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Coenzyme Q_{10} prevents high glucose-induced oxidative stress in human umbilical vein endothelial cells

Hiroshi Tsuneki [a,*], Naoto Sekizaki [a], Takashi Suzuki [a], Shinjiro Kobayashi [b,c], Tsutomu Wada [a], Tadashi Okamoto [d], Ikuko Kimura [a], Toshiyasu Sasaoka [a]

[a] Department of Clinical Pharmacology, University of Toyama, 2630 Sugitani, Toyama 930-0194, Japan
[b] Department of Clinical Pharmacology, Hokuriku University, 3-Ho Kanagawa-Machi, Kanazawa 920-1181, Japan
[c] Organization for Frontier Research in Preventive Pharmaceutical Sciences, Hokuriku University, 3-Ho Kanagawa-Machi, Kanazawa 920-1181, Japan
[d] Department of Health Sciences and Social Pharmacy, Faculty of Pharmaceutical Sciences, Kobe Gakuin University, Kobe, Japan

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Abstract

Hyperglycemia-induced oxidative stress plays a crucial role in the pathogenesis of vascular complications in diabetes. Although some clinical evidences suggest the use of an antioxidant reagent coenzyme Q_{10} in diabetes with hypertension, the direct effect of coenzyme Q_{10} on the endothelial functions has not been examined. In the present study, we therefore investigated the protective effect of coenzyme Q_{10} against high glucose-induced oxidative stress in human umbilical vein endothelial cells (HUVEC). HUVEC exposed to high glucose (30 mM) exhibited abnormal properties, including the morphological and biochemical features of apoptosis, overproduction of reactive oxygen species, activation of protein kinase Cβ2, and increase in endothelial nitric oxide synthase expression. Treatment with coenzyme Q_{10} strongly inhibited these changes in HUVEC under high glucose condition. In addition, coenzyme Q_{10} inhibited high glucose-induced cleavage of poly(ADP-ribose) polymerase, an endogenous caspase-3 substrate. These results suggest that coenzyme Q_{10} prevents reactive oxygen species-induced apoptosis through inhibition of the mitochondria-dependent caspase-3 pathway. Moreover, consistent with previous reports, high glucose caused upregulation of intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) in HUVEC, and promoted the adhesion of U937 monocytic cells. Coenzyme Q_{10} displayed potent inhibitory effects against these endothelial abnormalities. Thus, we provide the first evidence that coenzyme Q_{10} has a beneficial effect in protecting against the endothelial dysfunction by high glucose-induced oxidative stress *in vitro*.

Keywords: Coenzyme Q_{10}; Endothelial cell; Apoptosis; Adhesion; Glucose; Diabetes

1. Introduction

Diabetes mellitus is associated with vascular complications that contribute to the increased morbidity and mortality of the disease (Calles-Escandon and Cipolla, 2001; Feener and King, 2001). The diabetic complications include macrovascular diseases manifested by accelerated atherosclerosis and microvascular diseases such as retinopathy, nephropathy and neuropathy. Evidence indicates a causal link between diabetic hyperglycemia and the development of vascular complications (Way et al., 2001). Hyperglycemia is shown to cause the endothelial dysfunctions that include decreased endothelium-dependent vasodilation and accelerated thrombus formation through increased leukocyte–endothelial cell adhesion (Feener and King, 2001). In addition, high glucose induces apoptosis in vascular endothelial cells, which is also considered to be implicated in the development of diabetic complications (Risso et al., 2001; Zou et al., 2002; Liu et al., 2004; Sheu et al., 2005).

It has been reported that diabetic patients showed increased generation of reactive oxygen species, compared to control subjects (Dandona et al., 1996). Reactive oxygen species can cause oxidative damage to cellular constituents, such as DNA, lipids and proteins. In endothelial cells, glucose can enter freely through the cell membrane in an insulin-independent manner via a glucose transporter 1 (GLUT1), and the intracellular

* Corresponding author. Tel.: +81 76 434 7514; fax: +81 76 434 5067.
E-mail address: htsuneki@pha.u-toyama.ac.jp (H. Tsuneki).

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glucose elevation leads to overproduction of reactive oxygen species, particularly superoxide anion, in mitochondria at the level of complex II (succinate–coenzyme Q reductase) (Nishikawa et al., 2000; Ceriello, 2003). This is the initial step in activation of four major pathways of hyperglycemic damage, including activation of protein kinase C, increased advanced glycosylation end product formation, increased polyol pathway flux and hexosamine pathway flux (Brownlee, 2001; Ceriello, 2003; Feldman, 2003). Thus, oxidative stress is considered to play an important role in the etiology of diabetic vascular complications.

Coenzyme Q_{10}, also known as ubiquinone-10 or ubiquinol-10, is well defined as a crucial component in mitochondrial oxidative phosphorylation and adenosine triphosphate production (Crane, 2001). It has also been clarified that coenzyme Q_{10} located in the mitochondria, lysozomes, Golgi and plasma membranes provides an antioxidant action either by direct reaction with free radicals or by regeneration of tocopherol and ascorbate from their oxidized state (Crane, 2001). In the human lung, heart, spleen, liver and kidney, the coenzyme Q_{10} content is highest at 20 years of age, and then gradually decreases upon further aging (Kalén et al., 1989). Coenzyme Q_{10} levels are decreased in the myocardium of patients with cardiovascular disease, although conflicting results have been seen in plasma coenzyme Q_{10} levels (Sarter, 2002). In addition, circulating levels of coenzyme Q_{10} are shown to be significantly lower in patients with diabetes than control subjects (McDonnell and Archbold, 1996; Miyake et al., 1999). Therefore, decreased plasma coenzyme Q_{10} is considered to be associated with increased oxidative stress in diabetes.

Coenzyme Q_{10} has been clinically used for the treatment of congestive heart failure. Some evidences also suggest its use in diabetes, hypertension and coronal artery disease (Chagan et al., 2002; Sarter, 2002). In particular, dietary supplementation with coenzyme Q_{10} improves endothelial function of the brachial artery in patients with type 2 diabetes, in terms of flow-mediated dilatation of the artery (Watts et al., 2002). However, the direct effect of coenzyme Q_{10} on the endothelial functions has not been examined. In the present study, we therefore investigated the protective effect of coenzyme Q_{10} against high glucose-induced oxidative stress in human umbilical vein endothelial cells.

