were purchased from Molecular Probes (Eugene, OR); 5,58,6,68-tetraethylbenzimidazolcarbocyanine iodide (JC-1) and anti-active caspase 3 were obtained from BioVision (Palo Alto, CA); anti-vascular cell adhesion molecule 1 (VCAM-1), anti-intercellular adhesion molecules (ICAM-1) and anti-E-selectin, IL-8 and endothelin 1 (ET-1) ELISA kits and the annexin V apoptosis kit were purchased from R&D Systems (Minneapolis, MN); anti-eNOS, anti-iNOS, anti-nitrotyrosine, anti-cyclooxygenase II (COX-II), anti-P53, anti-phospho-P53, anti-cytochrome *c*, anti-Bcl 2 and anti-Bax were obtained from Transduction Laboratories (San Diego, CA).

2.2. Cell cultures

This study was approved by the Research Ethics Committee of the China Medical University Hospital. After receiving written informed consent from the parents, fresh human umbilical cords were obtained from neonates after birth, suspended in Hanks' balanced salt solution (Gibco) and stored at 4°C. Human umbilical vein endothelial cells (HUVECs) were isolated with collagenase and used at passages 2–3 as described previously [20]. THP-1, a human monocytic leukemia cell line, was obtained from ATCC (Rockville, MD) and cultured in RPMI with 10% FBS at a density of 2 to 5×10^6 cells/ml, as suggested in the product specification sheet provided by the vendor.

2.3. Lipoprotein separation

The protocol for LDL separation used in this study has been described previously [21]. Briefly, native LDL was isolated from fresh normolipidemic human serum by sequential ultracentrifugation (ρ=1.019–1.210 g/ml) in KBr solution containing 30 mM EDTA. Immediately before oxidation, LDL was separated from EDTA and from diffusible low molecular mass compounds by gel filtration on PD-10 Sephadex G-25 M gel (Pharmacia, St-Quentin, France) in 0.01 M phosphate-buffered saline (PBS; 136.9 mM NaCl, 2.68 mM KCl, 4 mM Na_2HPO_4, 1.76 mM KH_2PO_4, pH 7.4). Copper-modified LDL (1 mg protein/ml) was prepared by exposing LDL to 10 μM $CuSO_4$ for 16 h at 37°C. The oxLDL we studied had a TBARS value of 16–20 nM/mg protein of LDL protein; native LDL had no detectable TBARS.

2.4. Determination of cytotoxicity and induce of apoptosis

To determine the effect of CoQ10 on oxLDL-induced cytotoxicity, HUVECs were incubated with indicated concentrations of CoQ10 (chloroform-dissolved) for 2 h and then exposed to 130 μg/ml oxLDL for an additional 24 h. Cell viability was assessed by the MTT assay [22], CoQ10 was present during oxLDL exposure. Apoptotic cells were determined by annexin V and TUNEL assays. In preparation for flow cytometry, cells were harvested and stained with both annexin V and PI for 10 min. They were then washed in PBS, dissolved in HEPES buffer. Apoptotic cells assessed by the TUNEL assay were visualized under a fluorescence microscope or analyzed by flow cytometry [23].

2.5. Measurement of ROS production

HUVECs (10^4 cells/well) in 96-well plates were preincubated with various concentrations of CoQ10 for 2 h and then incubated with 10 μM DCF-AM for 1 h; the fluorescence intensity was measured with a fluorescence microplate reader (Labsystems, CA) calibrated for excitation at 485 nm and emission at 538 nm (before and after 2 h of stimulation with 130 μg/ml oxLDL), CoQ10 was present during oxLDL exposure. The percentage increase in fluorescence per well was calculated by the formula $[(Ft_2-Ft_0)/Ft_0]\times100$, where Ft_2 is the fluorescence at 2 h of oxLDL exposure and Ft_0 is the fluorescence at 0 min of oxLDL exposure.

2.6. Measurement of antioxidant enzyme activity

To determine the effects of CoQ10 after oxLDL exposure, SOD and catalase activity in the homogenate was determined by an enzymatic assay method using a commercial kit according to the manufacturer's instructions. Enzyme activity was converted to units per milligram of protein.

2.7. Immunoblotting

To determine whether CoQ10 could attenuate the oxLDL-induced expression of apoptosis-regulating proteins, HUVECs were pretreated with CoQ10 for 2 h and then stimulated with oxLDL for 24 h; CoQ10 was present during oxLDL exposure. At the end of stimulation, cells were lysed in RIPA buffer. Proteins were then separated by electrophoresis on SDS-polyacrylamide gel. After the proteins had been transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA), the blot was

incubated with blocking buffer (1× PBS and 5% nonfat dry milk) for 1 h at room temperature and then probed with primary antibodies (eNOS, iNOS, nitrotyrosine, COX-II, phosphorylated P53, P53, Bcl-2, Bax; 1:1000 dilutions) overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary antibody (1:5000) for 1 h. The intensities were quantified by densitometric analysis (Digital Protein DNA Imagineware, Huntington Station, NY).

2.8. Nitrite (NO_2^-) accumulation

NO_2^- accumulation was used as an indicator of NO production in the medium and was assayed by Gries reagent [24]. Briefly, 100 μl of Gries reagent (1% sulfanilamide–0.1% naphthylethylene diamine dihydrochloride–2.5% H_3PO_4) (Sigma) was added to 100 μl of each supernatant in triplicate wells of 96-well plates. The plates were read in a microplate reader (Molecular Devices, Palo Alto, CA) at 550 nm against a standard curve of $NaNO_2$ in culture medium.

2.9. NF-κB assay

To explore the effects of CoQ10 on oxLDL-induced NF-κB activation, HUVECs were pretreated with CoQ10 for 2 h and then stimulated with oxLDL (130 μg/ml) for 1 h. In some cases, HUVECs were incubated with specific eNOS inhibitor (L-NIO, 10 μM), specific iNOS inhibitor (1400W, 10 μM) or exogenous NO donor SNP (25 μM) for 1 h before the treatment with CoQ10 and oxLDL, CoQ10 was present during oxLDL exposure. To prepare nuclear extracts for the NF-κB assay, the cells were first resuspended in buffer containing 10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol and 0.2 mM phenylmethylsulfonyl fluoride (PMSF), followed by vigorous vortexing for 15 s. The samples were allowed to stand at 4°C for 10 min and then were centrifuged at 2000 rpm for 2 min. The pelleted nuclei were resuspended in 30 μL buffer containing 20 mM HEPES (pH 7.9), 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol and 0.2 mM PMSF and incubated for 20 min on ice. The nuclear lysates were then centrifuged at 15,000 rpm for 2 min. Supernatants containing the solubilized nuclear proteins were stored at −70°C for subsequent NF-κB assay. NF-κB activity was measured by an NF-κB p65 Active ELISA kit (Imgenex, San Diego, CA) according to the manufacturer's instructions. The absorbance at 405 nm was determined using a microplate reader (spectraMAX 340).

2.10. Assay for ET-1 and IL-8 secretion

HUVECs were pretreated with the indicated concentrations of CoQ10 for 2 h followed by treatment with oxLDL (130 μg/ml) for 24 h; CoQ10 was present during oxLDL exposure. At the end of the oxLDL incubation period, cell supernatants were removed and assayed for ET-1 and IL-8 concentrations using an ELISA kit obtained from R&D Systems. Data are expressed in nanograms per milliliter of duplicate samples.

2.11. Adhesion assay

HUVECs at 1×10^5 cells/ml were cultured in 96-well plates. Cells were pretreated with the indicated concentrations of CoQ10 for 2 h followed by oxLDL (130 μg/ml) for 24 h; CoQ10 was present during oxLDL exposure. The medium was then removed and 0.1 ml/well of THP-1 cells (prelabeled with BECF-AM 4 μM for 30 min in RPMI at 1×10^6 cell/ml density) were added to fresh RPMI. The cells were allowed to adhere at 37°C for 1 h in a 5% CO_2 incubator. Plates were washed three times with to remove the nonadherent cells by M199. The number of adherent cells was estimated by microscopic examination and then the cells were lysed with 0.1 ml 0.25% Triton X-100. The fluorescence intensity was measured with a fluorescence microplate reader (Lab System) calibrated for an excitation at 485 nm and for emission at 538 nm.

