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In Vitro Effects of the Reduced Form of Coenzyme Q$_{10}$ on Secretion Levels of TNF-α and Chemokines in Response to LPS in the Human Monocytic Cell Line THP-1

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Summary Ubiquinol-10 (QH$_2$), the reduced form of Coenzyme Q$_{10}$ (CoQ$_{10}$) serves as a potent antioxidant of lipid membranes. Because many antioxidants reveal potent anti-inflammatory effects, the influence of QH$_2$ on lipopolysaccharide (LPS)-induced pro-inflammatory cytokines and chemokines were determined in the human monocytic cell line THP-1. Stimulation of cells with LPS resulted in a distinct release of Tumour necrosis factor-alpha (TNF-α), Macrophage inflammatory protein-1 alpha (MIP-1α), Regulated upon activation, normal T cell expressed and secreted (RANTES) and Monocyte chemotattractant protein-1 (MCP-1). The LPS-induced responses were significantly decreased by pre-incubation of cells with QH$_2$ to $60.27 \pm 9.3\%$ ($p = 0.0009$), $48.13 \pm 6.93\%$ ($p = 0.0007$) and $74.36 \pm 7.25\%$ ($p = 0.008$) for TNF-α, MIP-1α and RANTES, respectively. In conclusion, our results indicate anti-inflammatory effects of the reduced form of CoQ$_{10}$ on various proinflammatory cytokines and chemokines *in vitro*.

***Key Words*: coenzyme Q$_{10}$, ubiquinol-10, inflammation, monocytes**

Introduction

Exposure of cells to the pro-inflammatory lipopoly-saccharide (LPS) triggers TLR4-dependent phosphorylation cascades which lead to activation of NFκB. This central transcription factor induces the expression and subsequent secretion of various pro-inflammatory cytokines and chemokines [1–3]. Reactive oxygen species (ROS) are important for the activity of the TLR4-signalling pathway [4]. Accordingly, antioxidants are described as anti-inflammatory agents [5, 6]. Because Coenzyme Q$_{10}$ (CoQ$_{10}$) is a potent antioxidant, we postulated that this molecule possesses anti-inflammatory properties. More recently was shown that CoQ$_{10}$ supplementation minimizes oxidative stress during statin drug therapy [7]. Indeed, we found a reduction of LPS-induced cytokine release by CoQ$_{10}$ in murine and human monocytic cell lines [8]. In the latter study, we treated cells with the oxidized form of CoQ$_{10}$ (ubiquinone-10) which is converted intracellular to ubiquinol-10 (QH$_2$). As only the reduced form of CoQ$_{10}$ can act as an antioxidant, here we studied effects on secretion of the cytokine TNF-α and different chemokines in LPS-stimulated THP-1 cells that were directly incubated with QH$_2$.

Material and Methods

Reagents

Lipopolysaccharide (LPS, *E.coli* O55:B5) was obtained from Sigma-Aldrich (Taufkirchen, Germany). The aqueous solutions of ubiquinol-10 (PEG-60 hydrogenated castor oil, ubiquinol-10, glycerol, water) and the corresponding vehicle (no ubiquinol-10 supplement) were received from KANEKA Corporation (Osaka, Japan).

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Table 1. Effect of ubiquinol-10 (QH₂) and the reference substances PDTC and NAC on viability of THP-1 cells.

	10% DMSO	Vehicle control	100 μM PDTC	100 μM NAC	1 μM QH₂	10 μM QH₂	100 μM QH₂
%	1.71	148.87	109.44	92.51	120.74	109.68	98.43
±SEM	±0.36	±15.09	±22.33	±21.38	±13.20	±15.70	±16.09

THP-1 cells were either treated with 1–100 μM QH₂ or 100 μM PDTC or NAC for 24 h. Medium was used as negative control (data not shown) and 10% dimethyl sulfoxide (DMSO) as positive control (poco, positive control). The applied amount of the vehicle (veco) was in accordance to 100 μM QH₂. The cell viability of the negative control was set to 100% and the other values (means ± SEM) were referenced to it. Three independent experiments were performed in triplicate.