2. Materials and methods

2.1. Materials

Coenzyme Q_{10} (ubiquinone-10) was donated by Nisshin Pharma Inc. (Tokyo, Japan). This compound was dissolved in dimethyl sulfoxide (DMSO, Sigma) and diluted in culture medium. Anti-β-actin antibody was purchased from Sigma (St. Louis, MO, USA). Hoechst 33258 was purchased from Wako Pure Chemicals Industries (Osaka, Japan). Anti-intercellular adhesion molecule 1 (ICAM-1) antibody, anti-nitric oxide synthase 3-antibody, anti-protein kinase Cβ2 antibody, anti-Ser^{660} phospho-specific protein kinase Cβ2/δ antibody, anti-vascular cell adhesion molecule 1 (VCAM-1) antibody, anti-poly(ADP-ribose) polymerase (PARP) antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Other standard reagents were purchased from Sigma or Wako, unless otherwise indicated.

2.2. Cell-culture conditions

Human umbilical vein endothelial cells (HUVEC, Clonetics, Cambrex Bio Science, Walkersville, MD, USA) were cultured as previously described (Tsuneki et al., 2005). In brief, the cells were plated on glass coverslips or culture dishes coated with type 1 collagen (BD Biosciences, Franklin Lakes, NJ, USA) and cultured with endothelial cell basal medium-2 (EBM-2, Cambrex) containing 5% fetal bovine serum (FBS, JRH Biosciences, Lenexa, KS, USA) at 37 °C in 5% CO_2, according to the supplier's recommendation. The medium was exchanged every other day. In experiments examining the influences of glucose at various concentrations (5 or 30 mM) on endothelial functions, culture medium was changed to medium 199 (Sigma). All the medium 199 used in this study was supplemented with 10% FBS, 50 μg/ml endothelial cell growth supplement, 2 mM L-glutamine, 25 mM HEPES, 100 units/ml penicillin (Banyu Pharmaceutical, Tokyo, Japan), 0.1 mg/ml streptomycin (Meiji Seika, Tokyo, Japan), 5 units/ml heparin.

2.3. Detection of apoptosis

Confluent HUVEC monolayers on collagen-coated glass coverslips or collagen-coated 35-mm-diameter dishes were pretreated with medium 199 in the presence or absence of coenzyme Q_{10} (10 μM) for 24 h. The endothelial cells were subsequently treated with medium 199 containing 5 mM (normal) glucose, 30 mM (high) glucose, 30 mM mannitol (osmotic control) or 5 mM glucose plus 25 mM mannitol (another osmotic control) for 7 days without changing the concentration of coenzyme Q_{10}. For morphological analysis, the cells on the coverslips were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), and stained with 10 μM Hoechst 33258 for 10 min under dark conditions at room temperature. Cells were observed under a fluorescence microscope (AX80, Olympus, Tokyo, Japan). For quantitative analysis of histone-associated DNA fragmentation during apoptosis, the cells were collected from culture dishes and subjected to the cell death detection enzyme-linked immunosorbent assay (ELISA) method (Roche Applied Science, Indianapolis, IN, USA).

2.4. Western blotting

Confluent HUVEC monolayers on 35-mm-diameter dishes were pretreated with medium 199 in the presence or absence of coenzyme Q_{10} (10 μM) for 24 h. Subsequently, the cells were treated with medium 199 containing 5 mM glucose, 30 mM glucose or 30 mM mannitol for indicated times without changing the concentration of coenzyme Q_{10} (10 μM). Then, HUVEC were lysed in a lysis buffer (30 mM Tris, 150 mM NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA), 0.5%

sodium deoxycholate, 1 mM Na_3VO_4, 160 mM NaF, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 120 μg/ml aprotinin and 1 μM leupeptin). The obtained cell lysates were centrifuged for 10 min at 14,000 rpm at 4 °C, and the supernatant was collected to remove cellular debris. After mixing with Laemmli solution [0.01% bromophenol blue, 50 mM sodium phosphate, 50% glycerol, 10% sodium dodecyl sulfate (SDS)] plus 7.5% (±)-dithiothreitol, proteins were denatured in boiling water for 5 min, separated by SDS–polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica MA, USA). The PVDF membrane was blocked for 1 h with 5% nonfat milk in Tris-buffered saline with Tween 20 (TBS-T; 10 mM Tris, 150 mM NaCl, 0.1% Tween 20, pH 7.5), and then incubated for 16 h at 4 °C with primary antibody dissolved in TBS-T containing 5% bovine serum albumin or in an immunoreaction enhancer solution (Can Get Signal™, Toyobo, Osaka, Japan). The PVDF membrane was washed and incubated with horseradish peroxidase-conjugated anti-rabbit IgG or horseradish peroxidase-conjugated anti-mouse IgG (Amersham Pharmacia Biotech, Piscataway, NJ, USA) at 25 °C for 1 h, followed by chemiluminescence detection using ECL Western blotting detection reagents according to the manufacturer's instruction (Amersham). The density of protein bands was quantified with a luminoimage analyzer (LAS-1000 Plus, Fuji Film, Tokyo, Japan). The expression level of ICAM-1, VCAM-1 and endothelial nitric oxide synthase (eNOS) was calculated as the ratio of the intensity of the band relative to that of β-actin in each sample. The phosphorylation level of protein kinase Cβ2 was calculated as the ratio of the intensity of the band relative to that of total protein kinase Cβ2 in each sample.

2.5. Intracellular reactive oxygen species

The intracellular reactive oxygen species production was detected, as previously described (Nishikawa et al., 2000). HUVEC (1.5×10^4 cells) were cultured in 96-well plates for 24 h, followed by incubation with medium 199 in the presence or absence of coenzyme Q_{10} (0.1, 1 or 10 mM) for 24 h. Subsequently, the cells were incubated with medium 199 containing 5 mM glucose, 30 mM glucose or 30 mM mannitol without changing the concentration of coenzyme Q_{10} for 3 h. Then, the cells were loaded with 10 μM 5-(and-6)-chloromethyl-2′,7′-dichlorodihydro-fluorescein diacetate acetyl ester (CM-H_2DCFDA, Molecular probes, Eugene, OR, USA) dissolved in Krebs–Ringer bicarbonate buffer (KRBB; 135 mM NaCl, 3.6 mM KCl, 10 mM HEPES, 5 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, 0.5 mM $MgCl_2$, pH 7.4) for 30 min, incubated with medium 199 for 2 h, and analyzed in a multifunctional plate reader (Genios, Wako).

