Effect of Thymoquinone on Mouse Dendritic Cells

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Key Words

Cytokines • Maturation • Phosphatidylserine • LPS

Abstract

Thymoquinone, a component of *Nigella sativa* is known to confer protection against tumour growth due to stimulation of tumour cell apoptosis. Moreover, thymoquinone has remarkable anti-inflammatory potency. Surprisingly, despite its powerful influence on inflammation and its immunomodulatory effects, little is known about its effect on dendritic cells (DCs), key players in the regulation of innate and adaptive immunity. DC maturation and cytokine release is triggered by bacterial components such as lipopolysaccharides (LPS). The present study explored whether thymoquinone modifies LPS-induced DC maturation, survival and cytokine release. To this end, mouse bone marrow derived DCs were treated with LPS and different concentrations of thymoquinone and the surface expression of CD11c, CD86, MHCII, CD54 and CD40 was determined by FACS analysis, the formation of the interleukins 10 (IL-10) and 12 (IL-12p70) as well as TNF- α by ELISA, caspase activation by FITC-labelled antibodies

(FACS), cell membrane scrambling by annexin V binding (FACS) and Akt and ERK1/2 phosphorylation by Western blotting. LPS increased the percentage of CD11c⁺CD86⁺, CD11c⁺MHCII⁺, CD11c⁺CD40⁺ and CD11c⁺CD54⁺ cells and stimulated the release of IL-10, IL-12p70 and TNF- α . These effects were blunted by thymoquinone in a concentration dependent manner (1-20 μ M). Moreover, LPS decreased and thymoquinone increased caspase 3 and caspase 8 activation and annexin V binding. Moreover, LPS-induced phosphorylation of prosurvival kinases Akt and ERK1/2 was abrogated by thymoquinone. In conclusion, thymoquinone compromises the maturation, cytokine release and survival of DCs.

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Introduction

The potent bioactive ingredient of *Nigella sativa*, thymoquinone [1, 2], exerts remarkable and diversified anticarcinogenic [3-8] and anti-inflammatory [8-15] properties. The anticarcinogenic effect is mediated, at least partially, by the stimulation of tumour cell apoptosis [16-19]. A wide range of signalling mechanisms may contribute to the proapoptotic effects of thymoquinone including

p53- [6, 16, 17] and NFκB- [14] dependent gene expression, suppression of Akt and extracellular signal-regulated kinase [20], decrease of reduced glutathione [21] and caspase activation [21, 22]. Moreover, thymoquinone influences the formation of prostaglandins and leukotrienes [10].

Despite the numerous studies on its potent anti-inflammatory activity, surprisingly little is known about the effect of thymoquinone on the survival and function of dendritic cells (DCs) [8], antigen-presenting cells involved in the initiation of both innate and adaptive immunity and thus critically important for the regulation of the immune response [23-25]. Following stimulation by bacterial components such as lipopolysaccharides (LPS), DCs undergo maturation [26-30] and release several cytokines such as IL-10, IL-12 and TNF-α [31-33]. DCs also prevent potentially harmful immune responses against nonpathogenic antigens, which is particularly important in the intestine [34]. DCs are in contact with the intestinal lumen [35] and are thus exposed to relatively high concentrations of different nutrients. Dysregulation in gut-induced tolerance and commensal bacterial handling affects both local and systemic compartments and can contribute to autoimmune disease [36].

The present study explored, whether thymoquinone influences maturation of, cytokine release from and/or survival of DCs. To this end, bone marrow derived mouse DCs have been treated with LPS in the absence and presence of thymoquinone.

Materials and Methods

Cell Culture

Dendritic cells (DCs) were cultured from bone marrow as previously described [37, 38], with slight modifications. Briefly, bone marrow cells were flushed out of the cavities from the femur and tibia of 7-12 weeks old C57/BL6 mice (Charles River, Sulzfeld, Germany) with PBS. Cells were washed twice with RPMI and seeded out at a density of 2×10^6 cells per 60-mm dish. Cells were cultured for 8 days in RPMI 1640 (GIBCO, Carlsbad) containing: 10% FCS, 1% penicillin/streptomycin, 1% glutamine, 1% non-essential amino acids (NEAA) and 0.05% β-mercaptoethanol. Cultures were supplemented with GM-CSF (35 ng/mL, Preprotech Tebu) and fed with fresh medium containing GM-CSF on days 3 and 6. Nonadherent and loosely adherent cells were harvested after 8 days of culture. At day 8, >80% of the cells expressed CD11c, which is a marker for mouse DCs. Experiments were performed on DCs stimulated with lipopolysaccharide (from *Escherichia coli* Sigma-Aldrich, Germany, LPS, 200 ng/mL, 24 h) in the absence or in the presence of different concentrations of thymoquinone (Sigma-Aldrich, Germany) at day 9.

