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Coenzyme Q_0 regulates NFκB/AP-1 activation and enhances Nrf2 stabilization in attenuation of LPS-induced inflammation and redox imbalance: Evidence from *in vitro* and *in vivo* studies

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ABSTRACT

Coenzyme Q (CoQ) analogs with variable number of isoprenoid units have been demonstrated as anti-inflammatory and antioxidant/pro-oxidant molecules. In this study we used CoQ_0 (2,3-dimethoxy-5-methyl-1,4-benzoquinone, zero isoprenoid side-chains), a novel quinone derivative, and investigated its molecular actions against LPS-induced inflammation and redox imbalance in murine RAW264.7 macrophages and mice. In LPS-stimulated macrophages, non-cytotoxic concentrations of CoQ_0 (2.5–10 μM) inhibited iNOS/COX-2 protein expressions with subsequent reductions of NO, PGE_2, TNF-α and IL-1β secretions. This inhibition was reasoned by suppression of NFκB (p65) activation, and inhibition of AP-1 (c-Jun., c-Fos, ATF2) translocation. Our findings indicated that IKKα-mediated I-κB degradation and MAPK-signaling are involved in regulation of NFκB/AP-1 activation. Furthermore, CoQ_0 triggered HO-1 and NQO-1 genes through increased Nrf2 nuclear translocation and Nrf2/ARE-signaling. This phenomenon was confirmed by diminished CoQ_0 protective effects in Nrf2 knockdown cells, where LPS-induced NO, PGE_2, TNF-α and IL-1β productions remained high. Molecular evidence revealed that CoQ_0 enhanced Nrf2 steady-state level at both transcriptional and translational levels. CoQ_0-induced Nrf2 activation appears to be regulated by ROS–JNK-signaling cascades, as evidenced by suppressed Nrf2 activation upon treatment with pharmacological inhibitors of ROS (N-acetylcysteine) and JNK (SP600125). Besides, oral administration of CoQ_0 (5 mg/kg) suppressed LPS-induced (1 mg/kg) induction of iNOS/COX-2 and TNF-α/IL-1β through tight regulation of NFκB/Nrf2 signaling in mice liver and spleen. Our findings conclude that pharmacological actions of CoQ_0 are mediated *via* inhibition of NFκB/AP-1 activation and induction of Nrf2/ARE-signaling. Owing to its potent anti-inflammatory and antioxidant properties, CoQ_0 could be a promising candidate to treat inflammatory disorders.

1. Introduction

Dysregulation of inflammatory and redox-signaling pathways are the two promising systems that are responsible for development and progression of many diseases. The inflammatory response is tightly regulated by both pro- and anti-inflammatory mediators, and any disruption in this homeostasis lead to morbidity and reduce lifespan [1,2]. Among several inflammatory mediators, macrophages play a central role in coordinating the immune response to invading pathogens through phagocytosis and cytokine secretion [3]. Macrophage activation by endotoxins/lipopolysaccharides (LPS) has been widely studied to investigate the inflammatory mechanism in both cell culture and

Abbreviations: AP-1, activator protein-1; ARE, antioxidant response element; ATF2, activating transcription factor 2; CoQ_0, coenzyme Q_0; COX-2, cyclooxygenase-2; ERK, extracellular signal-regulated kinase; GSH, glutathione; HO-1, heme oxygenase-1; I-κB, inhibitor-κB; IKKα, IκB kinase α; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; JNK, c-jun N-terminal kinase; Keap-1, Kelch-like ECH-associated protein 1; LPS, lipopolysaccharide; NAC, N-acetylcysteine; NFκB, nuclear factor κB; NO, nitric oxide; NQO-1, NAD(P)H: quinone acceptor oxidoreductase 1; Nrf2, nuclear factor erythroid-2 related factor-2; PGE_2, prostaglandin E_2; PI3K, phosphatidylinositol 3-kinases; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α.