Cell culture

Cultivation of THP-1 cells occurred routinely in RPMI medium 1640 supplemented with 10% FCS and 1% antibiotics (penicillin/streptomycin) in a humidified incubator containing 5% CO_2 at 37°C. For determination of TNF-α and chemokines, cells were plated at a density of 0.5×10^6 cells in a 12-well plate for 24 h before pre-incubation. Subsequently, cells were preincubated with either 10 μM ubiquinol-10 or the reference substances pyrrolidine-dithiocarbamate (PDTC) or N-acetyl-cysteine (NAC), or the respective vehicle control. After 24 h, cell culture medium was removed and fresh LPS-containing medium (1 μg/ml) was added for 4 h. Finally, for cytokine determination via ELISA, supernatants were kept and stored at −80°C. For protein determination via the BRADFORD method, cells were collected into NET-buffer.

Cytotoxicity

For determination of cell viability, the Cell-Titer Glo® Luminescent Assay was used. Thus, total ATP levels were measured as an index of the viable cell number. The luminescence was detected on a GloMax® (Promega, Mannheim, Germany).

Determination of TNF-α and chemokines

Using TNF-α as an internal control, this cytokine was determined by DuoSet ELISA (R&D Systems, Wiesbaden, Germany) as well as multiplex suspension array technology (BioRad, Munich, Germany) according to the manufacturer's instructions. The chemokines MCP-1, MIP-1α and RANTES were determined by the multiplex suspension array system.

Protein concentration

Cells were collected into NET-buffer (50 mM TRIS [pH 7.5], 150 mM NaCl, 1 mM EDTA [pH 8.0], 0.5% NP-40) and the cell suspension was treated with ultrasonics and then centrifugated by 14000 rpm at 4°C for 20 min. Determination of protein concentration occurred in the resulting supernatant by the Bradford method according to the manufacturer's instructions.

Statistics

All data are results of two (PDTC, NAC) or three (QH₂) independent biological experiments performed in duplicate and expressed as means ± standard error of the mean (SEM). Results were analyzed by an unpaired two-sided Student's t-test using SPSS 11.5 for Windows and GraphPad Prism 4.0 software. p-values less than or equal to 0.05 were considered statistically significant.