Immunostaining and flow cytometry

Surface expression of CD11c, CD86, MHCII, CD54 and CD40 was determined by FACS analysis. To this end, cells (4×10^5) were incubated in 100 μl FACS buffer (PBS plus 0.1% FCS) containing fluorochrome-conjugated antibodies at a concentration of 10 μg/mL. A total of 2×10^4 cells were analyzed. The following antibodies (all from BD Pharmingen, Heidelberg, Germany) were used for staining: FITC-conjugated anti-mouse CD11c, clone HL3 (Armenian Hamster IgG₁, λ2), PE-conjugated anti-mouse CD86, clone GL1 (Rat IgG_{2a}, κ), PE-conjugated rat anti-mouse I-A/I-E, clone M5/114.15.2 (IgG2b, κ), PE-conjugated anti-mouse CD40 clone 3E2 (Rat IgG_{2a}, κ and PE-conjugated anti mouse ICAM-1 (CD-54), clone 3E2 (Armenian Hamster IgG1, κ). After incubating with the antibodies for 60 min at 4°C, the cells were washed twice and resuspended in FACS buffer for flow cytometric analysis.

Cytokine Measurement

IL-10, IL-12p70 and TNF-α concentrations in DC culture supernatants were determined by using OptEIA ELISA kit (BD Pharmingen) according to the manufacturer's protocol.

Phosphatidylserine translocation

Apoptotic cell membrane scrambling was evidenced from annexin V binding to phosphatidylserine (PS) at the cell surface [39]. To this end, the percentage of PS-translocating cells was evaluated by staining with fluorescein isothiocyanate (FITC)-conjugated Annexin V. In brief 4×10^5 cells were harvested and washed twice with Annexin washing buffer (AWB, 10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 5 mM CaCl₂). The cell pellet was resuspended in 100 μl of Annexin-V-Fluos labelling solution (Roche) (20 μl Annexin-V-Fluos labelling reagent in 1 ml AWB) and incubated for 15 min at room temperature. After washing with AWB, the cells were analyzed by flow cytometry.

Caspase 8 and Caspase 3 activation assay

Caspase 8 and Caspase 3 activation was determined using kits from Biovision according to manufacturer's instruction. Briefly 1×10^6 cells were washed twice with cold PBS, fixed and permeabilized with 'Cytofix/Cytoperm' solution and then by washing twice with 'Perm/ Wash' buffer. Then cells were stained with FITC conjugated anti-active Caspase 8 or Caspase 3 antibody in 'Perm/ Wash' buffer for 60 minutes. After 2 washing steps, the cells were analyzed by flow cytometry (FACS Calibur, BD Biosciences).

Immunoblotting

DCs (2×10^6 cells) were left unpulsed (control) or pulsed with LPS in the absence or in the presence of thymoquinone (10 μM) for 1 h and then snap-frozen in dry-ice ethanol bath. Cell pellets were thawed on ice and washed twice with PBS, then solubilized in lysis buffer (Pierce) containing protease inhibitor cocktail (Sigma-Aldrich, Taufkirchen, Germany). Samples were stored at -80°C until use for Western blotting. Cell lysates were separated by 10% SDS-PAGE and blotted on nitrocellulose membranes. The blots were blocked with 5% nonfat-milk in triethanolamine-buffered saline (TBS) and 0.1%

