RESEARCH ARTICLE

Coenzyme Q10 suppresses oxLDL-induced endothelial oxidative injuries by the modulation of LOX-1-mediated ROS generation via the AMPK/PKC/NADPH oxidase signaling pathway

Kun-Ling Tsai[1,2], Li-Hsin Chen[2,3], Shih-Hwa Chiou[1,2,3], Guang-Yuh Chiou[2], Yu-Chih Chen[2,4], Hsiang-Yun Chou[2], Liang-Kung Chen[5], Hsiao-Yun Chen[1,2], Tsan-Hung Chiu[6], Chiou-Sheng Tsai[7], Hsiu-Chung Ou,**[8], and Chung-Lan Kao*[1,9]*

[1] Institute of Clinical Medicine, National Yang-Ming University, Taipei, Taiwan
[2] Department of Medical Research and Education, Taipei Veterans General Hospital, Taipei, Taiwan
[3] Department and Institute of Pharmacology, National Yang-Ming University, Taipei, Taiwan
[4] Insitute and Hospital and Health Care administration, National Yang-Ming Unicersity, Taipei, Taiwan
[5] Center for Geriatrics and Gerontology, Taipei Veterans General Hospital, Taipei, Taiwan
[6] Department of Obstetrics and Gynecology, China Medical University Hospital, Taichung, Taiwan
[7] Department of Pathology and Laboratory Medicine, Taichung Veterans General Hospital, Taichung, Taiwan
[8] Department of Physical Therapy and Graduate Institute of Rehabilitation Science, China Medical University, Taichung, Taiwan
[9] Department of Physical Medicine and Rehabilitation, Taipei Veterans General Hospital, Taipei, Taiwan

Received: March 4, 2011
Revised: May 28, 2011
Accepted: June 14, 2011

Scope: The lectin-like oxidized low-density lipoprotein receptor (LOX-1) is one pivot receptor for oxidized low-density lipoprotein (oxLDL) in human endothelial cells. Co-enzyme Q10 (Co Q10) has been widely used in clinical intervention. However, the molecular mechanisms underlying its protective effects against oxidative stress in endothelial cells are still largely unknown. This study was designed to test the hypothesis that Co Q10 mitigates oxLDL-induced endothelial oxidative injuries via modulation of LOX-1-mediated reactive oxygen species (ROS) generation and explored the role of AMP-activated protein kinase (AMPK), a negative regulator of NADPH oxidase.

Methods and results: Human umbilical vein endothelial cells (HUVECs) were pretreated with Co Q10 and then incubated with oxLDL for 24 h. Co Q10 attenuated oxLDL-elicited LOX-1 expression and ROS generation by suppression of NADPH oxidase activation. Co Q10 rescued dephosphorylation of AMPK caused by oxLDL that in turn led to an activation of NADPH oxidase by PKC. The results were confirmed using AMPK siRNA. Moreover, oxLDL-suppressed Akt/eNOS and enhanced p38 phosphorylation, which in turn activated NF-κB pathway. These detrimental events were ameliorated by Co Q10.

Conclusion: These results provide new highlight onto the possible molecular mechanisms of how Q10 suppresses oxLDL-induced endothelial oxidative injuries by the modulation of LOX-1-mediated ROS generation via the AMPK/PKC/NADPH oxidase signaling pathway.

Keywords:
AMPK / Co Q10 / Inflammation / NADPH oxidase / OxLDL

Correspondence: Dr. Chung-Lan Kao, Department of Physical Medicine and Rehabilitation, Taipei Veterans General Hospital, No. 201, Sec. 2, Shih-Pai Road, Taipei 11217, Taiwan
E-mail: clkao@vghtpe.gov.tw
Fax: +886-2-28757359

Abbreviations: **AMPK,** AMP-activated protein kinase; **Co Q10** co-enzyme Q10; **DHE,** dihydroethidium; **DPI,** diphenyleneiodonium; **eNOS,** endothelial NO synthase; **HUVEC,** human umbilical vein endothelial cell; **LOX-1,** low-density lipoprotein receptor; **MCP-1,** monocyte chemoattractant protein-1; **NO,** nitric oxide; **oxLDL,** oxidized low-density lipoprotein; **siRNA,** small-interfering RNA

*These authors contributed equally to this work.
**Additional Corresponding author: Dr. Hsiu-Chung Ou
E-mail: ouhc@mail.cmu.edu.tw

1 Introduction

Atherosclerosis is a chronic vascular inflammatory disease. It is widely accepted that oxidized low-density lipoprotein (oxLDL) plays a key role in the early stages of atherosclerotic lesions [1]. The lectin-like oxLDL receptor (LOX-1) has been identified as one of the main receptors for oxLDL [2]. LOX-1 is expressed in macrophages, smooth muscle cells, and vascular endothelial cells, all of which are cell types involved in the development of atherosclerosis. In addition, LOX-1 has been detected in atherosclerotic lesions [3]. In addition to oxLDL, LOX-1 has a variety of other ligands, including angiotensin II, endothelin-1, and tumor necrosis factor-α (TNF-α). Furthermore, LOX-1 is important for sensing shear stress [4]. In endothelial cells, it is well documented that the binding of oxLDL to LOX-1 rapidly elevates ROS levels through the activation of NADPH oxidase [5] and that the activation of NADPH oxidase can then further upregulate LOX-1 expression [6]. In addition, increased LOX-1 expression can trigger diverse downstream signals such as the activation of p38 mitogen-activated protein kinase (MAPK), the enhancement of nitric oxide (NO) catabolism through superoxide generation, and the decrease in NO release due to attenuated endothelial NO synthase (eNOS) [7], which activates NF-κB and subsequent downstream pro-inflammatory responses [8, 9].

The NOX family of NADPH oxidases is the major source of ROS in endothelial cells. NADPH oxidase is composed of the two membrane components, Nox2 (also called gp91) and $p22^{phox}$ in addition to the three cytoplasmic proteins, $p47^{phox}$, $p67^{phox}$, and the small GTPase Rac-1. The process by which the NADPH oxidase enzyme complex is activated begins with the phosphorylation of $p47^{phox}$, which causes the translocation of the $p47^{phox}$/$p67^{phox}$ complex to the plasma membrane. There, $p47^{phox}$ interacts with $p22^{phox}$, while $p67^{phox}$ acts as the NOX activator through direct protein–protein interaction. Studies have demonstrated that PKC plays an important role in the activation of NADPH oxidase through phosphorylation of $p47^{phox}$ in platelets [10], myocytes [11], pancreatic islet cells [12], and vascular cells [13]. Furthermore, the dietary flavonoid quercetin exerts anti-hypertensive effects through the suppression of PKC-activated NADPH oxidase [14]. In addition, pravastatin and 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors inhibit both the insulin-induced translocation of $p47^{phox}$ from the cytosol to the membrane as well as ROS generation through a PKCδ-dependent mechanism [15].

AMP-activated protein kinase (AMPK), a serine/threonine protein kinase, is uniquely expressed in mammalian cells and involved in the regulation of cellular metabolism and energy balance [16]. AMPK can also influence a number of signaling cascades that are expected to have anti-atherosclerotic effects, such as the improvement of NO bioavailability, the attenuation of free radical generation, and the activation of angiogenic factors [17]. Several lines of evidence have demonstrated that PKC, which is negatively regulated by AMPK, is required for the activation of NADPH oxidase [18, 19], and the inhibition of PKC contributes to the attenuation of NADPH oxidase-derived ROS production [20]. Natural compounds, such as resveratrol [21] and quercetin [22], have been reported to exert strong antioxidant and anti-inflammatory activities through the activation of AMPK.

Co-enzyme Q10 (Co Q10) is essential for the mitochondrial respiratory electron transport chain and possesses antioxidant effects. Co Q10 gradually decreases with age [23–25], and is widely utilized as a dietary supplement to prevent oxidative stress-related diseases [26–30]. Many biological effects of Co Q10 have been reported. For example, Co Q10 increases mitochondrial function, increases ATP formation [31] and protects against high-fat diet-induced hyperlipidemia [32]. Clinical studies have shown that the administration of Co Q10 in atherosclerotic patients has a positive effect on endothelial function [33]. In addition, Co Q10 improves clinical parameters in patients following coronary artery bypass surgery and other cardiovascular diseases [34–37]. Studies have demonstrated that Co Q10 supplementation lowers oxidative stress and inflammation both in vivo [38, 39] and in vitro [40], but the underlying mechanisms have yet to be elucidated. A recent study demonstrated that supplementation with Co Q10 lowers age-related NADPH oxidase levels in healthy subjects [41]. Therefore, we sought to explore whether Co Q10 could protect against LOX-1-mediated endothelial oxidative injuries through the modulation of NADPH oxidase, and if so, whether AMPK, a negative regulator of both PKC-activated NADPH oxidase and Akt/eNOS/NO signaling was involved in the process.

2 Materials and methods

2.1 Reagents

Fetal bovine serum, M199, and trypsin-EDTA were obtained from Gibco (Grand Island, NY, USA); low-serum growth supplement was obtained from Cascade (Portland, OR, USA); Co Q10, Compound C, Ro-32-0432, hispidin, diphenyleneiodonium (DPI), TEMPLO, NAC, trolox, ethylene diamine tetraacetic acid (EDTA), penicillin and streptomycin were obtained from Sigma (St. Louis, MO, USA); dihydroethidium (DHE) was purchased from Molecular Probes (Eugene, OR, USA); anti-monocyte chemoattractant protein-1 (MCP-1), anti-CXC chemokine receptor 6 (CXCR6), anti-LOX-1 and interleukin 6 (IL-6) ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA); anti-$p22^{phox}$ and anti-gp91 were obtained from Santa Cruz (CA, USA); and anti-NF-κB/p65, anti-I-κBα, anti-AMPK, anti-AMPK-α, anti-AKT, anti-phospho AKT, anti-phospho eNOS, anti-eNOS, anti-PCNA, anti-phospho p38, anti-p38, anti-PKC and anti-phospho PKCαβ were obtained from Transduction Laboratories (CA, USA). Anti-Rac-1 and anti-$p47^{phox}$ were obtained from BD Biosciences (NJ, USA).

