Effect of Thymoquinone on Cytosolic pH and Na⁺/H⁺ Exchanger Activity in Mouse Dendritic Cells

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Key Words
Nigella sativa • Cell volume • Reactive oxygen species • TNF-α • LPS

Abstract
The anti-inflammatory Nigella sativa component thymoquinone compromises the function of dendritic cells (DCs), key players in the regulation of innate and adaptive immunity. DC function is regulated by the Na⁺/H⁺ exchanger (NHE), which is stimulated by lipopolysaccharides (LPS) and required for LPS-induced cell swelling, reactive oxygen species (ROS) production, TNF-α release and migration. Here we explored, whether thymoquinone influences NHE activity in DCs. To this end, bone marrow derived mouse DCs were treated with LPS in the absence and presence of thymoquinone (10 µM). Cytosolic pH (pHi) was determined from 2'7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) fluorescence, NHE activity from the Na⁺-dependent realalkalinization following an ammonium pulse, cell volume from forward scatter in FACS analysis, ROS production from 2'7'-dichlorodihydrofluorescein (DCFDA) fluorescence, TNF-α production utilizing ELISA and DC migration with transwell migration assays. As a result, exposure of DCs to LPS (1 µg/ml) led within 4 hours to transient increase of NHE activity. Thymoquinone did not significantly modify cytosolic pH or cellular NHE activity in the absence of LPS, but abrogated the effect of LPS on NHE activity. Accordingly, in the presence of thymoquinone LPS-treatment resulted in cytosolic acidification. LPS further increased forward scatter and ROS formation, effects similarly abrogated by thymoquinone. Again, in the absence of LPS, thymoquinone did not significantly modify ROS formation and cell volume. LPS further triggered TNF-α release and migration, effects again blunted in the presence of thymoquinone. NHE1 inhibitor cariporide (10 µM) blunted LPS induced TNF-α release and migration. The effects of thymoquinone on NHE activity and migration were reversed upon treatment of the cells with t-butyl hydroperoxide (TBOOH, 5 µM). In conclusion, thymoquinone blunts LPS induced NHE activity, cell swelling, oxidative burst, cytokine release and migration of bone marrow derived murine dendritic cells. NHE inhibition may thus contribute to the antiinflammatory action of thymoquinone.
Introduction

Thymoquinone is a powerful bioactive component of *Nigella sativa* [1, 2] with anticarcinogenic [3-8] and anti-inflammatory [8-15] properties. The anticarcinogenic effect results, at least in part, from stimulation of tumor cell apoptosis [16-19]. Thymoquinone sensitive signaling includes p53- [6, 16, 17] and NFκB- [14] dependent gene expression, inhibition of protein kinase B (PKB/Akt) and extracellular signal-regulated kinase ERK [20], decrease of reduced glutathione [21], caspase activation [21, 22] and inhibition of eicosanoid formation [9].

The antiinflammatory effect of thymoquinone is at least partially due its influence on maturation, cytokine formation, and survival of dendritic cells (DCs) [23], antigen-presenting cells involved in the initiation of both innate and adaptive immunity and thus critically important for the regulation of the immune response [24-26]. Stimulation of DCs by bacterial components such as lipopolysaccharides (LPS) triggers DCs maturation [27-31] and the release of several cytokines such as IL-10, IL-12, and TNF-α [32-34]. DCs may prevent potentially harmful immune responses against nonpathogenic antigens, an effect particularly important in intestine [35]. DCs protrude into the intestinal lumen [36] and are thus exposed to relatively high concentrations of nutrients. Deranged tolerance and response to commensal bacteria may contribute to autoimmune disease [37].

The present study explored, whether the effect of thymoquinone on dendritic cells involved altered regulation of the Na⁺/H⁺ exchanger, which is expressed in DCs and is in DCs required for LPS induced cell swelling and oxidative burst [38, 39]. Bone marrow derived mouse DCs have thus been treated with LPS in the absence and presence of thymoquinone and cytosolic pH, Na⁺/H⁺ exchanger activity, cell volume, reactive oxygen species formation, TNF-α release and migration were determined.

