Inhibition of neuroinflammation by thymoquinone requires activation of Nrf2/ARE signalling

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Abstract

Thymoquinone is an antioxidant phytochemical that has been shown to inhibit neuroinflammation. However, little is known about the potential roles of intracellular antioxidant signalling pathways in its anti-inflammatory activity. The objective of this study was to elucidate the roles played by activation of the Nrf2/ARE antioxidant mechanisms in the anti-inflammatory activity of this compound. Thymoquinone inhibited lipopolysaccharide (LPS)-induced neuroinflammation through interference with NF-κB signalling in BV2 microglia. Thymoquinone also activated Nrf2/ARE signalling by increasing nuclear localisation, DNA binding and transcriptional activity of Nrf2, as well as increasing protein levels of HO-1 and NQO1. Suppression of Nrf2 activity through siRNA or with the use of trigonelline resulted in the loss of anti-inflammatory activity by thymoquinone. Taken together, our studies show that thymoquinone inhibits NF-κB-dependent neuroinflammation in BV2 microglia, by targeting antioxidant pathway involving activation of both Nrf2/ARE. We propose that activation of Nrf2/ARE signalling pathway by thymoquinone probably results in inhibition of NF-κB-mediated neuroinflammation.

Keywords

Thymoquinone; Neuroinflammation; Antioxidant; NF-κB; Nrf2/ARE

1. Introduction

Neuroinflammation is now widely accepted as one of the major hallmarks in the pathogenesis of neurodegenerative disorders such as Alzheimer's disease. Microglial cells, which are the resident macrophages of the CNS are known to play major roles in neuroinflammation, serving as a first line of immune defence in response to stimuli such as infection, traumatic brain injury, and other forms of stress.

NF-κB is a transcription factor that binds to DNA and activates gene transcription. The activation of NF-κB has been shown to be associated with inflammation in microglia in the CNS, where it has been found to mediate different responses [1]. Several genes are responsive to the transcriptional activity of NF-κB and promoting neuroinflammation. These include the pro-inflammatory cytokines [2-4], inducible nitric oxide synthase (iNOS) [5], cyclooxygenase-2 (COX-2) and microsomal prostaglandin E synthase-1 (mPGES-1) [6].

Harmful effects of oxidative and nitrosative stress is now known to cause damage to the biological system [7]. There is a strong link between oxidative stress and inflammation. An unbalanced redox state is known to contribute to the pathogenesis of inflammatory conditions, including ageing [8]. Furthermore, it is now widely accepted that inflammation triggers the generation of elevated levels of cellular reactive oxygen species that cause cellular oxidative damage [9]. On the other hand, inflammatory cells respond to oxidative stress by releasing various NF-κB-mediated pro-inflammatory mediators [10]. Oxidative stress has also been linked to neuroinflammation. Accumulating evidence indicates that reactive oxygen species (ROS) produced by the microglia have a significant impact on adjacent neurons, as well as modulating microglial activity [11]. It has been shown that the activated microglia M1 phenotype is associated with elevated levels of NADPH oxidase (NOX)-dependent ROS generation [12]. Consequently, oxidative stress is now recognised as an important contributor to neuroinflammation, and its resultant neuronal damage in neurodegenerative disorders.

The nuclear erythroid 2 related factor 2 (Nrf2) is a major regulator of endogenous defence mechanisms in the body [13]. In the nucleus, Nrf2 binds to the antioxidant response element (ARE) DNA sites to initiate the transcription of several

cytoprotective genes. Studies have linked the activation of Nrf2 to anti-inflammatory activity through mechanisms involving NF-κB [13]. For example, Nair et al. (2008) have identified NF-κB binding sites in the promoter regions of the Nrf2 gene, suggesting an interaction between the two transcription factors [14]. Further, neuroinflammation and microglia activation in response to LPS have been shown to be more pronounced in Nrf2-deficient mice when compared to normal animals [15]. Consequently, it would be interesting to establish the contribution of Nrf2 activation in the inhibition of neuroinflammation by pharmacological substances.

Accumulating evidence have suggested that nutritional factors might be important modifiable risk factors for AD [19], and can play an important role in delaying its onset or halting its progression and to improve cognitive function. Dietary factors that have been shown to inhibit neuroinflammation include punicalagin [16], tiliroside [17], and naringenin [18].

Thymoquinone (Figure 1) is the main constituent of the oil obtained from the seeds of *Nigella sativa* (black cumin seed oil), which has been used for medicinal purposes for centuries in Asia, Middle East, and Africa. This compound has been shown to produce anti-inflammatory [19-22] and antioxidant [23] properties. Previous studies have shown that thymoquinone protected dopaminergic neurons against MPP+ and rotenone-induced cell death [24], and produced neuroprotective effects in cultured rat primary neurons exposed to amyloid β [25, 26]. Studies have also shown that thymoquinone inhibited neuroinflammation in mixed glial cells [27] and BV2 microglia [28, 29].