2.2 Cell cultures

The Research Ethics Committee of China Medical University Hospital approved these experiments. After receiving written informed consent from parents, neonatal human umbilical cords were obtained after birth and suspended in Hanks' Balanced Salt Solution (HBSS) (Gibco) at 4°C. Human umbilical vein endothelial cells (HUVECs) were isolated with collagenase and used at passages 2 and 3 [42]. After dissociation, the cells were collected and cultured on gelatin-coated culture dishes in medium 199 with low-serum growth supplement, 100 IU/mL penicillin and 0.1 mg/mL streptomycin. Subcultures were performed with trypsin-EDTA. Media was refreshed every two days.

2.3 Lipoprotein separation

Human plasma was obtained from the Taichung Blood Bank (Taichung, Taiwan) and LDL was isolated using sequential ultracentrifugation ($\rho = 1.019–1.063$ g/mL) in KBr solution containing 30 mM EDTA. It was protected from light, stored at 4°C in a sterile environment and used within three days as previously described [43]. Immediately before the oxidation 154 tests, LDL was separated from EDTA and diffusible low–molecular-mass compounds by gel filtration on PD-10 Sephadex G-25 M gel (Pharmacia) in 0.01 mol/L phosphate-buffered saline (136.9 mmol/L NaCl, 2.68 mmol/L KCl, 4 mmol/L Na_2HPO_4, 1.76 mmol/L KH_2PO_4) at 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. $CuSO_4$ was removed by PD10 columns after oxidizing in the prepared oxLDL.

2.4 Measurement of ROS production

ROS are specific mediators of atherogenic stimuli that induce leukocyte adhesion to endothelial cells. In addition, the role of ROS in oxLDL-mediated cytotoxicity is speculated to be caused by the induction of apoptosis and the activation of the caspase cascade [44]. The effect of Co Q10 on ROS production in HUVECs was determined by DHE. Confluent HUVECs (10^4 cells/well) in 96-well plates were pre-incubated with various concentrations of Co Q10 for 2 h. oxLDL was then added to the medium in the absence or presence of Co Q10 for an additional 2 h. After removing medium from the wells, the cells were incubated with 10 μM DHE for 1 h. The fluorescence intensity was measured with a fluorescence microplate reader (Labsystem, CA, USA) calibrated with an excitation at 540 nm and emission at 590 nm. The percentage increase in fluorescence per well was calculated by the formula $[(Ft_2 - Ft_0)/Ft_0] \times 100$, where Ft_2 is the fluorescence at 2 h of oxLDL exposure, and Ft_0 is the fluorescence at 0 h of oxLDL exposure.

2.5 Isolation of mRNA and quantitative real-time PCR

Total RNA was isolated from HUVECs using the RNeasy kit (Qiagen, Valencia, CA, USA). Oligonucleotides for LOX-1 and β-actin were designed using the computer software package Primer Express 2.0 (Applied Biosystems, Foster City, CA, USA). All of the oligonucleotides were synthesized by Invitrogen (Breda, The Netherlands). Oligonucleotide specificity was determined by a homology search within the human genome (BLAST, National Center for Biotechnology Information, Bethesda, MD, USA) and confirmed by dissociation curve analysis. The oligonucleotide sequences were as follows: LOX-1 sense primer, 5′-GATGCCCCACTT-GTTCAGAT-3′; anti-sense primer, 5′-CAGAGTTCGCACC-TACGTCA-3′; β-actin sense primer, 5′-AGGTCATCACTA-TTGGCAACGA-3′; anti-sense primer, 5′-CACTTCATGA-TGGAATTGAATGTAGTT-3′. PCR was performed with SYBR Green in an ABI 7000 sequence detection system (Applied Biosystems) according to the manufacturer's guidelines.

2.6 Preparation of nuclear and cytosolic extracts

Nuclear and cytosolic extracts were isolated with a Nuclear and Cytoplasmic Extraction kit (Pierce Chemical, Rockford, IL, USA). After the incubation period, HUVECs were collected by centrifugation at $600 \times g$ for 5 min at 4°C. The pellets were washed twice with ice-cold PBS, followed by the addition of 0.2 mL of cytoplasmic extraction buffer A and vigorous mixing for 15 s. Ice-cold cytoplasmic extraction buffer B (11 μL) was added to the solution. After vortex mixing, nuclei and cytosolic fractions were separated by centrifugation at $16\,000 \times g$ for 5 min. The cytoplasmic extracts (supernatants) were stored at −80°C. Nuclear extraction buffer was added to the nuclear fractions (pellets), which were then mixed by vortexing on the highest setting for 15 s. The mixture was put on ice, and a 15-s vortex was performed every 10 min for a total of 40 min. Nuclei were centrifuged at $16\,000 \times g$ for 10 min. The nuclear extracts (supernatants) were stored at −80°C until use.

2.7 Preparation of membrane and cytosolic extracts

A cellular membrane fraction was prepared with Mem-PER (Pierce) according to the manufacturer's instructions. The Mem-PER system consists of three reagents: reagent A is a cell lysis buffer, reagent B is a detergent dilution buffer and reagent C is a membrane solubilization buffer. After the incubation period, HUVECs were collected by centrifugation at $600 \times g$ for 5 min at 4°C. Each cell pellet, containing 5×10^6 cells, was lysed at room temperature using Mem-PER reagent A. Membrane proteins were solubilized on ice

with Mem-PER reagent C diluted 2:1 with Mem-PER reagent B. Reagents A and B/C were supplemented with Halt protease inhibitor cocktail (Pierce Biotechnology). The solubilized protein mixture was centrifuged at 10 000 × *g* for 3 min at 4°C to remove cellular debris. The clarified supernatant was heated at 37°C for 10 min, followed by centrifugation at 10 000 × *g* for 2 min to produce separated membrane and hydrophilic protein fractions. The hydrophobic fraction of membrane proteins (bottom layer) was stored at −80°C until use.

2.8 Immunoblotting

To determine whether Co Q10 could ameliorate the oxLDL-induced activation LOX-1-mediated signaling pathways, HUVECs were grown to confluence, pre-treated with Co Q10 for 2 h, and then stimulated with oxLDL for 24 h. At the end of stimulation the cells were washed, scraped from dishes and 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, USA), the blot was incubated with blocking buffer for 1 h at room temperature and then probed with primary antibodies overnight at 4°C, followed by incubation with a horseradish peroxidase-conjugated secondary antibody for 1 h. To control equal loading of total protein in all lanes, blots were stained with mouse anti-β-actin antibody. The bound immunoproteins were detected by an enhancer chemiluminescent assay (ECL; Amersham, Berkshire, UK), and intensities were quantified by densitometric analysis (Digital Protein DNA Imagineware, Huntington Station, NY, USA).

2.9 Transfection with small-interfering RNA (siRNA)

ON-TARGETplus SMARTpool siRNAs for non-targeting controls was purchased from Dharmacon Research (Lafayette, CO, USA). AMPKα1 siRNA (sc-270142) was purchased from Santa Cruz. Transient transfection was carried out using INTERFERin™ siRNA transfection reagent (Polyplus Transfection, Huntingdon, UK) according to the manufacturer's guide. Three days after transfection, cells were treated with the indicated reagent for further experiments.

2.10 Statistical analyses

The results are expressed as mean ± SD. Differences between the groups were analyzed using one-way ANOVA followed by Student's *t* test. A *p*-value <0.05 was considered statistically significant.

3 Results

3.1 Co Q10 attenuated oxLDL-induced LOX-1 expression

LOX-1 mRNA (Fig. 1A) and protein expression (Fig. 1B and C) were increased after exposure to oxLDL (130 μg/mL) in HUVECs. However, pre-treatment of HUVECs for 2 h with Co Q10 (2.5–20 μM) before exposure to oxLDL for 24 h

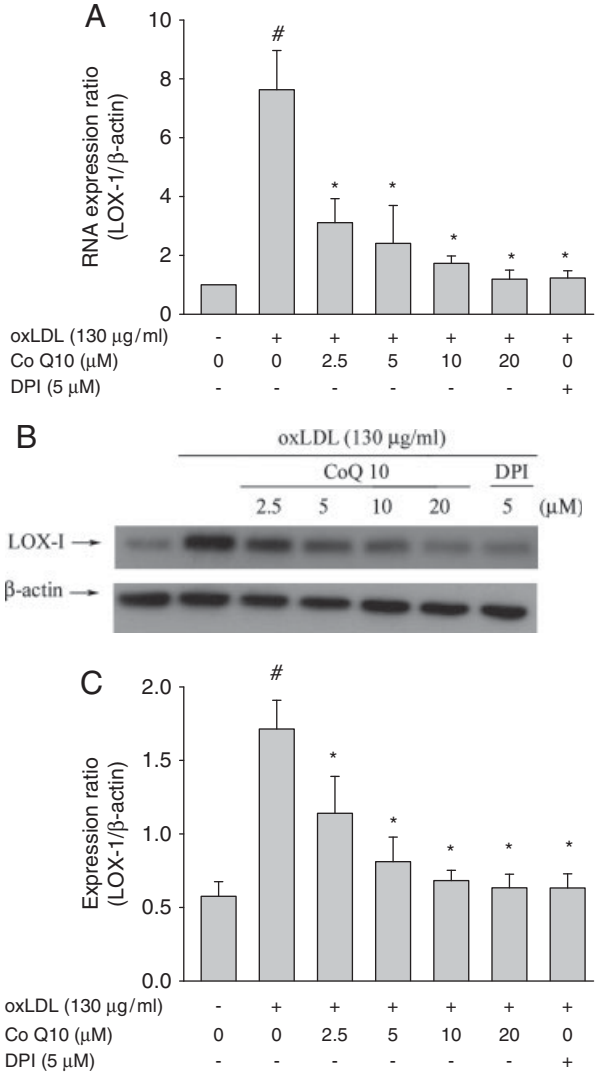

Figure 1. Inhibitory effect of Co Q10 on oxLDL-induced endothelial LOX-1 gene (A) and protein expression (B, C). HUVECs were pretreated with Co Q10 (2.5–20 μM) or DPI (5 μM) for 2 h, followed by exposure to oxLDL (130 μg/mL) for a further 24 h. At the end of the incubation period, cells were lysed and LOX-1 mRNA and protein were analyzed by real-time PCR and Western blotting, respectively. Both mRNA and protein levels of LOX-1 were normalized to the level of β-actin. Data are mean ± SD of three different experiments. $^{\#}p<0.05$ compared with untreated control HUVECs. $^{*}p<0.05$ compared with oxLDL-stimulated HUVECs.

resulted in the reduction of LOX-1 expression in a dose-dependent manner. Furthermore, pre-treatment the ROS inhibitor DPI markedly inhibited oxLDL-upregulated LOX-1 expression, indicating that ROS plays a key role in the increased expression of LOX-1.