2.6. Adhesion of U937 cells to HUVEC

The human lymphoma U937 cell line (European Collection of Cell Cultures, Wiltshire, UK) was grown in RPMI1640 medium (Invitrogen, Grand Island, NY, USA) containing 5% FBS. Then, the U937 cells were collected by centrifugation and suspended with FBS-free medium at a density of 2×10^5 cells/ml. In the meantime, confluent HUVEC monolayers on 35-mm-diameter dishes were pretreated with medium 199 in the presence or absence of coenzyme Q_{10} (10 μM) for 24 h. Subsequently, the endothelial cells were treated with medium 199 containing 5 mM glucose, 30 mM glucose or 30 mM mannitol without changing the concentration of coenzyme Q_{10} for 5 h. After washing the HUVEC twice with RPMI1640 medium, the U937 cell suspension prepared above was added and incubated for 30 min at 37 °C. Non-adherent U937 cells were removed by washing three times with PBS. Three microscopic fields per culture dish were photographed at random under phase contrast microscope (CK40, Olympus), and the average number of U937 cells adhering to HUVEC within these fields was determined.

2.7. Statistical analysis

Data are represented as means ± S.E.M. The significance of differences between two groups was assessed by Student's *t*-test, and the differences between multiple groups were assessed by one-way analysis of variance (ANOVA), followed by the Scheffé's multiple range test. Values of P less than 0.05 were considered to be significant.

3. Results

3.1. Coenzyme Q_{10} inhibits high glucose-induced apoptosis in HUVEC

To investigate the effect of coenzyme Q_{10} on high glucose-induced apoptosis in HUVEC, morphological changes were analyzed by Hoechst 33258 staining. After 7 days of exposure to high glucose concentration (30 mM), a number of cells showed apoptotic features such as chromatin condensation (Fig. 1A). Upon treatment with coenzyme Q_{10} (10 μM), however, the nuclear morphological changes induced by high glucose were disappeared. No apoptotic cells were observed in cultured with normal glucose concentration (5 mM) or 30 mM mannitol as an osmotic control.

We also monitored apoptotic death by analyzing DNA fragmentation using an ELISA method. During a 7-day exposure to 30 mM glucose, DNA fragmentation increased 1.3-fold over the levels in cells incubated with 5 mM glucose (Fig. 1B). Coenzyme Q_{10} (10 μM) did not affect the extent of DNA fragmentation at normal glucose concentration (5 mM). Under the high glucose condition, the increase in DNA fragmentation was inhibited by coenzyme Q_{10} (1–10 μM) in a concentration-dependent manner. In particular, coenzyme Q_{10} at 10 μM completely abolished the DNA fragmentation. There was no increase in DNA fragmentation by 7-day exposure to either 30 mM mannitol (not shown) or 5 mM glucose plus 25 mM mannitol (Fig. 1B).

To elucidate whether coenzyme Q_{10} inhibits caspase-dependent pathway to apoptosis, we examined the effect of coenzyme Q_{10} on proteolytic cleavage of PARP, a caspase substrate, by Western blotting. In the control HUVEC treated

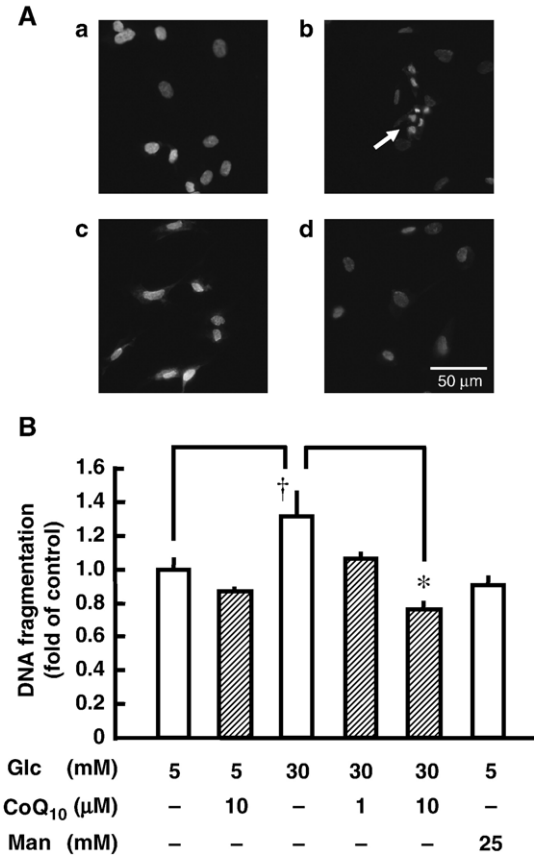

Fig. 1. Inhibitory effect of coenzyme Q_{10} on high glucose-induced apoptosis in HUVEC. HUVEC were incubated in the presence or absence of coenzyme Q_{10} (CoQ_{10}, 1–10 μM) for 24 h, and then exposed to 5 mM glucose (Glc), 30 mM glucose, 30 mM mannitol (Man), or 5 mM glucose plus 25 mM mannitol for 7 days without changing the concentration of coenzyme Q_{10}. (A) Hoechst 33258 staining for detection of apoptotic nuclear morphology under fluorescence microscope. Cells were treated with 5 mM glucose (a), 30 mM glucose (b), 30 mM glucose plus 10 μM coenzyme Q_{10} (c), or 30 mM mannitol (d, osmotic control). Arrow indicates apoptotic cells. (B) The extent of apoptosis assessed by DNA fragmentation ELISA. Each value was normalized to the mean apoptosis level in the absence of coenzyme Q_{10} at normal glucose concentration (5 mM). Data values are means±S.E.M. $n=6–12$ per group. †$P<0.05$ determined by *t*-test. *$P<0.05$ determined by ANOVA with Scheffé's test.

with 5 mM glucose, no fragment of PARP was observed (Fig. 2). In contrast, when HUVEC were treated with 30 mM glucose for 72 h, the fragment of PARP as an 85-kDa band was detected. However, the high glucose-induced generation of PARP fragment was significantly reduced upon treatment with 10 μM coenzyme Q_{10}. There was no generation of PARP fragment by 72-h exposure to 30 mM mannitol.