Materials and Methods

Animals

All animal experiments were conducted according to German law for the welfare of animals and were approved by local authorities. Dendritic cells (DCs) were cultured from bone marrow of 7-11 weeks old female C57/Bl-6 mice (Charles River, Sulzfeld, Germany) [40, 41]. Mice had free access to control diet (1314, Altromin Heidenau, Germany) and tap drinking water.

Cell Culture

Bone marrow-derived cells were flushed out of the cavities from the femur and tibia with PBS [42]. Cells were then washed twice with RPMI and seeded out at a density of 2 x 10⁶ cells per 60-mm dish. Cells were cultured for 6 days in RPMI 1640 (GIBCO, Carlsbad) containing: 10 % fetal calf serum (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. At day 7, ~80% of the cells expressed CD11c, which is a marker for mouse DCs. Experiments were performed at days 7-9 of DCs culture.

Immunostaining and flow cytometry

Cells (4 x 10⁶) were incubated in 100 μl FACS buffer (phosphate buffered saline (PBS) plus 0.1% FCS) containing fluoro-chrome-conjugated antibodies at a concentration of 10 μg/ml. A total of 2 x 10⁶ cells were analyzed. Staining with FITC-conjugated anti-mouse CD11c (BD Pharmingen, Heidelberg, Germany) was used as a positive marker for dendritic cells [43]. After incubating with the antibody for 60 minutes at 4°C, the cells were washed twice and resuspended in FACS buffer for flow cytometric analysis.

Treatments

Stock solutions of LPS were prepared in culture medium whereas thymoquinone was dissolved in DMSO and cariporide in sterile distilled water. The cells were treated by adding the substances to the cell suspension at the indicated final concentrations and incubating accordingly at 37°C in a humidified 5 % CO₂ atmosphere. Sham experiments were conducted by adding plain DMSO to the respective solutions at a final concentration of 0.1 %. Thymoquinone was used at a final concentration of 10 μM, which has previously been shown to be an effective concentration [23].

Determination of cell volume

Cell volume was determined by the forward scatter in flow cytometric analysis. Briefly, 2 x 10⁶ cells were taken in a culture dish and treated with LPS (with or without thymoquinone). After the treatment, cells were collected, centrifuged, the pellet was resuspended in FACS buffer and analysed with flow cytometry (FACS-calibur from Becton Dickinson; Heidelberg, Germany).

Determination of ROS production

ROS production in DCs was determined utilizing 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) [44]. Briefly, 2 x 10⁶ cells were taken in a culture dish and treated with LPS (with or without thymoquinone). After the treatment, cells were collected, centrifuged and the pellet was washed twice with ice-cold PBS. The pellet was then resuspended in FACS buffer and the fluorescence was analysed with flow cytometry (FACS-calibur from Becton Dickinson; Heidelberg, Germany). DCFDA fluorescence...
intensity was measured in FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Measurement of intracellular pH

For digital imaging of cytosolic pH (pHᵢ), the cells were incubated in a Hepes-buffered Ringer solution containing 10 mM 2',7'-Bis-(carboxyethyl)-5(6)-carboxyfluorescein-acetoxyethyl ester (BCECF-AM Molecular Probes, Leiden, The Netherlands) for 15 min at 37°C. After loading, the chamber was flushed for 5 min with Ringer solution to remove any deesterified dye. The perfusion chamber was mounted on the stage of an inverted microscope (Zeiss Axiovert 135), which was used in the epifluorescence mode with a 40 x oil immersion objective (Zeiss Neoplan, Germany). BCECF was successively excited at 490/10 and 440/10 nm, and the resultant fluorescent signal was monitored at 535/10 nm using an intensified charge-coupled device camera (Proxitronic, Germany) and specialized computer software (Metafluor, USA). Between 10-20 cells were outlined and monitored during the course of the measurements. The results from each cell were averaged and taken for final analysis. Intensity ratio (490/440) data were converted into pH values using the high-K⁺/nigericin calibration technique [45]. To this end, the cells were perfused at the end of each experiment for 5 minutes with standard high-K⁺/nigericin (10 µg/ml) solution (pH 7.0). The intensity ratio data thus obtained were converted into pH values using the rₘᵢₙ, rₘᵦ, pKᵥ values previously generated from calibration experiments to generate a standard nonlinear curve (pH range 5 to 8.5).