3.2 Co Q10 modulated oxLDL-induced membrane assembly of NADPH oxidase

Next, we determined the effects of Co Q10 on NADPH oxidase activation after exposure to oxLDL. Membrane translocation assays showed that the levels of $p47^{phox}$ and Rac-1 in membrane fractions of HUVECs were higher in cells treated with oxLDL for 1 h than in control cells. In addition, we found that the protein levels of gp91 and $p22^{phox}$ were significantly increased in HUVECs exposed to oxLDL for 24 h. However, pretreatment of oxLDL-exposed cells with Co Q10 led to a dose-dependent reduction in the membrane assembly of $p47^{phox}$ and Rac-1 as well as the suppression of gp91 and $p22^{phox}$ protein expression (Fig. 2A–C).

3.3 Co Q10 protected against oxLDL-facilitated ROS production

Fluorescence microscopy and flow cytometry were used to evaluate the protective effects of Co Q10 on oxLDL-induced ROS generation. Our data showed that exposure to oxLDL for 2 h resulted in a near 10-fold increase in ROS, while pre-treatment with Co Q10 (2.5–20 μM) significantly inhibited oxLDL-induced ROS generation in a dose-dependent manner (Fig. 3B, all $p<0.05$), fluorescence images were detected by fluorescence microscopy (Fig. 3A). In addition, pre-treatment with an LOX-1 monoclonal antibody (anti-LOX-1 mAb) and an inhibitor of NADPH oxidase (DPI) abrogated oxLDL-elicited ROS, suggesting that ROS generation (one of the earliest signals after oxLDL exposure) was largely dependent on the binding of oxLDL to LOX-1 and the subsequent activation of NADPH oxidase. To examine the relevance between Co Q10 and AMPK-suppressed PKC-mediated NADPH oxidase activation, pharmacological inhibitors of AMPK (compound C), PKC-α (Ro-32-0432) and PKC-β (hispidin) were added for 1 h prior to oxLDL treatment. As expected, the ability of Co Q10 to reduce ROS was abolished

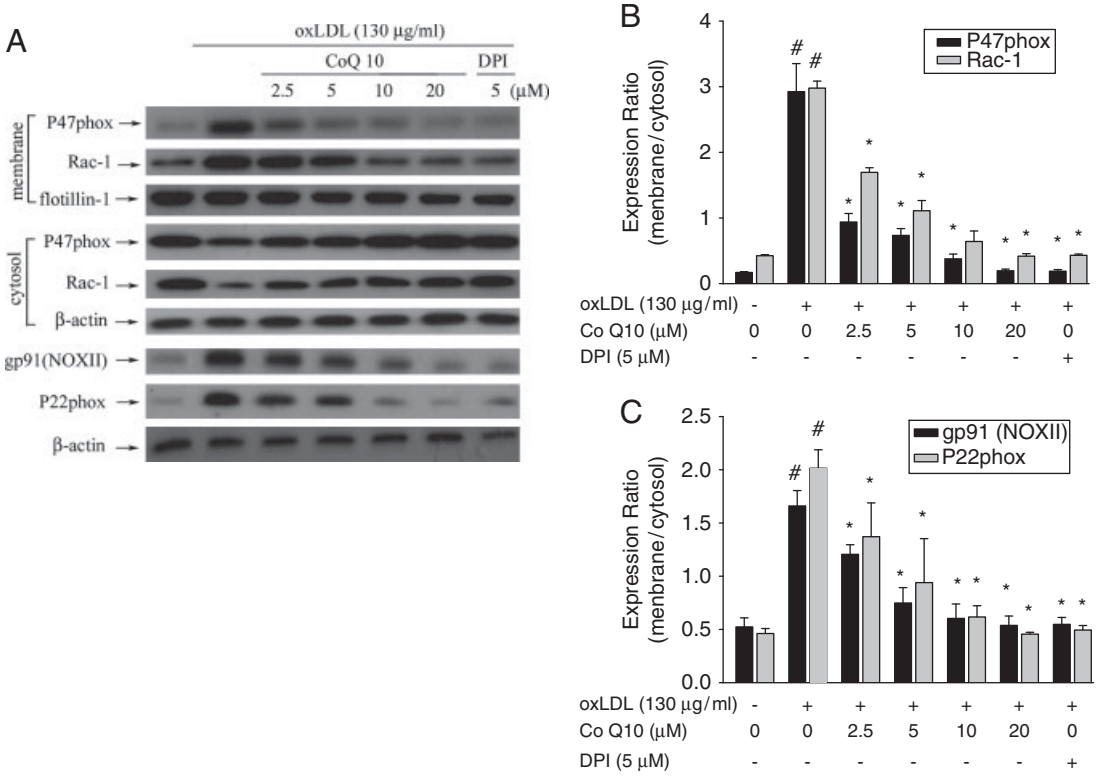

Figure 2. Co Q10 attenuated the level of NADPH oxidase membrane assembly. HUVECs were pretreated for 2 h with the indicated concentrations of Co Q10 followed by stimulation with oxLDL (130 μg/mL) for 1 or 24 h. Preparation of membrane and cytosolic proteins is described in Section 2. Representative Western blots (A) and summary data (B, C) showed that Co Q10 protected against oxLDL-induced $p47^{phox}$ and Rac-1 translocation to the plasma membrane, and gp91 as well as $p22^{phox}$ expression. The levels of cytosolic protein and membrane protein were normalized to the levels of β-actin and flotillin-1, respectively. Data are mean ± SD of three different experiments. $^{\#}p<0.05$ compared with untreated control HUVECs. $^{*}p<0.05$ compared with oxLDL-stimulated HUVECs.

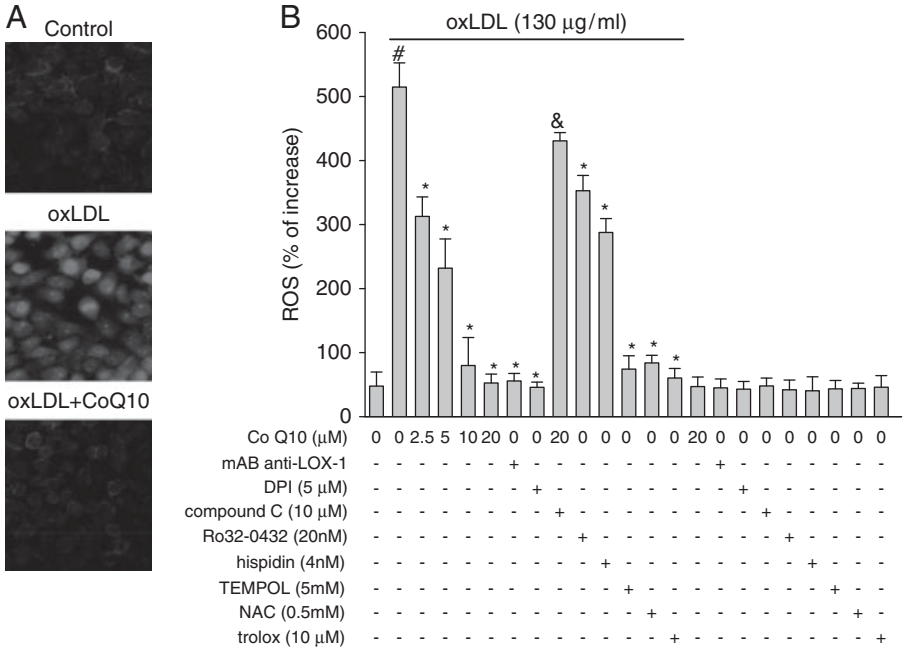

Figure 3. Inhibitory effects of Co Q10 on oxLDL-induced ROS generation in HUVECs. After pre-incubation for 2 h with the LOX-1 antibody, AMPK inhibitor (compound C), PKC-α inhibitor (Ro-32-0432), PKC-β inhibitor (hispidin), Co Q10, TEMPOL, NAC and trolox. Cells were treated with 130 μg/mL oxLDL for 2 h followed by a 1-h incubation with superoxide-sensitive fluorescent probe DHE (10 μM). (A) Fluorescence images show the ROS level in control cells and HUVECs stimulated with oxLDL alone and in the presence of Co Q10 and different inhibitors. (B) Fluorescence intensity of cells was measured with a fluorescence microplate reader. Fluorescence distribution of DHE oxidation is expressed as a percentage of increased intensity. Data are mean ± SD of three different experiments. $^{\#}p<0.05$ compared with untreated control HUVECs. $^{*}p<0.05$ compared with oxLDL-stimulated HUVECs. $^{\&}p<0.05$ compared with oxLDL+Co Q10 treatment.

in cells pre-treated with compound C, indicating that the activation of AMPK is involved in the effects of Co Q10. In addition, the inhibitors of PKC-α (Ro-32-0432) and PKC-β (hispidin) reduced oxLDL-induced ROS by 30 and 42%, respectively. Ultimately, several well-established anti-oxidant drugs, TEMPOL, trolox and NAC, were used to further compare the ROS inhibitive efcacy of Co Q10. Our results suggested that TEMPLO at the concentration of 5 mM, NAC at the concentration of 0.5 mM, trolox at the concentration of 10 μM, those concentrations were used to repress oxidative stress-induced injury, was not as potent as Co Q10 20 μM in ROS reduction.