In this study, we have shown further that thymoquinone inhibits neuroinflammation in LPS-stimulated cultured BV2 and rat primary microglia. We have further shown that inhibition of neuroinflammation by thymoquinone in BV2 microglia is related to its ability to activate both the Nrf2 antioxidant and LKB1/AMPK1 signalling pathways.

2. Materials and methods

2.1 Cell culture

BV2 mouse microglia cell line ICLC ATL03001 (Interlab Cell Line Collection, Banca Bilogica e Cell Factory, Italy) were maintained in RPMI1640 medium with 10% fetal bovine serum (FBS) (Sigma), 2 mM L-glutamine (Sigma), 100 U/ml penicillin and

100 mg/ml streptomycin (Sigma) in a 5% CO_2 incubator. Cells were treated with LPS (100 ng/ml; from *Salmonella typhimurium*, Sigma Aldrich).

Primary mixed glial cell cultures were established from cerebral cortices of postnatal day P0-P1 Sprague–Dawley rats. Briefly, forebrains were minced, dissociated and collected by centrifugation (1000 × g, 10 min, 4°C), re-suspended in DMEM, containing 10% foetal calf serum and antibiotics (40 U/mL penicillin and 40 µg/mL streptomycin) and cultured on 10 cm cell culture dishes (Falcon, Heidelberg, Germany) in 5% CO_2 at 37°C. After 12-14 days *in vitro*, floating microglia were harvested from mixed glia cultures and re-seeded into cell culture plates. The next day, medium was removed to get rid of non-adherent cell and fresh medium was added and after 1 h, cells were used for experiments. Cells were treated with LPS (10 ng/ml; from *Salmonella typhimurium*, Sigma Aldrich).

2.2 Cell viability assays

Cultured BV2 or primary rat microglia were incubated with or without LPS (100 ng/ml for BV2 or 10 ng/ml for rat primary microglia) in the absence or presence of thymoquinone (2.5–10 µM) for 24 h. Thereafter, cells were lysed and supernatants collected for LDH assay. LDH levels in supernatants were determined using the CytoTox 96® non-radioactive cytotoxicity assay kit (Promega, Southampton), according to the manufacturer's instructions.

2.3 Determination of TNFα, IL-6, PGE_2, and NO production in LPS-activated microglia

BV2 or rat primary microglia were incubated with or without LPS (100 ng/ml or 10 ng/ml)) in the absence or presence of thymoquinone (2.5–10 µM) for 24 h. After stimulation, cell supernatants were collected and centrifuged at 1000 g for 5 min at 4 C. Release of pro-inflammatory cytokines (TNFα, IL-1β and IL-6) from culture supernatants was determined using mouse or rat TNFα, IL-1β and IL-6 ELISA kits (Biolegend, UK). Nitrite production was quantified using the Griess assay kit (Promega, Southampton, UK), followed by colorimetric measurement at 540 nm. PGE_2 release was measured in cell supernatants with a commercially available enzyme immunoassay (EIA) kit (Arbor Assays, MI, USA), followed by measurement at 450 nm, according to the manufacturer's instructions.

2.4 DNA binding assays

An ELISA-based DNA binding assay (EMSA) was used to investigate the effects of thymoquinone on DNA binding of NF-κB, as earlier described [30, 31]. BV2 microglia were treated with thymoquinone (2.5-10 μM). Thirty minutes later, cells were stimulated with LPS (100 ng/ml). One hour later, nuclear extracts were prepared using EpiSeeker Nuclear Extraction Kit (Abcam), according to the manufacturer's instructions. DNA binding assay was carried on nuclear extracts using the TransAM NF-κB transcription factor EMSA kit containing immobilised NF-κB consensus site (5' GGGACTTTCC-3') (Activ Motif, Belgium) according the manufacturer's instructions. Briefly, 30 μl of complete binding buffer were added to each well, followed by 20 μg nuclear extract samples. The plate was covered and rocked (100 rpm) for 1 h at room temperature. This was followed by addition of NF-κB antibody (1:1000; 1 h) and HRP-conjugated antibody (1:1000; 1 h). Absorbance was read on a Tecan F50 microplate reader at 450 nm.

To investigate DNA binding of Nrf2, BV2 microglia were treated with thymoquinone (2.5-10 μM). Nuclear extracts were added to 96-well plates on which has been immobilised oligonucleotide containing the ARE consensus binding site (5' GTCACAGTGACTCAGCAGAATCTG-3'). Assay procedure was as described for NF-κB, using an Nrf2 antibody (1:1000; 1 h).