2.12. Adhesion molecule expression

To determine whether CoQ10 could attenuate the adhesion molecule expression induced by oxLDL, HUVECs were pretreated with CoQ10 for 2 h and stimulated with oxLDL (130 μg/ml) for 24 h; CoQ10 was present during oxLDL exposure. At the end of stimulation, HUVECs were harvested and incubated with fluorescein isothiocyanate (FITC)-conjugated antibody (R&D Systems) for 45 min at room temperature. Their immunofluorescence intensity was analyzed by flow cytometry using a Becton Dickinson FACScan flow cytometer (Mountain View, CA).

2.13. Measurement of $[Ca^{2+}]_i$

To determine the effect of CoQ10 on the oxLDL-induced increase in intracellular calcium concentration, HUVECs were seeded onto 24-mm glass coverslips, pretreated with CoQ10 for 2 h and then stimulated with oxLDL (130 μg/ml) for 24 h. The cells on the coverslips were loaded with 2 μM fura-2 AM (Molecular Probe) in M199 and allowed to stand for 30 min at 37°C. After loading, the cells were washed with PBS to remove excess fluorescent dye. Then, the fluorescence of the cells on each coverslip was measured and recorded using an inverted Olympus microscope IX-70. $[Ca^{2+}]_i$ in endothelial cells was monitored at an emission wavelength of 510 nm with excitation wavelengths alternating between 340 and 380 nm with the use of a cooled charge-coupled device (CCD) camera (MicroMAX, 782YHS; Roper Scientific, Trenton, NJ), recorded using SimplePCI 6.0 (Compix Institute, Cranberry Township, PA) and calculated using Grynkiewicz's method [25].

2.14. Measurement of mitochondrial membrane potential

The lipophilic cationic probe fluorochrome 5,58,6,68-tetraethylbenzimidazolcarbocyanine iodide (JC-1) was used to explore the effects of CoQ10 on mitochondria membrane potential ($\Delta\Psi m$). JC-1 exists either as a green fluorescent monomer at depolarized membrane potentials or as a red fluorescent J-aggregate at hyperpolarized membrane potentials. After treating cells with oxLDL (130 μg/ml) for 24 h in the presence or absence of various concentrations of CoQ10, cells were rinsed with M199 and JC-1 (5 μM) was loaded. After 20 min of incubation at 37°C, cells were examined under a fluorescence microscope. Determination of the $\Delta\Psi m$ was carried out using a FACScan flow cytometer [26].

2.15. Isolation of cytosolic fraction for cytochrome c analysis

After treating cells with oxLDL in the presence or absence of CoQ10, the cells were collected and lysed with lysis buffer for 20 min on ice. The samples were homogenized by 10 passages through two 2-gauge needle. The homogenate was centrifuged at 12,000 rpm for 20 min at 4°C. A volume of cell lysates containing 30 μg of protein was analyzed by Western blot analysis for cytochrome *c* (1:1000) and β-actin (1:50,000).

2.16. Measurement of active caspase 3

To explore the effects of CoQ10 on oxLDL-induced caspase 3 activation, HUVECs were pretreated with CoQ10 for 2 h and then stimulated with oxLDL (130 μg/ml) for 24 h. In some cases, HUVECs were incubated with specific eNOS inhibitor (L-NIO, 10 μM), specific iNOS inhibitor (1400W, 10 μM) or exogenous NO donor SNP (25 μM) for 1 h before the treatment with CoQ10 and oxLDL. The level of active caspase-3 was detected by flow cytometry using a commercial fluorescein active caspase kit (Mountain View, CA) under a fluorescence microscope. The activity of caspase 3 was also measured by an EnzChek caspase-3 assay kit according to the manufacturer's instructions (Molecular Probes). After being lysed by repeated freeze–thaw cycles. Equal amounts of protein (50 μg) were added to the reaction buffer containing 5 mM of caspase 3 substrate Z-DEVD-R110, and the mixture was incubated at room temperature for 30 min. The fluorescence generated from cleavage of the substrate by caspase 3 was monitored with a fluorescence microplate reader (Labsystems) calibrated for excitation at 496 nm and for emission at 520 nm.

2.17. Statistical analyses

Results are expressed as mean±S.E. Differences between groups were analyzed using one-way ANOVA followed by Bonferroni's post hoc test. A P value <0.05 was considered statistically significant.

3. Results

3.1. CoQ10 blocked phosphatidylserine translocation and DNA damage and cell death induced by oxLDL in HUVECs

After exposure to oxLDL, the number of shrunken cells or cells with blebbing membranes was significantly lower in HUVECs that had been pretreated with CoQ10 than in those that had not been exposed to CoQ10 (Fig. 1A). As seen in Fig. 1B, the viability of HUVECs was reduced by almost 55% after exposure to oxLDL; however, CoQ10 significantly increased the viability in a dose-dependent manner. Normal LDL (nLDL) was used as one negative control, as our finding, the viability of HUVECs was not repressed after exposure to nLDL.

In addition, CoQ10 treatment was not cytotoxic up to a concentration of 50 μM. The ED50 (half-maximal effective concentration) of CoQ10 was 4.2 μM after exposure to 130 μg/ml oxLDL for 24 h. Therefore, 130 μg/ml of oxLDL and 2.5–20 μM of CoQ10 were used in the following experiments.

The annexin V and TUNEL assays were used to ascertain the apoptotic effects of oxLDL and the antiapoptotic effects of CoQ10 in HUVECs. Flow cytometric analysis revealed that the percentage of apoptotic cells in untreated HUVECs was less than 3%. In HUVECs exposed to oxLDL, however, the percentage of apoptotic cells (20.4%) and necrotic cells (10.9%) was markedly higher. The percentage of apoptotic cells in HUVECs pretreated with 20 μM CoQ10 (4.9%) was close to that in untreated HUVECs (2.9%) (Fig. 1C).