3.4 Co Q10 reversed oxLDL-induced dephosphorylation of AMPK-α and phosphorylation of PKC-αβ

To further confirm whether AMPK activation was involved in the inhibitory effect of Co Q10 on oxLDL-ROS generation, the protein levels of phosphorylated AMPK were determined by Western blot. As shown in Fig. 4A and B, treatment of HUVECs with oxLDL (130 μg/mL) for 1 h led to a reduction of phosphorylated AMPK-α. In addition, Co Q10 blocked the oxLDL-induced suppression of AMPK-α phosphorylation in HUVECs pretreated with Co Q10 in a dose-dependent manner (2.5–20 μM, all $p<0.05$).

Previous studies indicate that PKC isoforms likely play an important role in the regulation of the NADPH subunit expression and, in particular, the translocation of $p47^{phox}$ from the cytosol to the membrane [45, 46]. AMPK-α can inhibit ROS production through the suppression of protein kinase C (PKC), which prevents the activation of NADPH oxidase [20]. We therefore sought to determine whether Co Q10 affected oxLDL-induced PKC activation. As shown in Fig. 4C and D, oxLDL markedly increased phosphorylation of PKC-αβ after 1 h exposure. However, there was a marked reduction in PKC-αβ phosphorylation in HUVECs that had been pre-treated with 20 μM Co Q10. In addition, oxLDL-induced PKC-αβ activation was abolished by adding the AMPK inhibitor (compound C) and partially abolished by adding an inhibitor of PKC-α (Ro-32-0432) or PKC-β (hispidin), suggesting that PKC-α/β is a target of Co Q10 downstream of AMPK activation.

3.5 Knockdown of AMPK impairs the inhibitory effects of Co Q10 on oxLDL-induced PKC-αβ and $p47^{phox}$ activation

To further determine whether AMPK was involved in the inhibitory effects of Co Q10 on PKC-αβ-mediated oxLDL-

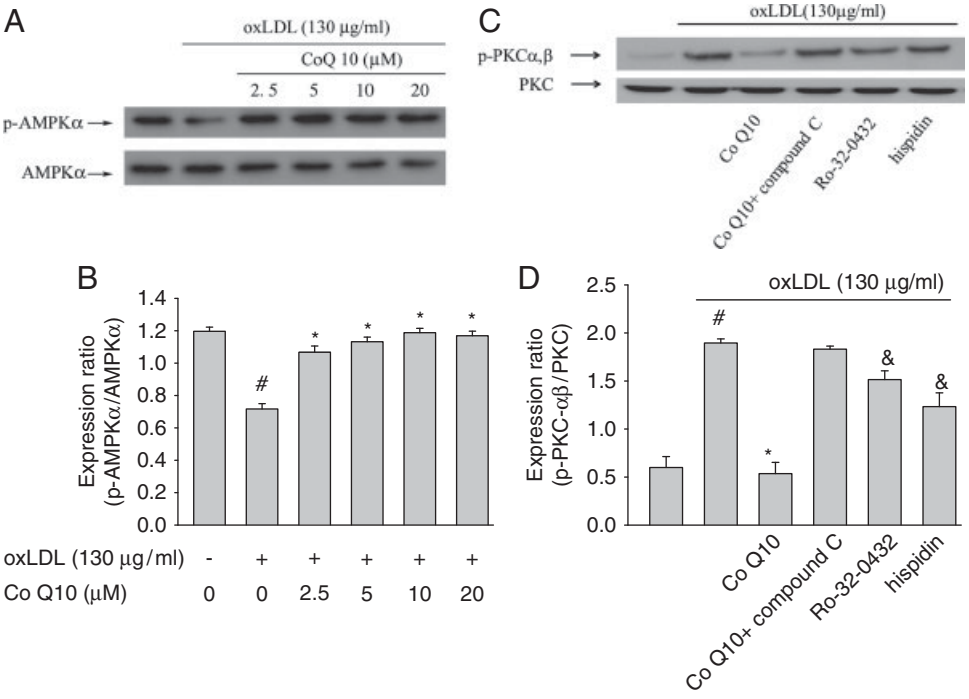

Figure 4. Inhibitory effect of Co Q10 on oxLDL-repressed endothelial AMPK activation (A, B) and oxLDL-induced PKC activation (C, D). HUVECs were pretreated with Co Q10 (2.5–20 μM) for 2 h, followed by exposure to oxLDL (130 μg/mL) for a further 2 h. In some cases, various pharmacological inhibitors such as AMPK inhibitor (10 μM compound C), PKC-α inhibitor (20 nM Ro-32-0432), PKC-β inhibitor (5 μM hispidin) were added for 1 h prior Co Q10 treatment. At the end of the incubation period, levels of both phosphorylated AMPK and PKC were determined by immunoblotting. The protein levels of p-AMPK-α were normalized to the level of AMPK-α. The protein levels of p-PKCα/β were normalized to the level of PKC. Data are mean ± SD of three different experiments. $p < 0.05$ compared with untreated control HUVECs. $^{*}p < 0.05$ compared with oxLDL-stimulated HUVECs. $^{\&}p < 0.05$ compared with oxLDL+Co Q10 treatment.

induced NADPH oxidase activation, we silenced AMPK-α1 gene expression using siRNA. As shown in Fig. 5A and B, transfection with AMPK-α1 siRNA for 72 h effectively reduced the protein levels of AMPK-α1 (Fig. 5C). Furthermore, the inhibitory effects of Co Q10 on oxLDL-induced membrane translocation of PKC-αβ and $p47^{phox}$ were abolished in HUVECs transfected with AMPK-α1 siRNA.

3.6 Co Q10-mediated protection involves Akt/eNOS activation

Akt plays a major role in promoting cell survival in response to various stimuli in addition to activating endothelial nitric oxide synthase (eNOS) that causes nitric oxide (NO) production. Studies have reported that oxLDL decreased Akt and eNOS phosphorylation in endothelial cells [47]. In contrast, activation of Akt and eNOS were shown to repress apoptosis and promote cell survival [48]. To investigate whether AMPK/Akt/eNOS signaling is involved in the protective effects of Co Q10, we performed a Western blot analysis using phosphor-specific Akt (Ser473) and phosphor-specific eNOS (Ser1177) antibodies. As expected, Co Q10 significantly reversed the dephosphorylation of Akt and eNOS (caused by oxLDL) in a dose-dependent manner (Fig. 6A–C, all $p < 0.05$).

3.7 Co Q10 protects against oxLDL-mediated NF-κB activation by modulation of p38 MAPK

The oxLDL-generated ROS can upregulate p38 MAPK as well as downregulate phosphoinositide 3-kinase (PI3K), both of which leads to the activation of NF-κB that subsequently triggers downstream pro-inflammatory responses [49]. As shown in Fig. 7, oxLDL induced phosphorylation of p38 MAPK was attenuated in cells pre-treated with Co Q10 in a dose-dependant manner.

NF-κB is a family of dimers composed of members of the Rel/NF-κB family [50]. Activation of NF-κB requires the dissociation of the inhibitory factor I-κB. Upon dissociation, NF-κB is rapidly translocated to the nucleus, where it presents as a p65/p50 heterodimer and binds directly to its cognate DNA sequence [51]. As shown in Fig. 7A and D, I-κB was degraded after exposure to oxLDL, thereby causing the nuclear translocation of NF-κB p65. In contrast, in cells pretreated with various concentrations of Co Q10 or SB203580, a specific inhibitor of p38MAPK, NF-κB activation was

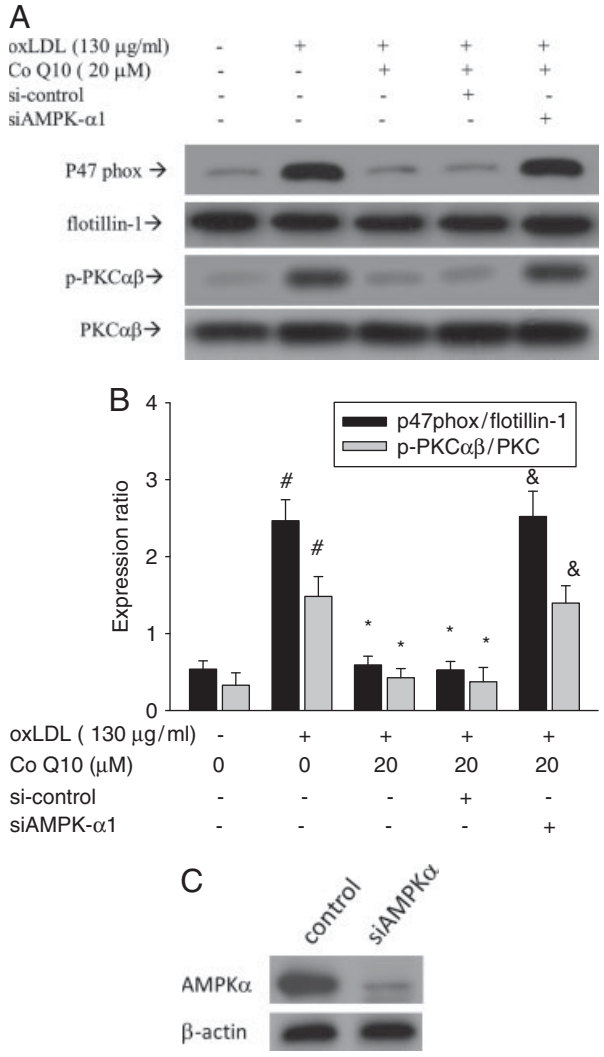

Figure 5. Inhibition of AMPK-α with siRNA antagonized the effects of Co Q10 on oxLDL-induced PKC and NADPH oxidase activation (A, B). HUVECs were transfected with AMPKα1 siRNA for 48 h and then treated with 20 μM of Co Q10 for 1 h followed by exposure to 130 μg/mL oxLDL for 1 h. Preparation of membrane and cytosolic proteins is described in Section 2. The levels of cytosolic and membrane protein were normalized to the levels of β-actin and flotillin-1, respectively. Western analysis of siAMPK-α knockdown efficiency (C). Both of cells transfected with siAMPK-α and control group were analyzed with Western blotting using anti-AMPK-α antibody. The AMPK-α expression level was normalized to the level of β-actin. Data are mean ± SD of three different experiments. $p<0.05$ compared with untreated control HUVECs. $^{*}p<0.05$ compared with oxLDL-stimulated HUVECs. $^{\&}p<0.05$ compared with oxLDL+Co Q10 treatment.