2.5 Immunofluorescence microscopy

Immunofluorescence experiments were carried out as described earlier [32]. BV2 microglia were cultured in 24 well plates. At confluence, cells were pre-treated with thymoquinone (2.5-10 μM) 30 min prior to LPS (100 ng/ml) stimulation. At the end of the stimulation, cells were fixed with ice-cold 100% methanol for 15 min at -20ºC and washed 3 times for 5 min with PBS. Non-specific binding sites were blocked by incubating cells in 5% BSA blocking solution (containing 10% horse serum in 1X TBS-T) for 60 min at room temperature followed by washing with PBS. Thereafter, the cells were incubated with rabbit anti-NF-κBp65 (Santa Cruz; 1:100) antibody overnight at 4ºC. Following overnight incubation, cells were washed 3 times with PBS and incubated for 2 h in dark with Alexa Fluor 488-conjugated donkey anti-rabbit IgG (Life Technologies; 1:500) secondary antibody. Thereafter, cells were washed with PBS and counterstained with 4', 6 diamidino-2-phenylindole

dihydrochloride (50 nm, DAPI; Invitrogen) for 5 min. After rinsing cells with PBS, excess buffer was removed and gold antifade reagent (Invitrogen) was added. All staining procedures were performed at room temperature. Representative fluorescence images were obtained using EVOS® FLoid® cell imaging station. Immunofluorescence was also used to detect nuclear localisation of Nrf2 in BV2 microglia treated with thymoquinone (2.5-10 μM), using rabbit Nrf2 antibody (Santa Cruz; 1:100).

2.6 Preparation of cytoplasmic and nuclear extracts

To obtain cytoplasmic extracts following treatments, cells were washed with PBS and lysed in 1.3× SDS-containing sample buffer without 1, 4-DTT or bromophenol blue containing 100 μM orthovanadate. Nuclear extracts were obtained using a commercially available nuclear extraction buffer (Abcam, UK). Following cytoplasmic and nuclear extraction, protein concentrations were determined.

2.7 Immunoblotting

For Western blotting, 20-40 μg of total protein from each sample was subjected to SDS-PAGE under reducing conditions. Proteins were then transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore). The membranes were blocked for 1 h at room temperature and then incubated overnight at 4°C with primary antibodies. Primary antibodies used were rabbit anti-COX-2 (Santa Cruz), rabbit anti-iNOS (Santa Cruz), rabbit anti-phospho-IκBα (Santa Cruz), rabbit anti-phospho-p65 (Cell Signalling), rabbit anti-p65 (Cell Signalling) rabbit anti-acetyl-p65 (Cell Signalling), rabbit anti-Nrf2 (Santa Cruz), rabbit anti-HO1 (Santa Cruz), rabbit anti-NQO1 (Santa Cruz), and rabbit anti-actin (Sigma). Primary antibodies were diluted in Tris-buffered saline (TBS), containing 0.1% Tween 20 (TBS-T) and 1 or 5% BSA. Membranes were incubated with the primary antibody overnight at 4°C. After extensive washing (three times for 15 min each in TBS-T), proteins were detected by incubation with Alexa Fluor 680 goat anti-rabbit secondary antibody (1:10000; Life Technologies) at room temperature for 1 h. Detection was done using a LICOR Odyssey Imager. All Western blot experiments were carried out at least three times.

2.8 Real-time quantitative PCR

RNA preparation was done by using RNAspin mini RNA isolation kit (GE Healthcare, Freiburg, Germany) and for cDNA synthesis, 1 μg of total RNA was reverse

transcribed using M-MLV reverse transcriptase and random hexamers (Biomers, Ulm, Germany). The synthesised cDNA was the template for the real-time PCR amplification that was carried out by the CFX96 real-time PCR detection system (Bio-Rad Laboratories, Inc.) using iQTM SYBRTM Green supermix (Bio-Rad Laboratories GmbH, Munich, Germany). Specific primers were designed by using Universal Probe Library Assay Design Center (Roche) and were obtained from Biomers (Ulm, Germany). Reaction conditions were 3 min at 95 °C, followed by 40 cycles of 15 s at 95°C, 30 s at 50°C, and 45 s at 72°C, and every cycle was followed by plate reading. After that, 1 min at 95°C, 1 min at 55°C, followed by melt curve conditions of 65°C, 95°C with increment of 0.5ºC for 5 s, followed by final plate reading. GAPDH served as an internal control for sample normalization, and the comparative cycle threshold Ct method was used for data quantification. The following primer sequences were used in the present study:

TNFα:	Fwd 5´-TGAACTTCGGGGTGATCG-3´
	Rev 5´-GGGCTTGTCACTCGAGTTTT-3´
IL-6:	Fwd 5´-CCTGGAGTTTGTGAAGAACAACT-3´
	Rev 5´-GGAAGTTGGGGTAGGAAGGA-3´
IL-1β:	Fwd 5´-TGTGATGAAAGACGGCACAC-3´
	Rev 5´-CTTCTTCTTTGGGTATTGTTTGG-3´
GAPDH:	Fwd 5´-TGGGAAGCTGGTCATCAAC-3´
	Rev: 5´- GCATCACCCCATTTGATCTT-3´