For acid loading, cells were transiently exposed to a solution containing 20 mM NH₄Cl leading to initial alkalinalization of cytosolic pH (pHᵢ) due to entry of NH₄⁺ and binding of H⁺ to form NH₄⁺ [46]. The acidification of cytosolic pH upon removal of ammonia allowed calculating the mean intrinsic buffering power (β) of the cells [46]. Assuming that NH₄⁺ and NH₃ are in equilibrium between cytosolic and extracellular fluid and that ammonia leaves the cells as NH₃:

\[ \beta = \Delta [\text{NH}_4^+] / \Delta \text{pH} \]

where ΔpH is the decrease of cytosolic pH (pHᵢ) following ammonia removal and Δ[\text{NH}_4^+] is the decrease of cytosolic NH₄⁺ concentration ([\text{NH}_4^+]), which is identical to [\text{NH}_3]ᵢ immediately before the entry of ammonia. The pK for NH₄⁺/NH₃ is 8.9 [47] and at an extracellular pH (pHᵢ) of 7.4 the NH₄⁺ concentration in extracellular fluid ([\text{NH}_4^+]ᵢ) is 19.37 mM [29/1 + 10^{pKᵢ-pHᵢ}]. [\text{NH}_3]ᵢ was calculated from:

\[ [\text{NH}_3]ᵢ = 19.37 \cdot 10^{pKᵢ-pHᵢ} \text{mM} \]

To calculate the ΔpH/min during re-alkalinization, a manual linear fit was placed over a narrow pH range (pH 6.7 to 6.9) which could be applied to all measured cells.

The solutions were composed of (in mM): standard Hepes: 115 NaCl, 5 KCl, 1 CaCl₂, 1.2 MgSO₄, 2 NaH₂PO₄, 10 glucose, 32.2 HEPES; sodium free Hepes: 132.8 NMDG, 5 KCl, 1 CaCl₂, 1.2 MgSO₄, 2 KH₂PO₄, 32.2 HEPES, 10 mannitol, 10 glucose (for sodium free ammonium chloride 10 mM NMDG and mannitol were replaced with 20 mM NH₄Cl); high K⁺ for calibration 105 KCl, 1 CaCl₂, 1.2 MgSO₄, 32.2 HEPES, 10 mannitol, 5 µM nigericin. The pH of the solutions was titrated to 7.4 or 7.0 with HCl/NaOH, HCl/NMDG and HCl/KOH, respectively, at 37°C.

Migration

For migration assays transwells inserts (BD Falcon 353097) and BD BioCoatTM MatrigelTM Invasion Chambers (BD Biosciences 354480) were used with a pore diameter size of 8 µm. The transwells were placed in a 24-well cell culture plate containing cell culture medium (750 µl) with or without CCL21 (250 ng/ml, Peprotech) in the lower chamber. The upper chambers were filled with 500 µl cell culture medium containing DCs in a concentration of 50000 cells/ml. The chamber was placed in a 5% CO₂, 37°C incubator for 4 hours. In the following, the non-migrated cells were removed by scrubbing with a cotton tipped swab for two times and washing with PBS. The membrane was removed with a scalpel and fixed in 4 % PFA for 15 mins. The migrated cells were then analyzed by staining with DAPI.

TNF-α Measurement

TNF-α concentration in DC culture supernatants was determined by using OptEIA ELISA kit (BD Pharmingen) according to the manufacturer’s protocol.