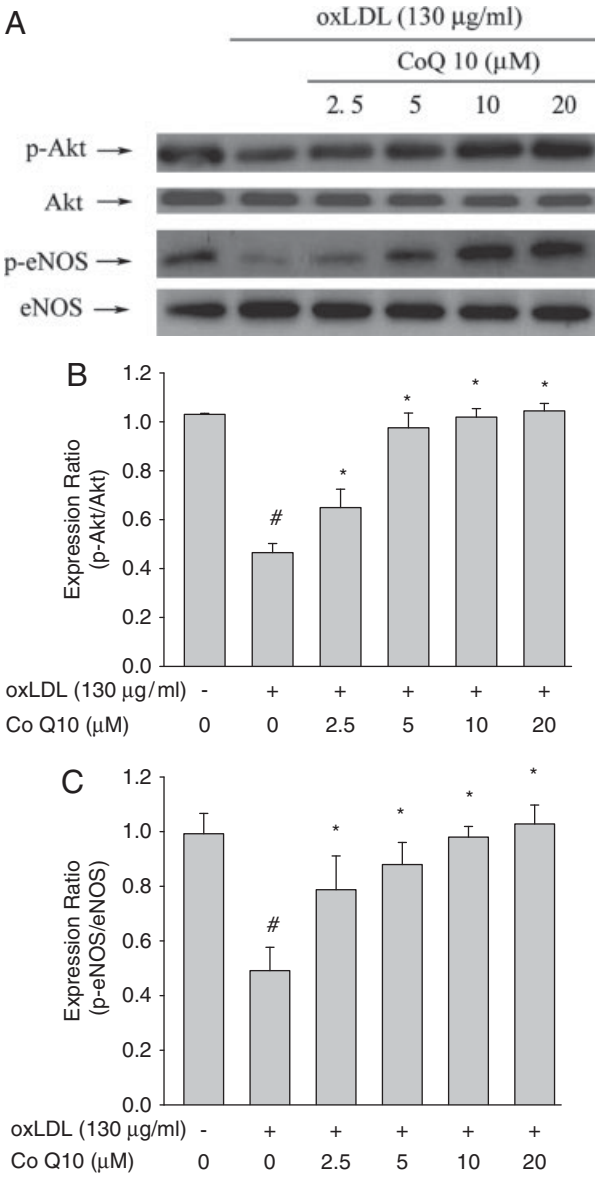

Figure 6. Co Q10 ameliorated the oxLDL-downregulated Akt and eNOS activation (A–C), HUVECs were pretreated for 2 h with the indicated concentrations of Co Q10 followed by stimulation with oxLDL (130 μg/mL) for another 1 h. At the end of the incubation period, level of both phosphorylated Akt and eNOS were determined by immunoblotting. Data are mean ± SD of three different experiments. $p<0.05$ compared with untreated control HUVECs. $^{*}p<0.05$ compared with oxLDL-stimulated HUVECs.

markedly inhibited (all $p<0.05$). Moreover, activated NF-κB could subsequently trigger serious inflammatory responses, including the release of cytokines and expression of adhesion molecules that could lead to endothelial oxidative injuries. Our results showed that oxLDL induced a significant increase of IL-6 and MCP-1. However, pretreatment with Co Q10 attenuated the release of IL-6 (Fig. S1A) and the expression level of adhesion molecules MCP-1 and CXCR-6 (Fig. S1B).

4 Discussion

LOX-1 was initially identified in endothelial cells as a molecule that induced endothelial dysfunction when

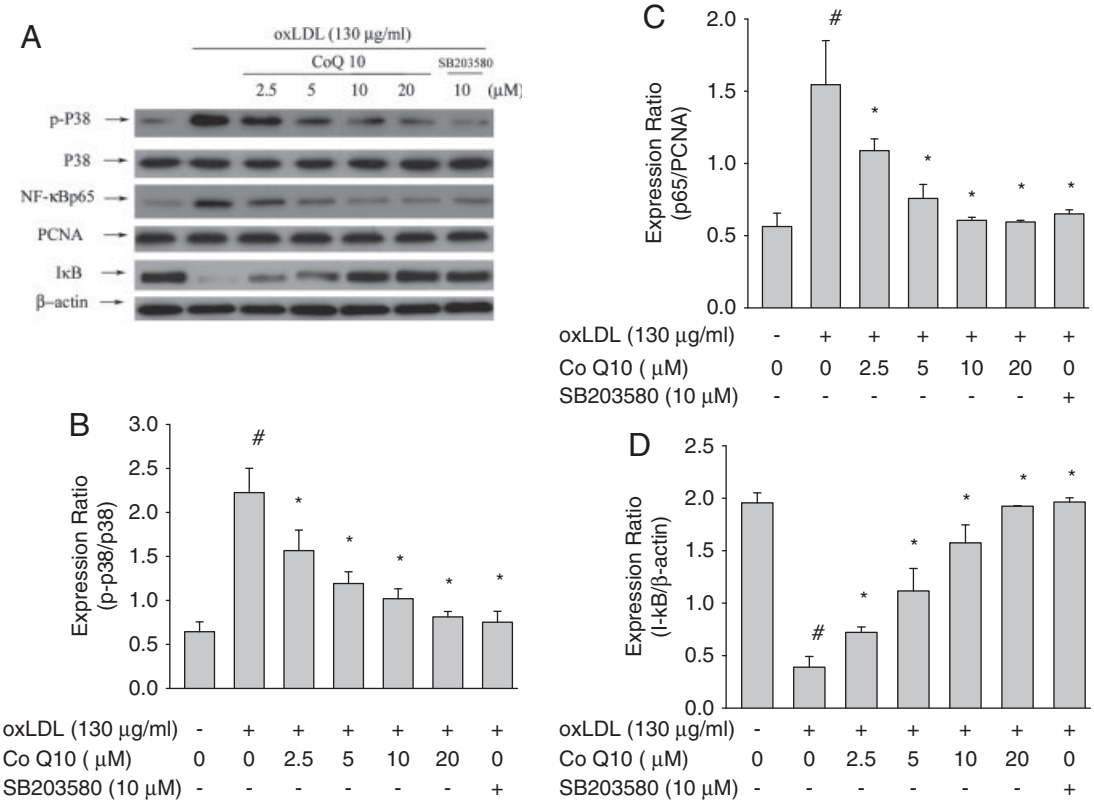

Figure 7. Effects of Co Q10 on phosphorylation of p38 MAPK and on the translocation of NF-κB. HUVECs were pretreated with indicated concentrations of Co Q10 for 2 h followed by exposure to 130 μg/mL oxLDL for 2 h (A–D). Western blot analysis was used to evaluate the expression of both phosphorylated and total p38 MAPK (B), and the activation of NF-κB. Anti-β-actin and anti-PCNA antibodies were used for normalization of cytosolic and nuclear proteins (C, D), respectively. Data are mean ± SD of three different experiments. $p < 0.05$ compared with untreated control HUVECs. *$p < 0.05$ compared with oxLDL-stimulated HUVECs.

triggered by oxLDL. When oxLDL binds to LOX-1 it induces rapid RhoA and Rac1 activation, resulting in NADPH oxidase activation and eNOS downregulation [52]. In this present study, we first demonstrated that Co Q10 decreased the expression of LOX-1 in HUVECs that had been exposed to oxLDL. Second, we showed that Co Q10 inhibited superoxide generation (one of the earliest signals in oxLDL stimulation), thereby blocking the LOX-1-mediated signaling pathway and the further generation of ROS. In addition, our results indicate that Co Q10 ameliorated oxLDL-induced dephosphorylation of AMPK, which led to PKC-αβ-mediated NADPH oxidase activation and subsequent superoxide generation in addition to impaired Akt/eNOS/NO signaling. Furthermore, Co Q10 reversed oxLDL-reduced bioavailability of NO, allowing p38-mediated NF-κB activation. Because the regulation of LOX-1 involves NF-κB [5], the inhibition of LOX-1 may result in a reduction in the oxidative stress-dependent activation of NF-κB.

Extensive reports have demonstrated that oxLDL strongly stimulates ROS production by NADPH oxidase [53], which further upregulates the expression of LOX-1 in endothelial cells leading to increased oxLDL uptake. In this study, we demonstrated that oxLDL-enhanced LOX-1 expression was inhibited by Co Q10 and the NADPH oxidase inhibitor DPI. In addition, oxLDL-enhanced ROS generation was attenuated by Co Q10 and a monoclonal antibody to LOX-1, indicating that Co Q10 has a crucial role of in NADPH oxidase-derived ROS and LOX-1 gene regulation. These findings were consistent with previous studies showing that resveratrol protected endothelial cells from oxLDL-induced oxidative stress through the inhibition of NADPH oxidase activity [54] and that the NADPH oxidase inhibitor apocynin reversed glucose-induced oxidative stress via NADPH oxidase activation and contributed to both LOX-1 upregulation and eNOS downregulation [6].