3.2. Coenzyme Q_{10} inhibits high glucose-induced intracellular reactive oxygen species generation in HUVEC

The increase in apoptosis of endothelial cells during exposure to high glucose is reported to be linked to the overproduction of reactive oxygen species by mitochondrial electron-transport chain (Quagliaro et al., 2003; Reusch, 2003). To clarify whether the observed antiapoptotic effect of coenzyme Q_{10} is attributed to the reduction of oxidative stress, we investigated the effect of coenzyme Q_{10} on the intracellular reactive oxygen species generation in HUVEC exposed to high glucose. As shown in Fig. 3, treatment with 30 mM glucose produced 2.5-fold increase in the reactive oxygen species generation, whereas the intracellular reactive oxygen species levels were unaffected by treatment with medium containing 5 mM glucose plus 25 mM mannitol (Fig. 3) or 30 mM mannitol (data not shown). Coenzyme Q_{10} (10 μM) alone did not significantly affect the basal level of reactive oxygen species generation at normal glucose concentration (5 mM). However, the reactive oxygen species generation by 30 mM glucose was inhibited by coenzyme Q_{10} (0.1–10 μM) in a concentration-dependent manner, and it was completely abolished at 10 μM.

3.3. Coenzyme Q_{10} inhibits high glucose-induced protein kinase C phosphorylation in HUVEC

Overproduction of reactive oxygen species is known to activate several pathways involved in the pathogenesis of diabetic complications, such as activation of protein kinase C (Ceriello, 2003). In particular, the activation of protein kinase Cβ isoform is considered to be important for vascular dysfunction in diabetes (Rask-Madsen and King, 2005). We therefore investigated the influence of coenzyme Q_{10} on the activation of protein kinase Cβ2 in HUVEC exposed to high glucose, by evaluating the phosphorylation level of protein kinase C as a potential measure of the activation. After treatment with 30 mM glucose for 1–6 h, transient increase in Ser^{660} phosphorylation of protein kinase Cβ2 was observed (Fig. 4). The phosphorylation levels maximally increased

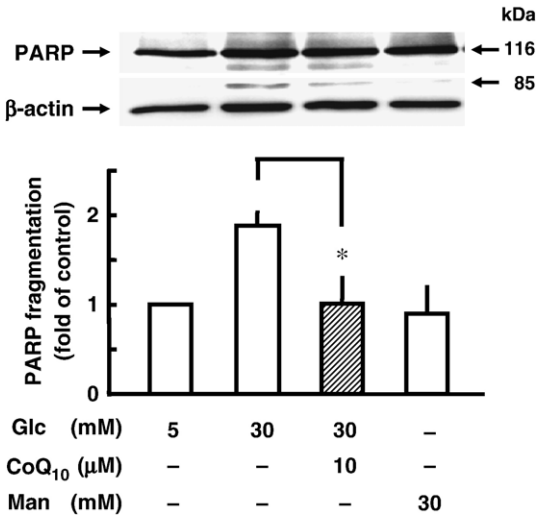

Fig. 2. Inhibitory effect of coenzyme Q_{10} on high glucose-induced PARP fragmentation in HUVEC. HUVEC were incubated in the presence or absence of coenzyme Q_{10} (CoQ_{10}, 10 μM) for 24 h, and then exposed to 5 mM glucose (Glc), 30 mM glucose, or 30 mM mannitol (Man) for 72 h without changing the concentration of coenzyme Q_{10}. The fragment of PARP (85 kDa) was detected by Western blotting. Blots are representative of four independent experiments. The histograms represent the amount of PARP fragment relative to β-actin, as determined by densitometric analysis. Each value was normalized to the mean level in the absence of coenzyme Q_{10} at normal glucose concentration (5 mM). Data values are means±S.E.M. *$P<0.05$ determined by *t*-test.

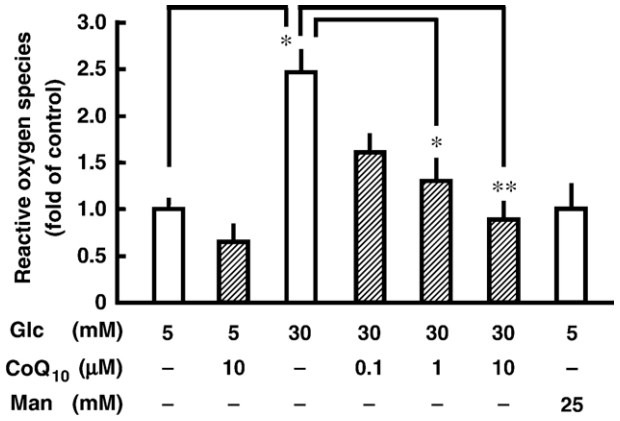

Fig. 3. Coenzyme Q_{10} prevents high glucose-induced intracellular reactive oxygen species generation in HUVEC. HUVEC were incubated in the presence or absence of coenzyme Q_{10} (CoQ_{10}, 0.1–10 μM) for 24 h, and then exposed to 5 mM glucose (Glc), 30 mM glucose, or 5 mM glucose plus 25 mM mannitol (Man) for 3 h without changing the concentration of coenzyme Q_{10}. Subsequently, the cells were loaded with the fluorescent probe CM-H_2DCFDA (10 μM) to estimate intracellular reactive oxygen species level. Each value was normalized to the mean reactive oxygen species level in the absence of coenzyme Q_{10} at normal glucose concentration (5 mM). Data values are means ± S.E.M. $n = 12–16$ per group. $^*P < 0.05$ and $^{**}P < 0.01$ determined by ANOVA with Scheffé's test.