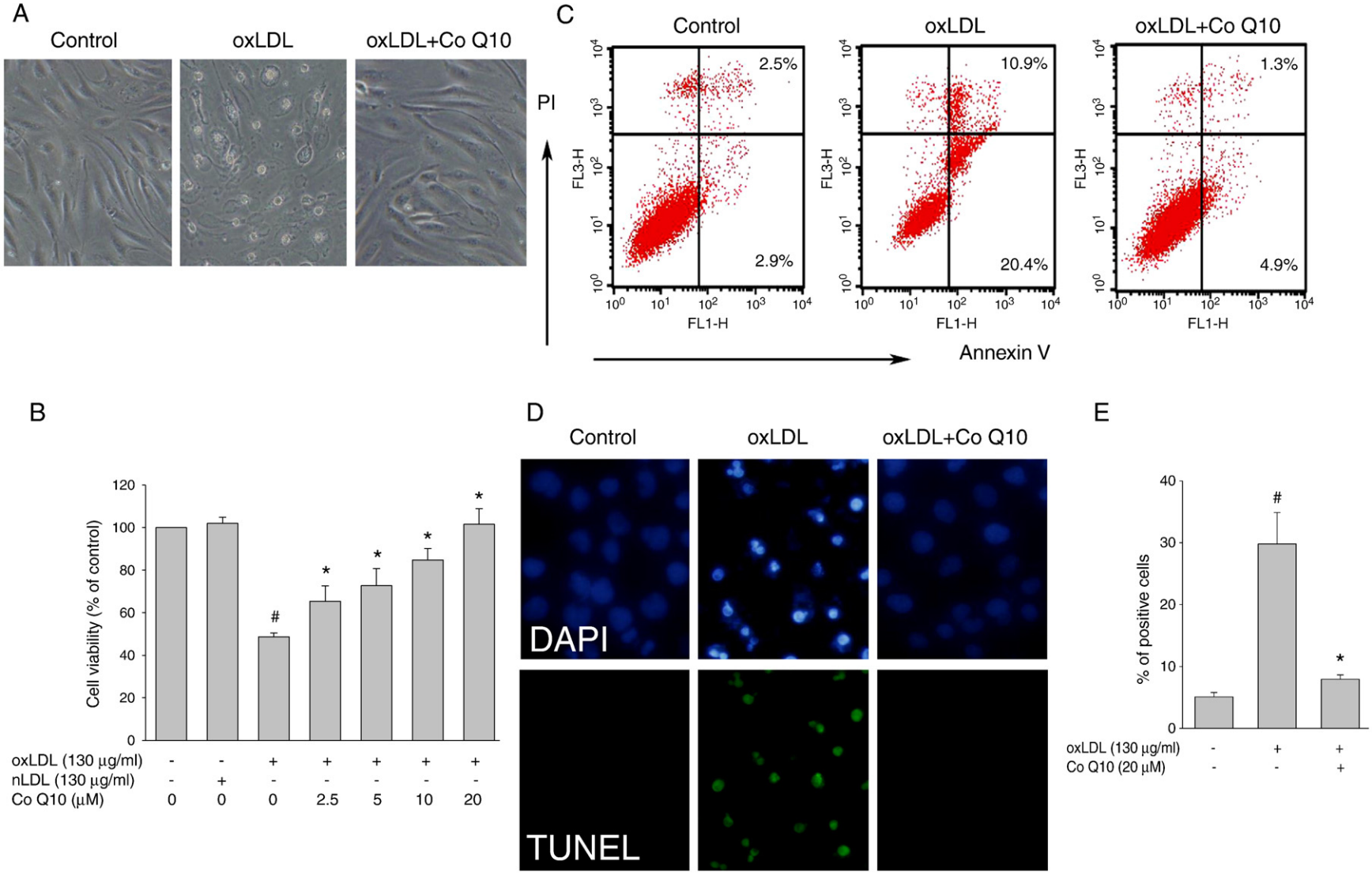

Fig. 1. Effects of CoQ10 on oxLDL-induced endothelial cell death. HUVECs were incubated with oxLDL (130 μg/ml) or nLDL in the absence or presence of indicated concentrations of CoQ10 for 24 h. Photomicrographs from phase-contrast microscopy (A). Viability was determined *via* MTT assay. Effects of CoQ10 on oxLDL-induced endothelial apoptosis (B). HUVECs were incubated with oxLDL (130 μg/ml) in the absence or presence of indicated concentrations of CoQ10 for 24 h. Apoptotic and necrotic death of oxLDL-exposed HUVECs were measured using a FITC-labeled annexin V assay and PI staining. Flow cytometry was used for confirmation (C). HUVECs were incubated with oxLDL in the absence (middle) or presence (right) of 10 μM CoQ10 for 24 h. The lower right quadrants represent the apoptotic cells and the upper right quadrants represent the necrotic cells. Late apoptotic death of oxLDL-exposed HUVECs was evaluated using the TUNEL assay (D). Data are expressed as the mean±S.E. of three independent analyses (E). #$P<.05$ vs. untreated control; *$P<.05$ vs. oxLDL treatment.

The TUNEL and DAPI staining assays were then used to clarify the protective effects of CoQ10 against oxLDL-induced DNA damage. As shown in Fig. 1D,E, cells incubated with oxLDL for 24 h showed typical features of apoptosis, including the formation of condensed nuclei. Those morphologic features were not observed in HUVECs pretreated with CoQ10.

3.2. CoQ10 inhibited oxLDL-induced intracellular ROS generation in HUVECs

To clarify whether the observed antiapoptotic effect of CoQ10 can be attributed to reduction in oxidative stress. We found that treatment with oxLDL for 2 h produced an eightfold increase in ROS generation. Pretreatment of HUVECs with CoQ10 (2.5–20 μM) led to a dose-dependent reduction in ROS (Fig. 2A, B, all $P<.05$). In this investigation, nLDL was used as one negative control, as our result, there are no significance difference between control group and nLDL group in intracellular ROS level.

To examine the mechanisms involved in the antioxidant action of CoQ10 in HUVECs exposed to oxLDL, we analyzed the activities of antioxidant enzyme activity in HUVECs treated with 130 μg/ml of oxLDL for 2 h. As shown in Fig. 2C,D, the activity of SOD and catalase were reduced by 48% and 68%, respectively, in cells exposed to oxLDL; in contrast, pretreatment of cells with CoQ10 (2.5–20 μM) significantly potentiated the activity of those antioxidant enzymes in a dose-dependent manner (all $P<.05$).

3.3. CoQ10 protects against oxLDL-induced release of ET-1 and suppression of NOS protein expression

To validate weather NO and NO synthase were involved in CoQ10 suppress oxLDL-induced injury. We, therefore, studied the effects of CoQ10 on protein expression of eNOS and iNOS as well as NO content and formation of nitrotyrosine. As shown in Fig. 3A,B, exposure to oxLDL resulted in significantly lower eNOS expression, higher iNOS expression and higher levels of nitrotyrosin than control cells; however, in HUVECs pretreated with 10 μM CoQ10, the levels of eNOS, iNOS and nitrotyrosin expression were close to those seen in control cells. Furthermore, the oxLDL-enhanced release of NO (Fig. 3C) was also suppressed in HUVECs pretreated with CoQ10 and the selective iNOS inhibitor 1400W but not eNOS inhibitor L-NIO.

It has been speculated that reduced NO release/bioavailability and enhanced release of ET-1 may contribute to oxLDL-induced endothelial dysfunction and the development of atherosclerosis [27]. Our results display that CoQ10 inhibited oxLDL-enhanced ET-1 secretion (Fig. 3D).

3.4. CoQ10-mediated protective function involving eNOS action and CoQ10 mitigated the NF-κB and NF-κB-related proinflammatory response in HUVECs

NF-κB is a vital player in the regulation of inflammatory response, apoptosis and cell survival [28]. In addition, NO inhibits cleavage of