Statistics

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

Results

BCECF fluorescence was utilized to determine cytosolic pH (pHᵢ) in bone marrow derived mouse dendritic cells (DCs). The Na⁺/H⁺ exchanger (NHE) activity was determined with the ammonium pulse technique. In a first step NH₄Cl was added to the perfusate leading to NH₄⁺ entry into the cells and subsequent binding of cytosolic H⁺ to form NH₄⁺ and thus to a transient cytosolic alkalinalization (Fig. 1). The subsequent removal of NH₄Cl led to exit of NH₄⁺ leaving H⁺ behind and thus resulting in cytosolic acidification (Fig. 1). In the absence of Na⁺, the pH did not recover from this acidification after an ammonium pulse (Table 1). Accordingly, the cells did not express a Na⁺ independent H⁺ extrusion mechanism. The addition of Na⁺ was, however, followed by a rapid pH recovery pointing to the operation of a Na⁺/H⁺ exchanger. Exposure to thymoquinone (10 µM) did not significantly modify the Na⁺/H⁺ exchanger activity or the intracellular pH of DCs prior to stimulation with LPS (Fig. 1).

NHE activity has previously been shown to be stimulated by treatment of the DCs with 1 µg/ml lipopolysaccharides (LPS) [38]. Thus, the influence of thymoquinone on the effect of LPS on pHᵢ and NHE activity was tested. Following treatment with LPS
In contrast, treatment of DCs with LPS in the presence of thymoquinone (10 µM) was followed by a significant decrease of the cytosolic pH (Table 1). Treatment of DCs with LPS (1 µg/ml) was followed by a significant increase (4 hours) of Na+/H+ exchanger activity (Fig 1). The administration of thymoquinone along with LPS virtually abrogated the stimulation of NHE activity by LPS (Fig. 1).

As shown earlier [38], the stimulation of NHE ac-
tivity in DCs by LPS was paralleled by and actually dependent on the generation of reactive oxygen species (ROS). To explore whether thymoquinone influenced the ROS formation following LPS treatment, an additional series of experiments was performed elucidating the effect of LPS on ROS production in the absence and presence of thymoquinone. As shown in Fig. 2, treatment of DCs with LPS was within four hours followed by a significant increase in the percentage of ROS positive cells, an effect significantly blunted in the presence of thymoquinone.

To further test, whether the inhibitory effect of thymoquinone on ROS formation contributed to the inhibitory effect of thymoquinone on NHE, the NHE activity was measured in DCs treated additionally with t-butyl hydroperoxide (TBOOH, 5 µM, 4 h). In the presence of LPS and thymoquinone, TBOOH treatment was followed by a significant increase of NHE activity (Fig. 3). Thus, the decrease of ROS formation could well contribute to the inhibitory effect of thymoquinone on NHE activity following stimulation with LPS.

A further series of experiments aimed to define the effect of thymoquinone on LPS induced cell swelling. To this end cell volume was studied utilizing forward scatter in FACS analysis. The exposure to LPS was within 4 hours followed by significant cell swelling, an effect abrogated in the presence of thymoquinone (Fig. 4). Administration of thymoquinone alone did not significantly modify cell volume (Fig. 4).

FITC-annexin V staining of the cells was employed to clarify, whether a 4-hour treatment with thymoquinone and LPS resulted in apoptosis. A 4-hour treatment of the DCs with thymoquinone (10 µM) did not significantly modify annexin V fluorescence intensity which amounted to 35.2 ± 5.8 % (n = 4) prior to and 34.8 ± 5.2 % (n = 4) 4 hours following thymoquinone treatment.

### Table 1. Cytosolic pH (pH$_i$), buffer capacity (BC, mM/ΔpH$_i$) Na$^+$-independent (0 Na$^+$) and Na$^+$-dependent (+Na$^+$) pH recovery (ΔpH units/min) following an ammonium pulse in bone marrow derived dendritic cells (DCs) prior to (control) and following treatment with lipopolysaccharide LPS (1 µg/ml) or thymoquinone TQ (10 µM) in isolation (LPS, TQ) or combination (LPS+TQ). *indicates significant difference to respective control value, †indicates significant difference to respective value in the presence of LPS alone.