~3-fold after 2-h treatment with 30 mM glucose. Upon treatment with 10 μM coenzyme Q_{10}, the phosphorylation levels after 2 h of the high glucose treatment were reduced to close to the basal levels.

3.4. Coenzyme Q_{10} inhibits high glucose-induced eNOS expression in HUVEC

We further investigated the influence of coenzyme Q_{10} on the eNOS expression induced by exposure to high glucose. Changing the glucose concentration from 5 mM to 30 mM resulted in a prolonged (1–4 h) increase in the expression of eNOS. The expression levels maximally increased 1.6-fold after treatment with 30 mM glucose for 1 h (Fig. 5). The upregulation of eNOS by 30 mM glucose was almost completely inhibited upon treatment with 10 μM coenzyme Q_{10}.

3.5. Coenzyme Q_{10} prevents the expression of adhesion molecules induced by exposure to high glucose in HUVEC

It has been shown that incubation of endothelial cells with high glucose leads to protein kinase Cβ2-dependent upregulation of adhesion molecules, which may account for vascular damage in diabetes (Kouroedov et al., 2004). To reveal whether coenzyme Q_{10} affects the adherence properties of HUVEC, we investigated the influences of coenzyme Q_{10} on the expression of ICAM-1 and VCAM-1 in HUVEC acutely exposed to high glucose. As shown in Fig. 6A, exposure of HUVEC to 30 mM glucose for 1 to 4 h resulted in increase of ICAM-1 protein expression in an incubation time-dependent manner. The expression levels maximally increased 2.7-fold after 4 h of the high glucose treatment. Upon treatment with 10 μM coenzyme Q_{10}, however, the high glucose-induced upregulation of ICAM-1 was significantly reduced. Similarly, the exposure of the cells to 30 mM glucose for 4 and 6 h led to 2.4- and 2.8-fold increase in the expression of VCAM-1 protein, respectively (Fig. 6B). Coenzyme Q_{10} (10 μM) also significantly inhibited these changes in the VCAM-1 expression.

Since the expression of adhesion molecules in HUVEC is reported to be increased after long-term exposure to high glucose (Verrier et al., 2004) in addition to short-term exposure (Takami et al., 1998), we further examined the influence of long-term exposure to high glucose on the expression of ICAM-1 and VCAM-1. After exposure to 30 mM glucose for 72 h, the expression level of ICAM-1 increased ~2-fold (Fig. 7A). However, the increase was significantly reduced upon treatment with 10 μM coenzyme Q_{10}. No such increase was observed after exposure to 30 mM mannitol for 72 h. Similarly, after exposure to 30 mM glucose for 72 h, the expression levels of VCAM-1 increased ~2-fold, and the upregulation was significantly inhibited by treatment with 10 μM coenzyme Q_{10} (Fig. 7B).

3.6. Coenzyme Q_{10} prevents adhesion of monocytes to HUVEC exposed to high glucose

To clarify the functional consequences of the reduced expression of adhesion molecules by coenzyme Q_{10}, we investigated the influence of coenzyme Q_{10} on adhesion of U937 human monocyte cell line to HUVEC exposed to high glucose. As shown in Fig. 8, the U937 cell adhesion was significantly promoted after exposure of HUVEC to 30 mM glucose for 5 h. The number of U937 cells adhering to HUVEC exposed to 30 mM glucose was ~4-fold larger than that

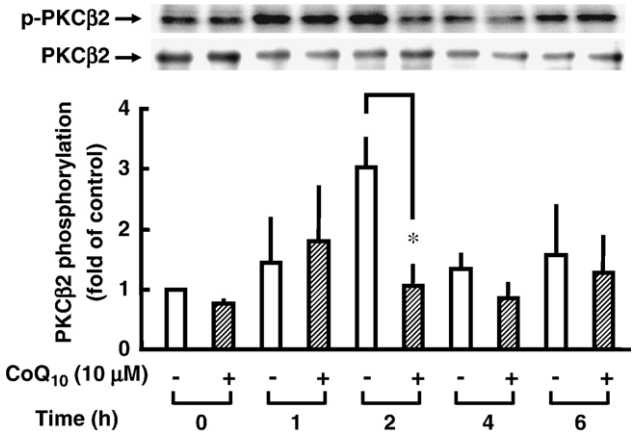

Fig. 4. Inhibition of high glucose-induced protein kinase Cβ2 phosphorylation by coenzyme Q_{10} in HUVEC. HUVEC were incubated in the presence or absence of coenzyme Q_{10} (CoQ_{10}, 10 μM) for 24 h, and then exposed to high glucose concentration (30 mM) for the indicated times without changing the concentration of coenzyme Q_{10}. The phosphorylation and expression of protein kinase Cβ2 (PKCβ2) were analyzed by Western blotting. Blots are representative of three independent experiments. The relative phosphorylation level was calculated as the ratio of the density of the phospho-PKCβ2 (p-PKCβ2) band to the total PKCβ2 band in each sample. Each value was normalized to the mean phosphorylation level in the absence of coenzyme Q_{10} before exposure to high glucose. Data values are means ± S.E.M. $n = 3$ per group. $^*P < 0.05$ compared with the levels in the absence of coenzyme Q_{10} at each time-point.

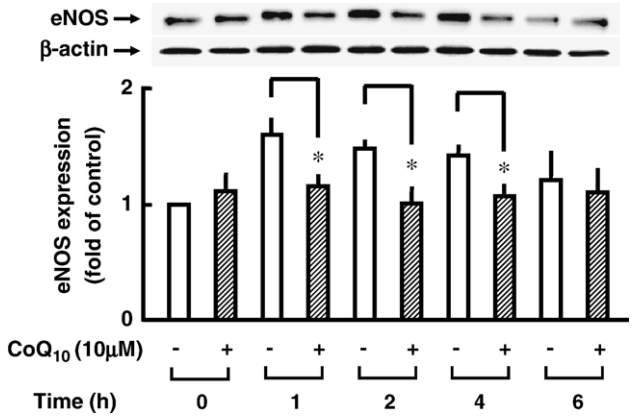

Fig. 5. Coenzyme Q_{10} blocks high glucose-induced eNOS expression in HUVEC. HUVEC were incubated in the presence or absence of coenzyme Q_{10} (CoQ_{10}, 10 μM) for 24 h, and then exposed to high glucose concentration (30 mM) for the indicated times without changing the concentration of coenzyme Q_{10}. The expression level of eNOS was analyzed by Western blotting. Blots are representative of six independent experiments. The histogram represents the amount of eNOS relative to β-actin, as determined by densitometric analysis. Each value was normalized to the mean eNOS level in the absence of coenzyme Q_{10} before exposure to high glucose. Data values are means±S.E.M. $n=5–6$ per group. *$P<0.05$ compared with the levels in the absence of coenzyme Q_{10} at each time-point.

incubated with 5 mM glucose. Treatment of HUVEC with 10 μM coenzyme Q_{10} before and during exposure to 30 mM glucose completely prevented the adhesion of U937 cells. No increase in the adherent cell numbers was observed after exposure of HUVEC to 30 mM mannitol.