2.9 Transient transfection and reporter assays

At 70-80% confluence, cultured BV2 microglia were transfected with pGL4.32[luc2P/NF-κB-RE/Hygro] vector (Promega, UK), using Fugene 6 (Promega) transfection reagent and incubated for 6 h at 37°C. Following transfection, media was changed to OPTI-MEM and incubated for a further 8 h. Thereafter, transfected cells were treated with thymoquinone (2.5-10 μM) 30 min prior to stimulation with LPS (100 ng/ml) for 6 h. At the end of the stimulation, NF-κB-mediated gene expression was measured with Dual-Glo® luciferase assay kit (Promega), according to the manufacturer's instructions.

To carry out the ARE-dependent reporter gene assay, BV2 microglia were seeded out and incubated in solid white 96-well at 37°C for 24 h. A transfection cocktail was made by diluting ARE vector (pGL4.37 [luc2P/ARE/Hygro]; Promega) at a concentration of 1 ng DNA/µl in Fugene 6 transfection reagent. The cocktail was incubated at room temperature for 20 min, and 8 µl to each well, followed by incubation for 18 h at 37°C. Thereafter, culture medium was changed to OPTI-MEM and incubated for 6 h at 37°C. Cells were then treated with thymoquinone (2.5-10 µM) and incubated at 37°C for 8 h. Thereafter, ARE-mediated gene expression was measured using Dual-Glo luciferase assay kit (Promega), according to the manufacturer's instructions.

2.10 Nrf2 knockdown: siRNA silencing experiments

Small interfering RNA (siRNA) targeted at Nrf2 (Santa Cruz Biotechnology) was used to knockout Nrf2. BV2 cells were cultured and incubated at 37°C in a 5% CO_2 incubator until 70-80 % confluent. Thereafter, 2 µl Nrf2 siRNA duplex were diluted into 100 µl of siRNA transfection medium (Santa Cruz Biotechnology). In a separate tube, 2 µl of transfection reagent (Santa Cruz biotechnology) was diluted into 100 µl of siRNA transfection medium. The dilutions were mixed gently and incubated for 30 min at room temperature. Next, cells were incubated in Nrf2 siRNA transfection cocktail for 6 h at 37°C. Control BV2 microglia were transfected with control siRNA. Following transfection, media was changed in Nrf2 siRNA and control siRNA transfected cells to complete media and incubated for a further 18 h. Effects of thymoquinone (10 µM) on NO, PGE_2, TNFα, IL-1β, IL-6 and ROS production in LPS-stimulated control siRNA and Nrf2-siRNA-transfected BV2 cells were then determined. Transfection efficiency was determined by preparing RNA from both control siRNA and Nrf2 siRNA-transfected BV2 cells, and qPCR carried out to determine Nrf2 expression. Similar experiments were carried out in AMPK-knockout BV2 microglia.

2.11 Statistical analysis

Values of all experiments were represented as a mean ± SEM of at least 3 experiments. Values were compared using one-way ANOVA followed by a post-hoc Student Newman-Keuls test.

3. Results

3.1 Thymoquinone treatment did not affect viability of LPS-stimulated BV2 and rat primary microglia

BV2 cells were treated with thymoquinone (2.5-10 µM) in the presence or absence of LPS for 24 h, followed by determination of cell viability. Results of LDH assay (Figure 2A) show that thymoquinone treatment did not produce toxicity in BV2 cells. Similar results were obtained with rat primary microglia treated with thymoquinone (2.5-10 µM) (Figure 2B). These data suggest that the compound did not affect cell viability at concentrations used in the study.

3.2 Thymoquinone produced NF-κB-mediated inhibition of neuroinflammation

We investigated the effects of thymoquinone on the release of pro-inflammatory cytokines in BV2 microglia following stimulation with LPS (100 ng/ml). Results in Figure 3 show that pre-treatment with thymoquinone (2.5, 5 and 10 µM) produced significant ($p<0.001$) and concentration-dependent reduction in the release of TNFα (Figure 3A), IL-6 (Figure 3B) and IL-1β (Figure 3C), following stimulation with LPS. Also, qPCR experiments in BV2 microglia revealed statistical ($p<0.01$) and concentration-dependent reduction in the mRNA levels of TNFα (Figure 4A), IL-6 (Figure 4B) and IL-1β (Figure 4C).