Results and Discussion

NFκB is a multisubunit transcription factor that is ubiquitously expressed in different cell types and can be activated by several agents such as LPS, TNF-α or the oxidant hydrogen peroxide (H_2O_2) [9]. This activation process includes phosphorylation of the IκB subunit and its dissociation from the inactive cytoplasmic complex. Thus, the active dimer of p50 and p65 translocates into the nucleus where specific target genes of pro-inflammatory mediators and cytokines become immediately up-regulated [9, 10]. However, this NFκB-activating cascade was shown to be inhibited by antioxidants such as PDTC and NAC. This has led to the hypothesis that oxygen radicals are key players in the activation of NFκB through an redox-dependent mechanism [9, 11, 12]. Because many antioxidants reveal potent anti-inflammatory effects, the influence of QH₂ on LPS-induced pro-inflammatory cytokines and chemokines was determined in the human monocytic cell line THP-1. All experiments were performed with the well known radical scavengers PDTC and NAC to validate the putative anti-inflammatory effects of QH₂. To implement culture conditions that do not lead to unspecific side effects, cell vitality was measured at different medium concentrations of QH₂. As shown in Table 1, no cytotoxic effects were found for PDTC (100 μM), NAC (100 μM) and QH₂ (1, 10, 100 μM). For further experiments we used 10 μM QH₂, because this concentration leads to a significantly higher intracellular QH₂ content in THP-1 cells and is also achievable in human serum through QH₂ supplementation (unpublished results). As shown in Figure 1A-D, unstimulated THP-1 monocytes secrete low amounts of the pro-inflammatory cytokine TNF-α and chemokines MIP-1α, RANTES and MCP-1 into the medium. However, stimulation with LPS induces approximately 58-(TNF-α), 7-(MIP-1α), 2-(RANTES) and 4-(MCP-1) fold higher levels of these pro-inflammatory
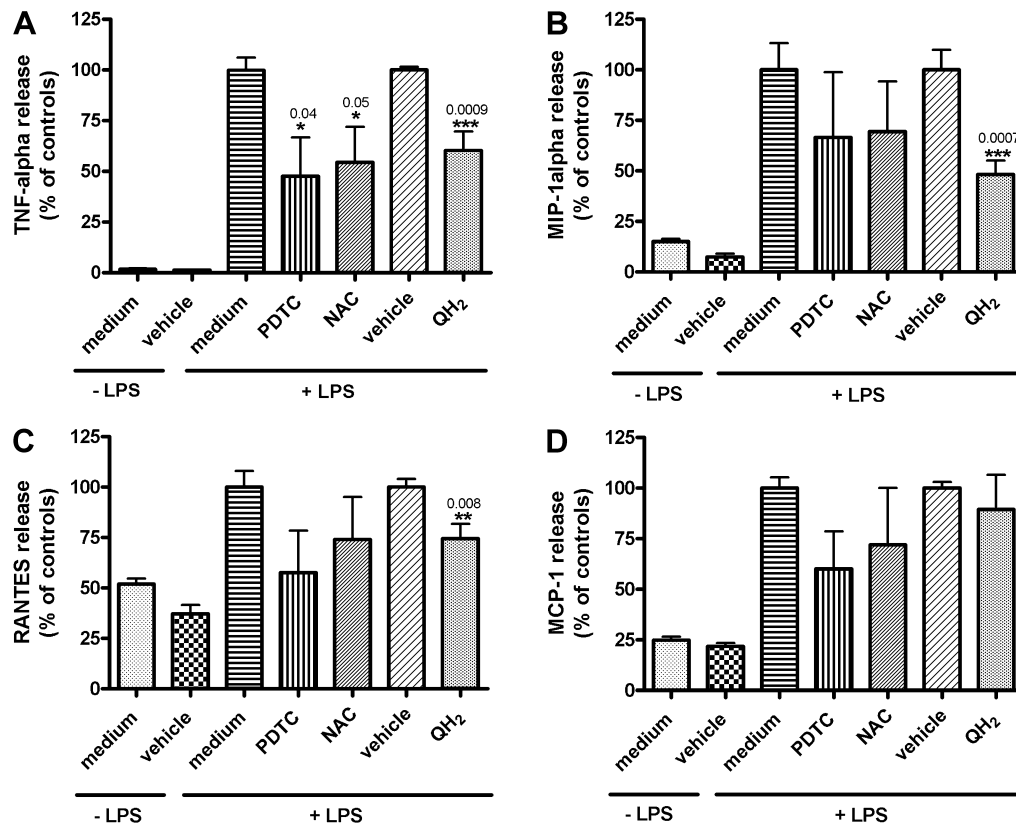
Fig. 1. Effects of pre-treatment of ubiquinol on LPS-induced release of TNF-α (A), MIP-1α (B), RANTES (C) and MCP-1 (D) in THP-1 cells.
Cells were either pre-treated with 10 μM QH₂ or the respective reference substances PDTC or NAC, or medium and vehicle for 24 h. Afterwards, media were removed and cells were treated with LPS (1 μg/ml medium) for 4 h. The resulting concentrations (pg/μg cellular protein) of TNF-α, MIP-1α, RANTES and MCP-1 of the vehicle controls (+LPS) were set to 100% for QH₂-pretreated cells and the other values were referenced to it. Values from PDTC- and NAC-pretreated cells were related to medium controls (+LPS) taken as 100%. Statistically significant data (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$) are means ± SEM of four (ubiquinol, vehicle) or two (PDTC, NAC, medium) independent experiments performed in duplicate.

agents in the cell culture medium within 4 h, respectively. Next, we tested the effect of pre-incubation of cells with 10 μM QH₂ for 24 h. Thus, as shown in Figure 1A–C, the LPS-induced responses were significantly decreased to 60.27 ± 9.3%, 48.13 ± 6.93% and 74.36 ± 7.25% for TNF-α, MIP-1α and RANTES, respectively (Fig. 1A–C). No significant effect was found for MCP-1 (Fig. 1D). Pre-incubation of cells with 10 μM PDTC or 10 μM NAC decreased TNF-α levels significantly to 47.69 ± 19.07% and 54.43 ± 17.64%, respectively (Fig. 1A). No significant effects of PDTC and NAC were found on LPS-induced secretion levels of other pro-inflammatory mediators.