4. Discussion

Hyperglycemia-induced oxidative stress plays a crucial role in the pathogenesis of both microvascular and macrovascular complications in diabetes (Ceriello, 2003). The increased oxidative stress contributes to endothelial dysfunctions, resulting in disturbance of microvascular autoregulation, activation of pro-inflammatory and pro-thrombotic pathways, and increased arterial stiffness (Chew and Watts, 2004). Antioxidant therapy is therefore considered to be a promising strategy to prevent the oxidative damage to the vascular endothelium in the early stage of atherosclerosis (Kaliora et al., 2006). In fact, considerable attention has been focused on several naturally occurring constituents, including polyphenols, carotenoids and coenzyme Q_{10}, because of their potent antioxidant activity (Kaliora et al., 2006). However, there is no report investigating the direct effect of coenzyme Q_{10} on endothelial cells *in vitro*. In the present study, we demonstrated for the first time that coenzyme Q_{10} prevents oxidative stress, apoptotic cell death and alteration of cell-adhesion properties in cultured endothelial cells under high glucose condition. The effective concentrations of coenzyme Q_{10} *in vitro* (1 to 10 μM) correspond to the plasma concentrations after treating with coenzyme Q_{10} in humans (Lu et al., 2003; Shults et al., 2004).

The exposure of endothelial cells to high glucose increases the production of reactive oxygen species at the mitochondrial level, leading to increased cellular apoptosis (Quagliaro et al., 2003; Reusch, 2003). Intermittent high glucose is more deleterious to endothelial cells than a constant high concentration of glucose (Risso et al., 2001). In the present study, we found that coenzyme Q_{10} strongly inhibited the production of reactive oxygen species in HUVEC exposed to stable high glucose, and prevented apoptotic morphological and biochemical changes. In addition, coenzyme Q_{10} inhibited high glucose-induced cleavage of PARP, an endogenous caspase-3 substrate, suggesting that coenzyme Q_{10} inhibits the caspase-dependent apoptosis which could be mediated by mitochondrial pathways. Similarly, coenzyme Q_{10} has been reported to cause antiapoptotic effect in other type of cells, such as keratocytes and SH-SY5Y neuronal cells, by inhibiting mitochondrial membrane depolarization, cytochrome *c* release and caspase activation (Papucci et al., 2003; Somayajulu et al., 2005). Exogenous

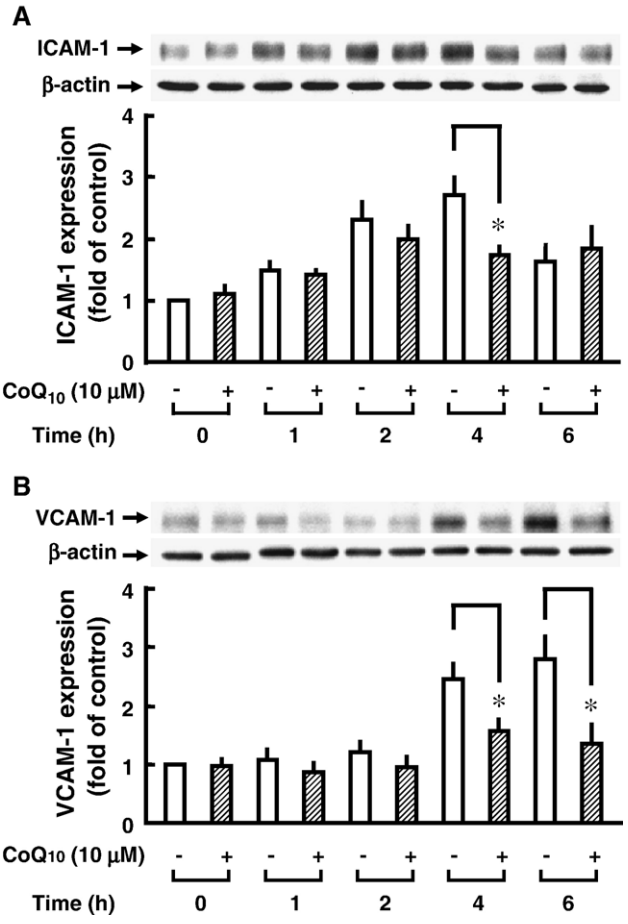

Fig. 6. Coenzyme Q_{10} prevents ICAM-1 and VCAM-1 expression induced by short-term exposure to high glucose in HUVEC. HUVEC were incubated in the presence or absence of coenzyme Q_{10} (10 μM) for 24 h, and then exposed to high glucose concentration (30 mM) for the indicated times without changing the concentration of coenzyme Q_{10}. (A) Western blots for ICAM-1 and β-actin. Blots are representative of five independent experiments. The histograms represent the amount of ICAM-1 relative to β-actin, as determined by densitometric analysis. (B) Western blots for VCAM-1 and β-actin. Blots are representative of six independent experiments. The histograms represent the amount of VCAM-1 relative to β-actin. Each value was normalized to the mean expression level in the absence of coenzyme Q_{10} before exposure to high glucose. Data values are means±S.E.M. *$P<0.05$ compared with the levels in the absence of coenzyme Q_{10} at each time-point.