We also tested effects of thymoquinone of the release and mRNA levels of pro-inflammatory cytokines in rat primary microglia stimulated with LPS (10 ng/ml). Results in Figures 5A-C show that activation of rat primary microglia resulted in marked secretion of TNFα, IL-6 and IL-1β. However, pre-treatment with thymoquinone (2.5-10 µM) produced a significant ($p<0.001$) reduction in the release of these pro-inflammatory cytokines. Similar to our observations in BV2 microglia, mRNA levels of TNFα, IL-6 and IL-1β were reduced by thymoquinone in rat primary microglia stimulated with LPS (10 ng/ml) (Figures 6A-C).

Experiments on other mediators of neuroinflammation revealed that thymoquinone produced significant ($p<0.05$) and concentration-dependent inhibition of PGE_2 production induced by LPS stimulation in BV2 microglia and rat primary microglia (Figures 7A and 8A). We further investigated whether the actions of thymoquinone on PGE_2 production were mediated by COX-2 and observed a similar trend in the effects of thymoquinone; western blotting show that thymoquinone pre-treatment

resulted in significantly reduced levels of COX-2 protein levels in LPS-activated BV2 and rat primary microglia (Figures 7B and 8B), thus suggesting that the effects of thymoquinone on PGE_2 were mediated through inhibition of this enzyme.

The effects of thymoquinone on nitrite production in LPS-activated microglia are shown in Figures 9A and 10A. Stimulation of BV2 cells and rat primary microglia with LPS (100 ng/ml and 10 ng/ml, respectively) resulted in marked release of nitrite from the cells. However, on treating cells with thymoquinone (2.5-10 µM) prior to LPS, nitrite production was significantly ($p<0.05$) reduced. We also observed that thymoquinone (2.5, 5 and 10 µM) significantly ($p<0.001$) prevented LPS-induced increase in iNOS protein levels in BV2 microglia (Figure 9B). In rat primary microglia stimulated with LPS (10 ng/ml), significant ($P<0.05$) reduction in iNOS protein was observed following treatment with 5 and 10 µM thymoquinone, but not with 2.5 µM of the compound (Figure 10 B).

Encouraged by the observed marked inhibition of neuroinflammation by thymoquinone, we decided to carry out further experiments in BV2 microglia to elucidate the role of NF-κB activation in its effects. Initially, we used western blotting to determine whether thymoquinone would affect the phosphorylation of IκB. Our results revealed that stimulation of BV2 microglia with LPS (100 ng/ml) resulted in marked increase of phospho-IκB protein (Figure 11A). In the presence of 2.5 µM of thymoquinone, there was a reduction in the level of phospho-IκB protein. However, this reduction was not statistically significant. On increasing the concentration of thymoquinone to 5 and 10 µM, we observed statistically significant ($p<0.05$) and concentration-dependent inhibition of IκB phosphorylation.

Since NF-κB activation involves steps culminating in nuclear translocation of the p65 subunit followed by p65 phosphorylation, acetylation and methylation, DNA binding, and gene transcription [35], we next investigated the effects of thymoquinone on LPS-induced nuclear translocation of p65 in BV2 microglia, and used western blotting and immunofluorescence to show that the compound prevented this step of NF-κB activation (Figure 11B and 11C). Encouraged by the pronounced effect of thymoquinone on nuclear translocation of the p65 subunit, we became interested in evaluating whether the compound would affect LPS-induced acetylation of NF-κB. Figure 11D shows that activation of BV2 microglia produced an increase in acetyl-

p65 immunostaining. On re-treating cells with thymoquinone (2.5 μM), there was a reduction in fluorescence, indicating reduced levels of p65 acetylation. Interestingly, on increasing the concentration of thymoquinone to 5 and 10 μM, acetylation of p65 was hardly detectable.

We further investigated the modulatory effect of thymoquinone on NF-κB DNA binding using an ELISA-based DNA binding assay. Analysis of nuclear extracts from BV2 microglia revealed that stimulation with LPS increased DNA binding of NF-κB, when compared with unstimulated cells. On pre-treating cells with thymoquinone (2.5, 5 and 10 μM), there was a significant ($p<0.01$) and concentration-dependent reduction in the binding of NF-κB to the DNA (Figure 11E).

In order to establish whether thymoquinone targets NF-κB-mediated gene transcription, we transfected BV2 cells with NF-κB-luciferase reporter plasmid. Stimulation of transfected cells with LPS (100 ng/ml) resulted in marked luciferase activity (Figure 11F), which was significantly ($p<0.001$) repressed by thymoquinone (2.5-10 μM) in a concentration-dependent manner.

3.3 Thymoquinone activates the Nrf2/ARE antioxidant mechanism and increases HO-1 and NQO-1 protein levels in BV2 microglia

Encouraged by the results showing antioxidant action of thymoquinone, we aimed to further determine whether the compound has any effect on the Nrf2/ARE antioxidant protective mechanism. We treated BV2 microglia with thymoquinone and subjected nuclear extracts to western blotting. Figure 12A shows that nuclear accumulation of Nrf2 protein was significantly elevated in the presence of 2.5, 5 and 10 μM thymoquinone. Similarly, immunofluorescence experiments show an increase in nuclear staining of Nrf2 in cells treated with thymoquinone (10 μM), when compared with untreated cells (Figure 12B).