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animal models [4–6]. The activated macrophages subsequently induce the activation of redox-sensitive transcription factors, such as nuclear factor κB (NFκB) and activator protein-1 (AP-1) by phosphorylating the inhibitor-κB (I-κB) and their up-stream kinase inhibitor-κB kinase (IKK), and/or mitogen-activated protein kinases (MAPKs), including JNK, ERK and p38 [7,8]. These activated transcription factors then trigger various pro-inflammatory molecules, such as nitric oxide (NO), prostaglandin E_2 (PGE_2), tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) *via* induction of their corresponding mediator genes, inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) [4,9].

Besides, excessive production of pro-inflammatory mediators ultimately ruins the cellular redox-balance and provoke to progression of several diseases [10,11]. On the other hand, generation of reactive oxygen species (ROS) by external stimuli, including LPS has been shown to promote inflammatory response in cultured macrophages and animal tissues [6,12]. In this scenario, inhibition of ROS and/or activation of antioxidant genes, such as hemeoxygenase-1 (HO-1) and NAD(P)H:quinone acceptor oxidoreductase-1 (NQO-1) could suppress the inflammatory response in macrophages through suppression of NFκB/AP-1 activation. It has been shown that activation of antioxidant genes occurs *via* nuclear factor (erythroid-2) related factor-2 (Nrf2)/antioxidant response element (ARE)-signaling pathway under stress conditions to protect the cells [13,14]. Thus, treatment with anti-inflammatory and/or antioxidant molecules could be a suitable approach to minimize or prevent the progression of inflammatory diseases.

A large number of preclinical and clinical studies demonstrated that coenzyme Q (CoQ) or ubiquinone analogs have potential therapeutic effects for inflammation, cancer and several metabolic disorders [15–17]. CoQ is a lipophilic molecule present in the inner side of mitochondrial membrane of most aerobic organisms from bacteria to mammals. CoQ is composed of a redox active benzoquinone ring conjugated to an isoprenoid chain. The subscript in CoQ molecule refers to the number of isoprene repeat units, which included CoQ_0 to CoQ_{10} [16]. Various analogs of CoQ have been shown to exhibit either pro-oxidant or antioxidant properties. Antioxidant property was represented by preventing oxidative stress-induced cells death [18,19]. While CoQ_0, a redox-active compound without isoprenoid side-chain that predominantly accumulates in mitochondria, has been shown to inhibit the activity of complex 1 of mitochondrial respiratory chain and prevent opening of the mitochondrial permeability transition pore [20]. CoQ_0 is toxic to insulin producing cells, in which 1 μM of CoQ_0 caused cell death in pancreatic cells [21]. The potent cytotoxicity of CoQ_0 towards human breast cancer MDA-MB-231 and SKBr3 cells was shown with an IC_{50} of 1.7 μM and 3.1 μM respectively, through induction of apoptosis and cell-cycle arrest [15].

Despite its cytotoxicity, some *in vivo* studies demonstrated no deleterious effects when CoQ_0 combined with other nutrients. Particularly administration of CoQ_0 mixture to rodents inhibited the oxidative damage in blood, heart, liver, kidney and spleen [19,22]. However, the key pharmacological efficacies of a single CoQ_0 molecule against inflammation and redox imbalance have not been thoroughly investigated, and involved precise signaling pathways are largely unknown. Therefore, we investigated whether CoQ_0 (Fig. 1A) treatment could attenuate the LPS-induced inflammatory response and redox imbalance in RAW264.7 macrophages (*in vitro*) and LPS-challenged mice (*in vivo*). Furthermore, the crucial role of NFκB and Nrf2/ARE-signaling pathways behind the protective effects of CoQ_0 has been investigated to elucidate the underlying molecular mechanism.