Inflammation has been related to the pathogenesis of various diseases, such as atherosclerosis [13]. Monocytes play an important role in the response to inflammatory agents, particularly to those derived from gut bacteria and are able to enter the circulation, such as bacterial endotoxins. Thus, endotoxins circulate at low concentrations in the blood of all healthy individuals, but are also increased after

a high-fat meal [14]. However, elevated levels are associated with an increased risk of atherosclerosis or sepsis [14–16]. For our experiments we used LPS, a compound of gram-negative bacteria that is also relevant *in vivo* to trigger a serious medical inflammatory process *in vitro*. Finally, stimulation of monocytes with LPS induces production of ROS, which in turn activate the transcription factor NFκB [4, 9] that triggers a large amount of genes encoding for inflammatory mediators and cytokines [17]. Numerous studies in monocytes revealed natural occurring antioxidants as compounds with anti-inflammatory effects [18, 19]. Here we used CoQ₁₀ in order to study its putative anti-inflammatory effect in the human monocytic cell line THP-1. For this purpose, we used the reduced form of CoQ₁₀, QH₂, which functions as an antioxidant. To our knowledge, effects of QH₂ on inflammatory markers have not been investigated so far, because this form of CoQ₁₀ is not commonly available. Thus, only a few studies were published using QH₂ *in vivo*. These studies indicate effects

of QH$_2$ on safety and bioavailability [20], sperm kinetic features [21], oxidative imbalance in children with Trisomy 21 [22] and neuroprotection in an animal Parkinson model [23]. In THP-1 cells, we found that QH$_2$ reduces significantly the secretion of the pro-inflammatory agents TNF-α, MIP-1α, and RANTES in response to LPS. This putative anti-inflammatory effect of QH$_2$ could be due to its antioxidant property in cell membranes, because LPS-induced ROS production occurs very closely to the membrane [4]. One important membrane-associated complex that is relevant for generation of ROS in monocytic cells [24] is the NADPH oxidase. This complex is described to consist of four proteins, whereas Rac is the most critical component for a functional NADPH oxidase. Rac is regulated by small GTP-binding proteins [25]. In this context it was shown that LPS induced Rac activity and moreover, the NADPH oxidase-dependent ROS formation [26]. Thus it seems that LPS directly initiates the NADPH oxidase activity by downstream signalling pathways. Results from a further study indicate an up-regulation of the NADPH oxidase complex through a NFκB-dependent TNF-α activation process which finally leads to enhanced ROS production and further NFκB-activation [24]. This in turn might contribute to sustained releases of pro-inflammatory cytokines and mediators. In this context it was also shown that the well known antioxidant and radical scavenger NAC inhibited NFκB-activation via reduction of H$_2$O$_2$ [9], an important reactive oxygen intermediate (ROI) of the NADPH oxidase pathway. These strong radical scavenging effects are also described for PDTC [27], which we used as an additional internal control to describe the putative anti-inflammatory effects of the reduced form of CoQ$_{10}$ (QH$_2$), a compound with strong antioxidant properties. In general it seems that QH$_2$ mediates stronger anti-inflammatory effects on the tested pro-inflammatory compounds than PDTC and NAC, two well known radical scavengers mediating its anti-inflammatory properties through a diminished NFκB activation. Thus it seems that the reduced form of CoQ$_{10}$ (QH$_2$) mediates its anti-inflammatory effects at least in part through its strong antioxidant properties. However, these effects may be additionally mediated by gene expression. It has been shown in skeletal muscle of humans [28], heart of mice [29], CaCo-2 [30], and HeLa cells [31] that CoQ$_{10}$ influences the expression of different genes. These hypotheses should be tested in future studies. In conclusion, our results indicate anti-inflammatory effects of the reduced form of CoQ$_{10}$ on various proinflammatory cytokines and chemokines *in vitro*.

Acknowledgment

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