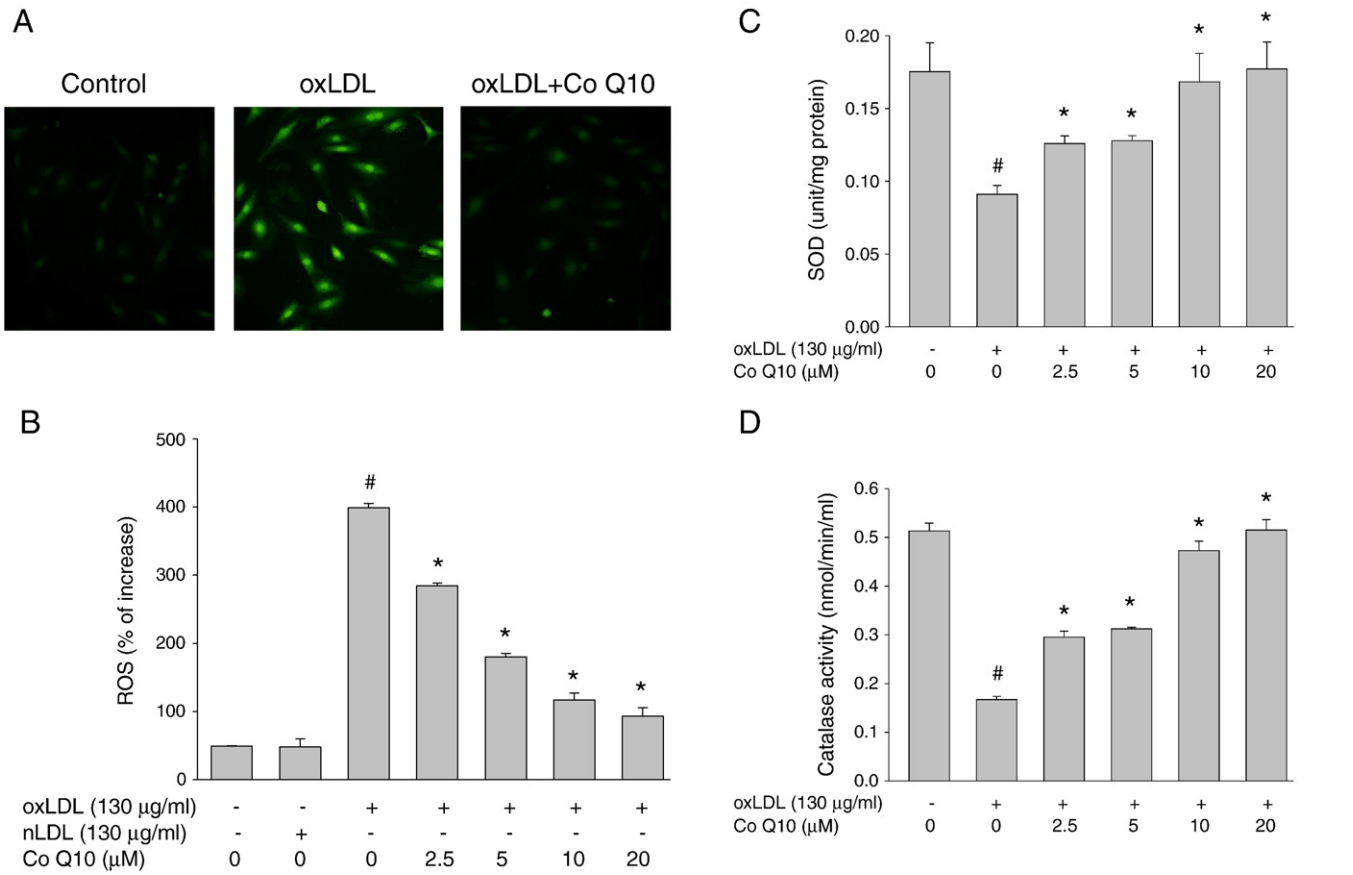

Fig. 2. The protective effects of CoQ10 on oxLDL-mediated ROS generation in HUVECs. After preincubation for 2 h with the indicated concentrations of CoQ10 (2.5–20 μM), HUVECs were incubated with the H_2O_2-sensitive fluorescent probe DCF-AM (10 μM) for 1 h, followed by treatment with 130 μg/ml oxLDL or nLDL for 2 h. (A) Fluorescence images exhibited the ROS level in control cells (left) and HUVECs stimulated with oxLDL (middle) in the presence of 10 μM CoQ10 (right). (B) Fluorescence intensity of HUVECs was measured with a fluorescence microplate reader. Fluorescence distribution of DCF-AM oxidation was expressed as a percentage of increased intensity. The activity of (C) SOD and (D) catalase in HUVECs stimulated with oxLDL in the absence or presence of indicated concentrations of CoQ10 were determined. Data are expressed as the mean±S.E. of three independent analyses. #$P<.05$ vs. untreated control; *$P<.05$ compared with oxLDL treatment.

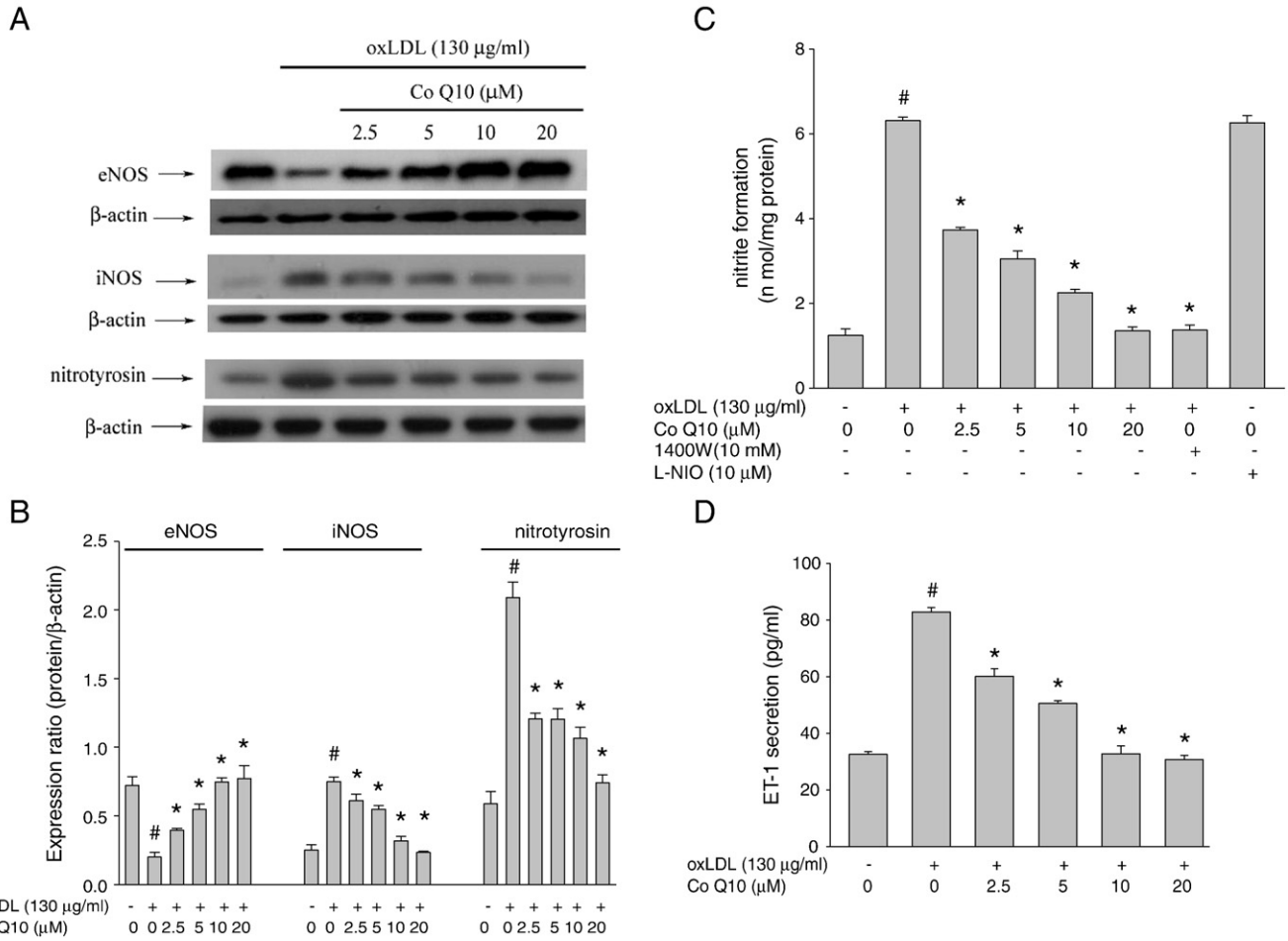

Fig. 3. CoQ10 suppressed the oxLDL-induced down-regulation of eNOS, up-regulation of iNOS protein expression (A, B) and oxLDL-enhanced NO release (C). In addition, CoQ10 attenuated the oxLDL-enhanced secretion of ET-1 (D). HUVECs were pretreated for 2 h with the indicated concentrations of CoQ10 followed by stimulation with oxLDL (130 μg/ml) for 24 h. At the end of the incubation period, level of eNSO, iNOS and nitrotyrosine protein were determined by immunoblotting; content of NO was assayed using Griess reagent; ET-1 secretion was measured by ELISA. The values represent means±S.E. from three separate experiments. #P<.05 vs. untreated control; *P <0.05 vs. oxLDL treatment.

IκB and NF-κB activation [29]. We hypothesized that oxLDL induces NF-κB activation by reducing the bioavailability of NO and that oxLDL-induced NF-κB activation could be reversed by CoQ10. As shown in Fig. 4A, pretreatment of HUVECs with CoQ10 conspicuously inhibited the oxLDL-induced activation of NK-κB. Moreover, cells pretreatment with L-NIO partially antagonized the inhibitory effect of CoQ10. Additionally, cells pretreated with 1400W or exogenous donor of NO (SNP) showed a marked reduction in the activation of NF-κB (all P<.05).