We next determined whether thymoquinone would increase DNA binding Nrf2 in BV2 microglia. As shown in Figure 12C, thymoquinone (2.5-10 μM) treatment induced significant ($p<0.001$) and concentration-dependent increase in the binding of Nrf2 to the ARE consensus binding site. We further show that incubation of ARE reporter-transfected BV2 cells with thymoquinone (2.5-10 μM) resulted in significant increase in luciferase activity, suggesting an increase in ARE transcriptional activity (Figure 12D).

Binding of Nrf2 to ARE consensus sites in the DNA results in transcription of cytoprotective genes such as HO-1 and NQO1. We were therefore interested in investigating the potential effects of thymoquinone on protein levels of both HO-1 and NQO1. Incubation of thymoquinone with BV2 microglia for 12 h produced a significant increase in both HO-1 and NQO1 protein levels, when compared to untreated cells (Figures 12E and 12F).

3.4 Inhibition of neuroinflammation by thymoquinone in LPS-stimulated BV2 microglia is reversed in the presence Nrf2 siRNA and trigonelline

Having established that thymoquinone inhibits neuroinflammation and produces Nrf2/ARE-mediated antioxidant activity in microglia, it became necessary to elucidate whether activation of Nrf2/ARE mechanism contributes to the anti-inflammatory effect of the compound. This question was asked using two approaches. Firstly, we transfected BV2 cells with small interfering RNA (siRNA) specific for Nrf2 to achieve gene knockdown, followed by thymoquinone treatment and LPS stimulation. Levels of inflammatory mediators were then measured in culture supernatants. In cells transfected with control siRNA, treatment with thymoquinone (10 µM) prior to LPS stimulation resulted is significant reduction in production of TNFα (Figure 13A), IL-6 (Figure 13B), IL1β (Figure 13C), nitrite (Figure 13D), and PGE_2 (Figure 13E). However, in Nrf2 siRNA transfected cells, the inhibitory effect of thymoquinone on inflammatory mediator release was significantly reversed, when compared with control siRNA-transfected cells. Similarly, we show that the ability of thymoquinone (10 µM) to inhibit DNA binding by NF-κB was significantly diminished in nuclear extracts of Nrf2 siRNA-transfected BV2 microglia (Figure 13F). We used western blotting to monitor the efficiency of Nrf2 knockdown and show that in nuclear extracts of Nrf2 siRNA transfected cells, protein levels of Nrf2 were markedly reduced (Figure 13G).

Secondly, trigonelline (an Nrf2 inhibitor) was added to BV2 cells 60 min prior to treatment with thymoquinone (10 µM) and stimulation with LPS. Analyses of culture supernatants show that anti-inflammatory activity of thymoquinone on the release of pro-inflammatory mediators was almost completely abolished in the presence of trigonelline (100 µM) (Figures 14A-E). Thymoquinone (10 µM) pre-treatment caused an inhibition of binding DNA binding by NF-κB. In contrast, incubation with

trigonelline 1 h prior to thymoquinone treatment did not result in an inhibition of DNA binding (Figure 14F).

4. Discussion

Studies have suggested that thymoquinone inhibits neuroinflammation in the microglia. Alemi et al. reported that the compound inhibited nitrite production from mix glia cells stimulated with LPS [27]. Similarly, thymoquinone was shown to reduce the levels of pro-inflammatory cytokines and chemokines such as IL-6, IL-12p40/70, CCL12 /MCP-5, CCL2/MCP-1, and G-CSF as well as iNOS in LPS-stimulated microglia [28]. More detailed experiments by Wang et al. (2015) revealed that this compound prevented the release of nitrite, PGE_2, TNFα and IL-1β from LPS-stimulated BV2 microglia. In this report, thymoquinone was also shown to block IκB-mediated activation of p65 subunit [29].

In this study, we have also shown that treatment with thymoquinone suppressed the release of the pro-inflammatory cytokines TNFα, IL-1β and IL-6. However, unlike previous studies on this compound, we have further demonstrated that the reduction of LPS-induced production of nitrite and PGE_2 by thymoquinone was mediated by enzymatic activities of both iNOS and COX-2, respectively. COX-2 has been shown to be upregulated in neuroinflammation involving both the neurons and reactive microglia [33, 34], and linked to PGE_2 production in the activated microglia [34]. Also, activation of microglia results in elevated expression of iNOS protein which mediates excessive production of nitric oxide [35].