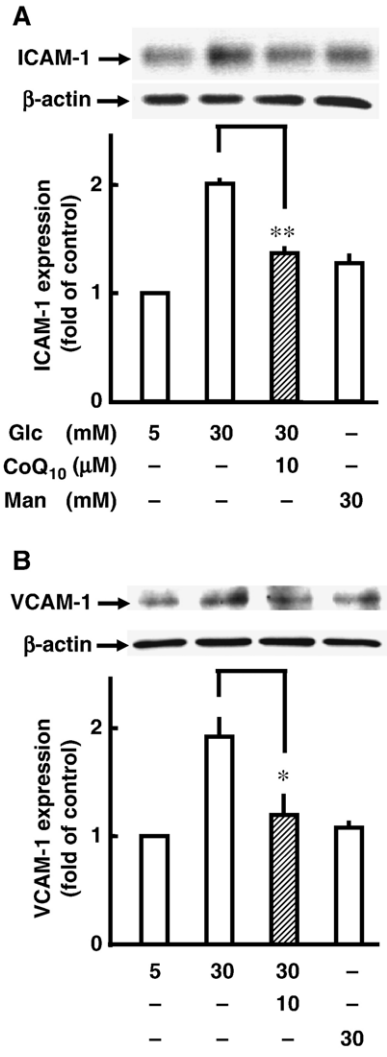

Fig. 7. Coenzyme Q_{10} prevents ICAM-1 and VCAM-1 expression induced by a long-term exposure to high glucose in HUVEC. HUVEC were incubated in the presence or absence of coenzyme Q_{10} (CoQ_{10}, 10 μM) for 24 h, and then exposed to 5 mM glucose (Glc), 30 mM glucose, or 30 mM mannitol (Man) for 72 h without changing the concentration of coenzyme Q_{10}. (A) Western blots for ICAM-1 and β-actin. Blots are representative of three independent experiments. The histograms represent the amount of ICAM-1 relative to β-actin, as determined by densitometric analysis. (B) Western blots for VCAM-1 and β-actin. Blots are representative of three independent experiments. The histograms represent the amount of VCAM-1. Each value was normalized to the mean expression level in the absence of coenzyme Q_{10} at normal glucose concentration (5 mM). Data values are means±S.E.M. $^{*}P<0.05$ and $^{**}P<0.01$ determined by ANOVA with Scheffé's test.

administration of coenzyme Q_{10} results in its transfer in an intact form to the inner membrane of mitochondria, where it serves as a cofactor in the electron-transport chain (Sarter, 2002; Sandhu et al., 2003). The mitochondrial permeability transition pore (PTP) is a ubiquinone-binding site, which plays a pivotal role in triggering apoptosis (Fontaine et al., 1998). Taken together, it is possible that coenzyme Q_{10} prevents the endothelial cells from high glucose-induced apoptosis, by interrupting the overproduction of reactive oxygen species at mitochondrial level.

Increased protein kinase C activity plays a key role in the development of microvascular and macrovascular complications in diabetes (Rask-Madsen and King, 2005). In this regard, high glucose concentrations in diabetes causes activation of protein kinase C in vascular cells by several mechanisms, including de novo synthesis of diacylglycerol (DAG), activation of phospholipase C, and inhibition of DAG kinase (King and Loeken, 2004; Rask-Madsen and King, 2005). As a result, high glucose stimulates reactive oxygen species production for oxidative stress through protein kinase C-dependent activation of NADPH oxidase in vascular endothelial cells (Inoguchi et al., 2000). It is also known that high glucose-induced protein kinase C activation alters vascular blood flow, extracellular matrix deposition, basement membrane thickening, increased permeability and neovascularization (Way et al., 2001).

Protein kinase C is classified into three groups: conventional protein kinase C (α, β2 and the splice variant β1, and γ), novel protein kinase C (δ, ε, η, and θ) and atypical protein kinase C (ξ and ι/λ). The protein kinase Cβ isoform exhibits the most significant increase in vascular tissues in response to hyperglycemia, compared to the other isoforms (Way et al., 2001). All the isoforms share three conserved phosphorylation motifs, namely, activation loop, turn motif and hydrophobic motif,

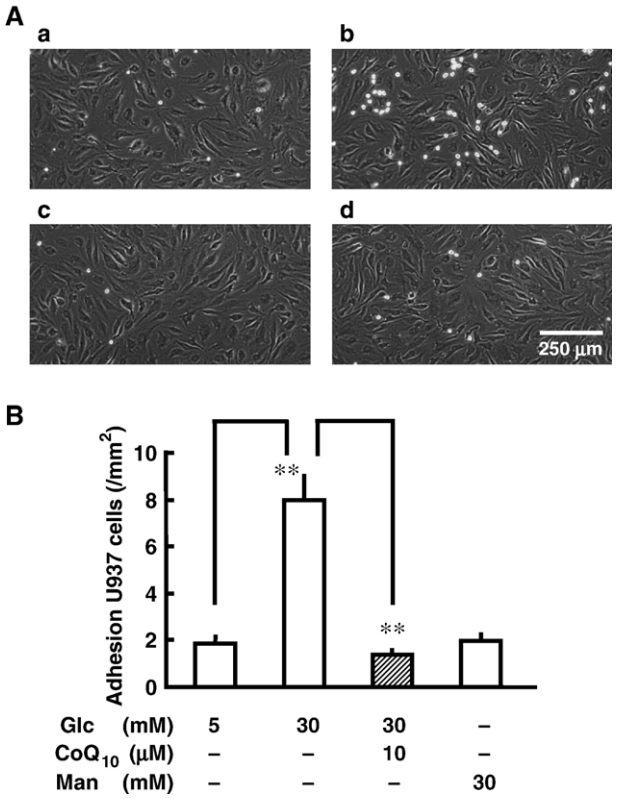

Fig. 8. Coenzyme Q_{10} inhibits high glucose-induced adhesion of monocytes to HUVEC. HUVEC were incubated in the presence or absence of coenzyme Q_{10} (CoQ_{10}, 10 μM) for 24 h, and then exposed to 5 mM glucose (Glc), 30 mM glucose, or 30 mM mannitol (Man) for 5 h without changing the concentration of coenzyme Q_{10}. The U937 cell suspension was subsequently added and incubated for 30 min. (A) Adhesion of U937 cells to the HUVEC exposed to 5 mM glucose (a), 30 mM glucose (b), 30 mM glucose plus coenzyme Q_{10} (c), or 30 mM mannitol (d). (B) The number of U937 cells adhering to HUVEC, determined by counting three randomly selected fields per culture dish. Data values are means±S.E.M. $n=3$–4 per group. $^{**}P<0.01$ determined by ANOVA with Scheffé's test.