Proinflammatory cytokines, COX-II and adhesion molecules were mediated by NF-κB. All of the proinflammatory events subsequently lead to the tethering and adherence of monocytic cells to endothelial cells. Our results showed that pretreatment with CoQ10 attenuated the release of IL-8 (Fig. 4B), the expression of COX-II (Fig. 4C,D), the adhesion of monocytic THP-1 cells to HUVECs exposed to oxLDL (Fig. 4E, F) and the expression of adhesion molecules (ICAM-1, VCAM-1 and E-selectin) (Fig. 4G).

3.5. CoQ10 suppressed the oxLDL-induced apoptic responses

Intracellular calcium rise is involved in oxLDL-induced endothelial apoptosis [30]. To validate the protective effect of chronic exposure of HUVECs to a detrimental concentration of oxLDL on intracellular calcium concentration, we incubated HUVECs with oxLDL (130 μg/ml) in the absence or presence of different concentrations of CoQ10. Our finding that the 340/380 ratio of $[Ca^{2+}]_i$ elevated in oxLDL-exposed cells after 24 h was reduced in cells pretreatment of CoQ10 (Fig. 5A, all P<.05).

The increase in intracellular Ca^{2+} triggers the activation of several calcium-dependent d proapoptotic signaling transduction pathways. In addition, calcium is the most important signal for opening of the mitochondrial permeability transition pore (PTP), a mechanism that triggers apoptosis. As a consequence of both the dysfunction of the electrochemical gradient caused by pore opening and rupture of the outer mitochondrial membrane, the mitochondrial membrane potential (Ψ_m) generally collapses. We, therefore, determined the mitochondrial permeability to understand whether CoQ10 preserves mitochondrial stability after exposure to oxLDL. As shown in Fig. 5B, oxLDL depolarized the mitochondrial transmembrane potential in HUVECs, as shown by the increase in green fluorescence (middle panel); however, pretreatment with CoQ10 contributed to the maintenance of mitochondrial transmembrane potential, as indicated by repression of green fluorescence and restoration of red fluorescence (right panel). The results of flow cytometry supported those findings (Fig. 5C).

Apoptosis regulator proteins in the Bcl-2 family govern mitochondrial outermembrane permeabilization and can be either proapoptotic or antiapoptotic. Cheng et al. [31] reported that oxLDL-induced generation of ROS in endothelial cells leads to the activation of P53, which subsequently induces a conformational change in Bax that

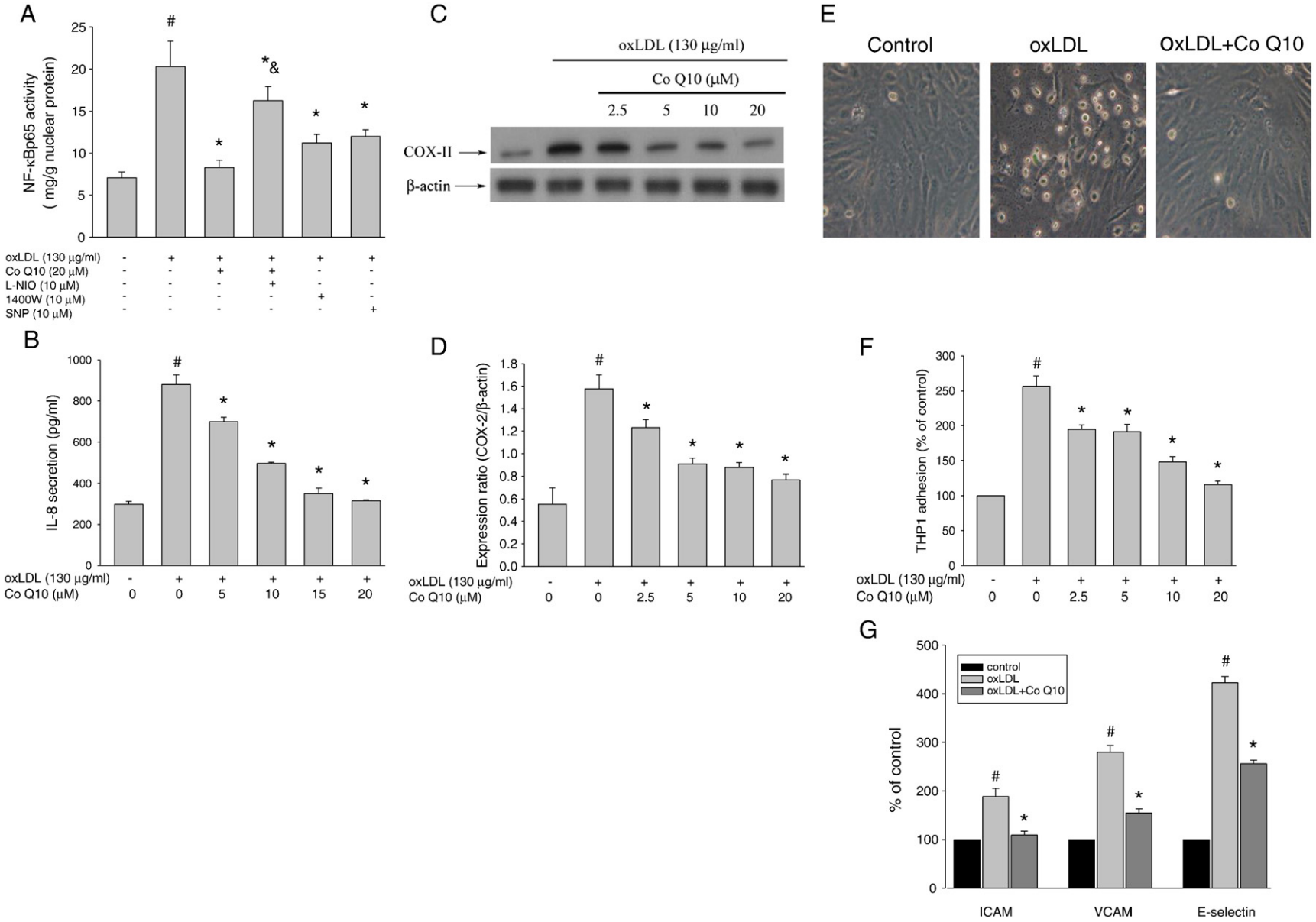

Fig. 4. Effects of CoQ10 on oxLDL-induced NF-κB activation. HUVECs were pretreated with each inhibitor 1 h before incubated for additional 1 h with oxLDL (130 μg/ml). Nucleic proteins were extracted for nuclear translocation assay of NF-κBp65 (A). Effects of CoQ10 on oxLDL-induced IL-8 release, COX-II expression, adhesion molecule expression and adhesiveness of THP-1 monocytic cells to HUVECs. HUVECs were pretreated with indicated concentrations of CoQ10 for 2 h followed by stimulation with oxLDL (130 μg/ml) for another 24 h. (B) Media were collected for ELISA assay of IL-8 protein levels. (C, D) Protein levels of COX-II were assessed by Western blotting. (E, F) THP-1 cells preloaded with BECEF were incubated with HUVECs for 1 h. The adhesiveness of HUVECs to THP-1 was measured as described in the Materials and Methods. (G) Cell surface expression of ICAM-1, VCAM-1 and E-selectin was determined by flow cytometry. The values represent means±S.E. from three separate experiments. $^{\#}P<.05$ vs. untreated control; $^{*}P<0.05$ vs. oxLDL treatment. $^{\&}P<.05$ vs. oxLDL+CoQ10 treatment.