NF-κB activation is known to involve steps culminating in nuclear translocation of the p65 subunit followed by p65 phosphorylation, acetylation and methylation, DNA binding, and gene transcription [32]. Further mechanistic studies by us revealed that thymoquinone inhibited both IκB as well as p65 phosphorylation and nuclear translocation following activation of BV2 microglia with LPS. These results are consistent with those published by Wang et al. [29]. However, in this study we have further shown that thymoquinone prevented LPS-induced acetylation of NF-κB, through inhibition of acetyl-p65 protein. Following acetylation, activated NF-κB is known to bind to specific DNA sequences in target genes, thereby regulating the transcription of genes involved in neuroinflammation, such as the pro-inflammatory

cytokines (TNFα, IL-6 and IL-1β), COX-2 and iNOS. Results of this study shows that pre-treatment of BV2 microglia with thymoquinone resulted in marked inhibition of binding by active NF-κB to its consensus site, following LPS stimulation. Furthermore, NF-κB-mediated gene transcription was inhibited by thymoquinone through suppression of luciferase activity in BV2 microglia which were transiently transfected with the NF-κB-luciferase reporter plasmid and stimulated with LPS. Our results suggest that thymoquinone inhibits neuroinflammation in BV2 microglia through steps involving IκB phosphorylation,

New findings have linked activation of the antioxidant transcription factor, Nrf2 to anti-inflammatory effects via interactions with NF-κB [13]. Consequently, we carried out investigations to determine whether thymoquinone would activate Nrf2, and whether Nrf2 activation contributed to its anti-inflammatory activity in the microglia. Treatment of BV2 microglia with thymoquinone resulted in increased nuclear localisation and DNA binding of Nrf2, as well as enhancing transcriptional activity of the antioxidant responsive element (ARE). We also observed an increase in the protein levels of HO-1 and NQO1 after treatment with the compound. These results suggest that thymoquinone activation of Nrf2 antioxidant mechanism probably contributes to the anti-inflammatory activity of thymoquinone in the microglia. Similar observations were made in experiments showing that thymoquinone induces HO-1 in HaCaT cells through Nrf2/ARE activation [36]. We further show that inhibition of inflammatory mediator release and DNA binding by NF-κB by thymoquinone was significantly reversed in the presence of either Nrf2 siRNA or trigonelline (a specific inhibitor of Nrf2). Taken together, we propose that thymoquinone inhibits neuroinflammation through mechanisms that are dependent on microglial Nrf2 activation.

Taken together, our studies show that thymoquinone inhibits NF-κB-dependent neuroinflammation in BV2 microglia, by targeting antioxidant pathway involving activation of Nrf2/ARE signalling. We propose that activation of Nrf2/ARE signalling pathway by thymoquinone probably results in inhibition of NF-κB-mediated neuroinflammation.

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Conflicts of interest

No conflict of interest.

Author Contributions

Ravikanth Velagapudi and Asit Kumar contributed equally to this study.

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Figure Legends

Figure 1

Structure of thymoquinone

Figure 2

Thymoquinone (2.5-10 μM) treatment did not affect the viability of BV2 microglia stimulated with LPS for 24 h.

Figure 3

Thymoquinone prevented the release of pro-inflammatory cytokines from LPS-activated BV2 microglia. BV2 cells were treated with vehicle or thymoquinone (2.5-10 μM) for 30 min prior to LPS stimulation for 24 h. Culture supernatants were analysed for TNFα (A), IL-6 (B) and IL-1β (C) using mouse ELISA kits. Bar graph shows compiled data from three independent experiments expressed as % production compared to LPS control (Mean ± SEM; ***p<0.001; one-way ANOVA)

Figure 4

Thymoquinone reduced TNF α (a), IL-6 (b) and IL-1β (C) mRNA levels in LPS-activated BV2 microglia. Cells were stimulated with LPS (100 ng/mL) in the presence or absence of thymoquinone (2.5–10 μM) for 4 h. All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post-hoc Student Newman-Keuls test, **p<0.01, ***p<0.001 in comparison with LPS control.

Figure 5

Thymoquinone prevented the release of pro-inflammatory cytokines from LPS activated rat primary microglia. Microglia cells were treated with vehicle or thymoquinone (2.5-10 μM) for 30 min prior to LPS (10 ng/ml) stimulation for 24 h. Culture supernatants were analysed for TNFα (A), IL-6 (B) and IL-1β (C) using rat ELISA kits. All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post-hoc Student Newman-Keuls test, ***p<0.001 in comparison with LPS control.

Figure 6

Thymoquinone reduced TNF α (A), IL-6 (B) and IL-1β (C) mRNA levels in LPS-activated rat primary microglia. Cells were stimulated with LPS (10 ng/ml) in the presence or absence of thymoquinone (2.5–10 μM) for 4 h. All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post-hoc Student Newman-Keuls test, **p<0.01, ***p<0.001 in comparison with LPS control.