which critically regulate their function (Newton, 2003). In particular, phosphorylation of the hydrophobic motif at C-terminus of protein kinase C results in conformational rearrangements that lock protein kinase C into a more stable and adequate conformation for catalysis (Newton, 2003). In this study, we observed that high glucose concentrations caused the increase in phosphorylation of Ser^{660} located in the hydrophobic motif at the C-terminus of protein kinase Cβ2 in HUVEC, and treatment with coenzyme Q_{10} effectively inhibited the phosphorylation. Since the unphosphorylated protein kinase C is catalytically inactive (Newton, 2003), treatment with coenzyme Q_{10} is likely to prevent high glucose-induced activation of protein kinase Cβ2 in the endothelial cells. High glucose-induced mitochondrial production of the superoxide anion (O_2^-) is considered to inhibit activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), leading to activation of the de novo DAG synthetic pathway, which further activates protein kinase C (King and Loeken, 2004). Given that protein kinase C promotes the reactive oxygen species production (Inoguchi et al., 2000) as mentioned above, we anticipate that coenzyme Q_{10} can ameliorate the vicious cycle in abnormal activation of protein kinase C under high glucose condition, by reducing superoxide anion production. Thus, coenzyme Q_{10} seems to be potentially valuable in protecting against damage of vascular endothelium exposed to hyperglycemia in diabetes.

Nitric oxide (NO) is a crucial mediator in the endothelial vasodilator function. The expression of eNOS is increased through protein kinase C activation during high glucose condition (Shen et al., 1999; Cosentino et al., 2003). In this process, reactive oxygen species-induced activation of phosphatidylinositol 3-kinase/Akt pathway mediates rapid upregulation of eNOS (Ho et al., 2006). Since protein kinase C activation increases eNOS transcription and eNOS mRNA half-life through promotion of oxidative stress (Rask-Madsen and King, 2005), the eNOS upregulation under high glucose condition may occur mainly at transcription level. Nevertheless, impairment of endothelium-dependent vasodilation is seen due to hyperglycemia-reduced NO availability (De Vriese et al., 2000; Ceriello, 2003). Although the mechanism underlying this 'uncoupling' of NO production in endothelial cells has not been clarified, the quenching of NO by superoxide or reactive oxygen species may be involved in the reduced bioactivity of NO (Ceriello, 2003). Moreover, redox imbalance, oxidation of an essential eNOS cofactor tetrahydrobiopterin (BH_4), and protein kinase C-dependent phosphorylation of eNOS may explain this mechanism (Chew and Watts, 2004; Rask-Madsen and King, 2005). Nitric oxide reacting with superoxide produces peroxynitrite radical ($ONOO^-$). Peroxynitrite is the potent cytotoxic substance, and causes abnormal endothelial functions, including overexpression of vascular endothelial growth factor or apoptotic cell death (Cosentino et al., 2003; Walford et al., 2004; Platt et al., 2005). In the present study, we demonstrated that treatment with coenzyme Q_{10} inhibited the increase in eNOS expression in HUVEC exposed to high glucose. This inhibitory effect seems to be at least in part due to the inhibition of protein kinase C, probably through interruption of superoxide produced under high glucose condition, although precise mechanism remains unknown. We therefore suggest that coenzyme Q_{10} acts to scavenge reactive oxygen species that interact with NO to form the potent cytotoxic peroxynitrite, and prevent the peroxynitrite-induced apoptosis.

Adhesion of monocytes to the endothelium, followed by transendothelial migration, occurs during the initial steps of atherosclerosis. This process is predominantly mediated by adhesion molecules expressed on the vascular endothelium and on circulating leukocytes in response to inflammatory stimuli (Blankenberg et al., 2003; Boyle, 2005). High glucose-induced activation of protein kinase Cβ2 causes rapid activation of a transcription factor, nuclear factor-κB, which stimulates VCAM-1 expression in human endothelial cells (Kouroedov et al., 2004). The induction of ICAM-1 by high glucose is also dependent on the activation of protein kinase Cβ in HUVEC (Quagliaro et al., 2005), although the underlying mechanism remains unidentified (Rask-Madsen and King, 2005). In fact, retinal and choroidal expression of adhesion molecules, such as ICAM-1, is enhanced in patients with diabetes (McLeod et al., 1995). In this study, VCAM-1 and ICAM-1 proteins were increased probably through the enhancement of transcription even after short exposure to high glucose in HUVEC. In addition, the adhesion of U937 monocytic cells was promoted in HUVEC exposed to high glucose. These changes under high glucose condition were greatly inhibited by treatment with coenzyme Q_{10}, suggesting that coenzyme Q_{10} can prevent oxidative stress and endothelial damage in the early stage of atherosclerosis. Our results from *in vitro* experiments support previous evidences that the benefits of coenzyme Q_{10} supplementation are best seen in diabetic subjects who have not yet developed established vascular complications (Chew and Watts, 2004).

In conclusion, we provide the first evidence that coenzyme Q_{10} has a beneficial effect in protecting against the endothelial dysfunction by high glucose-induced oxidative stress *in vitro*. Since hyperglycemia-induced overproduction of superoxide by the mitochondrial electron-transport chain has been implicated in the pathogenesis of diabetes-specific microvascular disease and diabetes-accelerated atherosclerosis (Brownlee, 2001; Du et al., 2003), clinical trials examining the efficacy of dietary antioxidants in prevention of the vascular disease development have been intensively conducted (Kaliora et al., 2006). Although there are a limited number of intervention trials to investigate the therapeutic potential of coenzyme Q_{10} so far, a recent report demonstrates that oral coenzyme Q_{10} supplementation improved endothelial function of the brachial artery in patients with type 2 diabetes (Watts et al., 2002). Further clinical trials are required to determine the effect of coenzyme Q_{10} on the development of endothelial dysfunction in diabetes.

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