Fig. 1. Effect of thymoquinone on LPS-induced CD86, MHCII and CD40 surface expression in mouse bone marrow derived dendritic cells (DCs). A., B., C., D. Original dot plots of CD11c⁺CD86⁺ (A), CD11c⁺MHCII⁺ (B), CD11c⁺CD40⁺ (C) and CD11c⁺CD54⁺ (D) DCs cultured for 24 h without (control, 1st panel) or with LPS (200 ng/ml) in the absence (2^d panel) and presence (3^d - 6th panels) of thymoquinone (1, 5, 10, 20 μ M). Numbers depict the percent of cells in the respective quadrants. Dead cells were gated in the flow cytometry analysis. E., F., G., H. Arithmetic means $\pm$ SEM (n = 4-6 each) of CD86, MHCII, CD40 and CD54 expression in DCs cultured without (control, white bar) and with LPS (200 ng/ml) in the absence (dotted bar) or presence (black bars) of thymoquinone (1 -20 μ M). * (p<0.05), ** (p<0.01) and *** (p<0.001) show statistically significant differences between thymoquinone-untreated and -treated LPS-stimulated DCs, ANOVA.

Tween-20. Then the blots were probed overnight with anti-phospho (p)-ERK, anti-ERK (Cell Signaling), anti p-Akt, anti-Akt (Cell Signaling) antibodies diluted in 5% milk in PBS and 0.1% Tween-20, washed 5 times, probed with secondary antibodies conjugated with horseradish peroxidase for 1 h at room temperature, and washed final 5 times. Antibody binding was detected with the enhanced chemiluminescence (ECL) kit (Amersham, Freiburg, Germany). Densitometer scans of the blots were performed using Quantity One (BioRad, Munich, Germany).

Statistics

Data are provided as means $\pm$ SEM, *n* represents the number of independent experiments. Differences were tested for significance using ANOVA. *P*<0.05 was considered statistically significant.

Results

Thymoquinone inhibits maturation of LPS-stimulated DCs

Mouse bone marrow derived DCs were treated with LPS (200 ng/ml, 24h) in the absence and presence of thymoquinone (1 - 20 μ M) and surface expression of CD11c, MHCII, costimulatory molecules CD86 and CD40 and adhesion molecule CD54, mediating DC-T cell clustering, was determined by FACS analysis. LPS increased the percentage of CD11c⁺CD86⁺, CD11c⁺MHCII⁺, CD11c⁺CD40⁺ and CD11c⁺CD54⁺ cells (Fig. 1), an effect blunted in the presence of thymoquinone. Thus, thymoquinone inhibits

Fig. 2. Effect of thymoquinone on LPS-induced formation of IL-10, IL-12 and TNF- α in mouse DCs. Concentrations of IL-10 (A), IL-12p70 (B) and TNF- α (C) in the supernatant of DCs cultured for 24 h without (control, white bars) and with LPS (200 ng/ml) in the absence (dotted bar) or presence (black bars) of thymoquinone (1–20 μ M). The cytokine concentrations were determined by ELISA. Arithmetic means $\pm$ SEM were calculated from 4–9 independent experiments, each reflecting the mean of a duplicate measurement (n.d. - non-detectable). * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$) show differences between thymoquinone-untreated and -treated LPS-stimulated DCs, ANOVA.

Fig. 3. Effect of thymoquinone and LPS on phosphatidylserine exposure at the surface of mouse DCs. A. Representative FACS histograms depicting the expression of annexin V in DCs cultured for 24 h without (control, 1st panel) or with LPS (200 ng/ml) in the absence (2^d panel) and presence (3^d–6th panels) of thymoquinone (1, 5, 10, 20 μ M). B: Arithmetic means $\pm$ SEM (n = 4–6 each) of the percentage of annexin V-positive DCs following a 24 h culture without (control, white bar) and with LPS (200 ng/ml) in the absence (dotted bar) or presence (black bars) of thymoquinone (1–20 μ M). ** ($p < 0.01$) and *** ($p < 0.001$) show differences between thymoquinone-untreated and -treated LPS-stimulated DCs, ANOVA. C. Arithmetic means $\pm$ SEM (n = 3–8 each) of the percentage of annexin V-positive DCs following a 24 h culture in the absence (open bar) or presence (grey bars) of thymoquinone (1–20 μ M). *** ($p < 0.001$) shows differences between thymoquinone-untreated and -treated DCs, ANOVA.

LPS-induced differentiation and maturation of DCs.