AMPK is activated by various types of stress associated with ATP depletion such as hypoxia, heat shock, metabolic poisoning, and in muscle, exercise. AMPK phosphorylates multiple targets that switch off anabolic pathways and switch on alternative catabolic pathways [55]. AMPK has been reported to prevent endothelial dysfunction by upregulating the Akt/eNOS/NO pathway [56] and by suppressing the PKC-mediated activation of NADPH oxidase in vascular tissues [20]. Recently, Dong et al. demonstrated that tempol, a potent anti-oxidant, attenuated oxLDL triggered endoplasmic reticulum stress via the activation of AMPK

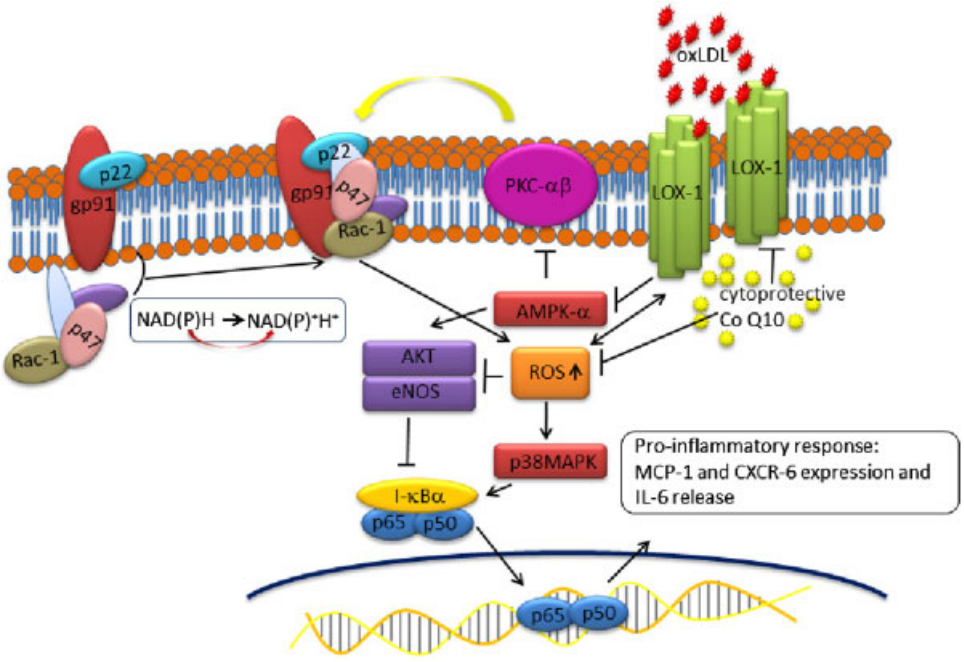

Figure 8. Schematic diagram showing cytoprotective signaling of Co Q10 in oxLDL-induced endothelial dysfunction. As depicted, Co Q10 mitigates oxLDL-induced LOX-1 expression, AMPK-α deactivation, PKC phosphorylation, NADPH oxidase activation and ROS generation and attenuates oxidative stress-related signaling transduction pathways, the → indicates activation or induction, and indicates inhibition or blockade.

[57]. In this present article, we revealed that Co Q10 reduces oxLDL-activated LOX-1 expression thereby promoting inflammatory responses in human endothelial cells. The mechanism of Co Q10-mediated anti-inflammation involves NADPH oxidase and PKC activation through AMPK dependent effect. Activation of AMPK function protects against oxidative damage as well as maintains endothelial function in oxidative situation [58–60]. In clinical strategies, AMPK activators are used as one cardiovascular disease for a long time, such as statins or rosiglitazone [61, 62]. Statins are well-established cardiovascular medicines to repress cholesterol levels acting by attenuating 3-hydroxy-3-methyl-glutaryl-Co enzyme A (HMG-CoA) reductase. The inhibition of mevalonate formatted from HMG-CoA leads a reduction effect in the cholesterol synthesis. In addition, the AMPK activators, such as statins, are reported as a protector to improve endothelial function, suppress endothelial free radical generation and reduce plaque rapture [63, 64]. Rosiglitazone, is another AMPK activator, had been used as anti-diabetic agents and anti-insulin resistance [65, 66]. Clinical parameters confirmed that rosiglitazone reduces LDL, increase HDL as well as reduces triglycerides in human plasma [67]. Besides, previous study reported rosiglitazone protect against sodium arsenite-induced vascular endothelial dysfunction and intracellular ROS generation, indicating that rosiglitazone acts the role of anti-oxidant [68]. In addition, metformin, an AMPK activators also, was reported have positive effects of endothelial parameters in DM patients [69, 70]. As mentioned earlier, AMPK activators are considered as anti-atherogenic properties.

Recently, people are interesting in natural products or anti-oxidant extracts as a daily supplement to protect against cardiovascular disease [71], at the same time, the results of clinical trials confirmed dietary anti-oxidants supplement had positive outcomes in preventing atherogenesis [72, 73]. Several natural products or food extracts are able to modulate AMPK function, for example, uaringenin, resveratrol, and kaempferol are able to upregulate AMPK expression level, thereby promoting cells survival and cells growth [74–77]. However, there are no studies interpreting the relationship between Co Q10 and AMPK, our group firstly make a cross-link between Co Q10 and AMPK. According to our data, AMPK knockdown impaired the inhibitory effects of Co Q10 on oxLDL-facilitated NADPH oxidase activation as well as PKC phosphorylation, providing a link between the Co Q10 and AMPK activation. These findings are in agreement with previous studies that demonstrated that AMPK acts as a negative regulator of NADPH oxidase. For example, AMPK negatively regulates NOX4-dependent activation of p53 and epithelial cell apoptosis [78], in addition to preventing the serine phosphorylation and membrane translocation of $p47^{phox}$ [55]. These new findings identify Co Q10 as a novel molecule that mitigates oxLDL-induced endothelial oxidative damage, suggesting that Co Q10 may be used as a newer pharmacological agent to treat or control chronic inflammation diseases in cardiovascular system.

AMPK promotes endothelial function by suppressing NADPH oxidase-derived superoxide production and by regulating eNOS through the phosphorylation of Ser1177 and Ser635 [79, 80]. NO produced by eNOS have anti-inflammatory and protective effects mediated by the inhibition of NF-κB activation through the stabilization of the IκB-α protein in addition to mediating the nitrosylation of Cys 62 on the p50 subunit [81]. ROS derived from NADPH oxidase not only reduced the bioavailability of NO, but also activated p38 MAPK, both of which caused NF-κB activation

and the nuclear translocation and subsequent regulation of pro-inflammatory molecules including cytokines, chemokines, enzymes and adhesion molecules [5]. In the present study, we found that some of the adverse effects of oxLDL, namely the inhibition of AMPK/Akt/eNOS and the activation of p38 MAPK and NF-κB, were abrogated by Co Q10 treatment, suggesting that the protective effect of Co Q10 is due, at least in part, to its ability to upregulate the AMPK/Akt/eNOS/NO signaling pathway.

Daily intake of Co Q10 is safe and tolerable. The maximum dosage for clinical trial studies was 3000 mg/day/person, and the plasma Co Q10 concentration after daily intake of Co Q10 was 8.69 μM [82]. In our study, results demonstrated that the lowest concentration of Co Q10 pretreatment (2.5 μM) still provided protection from oxLDL-related injury. The concentration of Co Q10 we used to attenuate oxLDL-mediated injury is easily physiologically achieved.

In summary, Co Q10 prevents the oxLDL-induced LOX-1-mediated ROS generation that is closely linked to endothelial dysfunction. Co Q10 reverses oxLDL-repressed AMPK-α activation as well PKC-activated NADPH oxidase, thereby maintaining the function Akt/eNOS and mitigating p38 MAPK and NF-κB activation (Fig. 8). This study was performed in vitro, and as such, further studies are required to confirm the extent to which Co Q10 suppresses oxLDL-facilitated pro-atherogenic effects as well as the effectiveness of Co Q10 in vivo. Our findings add to a growing body of evidence that Co Q10 has beneficial effects on cardiovascular health.

This study was supported by grants from The National Science Council, (NSC 98-2320-B-039-020-MY3, NSC 97-3111-B-075-001-MY3, 97-2320-B-075-003-MY3), China Medical University (CMU99-S-13), Taiwan, ROC. Taipei Veterans General Hospital (99E1), the Joint Projects of UTVGH (VGHUST 98-p1-01), Yen-Tjing-Ling Medical Foundation (96/97/98), National Yang-Ming University (Ministry of Education, Aim for the Top University Plan) and Genomic Center Project, Institute of Biological medicine (IBMS-CRC99-p01), Academia Sinica, and Center of Excellence for Cancer Research at Taipei Veterans General Hospital (DOH99-TD-C-111-007), Taiwan. This study is supported in part by Taiwan Department of Health Clinical Trial and Research Center of Excellence (DOH100-TD-B-111-004).

The authors have declared no conflict of interest.

5 References

[1] Ross, R., The pathogenesis of atherosclerosis: a perspective for the 1990s. *Nature* 1993, *362*, 801–809.

[2] Mehta, J. L., Chen, J., Hermonat, P. L., Romeo, F., Novelli, G., Lectin-like, oxidized low-density lipoprotein receptor-1 (LOX-1): a critical player in the development of atherosclerosis and related disorders. *Cardiovasc. Res.* 2006, *69*, 36–45.

[3] Murphy, J. E., Tacon, D., Tedbury, P. R., Hadden, J. M. et al., LOX-1 scavenger receptor mediates calcium-dependent recognition of phosphatidylserine and apoptotic cells. *Biochem. J.* 2006, *393*, 107–115.