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco's Modified Eagle's medium (DMEM), fetal bovine serum (FBS), glutamine, and penicillin–streptomycin were purchased from the Invitrogen/GIBCO BRL (Grand Island, NY, USA). Coenzyme Q_0 (2,3 dimethoxy-5-methyl-1,4 benzoquinone), LPS (from *Escherichia coli* 055:B5), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2′,7′-dihydrofluorescein-diacetate ($DCFH_2$-DA), cycloheximide, and *N*-acetyl-cysteine (NAC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse monoclonal antibodies against TNF-α, IL-1β and HO-1 were purchased from Abcam (Cambridge, UK). Antibodies against iNOS, COX-2, I-κBα, IKKα, Nrf2, NQO-1, Keap-1 and β-actin were purchased from Santa Cruz (Heidelberg, Germany). Antibodies against p65 NFκB, p-IKKα, p-c-Jun., p-c-Fos, p-ATF2 (p-activating transcription factor 2), p-PI3K, PI3K, p-AKT, AKT, p-JNK, JNK, p-p38 MAPK, p38 MAPK, p-ERK, ERK and histone H3 were obtained from Cell Signaling Technology Inc. (Danvers, MA). 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI), p38 MAPK inhibitor (SB203580), ERK inhibitor (PD98059), JNK inhibitor (SP600125), PI3K/AKT inhibitor (LY294002), and PKC inhibitor (GF109203X) were obtained from Calbiochem (La Jolla, CA, USA). The protease inhibitor MG132 was purchased from Merck KGaA (Darmstadt, Germany). PGE_2, TNF-α and IL-1β ELISA kits, and actinomycin D were purchased from R&D Systems Inc. (Minneapolis, MN, USA). All other chemicals were of the highest commercially available grade and were supplied either by Merck (Darmstadt, Germany) or Sigma (St. Louis, MO, USA).

2.2. Cell culture and sample treatment

The murine macrophage (RAW264.7) cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in DMEM containing 2 mM glutamine, 1% penicillin–streptomycin, and 10% heat-inactivated FBS at 37 °C in a humidified atmosphere containing 5% CO_2. The culture supernatant was removed after 1 h incubation with CoQ_0, the cells were washed with phosphate buffer saline (PBS), and culture medium was replaced with new medium with or without LPS (1 μg/mL) for the indicated time. The LPS was dissolved in PBS (pH 7.2).

2.3. MTT assay

Cell viability was monitored by the MTT colorimetric assay. RAW264.7 cells (4×10^5 cells/well) were grown to confluence on 12-well cell culture plates, and incubated with increasing concentrations of CoQ_0 (2.5–20 μM) for 24 h. After treatment, cells were incubated with 400 μL of 0.5 mg/mL of MTT in PBS for 2 h. The culture supernatant was removed, the remaining MTT formazan crystals were dissolved in 400 μL of isopropanol, and the absorbance was measured at 570 nm using an ELISA micro-plate reader (Bio-Tek Instruments, Winooski, VT, USA). The effect of CoQ_0 on cell viability was assessed as the percentage of viable cells compared with the vehicle-treated control cells, which were arbitrarily designated as 100%.

2.4. Preparation of cell extracts and Western blot analysis

RAW264.7 cells (4×10^6 cells/dish) were seeded into 6 cm dish, pretreated with CoQ_0 (2.5–10 μM) in the presence or absence of LPS (1 μg/mL) stimulation for 0.5–18 h. After treatment, cells were detached, washed once in cold PBS, and the total, cytoplasmic, and nuclear extracts were prepared according to the protocols of extraction reagents (Pierce Biotechnology, Rockfort, IL, USA). The protein content in each sample was determined using Bio-Rad protein assay reagent, with bovine serum albumin as the standard (Bio-Rad, Hercules, CA, USA). Equal amounts (50 μg) of denatured protein samples were electrophoresed by 8–15% SDS-PAGE, followed by transfer onto PVDF membranes for overnight. The membranes were blocked with 5% non-fat dry milk for 30 min at room temperature and then reacted with primary antibodies for 2 h. They were then incubated with a horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibody for 2 h and developed using the enhanced chemiluminescence substrate (Pierce