Thymoquinone impairs cytokine secretion by LPS-stimulated DCs

We next examined the formation of IL-10, IL-12p70 and TNF- α utilizing ELISA. DCs were stimulated with LPS (200 ng/ml, 24 h) in the absence and presence of thymoquinone (1–20 μ M). As illustrated in Fig. 2, LPS further stimulated the release of IL-10, IL-12p70 and TNF- α . The effect of LPS on cytokine release was again significantly blunted in the presence of thymoquinone. The effect reached statistical significance at ≥ 1 μ M thymoquinone.

Thymoquinone enhances the percentage of annexin V-binding DCs

DC apoptosis is characterized by phosphatidylserine exposure at the cell surface, which is identified by annexin V-binding in FACS analysis. To explore whether thymoquinone stimulates DC apoptosis, annexin V-binding was determined in DCs stimulated with LPS (200 ng/ml, 24 h) in the absence and presence of thymoquinone (1–20 μ M). As illustrated in Fig. 3A,B, LPS decreased the percentage of phosphatidylserine exposing cells, an effect reversed by thymoquinone. The effect reached statistical significance at ≥ 5 μ M thymoquinone. Phosphatidylserine exposure was also

Fig. 4. Effect of thymoquinone on caspase 3 and caspase 8 activity in DCs. Arithmetic means ($n = 3-8$) of the percentage of DCs with activated caspase 3 (A) or caspase 8 (B) following a 24 h culture without (white and grey bars) and with LPS (200 ng/ml, dotted and black bars) in the absence (white and dotted bars) or presence (grey and black bars) of thymoquinone (1-20 μ M). *** ($p < 0.001$) represents statistically significant difference between thymoquinone-untreated and -treated LPS-stimulated DCs; # ($p < 0.05$), ## ($p < 0.01$) and ### ($p < 0.001$) show differences between thymoquinone-untreated and -treated DCs in the absence of LPS, ANOVA.

enhanced when thymoquinone was applied to the cells in the absence of LPS (Fig. 3C).

Thymoquinone leads to caspase activation in DCs

Another marker of apoptosis is activation of caspases. The administration of thymoquinone was followed by activation of caspase 3 and caspase 8 in DCs, an effect reaching statistical significance at the concentration of 10 μ M (Fig. 4). LPS decreased the percentage of cells with activated caspases and again this effect was reversed by thymoquinone.

Thymoquinone impairs the LPS-induced phosphorylation of Akt and extracellular signal-regulated kinases ERK1/2 in DCs

LPS-induced maturation of DCs is known to include the activation of mitogen-induced protein kinases (MAPKs) and phosphoinositide 3-OH kinase (PI3 kinase)/Akt in DCs [40]. To gain insight into thymoquinone-induced apoptosis, we examined phosphorylation (and thus activation) of prosurvival kinases Akt and ERK1/2 following stimulation with LPS in the absence or in the presence of thymoquinone (Fig. 5). As a result, LPS stimulation fostered Akt and ERK1/2 activation, an effect abrogated by thymoquinone.

Discussion

The present study reveals a completely novel effect of thymoquinone, i.e. its ability to modify the maturation of dendritic cells (DCs). LPS leads to the upregulation of several maturation markers, such as CD86 [30], CD54 [28] and CD40 [26, 29]. The LPS induced stimulation of CD86, CD54 and CD40 expression is significantly blunted

Fig. 5. Effect of thymoquinone on LPS-induced phosphorylation of Akt and ERK1/2 in mouse DCs. Original Western blots (representative for 3 similar experiments) of DCs stimulated with LPS (200 ng/ml) in the absence or in the presence of thymoquinone (10 μ M) for 1 h or left untreated (control). Protein extracts were analyzed using antibodies directed to phosphorylated (p) Akt and p-ERK1/2. Equal protein loading was controlled by anti-Akt and anti-ERK1/2 antibodies. B., C. Arithmetic means $\pm$ SEM ($n = 3$) of the abundance of phosphorylated Akt as the ratio to total Akt (B) and of phosphorylated ERK1/2 as the ratio to total ERK1/2 (C) * ($p < 0.05$) indicates significant difference from LPS-stimulated cells; ANOVA.

at higher concentrations of thymoquinone. A decrease of CD86 and CD40 is typical for a tolerogenic DC phenotype. LPS further leads to upregulation of MHCII, which is critically important for antigen presentation [27]. Again, thymoquinone inhibits the effect on MHCII expression. Accordingly, thymoquinone is expected to blunt the stimulation of the immune response by LPS. To our knowledge, this is the first study demonstrating an effect of thymoquinone on maturation of the immune cells.