| Treatment | pH$_i$ ± SE | BC ± SE | 0 Na$^+$ ΔpH units/min | + Na$^+$ ΔpH units/min | Number of cells |
|-----------|-------------|---------|------------------------|------------------------|-----------------|
| control   | 7.32 ± 0.01 | 14.5 ± 1.3 | -0.10 ± 0.03          | 0.148 ± 0.010         | 299             |
| TQ (4 hr) | 7.34 ± 0.02 | 14.5 ± 1.1 | -0.07 ± 0.01          | 0.166 ± 0.020         | 135             |
| LPS (4 hrs)| 7.36 ± 0.01 | 14.8 ± 1.8 | -0.06 ± 0.01          | 0.275 ± 0.020         | 177             |
| LPS + TQ (4 hrs) | 7.31 ± 0.01$^b$ | 17.1 ± 3.2 | -0.08 ± 0.01          | 0.155 ± 0.011$^b$     | 201             |

**Fig. 2.** Effect of LPS and thymoquinone on ROS formation in mouse bone marrow derived dendritic cells (DCs). A. Representative FACS histograms depicting ROS-dependent DCFDA fluorescence in DCs without treatment (control, black line) as well as after a 4 hour treatment LPS (1 µg/ml) in the absence (green line) and presence (blue line) of thymoquinone (10 µM) or in the presence of thymoquinone alone (pink line) . B. Arithmetic means ± SEM (n = 5 independent experiments) of the percentage of ROS positive DCs incubated for 4 hours without (white bar) or with LPS (1 µg/ml) in the absence (black bar) or presence (dark grey bar) of thymoquinone (10 µM) or in the presence of thymoquinone (10 µM) alone (light grey bar). *(p<0.05) indicates significant difference from absence of LPS; #(p<0.05) indicates significant difference from absence of thymoquinone.
Formation of TNF-α was then examined utilizing ELISA. DCs were stimulated with LPS (1 µg/ml, 4 h) in the absence and presence of thymoquinone (10 µM). As illustrated in Fig. 4, LPS stimulated the release of TNF-α, an effect significantly blunted in the presence of thymoquinone. Inhibition of NHE activity by cariporide was previously shown to abrogate the production of ROS [38]. To explore the impact of NHE activity on TNF-α release, LPS induced TNF-α release was studied in the absence and presence of cariporide. As illustrated in Fig. 5, cariporide (10 µM, 4h) treatment significantly blunted TNF-α release. To test, whether the effect of thymoquinone on TNF-α release was due to its inhibitory effect on ROS production, experiments were performed in the presence of TBOOH (5 µM, 4 h). TBOOH exposure tended to increase TNF-α release from LPS and thymoquinone treated DCs, an effect, however, not reaching statistical significance (Fig. 5).

A transwell migration assay was performed to study the effect of thymoquinone on LPS-induced DC migration. As shown in Fig. 6 the presence of thymoquinone led to significant reduction in the number of migrated cells disclosing an inhibitory effect of thymoquinone on DC migration. In order to test, whether DC migration was dependent on the NHE activity and whether the effect of thymoquinone on DC migration was ROS dependent, experiments were performed in the presence of cariporide.
Fig. 5. Effect of thymoquinone on LPS-induced TNF-α release in mouse DCs. A. Arithmetic means ± SEM (n = 5-6 independent experiments) of concentration of TNF-α in the supernatant of DCs cultured for 4 h with LPS (1 µg/ml) in the absence (black bar) or presence of 10 µM thymoquinone (grey bar) or cariporide (striped bar) or in presence of thymoquinone and TBOOH (5 µM, crossed bar). #(p<0.05) indicate significant difference to respective value in absence of LPS.

Fig. 6. Effect of thymoquinone on LPS-induced migration in mouse DCs. B. Arithmetic means ± SEM (n = 5 regions out of 5-6 independent experiments each) of the normalized migration of DCs following a 4 hours treatment with LPS in the absence (black bar) or the presence of 10 µM thymoquinone (grey bar) or cariporide (striped bar) or in presence of thymoquinone and TBOOH (5 µM, crossed bar). #(p<0.05) indicate significant difference to respective value in absence of LPS; §(p<0.05) indicate significant difference to respective value in absence of TBOOH.