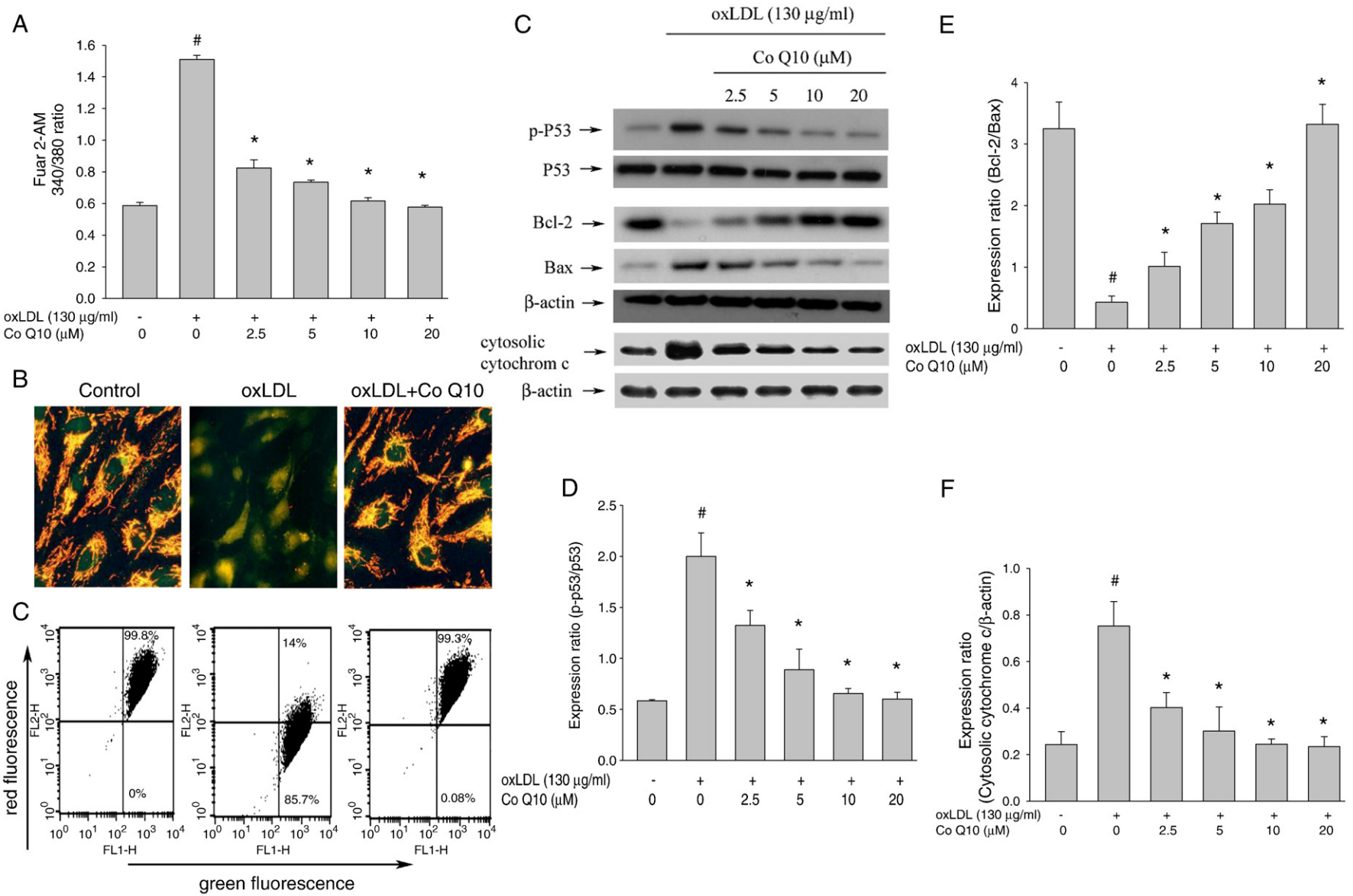

Fig. 5. Effects of CoQ10 on oxLDL-triggered intracellular calcium rise (A). HUVECs were pretreated with indicated concentrations of CoQ10 for 2 h followed by stimulation with oxLDL (130 μg/ml) for another 24 h. The measurement of intracellular calcium is described in the Materials and Methods. The influence of CoQ10 on mitochondrial transmembrane permeability transition induced by oxLDL. (B) ΔΨm was inspected with the signal from monomeric and J-aggregate JC-1 fluorescence, as described in the Materials and Methods. (left) No treatment; (middle) oxLDL; (right) oxLDL + CoQ10. (C) JC-1 fluorescence was confirmed by flow cytometry. (D–F) Immunoblotting analysis of apoptotic cells in HUVECs exposed to oxLDL and CoQ10. HUVECs were incubated with 130 μg/ml oxLDL in the absence or presence of indicated concentrations (2.5–20 μM) of CoQ10 for 24 h. Representative Western blots and summary data showing that oxLDL up-regulated proapoptotic (phosphorylated P53, Bax) and down-regulated antiapoptotic (Bcl-2) proteins and led to an increase in the concentration of cytosolic cytochrome *c*. Intervention with CoQ10 suppressed the above-mentioned oxLDL-induced responses. Results were confirmed by densitometric analysis; the values are presented as means±S.E. of three separate experiments. $^{\#}P<.05$ vs. untreated control; $^{*}P<.05$ vs. oxLDL treatment.

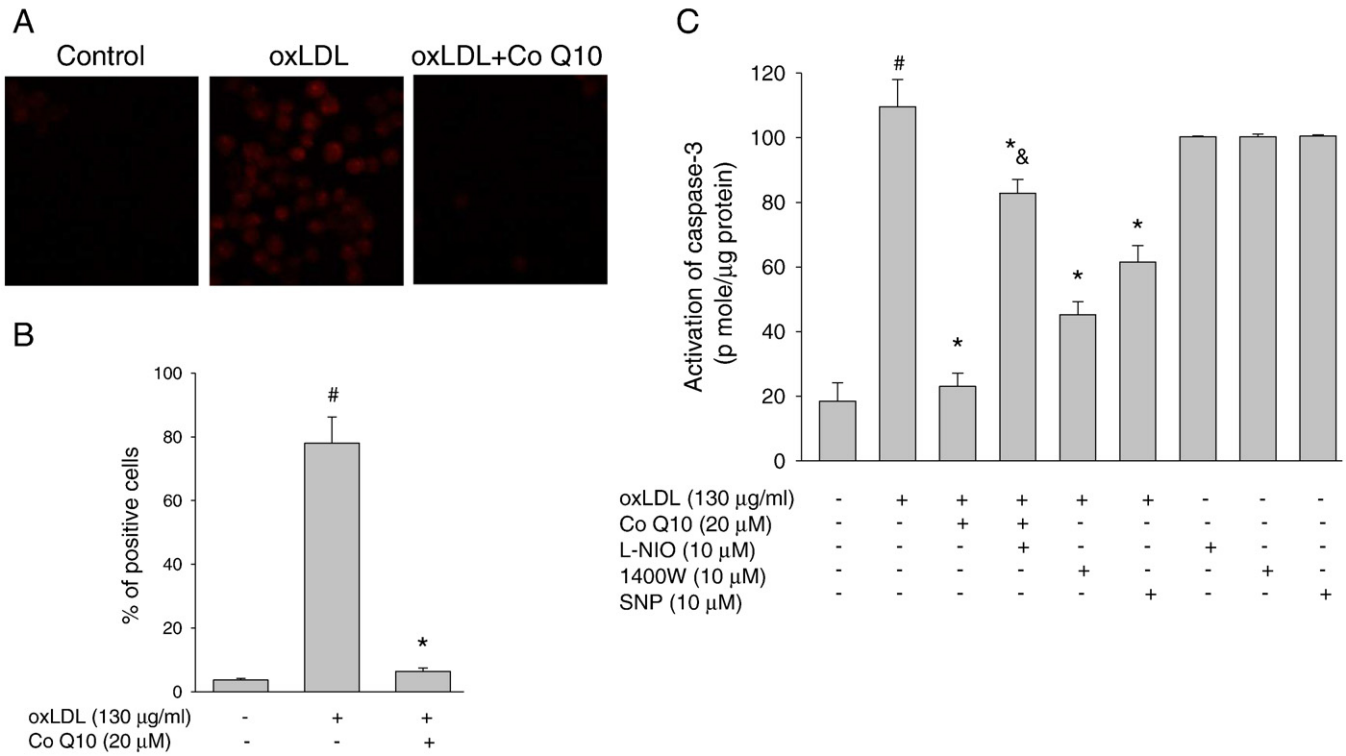

Fig. 6. Effects of CoQ10 on oxLDL-induced caspase 3 activation. (A) HUVECs were incubated for 1 h with indicated concentrations of CoQ10, followed by exposure to oxLDL (130 μg/ml) for another 24 h. (B) Fluorescent images show the activated caspase 3 level in control cells (left), HUVECs stimulated with oxLDL (middle), and in the presence of 10 μM CoQ10 (right). (C) Fluorescence intensity of cells was measured by flow cytometry. The activity of caspase 3 was measured by EnzCaspase-3 assay kit. Data are expressed as the mean±S.E. of three independent analyses. $^{\#}P<.05$ vs. untreated control; $^{*}P<.05$ vs. oxLDL treatment. $^{\&}P<.05$ vs. oxLDL+CoQ10 treatment.

enables the mitochondrial translocation of that proapoptotic protein. Our results showed that CoQ10 significantly reduced the activation of P53 and the expression of Bax and significantly increased the expression of the antiapoptotic protein Bcl-2 in a concentration-dependent manner (Fig. 5C–E).