LPS exposure further stimulates the formation of IL-12, TNF- α and IL-10 [31-33, 41-43]. Similar to LPS-induced maturation, the LPS-induced release of IL-12, TNF- α and IL-10 was blunted in the presence of thymoquinone. For a statistically significant blunting of cytokine release, thymoquinone concentrations of as little as 1 μ M were required. Thus, it appears that the cytokine release is particularly sensitive to thymoquinone. IL-12, which is produced mainly by activated myeloid DCs, plays a pivotal role in the differentiation of Th1 cells [31]. In animal models, predisposition to autoimmunity can be explained by abnormal levels of IL-12 secreted by antigen-presenting cells [31]. Similarly, TNF- α dysregulation is well known to contribute to the pathogenesis of various autoimmune disorders [31]. Interestingly, thymoquinone suppressed also the production of anti-inflammatory cytokine IL-10 which operates primarily as a feedback inhibitor of excessive T cell responses to microbial antigens [33]. IL-10 is released from Th1, Th2, Th17 and CD8⁺ T cells, as well as from CD4⁺Foxp3⁺Treg cells and CD4⁺ Tr1 cells. In addition, antigen-presenting cells are known to produce IL-10 in response to pathogen infection [33].

Inhibition of LPS-induced cytokine production by thymoquinone was also demonstrated for the mast cell line RBL-2H3 [11]. In LPS-activated RBL-2H3 cells, thymoquinone at concentration of 10 μ M suppressed the production of Th2-associated cytokines IL-5 and IL-13 (but not IL-10), which play a role in the pathogenesis of allergic inflammation [11] and the production of TNF- α [12]. Thymoquinone inhibited IL-5 and IL-13 cytokine production by blocking GATA transcription factor expression and binding to the cytokine gene promoter [11] and TNF- α production by modulating nuclear transactivation of NF- κ B [12]. Thymoquinone suppressed NF- κ B activation through modulation of the p65 subunit of NF- κ B and inhibition of IKK also in human chronic myeloid leukaemia cells KBM-5 [14]. In these cancer cells suppres-

sion of NF- κ B and downstream gene products impaired cell survival, proliferation as well as invasion and potentiated the apoptosis following treatment with TNF and chemotherapeutic agents [14].

Other potent anti-inflammatory effects of thymoquinone include inhibition of cyclooxygenase 2 (COX-2) expression and prostaglandin production in a mouse model of allergic airway inflammation [13]. Suppression of the inflammatory mediators prostaglandins and leukotriens by thymoquinone have been also shown in other inflammation-based models including experimental encephalomyelitis, colitis, peritonitis, edema, and arthritis [8, 15].

Thymoquinone stimulated DC apoptosis. Proapoptotic potency of thymoquinone has previously been shown in tumour cells [17, 19, 21, 44] and is assumed to account for the anticarcinogenic potency of the food component [3-8]. Thymoquinone has previously been shown to induce apoptosis and to potentiate the apoptotic effects of cytokines and chemotherapeutic agents at least in part by down-regulating the expression of several antiapoptotic genes, including cIAP1/2, XIAP, Bcl-2, Bcl-xL, and survivin [8, 14]. In the present study we demonstrate that thymoquinone led to caspase activation and reduced the activation of prosurvival kinases Akt and ERK1/2. The present data suggest that the thymoquinone-induced apoptosis may similarly contribute to its anti-inflammatory potency.

In conclusion, the present study reveals that thymoquinone inhibits LPS-induced DC maturation, cytokine formation and survival. The observations disclose mechanisms most likely contributing to the known anti-inflammatory effect of this valuable nutrient.

Abbreviations

DC (Dendritic cells); LPS (Lipopolysaccharides); IL (interleukin); PS (phosphatidylserine); FITC (fluorescein isothiocyanate); FACS (Fluorescence activated cell sorting).

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