Discussion

The present study reveals that exposure of murine bone marrow derived dendritic cells (DCs) to thymoquinone virtually abrogates the stimulation of the Na\(^+/\)H\(^+\) exchanger by lipopolysacharides, an effect paralleled by an inhibitory effect of thymoquinone on LPS-induced cell swelling, generation of reactive oxygen species (ROS), TNF-α release and migration. Within the experimental period of 4 hours, we did not see appreciable cell membrane scrambling. A 24 hours incubation with thymoquinone does, however, trigger apoptosis [23].

LPS-induced activation of the Na\(^+/\)H\(^+\) exchanger leads to cell swelling. In parallel to the Cl\(^-/\)HCO\(_3^-\) exchanger the Na\(^+/\)H\(^+\) exchanger accomplishes regulatory cell volume increase [48, 49]. The tandem leads to entry of NaCl together with osmotically obliged water. The H\(^+\) and HCO\(_3^-\) exiting in exchange for NaCl do not lead to a loss of cytosolic osmolarity as they are replenished in the cell by cytosolic formation from CO\(_2\), which can easily pass the cell membrane. As LPS-induced cell swelling is abolished in the presence of cariporide, activation of the Na\(^+/\)H\(^+\) exchanger apparently accounts for the cell swelling following LPS exposure.

Stimulation of the Na\(^+/\)H\(^+\) exchanger is a prerequisite for LPS-induced ROS formation [38]. ROS production is sensitive to cytosolic pH [50, 51] and thus requires parallel extrusion of H\(^+\). Accordingly, activation of the Na\(^+/\)H\(^+\) exchanger supports ROS production. Conversely, activation of Na\(^+/\)H\(^+\) exchanger by LPS is dependent on ROS formation [38]. The Na\(^+/\)H\(^+\) exchanger activity is regulated by cytosolic pH and is stimulated by cytosolic acidification [52]. Possibly, Na\(^+/\)H\(^+\) exchanger activity is activated by local acidification generated during ROS formation. Thymoquinone has antioxidant properties [53], which could at least in theory contribute to its effect on ROS formation and Na\(^+/\)H\(^+\) exchanger activity. On the other
had, oxidative stress has been shown to inhibit Na⁺/H⁺ exchanger activity in other cell types [54, 55].

ROS production is critically important for the killing of pathogens and is thus an essential element in the innate immune response [56]. As ROS production depends on Na⁺/H⁺ exchanger activity, the carrier may be similarly required for the killing of pathogens.

The Na⁺/H⁺ exchanger is further well known to participate in the regulation of migration [39, 57-59]. Thus, inhibition of the Na⁺/H⁺ exchanger may contribute to the inhibitory effect of thymoquinone on DC migration following stimulation with LPS.

The present observations further reveal that thymoquinone blunts the effect of LPS on TNF-α release. An effect of thymoquinone on TNF-α release has been observed previously [23]. The present observations demonstrate that the effect is paralleled and mimicked by Na⁺/H⁺ exchanger inhibition.

A previous study [23] revealed that thymoquinone counteracted DC maturation. LPS exposure is followed by the upregulation of several maturation markers, such as CD86 [31], CD54 [29], CD40 [27, 30], and MHCII [28], an effect blunted in the presence of thymoquinone [23]. Thymoquinone further blunts the LPS-induced formation of interleukin 10 and interleukin 12 [23], potent regulators of the immune response [32-34].

Similar to what has been shown in other cells [17, 19, 21, 60], thymoquinone stimulates DC apoptosis [23]. Notably, apoptosis may be paralleled and fostered by inhibition of the Na⁺/H⁺ exchanger and subsequent cytosolic acidification [49].

In conclusion, thymoquinone inhibits LPS-induced stimulation of Na⁺/H⁺ activity, an effect resulting in blunted LPS induced cell swelling, ROS formation, TNF-α release and migration. The effect of thymoquinone on Na⁺/H⁺ activity may contribute to the known anti-inflammatory effect of this nutrient.

**Abbreviations**

Na⁺/H⁺ exchanger (NHE); Dendritic cells (DC); Lipopolysaccharides (LPS); Tumor necrosis factor alpha (TNF-α); Fluorescence activated cell sorting (FACS).

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