[4] Ogura, S., Kakino, A., Sato, Y., Fujita, Y., et al., Lox-1: the multifunctional receptor underlying cardiovascular dysfunction. *Circ. J.* 2009, *73*, 1993–1999.

[5] Chen, X. P., Xun, K. L., Wu, Q., Zhang, T. T. et al., Oxidized low density lipoprotein receptor-1 mediates oxidized low vein endothelial cells: role of reactive oxygen species. *Vascul. Pharmacol.* 2007, *47*, 1–9.

[6] Taye, A., Saad, A. H., Kumar, A. H. S., Morawietz, H., Effect of apocynin on NADPH oxidase-mediated oxidative stress-LOX-1-eNOS pathway in human endothelial cells exposed to high glucose. *Eur. J. Pharmacol.* 2010, *627*, 42–48.

[7] Sugimoto, K., Ishibashi, T., Sawamura, T., Inoue, N. et al., LOX-1-MT1-MMP axis is crucial for RhoA and Rac1 activation induced by oxidized low-density lipoprotein in endothelial cells. *Cardiovasc. Res.* 2009, *84*, 127–136.

[8] Navarra, T., Del Turco, S., Berti, S., Basta, G., The lectin-like oxidized low-density lipoprotein receptor-1 and its soluble form: cardiovascular implications. *J. Atheroscler. Thromb.* 2010, *17*, 317–331.

[9] Li, D., Mehta, J. L., Intracellular signaling of LOX-1 in endothelial cell apoptosis. *Circ. Res.* 2009, *104*, 566–568.

[10] Simon, F., Stutzin, A., Protein kinase C-mediated phosphorylation of p47 phox modulates platelet-derived growth factor-induced H_2O_2 generation and cell proliferation in human umbilical vein endothelial cells. *Endothelium* 2008, *15*, 175–188.

[11] White, C. N., Figtree, G. A., Liu, C. C., Garcia, A. et al., Angiotensin II inhibits the Na^+-K^+ pump via PKC-dependent activation of NADPH oxidase. *Am. J. Physiol. Cell Physiol.* 2009, *296*, C693–C700.

[12] Graciano, M. F., Santos, L. R., Curi, R., Carpinelli, A. R., NAD(P)H oxidase participates in the palmitate-induced superoxide production and insulin secretion by rat pancreatic islets. *J. Cell Physiol.* 2011, *226*, 1110–1117.

[13] Ungvari, Z., Csiszar, A., Huang, A., Kaminski, P. M. et al., High pressure induces superoxide production in isolated arteries via protein kinase C-dependent activation of NAD(P)H oxidase. *Circulation* 2003, *108*, 1253–1258.

[14] Romero, M., Jimenez, R., Sanchez, M., Lopez-Sepulveda, R., et al., Quercetin inhibits vascular superoxide production induced by endothelin-1: role of NADPH oxidase, uncoupled eNOS and PKC. *Atherosclerosis* 2009, *202*, 58–67.

[15] Ceolotto, G., Papparella, I., Lenzini, L., Sartori, M. et al., Insulin generates free radicals in human fibroblasts ex vivo by a protein kinase C-dependent mechanism, which is inhibited by pravastatin. *Free Radic. Biol. Med.* 2006, *41*, 473–483.

[16] Hardie, D. G., AMP-activated/SNF1 protein kinases: conserved guardians of cellular energy. *Nat. Rev. Mol. Cell. Biol.* 2007, *8*, 774–785.

[17] Fisslthaler, B., Fleming, I., Activation and signaling by the AMP-activated protein kinase in endothelial cells. *Circ. Res.* 2009, *105*, 114–127.

[18] Leverence, J. T., Medhora, M., Konduri, G. G., Sampath, V., Lipopolysaccharide-induced cytokine expression in alveolar epithelial cells: role of PKCzeta-mediated p47phox phosphorylation. *Chem. Biol. Interact* 2011, *189*, 72–81.

[19] Zeng, Q., Han, Y., Bao, Y., Li, W. et al., 20-HETE increases NADPH oxidase-derived ROS production and stimulates the L-type Ca^{2+} channel via a PKC-dependent mechanism in cardiomyocytes. *Am. J. Physiol. Heart Circ. Physiol.* 2010, *299*, H1109–H1117.

[20] Ceolotto, G., Gallo, A., Papparella, I., Franco, L. et al., Rosiglitazone reduces glucose-induced oxidative stress mediated by NAD(P)H oxidase via AMPK-dependent mechanism. *Arterioscler. Thromb. Vasc. Biol.* 2007, *27*, 2627–2633.

[21] Shin, S. M., Cho, I. J., Kim, S. G., Resveratrol protects mitochondria against oxidative stress through AMP-activated protein kinase-mediated glycogen synthase kinase-3beta inhibition downstream of poly(ADP-ribose)polymerase-LKB1 pathway. *Mol. Pharmacol.* 2009, *76*, 884–895.

[22] Lu, J., Wu, D. M., Zheng, Y. L., Hu, B. et al., Quercetin activates AMP-activated protein kinase by reducing PP2C expression protecting old mouse brain against high cholesterol-induced neurotoxicity. *J. Pathol.* 2010, *222*, 199–212.

[23] Littarru, G. P., Tiano, L., Bioenergetic and antioxidant properties of coenzyme Q10: recent developments. *Mol. Biotechnol.* 2007, *37*, 31–37.

[24] Hoppe, U., Bergemann, J., Diembeck, W., Ennen, J. et al., Coenzyme Q10, a cutaneous antioxidant and energizer. *Biofactors* 1999, *9*, 371–378.

[25] Kalen, A., Appelkvist, E. L., Dallner, G., Age-related changes in the lipid compositions of rat and human tissues. *Lipids* 1989, *24*, 579–584.

[26] Caso, G., Kelly, P., McNurlan, M. A., Lawson, W. E., Effect of coenzyme q10 on myopathic symptoms in patients treated with statins. *Am. J. Cardiol.* 2007, *99*, 1409–1412.

[27] Dhanasekaran, M., Ren, J., The emerging role of coenzyme Q-10 in aging, neurodegeneration, cardiovascular disease, cancer and diabetes mellitus. *Curr. Neurovasc. Res.* 2005, *2*, 447–459.

[28] Hodgson, J. M., Watts, G. F., Playford, D. A., Burke, V., Croft, K. D., Coenzyme Q10 improves blood pressure and glycaemic control: a controlled trial in subjects with type 2 diabetes. *Eur. J. Clin. Nutr.* 2002, *56*, 1137-1142.

[29] Hunter, D. A., Coenzyme Q10 in early Parkinson disease. *Arch. Neurol.* 2003, *60*, 1170; author reply 1172–1173.

[30] Shults, C. W., Oakes, D., Kieburtz, K., Beal, M. F. et al., Effects of coenzyme Q10 in early Parkinson disease: evidence of slowing of the functional decline. *Arch. Neurol.* 2002, *59*, 1541–1550.

[31] Gonzalez, R., Ferrin, G., Hidalgo, A. B., Ranchal, I. et al., *N*-Acetylcysteine, coenzyme Q10 and superoxide dismutase mimetic prevent mitochondrial cell dysfunction and cell death induced by d-galactosamine in primary culture of human hepatocytes. *Chem. Biol. Interact.* 2009, *181*, 95–106.

[32] Ratnam, D. V., Chandraiah, G., Meena, A. K., Ramarao, P., Kumar, M. N., The co-encapsulated antioxidant nanoparticles of ellagic acid and coenzyme Q10 ameliorates hyperlipidemia in high fat diet fed rats. *J. Nanosci. Nanotechnol.* 2009, *9*, 6741–6746.

[33] Chapidze, G. E., Kapanadze, S. D., Dolidze, N. K., Latsabidze, N. E., Bakhutashvili, Z. V., Combination treatment with coenzyme Q10 and simvastatin in patients with coronary atherosclerosis. *Kardiologiia* 2006, *46*, 11–13.

[34] Makhija, N., Sendasgupta, C., Kiran, U., Lakshmy, R. et al., The role of oral coenzyme Q10 in patients undergoing coronary artery bypass graft surgery. *J. Cardiothorac. Vasc. Anesth.* 2008, *22*, 832–839.

[35] Celik, T., Iyisoy, A., Coenzyme Q10 and coronary artery bypass surgery: what we have learned from clinical trials. *J. Cardiothorac. Vasc. Anesth.* 2009, *23*, 935–936.

[36] Rosenfeldt, F. L., Pepe, S., Linnane, A., Nagley, P., et al., Coenzyme Q10 protects the aging heart against stress: studies in rats, human tissues, and patients. *Ann. NY Acad. Sci.* 2002, *959*, 355–359, discussion 463–355.

[37] Sarter, B., Coenzyme Q10 and cardiovascular disease: a review. *J. Cardiovasc. Nurs.* 2002, *16*, 9-20.

[38] Sohet, F. M., Neyrinck, A. M., Pachikian, B. D., de Backer, F. C. et al., Coenzyme Q10 supplementation lowers hepatic oxidative stress and inflammation associated with diet-induced obesity in mice. *Biochem. Pharmacol.* 2009, *78*, 1391–1400.

[39] Schmelzer, C., Kubo, H., Mori, M., Sawashita, J. et al., Supplementation with the reduced form of Coenzyme Q10 decelerates phenotypic characteristics of senescence and induces a peroxisome proliferator-activated receptor-α gene expression signature in SAMP1 mice. *Mol. Nutr. Food Res.* 2010, *54*, 805–815.

[40] Schmelzer, C., Lorenz, G., Rimbach, G., Doring, F., In vitro effects of the reduced form of coenzyme Q(10) on secretion levels of TNF-alpha and chemokines in response to LPS in the human monocytic cell line THP-1. *J. Clin. Biochem. Nutr.* 2009, *44*, 62–66.

[41] Morre, D. M., Morre, D. J., Rehmus, W., Kern, D., Supplementation with CoQ10 lowers age-related (ar) NOX levels in healthy subjects. *Biofactors* 2008, *32*, 221–230.