Disintegration of mitochondrial membrane function contributes to the release of cytochrome *c* from the mitochondria. As shown in Fig. 5C,F, CoQ10 reduced the cytosolic cytochrome *c* level in oxLDL-exposed HUVECs.

3.6. CoQ10 prevented oxLDL-induced caspase 3 activation

To examine whether CoQ10 ultimately influences the activity of this apoptotic effector in modulating apoptosis, we studied the effects of CoQ10 on oxLDL-induced activation of caspase 3 using fluorescence microscopy and flow cytometry. As shown in Fig. 6A,B, CoQ10 inhibited oxLDL-induced caspase 3 activation. We also determined the activity of caspase 3 using the EnzCaspase-3 assay kit. As seen in Fig. 6C, CoQ10 effectively inhibited the cleavage of caspase 3 activated by oxLDL. Simultaneous treatment of HUVECs with CoQ10 and eNOS inhibitor partially abolished the inhibitory effects of CoQ10 on caspase 3 activity. Furthermore, the addition of 1400W or SNP definitely inhibited oxLDL-induced activation of caspase 3.

4. Discussion

In the present study, we demonstrated that CoQ10 ameliorated oxLDL-induced endothelial dysfunction by inhibiting inflammatory and oxidative damage that leads to cellular apoptosis. Specifically, CoQ10 suppressed the generation of ROS, which subsequently attenuated the oxLDL-impaired expression of antioxidant enzymes, increased the bioavailability of NO, reduced ET-1 secretion, stabilized the mitochondrial membrane and maintained the endothelial $[Ca^{2+}]_i$ level, thereby preventing the release of cytochrome *c*, a molecule required for the activation of the proapoptotic protein caspase 3.

The generation of ROS and the activities of antioxidant enzymes must be kept in balance to preserve homeostasis and a stable intracellular redox state for normal cell function. Consistent with findings from previous studies that CoQ10 supplementation reversed the oxidative stress-induced suppression of antioxidant enzyme expression [13,32], our findings show that pretreatment with CoQ10 suppressed the oxLDL-induced reduction in SOD and catalase activities and resulted in decreased ROS generation in endothelial cells exposed to oxLDL (Fig. 2).

The relationship between NO and cellular inflammation is complex because NO is cytotoxic at high concentrations and has a protective effect at low concentrations. High levels of NO have the opportunity to react with superoxide leading to peroxynitrite formation and cell toxicity [33]. It has been shown that iNOS-derived overproduction of NO can lead to activation of NF-κB, which in turn leads to the up-regulation of several major proinflammatory mediators such as COX-2, iNOS and the adhesion molecules. In the present study, we found that pretreatment of CoQ10 suppressed the oxLDL-induced down-regulation of eNOS and up-regulation of iNOS. Furthermore, our data revealed that 1400W but not L-NIO represses NO formation (Fig. 3C), indicating that oxLDL-facilitated NO generation mainly through iNOS. In addition, pretreatment with L-NIO partially antagonized the effect of CoQ10 on inhibition of NF-κB, and pretreatment with 1400W or SNP partially reversed the oxLDL-induced activation of NF-κB, suggesting that the protective effect of CoQ10 is due to its ability to modulate the NO-mediated signaling pathway (Fig. 5A).

Intracellular calcium signaling is associated with the development of atherosclerosis and several cardiovascular diseases. Vindis et al.

[34] showed that oxLDL-induced apoptosis in endothelial cells involves two distinct calcium-dependent pathways, the first mediated by calpain/mPTP/cytochrome *c*/caspase-3 and the second mediated by apoptosis-inducing factor, which is caspase-independent. Clinical studies have shown that calcium channel blockers, such as azelnidipine and dihydropyridine, protect against oxidative stress-related injuries by inhibiting intracellular ROS generation, mitigating NF-κB activation and inhibiting the expression of adhesion molecules and proinflammatory factors [35,36]. We found that CoQ10 protects against oxLDL-induced apoptosis by suppressing the oxLDL-induced rise in intracellular calcium.

Endothelial cell damage induced by oxLDL was mediated through the mitochondrial-dependent apoptotic pathway. There is evidence that NF-κB activation is accompanied by elevated P53 levels, indicating that NF-κB might play a critical role in regulating P53 [37], which subsequently induces a conformational change in Bax that enables the mitochondrial translocation of that proapoptotic protein [31]. Our results showed that CoQ10 significantly reduced the activation of NF-κB, suppressed the expression of P53 and the expression of Bax and led to a significant increase in expression of the antiapoptotic protein Bcl-2, which prevented the release of cytochrome *c* and further activation of caspase 3. It has been linked to the inhibition of caspase activation and prevention of endothelial apoptosis caused by oxLDL [38]. Our findings that CoQ10 reduced the activity of caspase 3 in oxLDL-treated HUVECs and that inhibitors of eNOS partially blocked this effect clearly identify the antiapoptotic effect of CoQ10 is due, at least in part, to its ability to modulate the NO-mediated signaling pathway.

The concentrations (2.5–20 μM) we used to attenuate oxLDL-induced endothelial cell dysfunction are similar to those investigated to mitigate other oxidative stress-related responses. For example, 10 μM of CoQ10 was shown to inhibit high glucose-induced endothelial cell adhesion molecule expression, restore high glucose-mitigated eNOS expression and attenuate high-glucose-induced ROS generation and endothelial cell apoptosis [39]. The dosage of 3000 mg/day, which reach a plasma concentration of 8.69 μM, is safe and tolerable in patients with Parkinson's disease [40]. In this study, pretreatment with 2.5 μM, a physiologically achievable concentration, was enough to mitigate oxLDL-induced ROS generation, restore the suppression of antioxidant enzyme activities induced by oxLDL and suppress the activation of ROS-mediated proapoptotic signaling pathways.

In summary, we have demonstrated that CoQ10 suppressed oxidative stress-related responses by modulating NO-related signaling. Our results provide insight into some of the mechanisms by which CoQ10 protects against endothelial damage.

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References

[1] Lum H, Roebuck KA. Oxidant stress and endothelial cell dysfunction. Am J Physiol Cell Physiol 2001;280(4):C719–41.
[2] Chen XL, Zhang Q, Zhao R, Ding X, Tummala PE, Medford RM. Rac1 and superoxide are required for the expression of cell adhesion molecules induced by tumor necrosis factor-alpha in endothelial cells. J Pharmacol Exp Ther 2003;305(2):573–80.
[3] Chen XL, Zhang Q, Zhao R, Medford RM. Superoxide, H_2O_2, and iron are required for TNF-alpha-induced MCP-1 gene expression in endothelial cells: role of Rac1 and NADPH oxidase. Am J Physiol Heart Circ Physiol 2004;286(3):H1001–7.
[4] Cominacini L, Pasini AF, Garbin U, Davoli A, Tosetti ML, Campagnola M, et al. Oxidized low density lipoprotein (ox-LDL) binding to ox-LDL receptor-1 in endothelial cells induces the activation of NF-kappaB through an increased production of intracellular reactive oxygen species. J Biol Chem 2000;275(17):12633–8.
[5] Dimmeler S, Zeiher AM. Reactive oxygen species and vascular cell apoptosis in response to angiotensin II and pro-atherosclerotic factors. Regul Pept 2000;90(1–3):19–25.
[6] Crane FL. Biochemical functions of coenzyme Q10. J Am Coll Nutr 2001;20(6):591–8.
[7] Kalen A, Appelkvist EL, Dallner G. Age-related changes in the lipid compositions of rat and human tissues. Lipids 1989;24(7):579–84.
[8] Sarter B. Coenzyme Q10 and cardiovascular disease: a review. J Cardiovasc Nurs 2002;16(4):9–20.
[9] Potgieter M, Pretorius E, Oberholzer HM. Qualitative electron microscopic analysis of cultured chick embryonic cardiac and skeletal muscle cells: the cellular effect of coenzyme q10 after exposure to triton x-100. Ultrastruct Pathol 2009;33(3):93–101.
[10] Schmelzer C, Lindner I, Rimbach G, Niklowitz P, Menke T, Doring F. Functions of coenzyme Q10 in inflammation and gene expression. Biofactors 2008;32(1–4):179–83.
[11] Kon M, Kimura F, Akimoto T, Tanabe K, Murase Y, Ikemune S, et al. Effect of coenzyme Q(10) supplementation on exercise-induced muscular injury of rats. Exerc Immunol Rev 2007;13:76–88.
[12] Murad LB, Guimaraes MR, Vianna LM. Effects of decylubiquinone (coenzyme Q10 analog) supplementation on SHRSP. Biofactors 2007;30(1):13–8.
[13] Wang H, Zhao X, Yin S. Effects of coenzyme Q10 or combined with micronutrients on antioxidant defense system in rats. Wei Sheng Yan Jiu 2008;37(3):311–3.
[14] Sohet FM, Neyrinck AM, Pachikian BD, de Backer FC, Bindels LB, Niklowitz P, et al. Coenzyme Q10 supplementation lowers hepatic oxidative stress and inflammation associated with diet-induced obesity in mice. Biochem Pharmacol 2009.
[15] Hamilton SJ, Chew GT, Watts GF. Coenzyme Q10 improves endothelial dysfunction in statin-treated type 2 diabetic patients. Diabetes Care 2009;32(5):810–2.
[16] Rosenfeldt FL, Pepe S, Linnane A, Nagley P, Rowland M, Ou R, et al. Coenzyme Q10 protects the aging heart against stress: studies in rats, human tissues, and patients. Ann NY Acad Sci 2002;959:355–9 [discussion 463–355].
[17] Mabuchi H, Nohara A, Kobayashi J, Kawashiri MA, Katsuda S, Inazu A, et al. Effects of CoQ10 supplementation on plasma lipoprotein lipid, CoQ10 and liver and muscle enzyme levels in hypercholesterolemic patients treated with atorvastatin: a randomized double-blind study. Atherosclerosis 2007;195(2):e182–9.
[18] Chapidze G, Kapanadze S, Dolidze N, Bachutashvili Z, Latsabidze N. Prevention of coronary atherosclerosis by the use of combination therapy with antioxidant coenzyme Q10 and statins. Georgian Med News 2005(118):20–5.
[19] Chapidze GE, Kapanadze SD, Dolidze NK, Latsabidze NE, Bakhutashvili ZV. Combination treatment with coenzyme Q10 and simvastatin in patients with coronary atherosclerosis. Kardiologiia 2006;46(8):11–3.
[20] Ou HC, Song TY, Yeh YC, Huang CY, Yang SF, Chiu TH, et al. EGCG protects against oxidized LDL-induced endothelial dysfunction by inhibiting LOX-1-mediated signaling. J Appl Physiol 2010;108(6):1745–56.
[21] Kuo WW, Huang CY, Chung JG, Yang SF, Tsai KL, Chiu TH, et al. Crude extracts of Solanum lyratum protect endothelial cells against oxidized low-density lipoprotein-induced injury by direct antioxidant action. J Vasc Surg 2009;50(4):849–60.
[22] Denizot F, Lang R. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. J Immunol Methods 1986;89(2):271–7.
[23] Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 1992;119(3):493–501.
[24] Ding AH, Nathan CF, Stuehr DJ. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. J Immunol 1988;141(7):2407–12.
[25] Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca2+ indicators with greatly improved fluorescence properties. J Biol Chem 1985;260(6):3440–50.
[26] Bedner E, Li X, Gorczyca W, Melamed MR, Darzynkiewicz Z. Analysis of apoptosis by laser scanning cytometry. Cytometry 1999;35(3):181–95.
[27] Lubrano V, Baldi S, Ferrannini E, L'Abbate A, Natali A. Role of thromboxane A2 receptor on the effects of oxidized LDL on microvascular endothelium nitric oxide, endothelin-1, and IL-6 production. Microcirculation 2008;15(6):543–53.
[28] Robbesyn F, Salvayre R, Negre-Salvayre A. Dual role of oxidized LDL on the NF-kappaB signaling pathway. Free Radic Res 2004;38(6):541–51.
[29] Marshall HE, Merchant K, Stamler JS. Nitrosation and oxidation in the regulation of gene expression. FASEB J 2000;14(13):1889–900.
[30] Sanson M, Ingueneau C, Vindis C, Thiers JC, Glock Y, Rousseau H, et al. Oxygen-regulated protein-150 prevents calcium homeostasis deregulation and apoptosis induced by oxidized LDL in vascular cells. Cell Death Differ 2008;15(8):1255–65.
[31] Cheng J, Cui R, Chen CH, Du J. Oxidized low-density lipoprotein stimulates p53-dependent activation of proapoptotic bax leading to apoptosis of differentiated endothelial progenitor cells. Endocrinology 2007;148(5):2085–94.
[32] Rauscher FM, Sanders RA, Watkins III JB. Effects of coenzyme Q10 treatment on antioxidant pathways in normal and streptozotocin-induced diabetic rats. J Biochem Mol Toxicol 2001;15(1):41–6.
[33] Zou MH, Leist M, Ullrich V. Selective nitration of prostacyclin synthase and defective vasorelaxation in atherosclerotic bovine coronary arteries. Am J Pathol 1999;154(5):1359–65.
[34] Vindis C, Elbaz M, Escargueil-Blanc I, Auge N, Heniquez A, Thiers JC, et al. Two distinct calcium-dependent mitochondrial pathways are involved in

oxidized LDL-induced apoptosis. Arterioscler Thromb Vasc Biol 2005;25(3): 639–45.
[35] Naito Y, Shimozawa M, Manabe H, Nakabe N, Katada K, Kokura S, et al. Azelnidipine, a new calcium channel blocker, inhibits endothelial inflammatory response by reducing intracellular levels of reactive oxygen species. Eur J Pharmacol 2006;546(1–3):11–8.
[36] Takayama M, Yao K, Wada M. The dihydropyridine calcium channel blocker benidipine prevents lysophosphatidylcholine-induced endothelial dysfunction in rat aorta. J Biomed Sci 2009;16:57.
[37] Dentelli P, Rosso A, Zeoli A, Gambino R, Pegoraro L, Pagano G, et al. Oxidative stress-mediated mesangial cell proliferation requires RAC-1/reactive oxygen species production and beta4 integrin expression. J Biol Chem 2007;282(36): 26101–10.
[38] Hoffmann J, Haendeler J, Aicher A, Rossig L, Vasa M, Zeiher AM, et al. Aging enhances the sensitivity of endothelial cells toward apoptotic stimuli: important role of nitric oxide. Circ Res 2001;89(8):709–15.
[39] Tsuneki H, Sekizaki N, Suzuki T, Kobayashi S, Wada T, Okamoto T, et al. Coenzyme Q10 prevents high glucose-induced oxidative stress in human umbilical vein endothelial cells. Eur J Pharmacol 2007;566(1–3):1–10.
[40] Shults CW, Flint Beal M, Song D, Fontaine D. Pilot trial of high dosages of coenzyme Q10 in patients with Parkinson's disease. Exp Neurol 2004;188(2): 491–4.