[42] Kuo, W. W., Huang, C. Y., Chung, J. G., Yang, S. F. 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*, 849–860.

[43] Havel, R. J., Eder, H. A., Bragdon, J. H., The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* 1955, *34*, 1345–1353.

[44] Hsieh, C. C., Yen, M. H., Yen, C. H., Lau, Y. T., Oxidized low density lipoprotein induces apoptosis via generation of reactive oxygen species in vascular smooth muscle cells. *Cardiovasc. Res.* 2001, *49*, 135-145.

[45] Bey, E. A., Xu, B., Bhattacharjee, A., Oldfield, C. M. et al., Protein kinase C delta is required for p47phox phosphorylation and translocation in activated human monocytes. *J. Immunol.* 2004, *173*, 5730–5738.

[46] Ceolotto, G., Papparella, I., Lenzini, L., Sartori, M. et al., Insulin generates free radicals in human fibroblasts ex vivo by a protein kinase C-dependent mechanism, which is inhibited by pravastatin. *Free Radic. Biol. Med.* 2006, *41*, 473–483.

[47] Chavakis, E., Dernbach, E., Hermann, C., Mondorf, U. F. et al., Oxidized LDL inhibits vascular endothelial growth factor-induced endothelial cell migration by an inhibitory effect on the Akt/endothelial nitric oxide synthase pathway. *Circulation* 2001, *103*, 2102–2107.

[48] Amaravadi, R., Thompson, C. B., The survival kinases Akt and Pim as potential pharmacological targets. *J. Clin. Invest.* 2005, *115*, 2618–2624.

[49] Chen, X. P., Zhang, T. T., Du, G. H., Lectin-like oxidized low-density lipoprotein receptor-1, a new promising target for the therapy of atherosclerosis?. *Cardiovasc. Drug Rev.* 2007, *25*, 146–161.

[50] Ghosh, S., Baltimore, D., Activation in vitro of NF-kappa B by phosphorylation of its inhibitor I kappa B. *Nature* 1990, *344*, 678–682.

[51] Urban, M. B., Schreck, R., Baeuerle, P. A., NF-kappa B contacts DNA by a heterodimer of the p50 and p65 subunit. *EMBO J.* 1991, *10*, 1817–1825.

[52] Sawamura, T., Kume, N., Aoyama, T., Moriwaki, H. et al., An endothelial receptor for oxidized low-density lipoprotein. *Nature* 1997, *386*, 73–77.

[53] Heinloth, A., Heermeier, K., Raff, U., Wanner, C., Galle, J., Stimulation of NADPH oxidase by oxidized low-density lipoprotein induces proliferation of human vascular endothelial cells. *J. Am. Soc. Nephrol.* 2000, *11*, 1819–1825.

[54] Chow, S. E., Hshu, Y. C., Wang, J. S., Chen, J. K., Resveratrol attenuates oxLDL-stimulated NADPH oxidase activity and protects endothelial cells from oxidative functional damages. *J. Appl. Physiol.* 2007, *102*, 1520–1527.

[55] Alba, G., El Bekay, R., Alvarez-Maqueda, M., Chacon, P. et al., Stimulators of AMP-activated protein kinase inhibit the respiratory burst in human neutrophils. *FEBS Lett.* 2004, *573*, 219–225.

[56] Michell, B. J., Chen, Z., Tiganis, T., Stapleton, D. et al., Coordinated control of endothelial nitric-oxide synthase phosphorylation by protein kinase C and the cAMP-dependent protein kinase. *J. Biol. Chem.* 2001, *276*, 17625–17628.

[57] Dong, Y., Zhang, M., Wang, S., Liang, B. et al., Activation of AMP-activated protein kinase inhibits oxidized LDL-triggered endoplasmic reticulum stress in vivo. *Diabetes* 2010, *59*, 1386–1396.

[58] Schuhmacher, S., Foretz, M., Knorr, M., Jansen, T. et al., alpha1AMP-activated protein kinase preserves endothelial function during chronic angiotensin II treatment by limiting Nox2 upregulation. *Arterioscler. Thromb. Vasc. Biol.* 2011, *31*, 560–566.

[59] Rubin, L. J., Magliola, L., Feng, X., Jones, A. W., Hale, C. C., Metabolic activation of AMP kinase in vascular smooth muscle. *J. Appl. Physiol.* 2005, *98*, 296–306.

[60] Colombo, S. L., Moncada, S., AMPKalpha1 regulates the antioxidant status of vascular endothelial cells. *Biochem. J.* 2009, *421*, 163–169.

[61] Elrod, J. W., Lefer, D. J., The effects of statins on endothelium, inflammation and cardioprotection. *Drug News Perspect.* 2005, *18*, 229–236.

[62] Perampaladas, K., Parker, J. D., Gori, T., The impact of rosiglitazone on nitric oxide bioavailability and endothelial function. *Clin. Hemorheol. Microcirc.* 2010, *45*, 325–328.

[63] Ohkawara, H., Ishibashi, T., Saitoh, S., Inoue, N. et al., Preventive effects of pravastatin on thrombin-triggered vascular responses via Akt/eNOS and RhoA/Rac1 pathways in vivo. *Cardiovasc. Res.* 2010, *88*, 492-501.

[64] Massaro, M., Zampolli, A., Scoditti, E., Carluccio, M. A. et al., Statins inhibit cyclooxygenase-2 and matrix metalloproteinase-9 in human endothelial cells: anti-angiogenic actions possibly contributing to plaque stability. *Cardiovasc. Res.* 2010, *86*, 311–320.

[65] Mooradian, A. D., Chehade, J., Thurman, J. E., The role of thiazolidinediones in the treatment of patients with type 2 diabetes mellitus. *Treat. Endocrinol.* 2002, *1*, 13–20.

[66] Song, G. Y., Gao, Y., Wang, C., Hu, S. G., Wang, J., Qu, D. M., Ma, H. J., Rosiglitazone reduces fatty acid translocase and increases AMPK in skeletal muscle in aged rats: a possible mechanism to prevent high-fat-induced insulin resistance. *Chin. Med. J.* 2010, *123*, 2384–2391.

[67] Campbell, I. W., The clinical significance of PPAR gamma agonism. *Curr. Mol. Med.* 2005, *5*, 349-363.

[68] Kaur, T., Goel, R. K., Balakumar, P., Effect of rosiglitazone in sodium arsenite-induced experimental vascular endothelial dysfunction. *Arch. Pharm. Res.* 2010, *33*, 611–618.

[69] Engelen, L., Lund, S. S., Ferreira, I., Tarnow, L. et al., Improved glycemic control induced by both metformin and repaglinide is associated with a reduction in blood levels of 3-deoxyglucosone in nonobese patients with type 2 diabetes. *Eur. J. Endocrinol.* 2011, *164*, 371–379.

[70] Chen, L. L., Liao, Y. F., Zeng, T. S., Yu, F. et al., Effects of metformin plus gliclazide compared with metformin alone on circulating endothelial progenitor cell in type 2 diabetic patients. *Endocrine* 2010, *38*, 266–275.

[71] Sirtori, C. R., Galli, C., Anderson, J. W., Sirtori, E., Arnoldi, A., Functional foods for dyslipidaemia and cardiovascular risk prevention. *Nutr. Res. Rev.* 2009, *22*, 244-261.

[72] Kaliora, A. C., Dedoussis, G. V., Schmidt, H., Dietary antioxidants in preventing atherogenesis. *Atherosclerosis* 2006, *187*, 1–17.

[73] Watts, G. F., Playford, D. A., Croft, K. D., Ward, N. C. et al., Coenzyme Q(10) improves endothelial dysfunction of the brachial artery in Type II diabetes mellitus. *Diabetologia* 2002, *45*, 420–426.

[74] Zygmunt, K., Faubert, B., MacNeil, J., Tsiani, E., Naringenin, a citrus flavonoid, increases muscle cell glucose uptake via AMPK. *Biochem. Biophys. Res. Commun.* 2010, *398*, 178–183.

[75] Zhang, Z. F., Li, Q., Liang, J., Dai, X. Q. et al., Epigallocatechin-3-*O*-gallate (EGCG) protects the insulin sensitivity in

rat L6 muscle cells exposed to dexamethasone condition. *Phytomedicine* 2010, *17*, 14–18.

[76] Filomeni, G., Desideri, E., Cardaci, S., Graziani, I. et al., Carcinoma cells activate AMP-activated protein kinase-dependent autophagy as survival response to kaempferol-mediated energetic impairment. *Autophagy* 2010, *6*, 202–216.

[77] Brito, P. M., Devillard, R., Negre-Salvayre, A., Almeida, L. M. et al., Resveratrol inhibits the mTOR mitogenic signaling evoked by oxidized LDL in smooth muscle cells. *Atherosclerosis* 2009, *205*, 126–134.

[78] Eid, A. A., Ford, B. M., Block, K., Kasinath, B. S. et al., AMPK negatively regulates NOX4-dependent activation of p53 and epithelial cell apoptosis in diabetes. *J. Biol. Chem.* 2010, *285*, 37503–37512.

[79] Zou, M. H., Hou, X. Y., Shi, C. M., Nagata, D. et al., Modulation by peroxynitrite of Akt- and AMP-activated kinase-dependent Ser1179 phosphorylation of endothelial nitric oxide synthase. *J. Biol. Chem.* 2002, *277*, 32552–32557.

[80] Chen, Z., Peng, I. C., Sun, W., Su, M. I. et al., AMP-activated protein kinase functionally phosphorylates endothelial nitric oxide synthase Ser633. *Circ. Res.* 2009, *104*, 496–505.

[81] Peng, H. B., Libby, P., Liao, J. K., Induction and stabilization of I kappa B alpha by nitric oxide mediates inhibition of NF-kappa B. *J. Biol. Chem.* 1995, *270*, 14214–14219.

[82] Shults, C. W., 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*, 491–494.