Figure 7

Thymoquinone produced COX-2-mediated inhibition of PGE_2 production in LPS-stimulated BV2 microglia. (A) BV2 cells were treated with vehicle or thymoquinone (2.5-10 μM) for 30 min prior to LPS (100 ng/ml) stimulation for 24 h. Culture supernatants were analysed for PGE_2 production. All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post-hoc Student Newman-Keuls test, ***p<0.001 in comparison with LPS control. (B) BV2 cells were treated with vehicle or thymoquinone (2.5-10 μM) for 30 min prior to LPS (100 ng/ml) stimulation for 24 h. Cell lysates were analysed using immunoblotting for COX-2 and actin. Representative blots and densitometric analyses of three independent experiments are shown (Mean ± SEM; **p<0.01, ***p<0.001; one-way ANOVA with post-hoc Newman-Keuls test).

Figure 8

Thymoquinone produced COX-2-mediated inhibition of PGE_2 production in LPS-stimulated rat primary microglia. (A) Cells were treated with vehicle or thymoquinone (2.5-10 μM) for 30 min prior to LPS (10 ng/ml) stimulation for 24 h. Culture supernatants were analysed for PGE_2 production. All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post-hoc Student Newman-Keuls test, ***p<0.001 in comparison with LPS control. (B) Primary microglia cells were treated with vehicle or thymoquinone (2.5-10 μM) for 30 min prior to LPS (10 ng/ml) stimulation for 24 h. Cell lysates were analysed using immunoblotting for COX-2 and actin. Representative blots and densitometric analyses of three independent experiments are shown (Mean ± SEM; ***p<0.001; one-way ANOVA with post-hoc Newman-Keuls test).

Figure 9

Thymoquinone produced iNOS-mediated inhibition of nitrite production in LPS stimulated BV2 microglia. (A) BV2 cells were treated with vehicle or thymoquinone (2.5-10 µM) for 30 min prior to LPS (100 ng/ml) stimulation for 24 h. Culture supernatants were analysed for nitrite production. All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post-hoc Student Newman-Keuls test, ***p<0.001 in comparison with LPS control. (B) BV2 cells were treated with vehicle or thymoquinone (2.5-10 µM) for 30 min prior to LPS (100 ng/ml) stimulation for 24 h. Cell lysates were analysed using immunoblotting for iNOS and actin. Representative blots and densitometric analyses of three independent experiments are shown (Mean ± SEM; ***p<0.001; one-way ANOVA with post-hoc Newman-Keuls test).

Figure 10

Thymoquinone produced iNOS-mediated inhibition of nitrite production in LPS stimulated rat primary microglia. (A) Primary microglia were treated with vehicle or thymoquinone (2.5-10 µM) for 30 min prior to LPS (10 ng/ml) stimulation for 24 h. Culture supernatants were analysed for nitrite production. All values are expressed as mean ± SEM for three independent experiments. Data were analysed using one-way ANOVA for multiple comparison with post-hoc Student Newman-Keuls test, ***p<0.001 in comparison with LPS control. (B) Primary microglia were treated with vehicle or thymoquinone (2.5-10 µM) for 30 min prior to LPS (10 ng/ml) stimulation for 24 h. Cell lysates were analysed using immunoblotting for iNOS and actin. Representative blots and densitometric analyses of three independent experiments are shown (Mean ± SEM; *p<0.05, ***p<0.001; one-way ANOVA with post-hoc Newman-Keuls test).

Figure 11

Inhibition of neuroinflammation by thymoquinone is mediated by targeting NF-κB signalling. (A) BV2 cells were treated with vehicle or thymoquinone (2.5-10 µM) for 30 min prior to LPS stimulation for 24 h. Cell lysates were analysed using immunoblotting for phospho-IκB and actin. Representative blots and densitometric analyses of three independent experiments are shown (Mean ± SEM; *P<0.05, **p<0.01; one-way ANOVA with post-hoc Newman-Keuls test). (B) Thymoquinone

reduced phosphorylation of p65 sub-unit in LPS-activated BV2 microglia. BV2 cells were treated with thymoquinone (2.5-10 μM) prior to stimulation with LPS for 1 h. Cell lysates were analysed using immunoblotting for phospho-p65 and actin. Representative blots and densitometric analyses of three independent experiments are shown (Mean ± SEM; **p<0.01; one-way ANOVA with post-hoc Newman-Keuls test). (C) Thymoquinone prevented nuclear localisation of p65 subunit in LPS-stimulated BV2 microglia. BV2 cells were treated with thymoquinone (2.5-10 μM) prior to LPS (100 ng/ml) for 1 h. Immunofluorescence experiments were carried out to detect p65 protein localisation using an anti-p65 antibody and Alexa Fluor 488-conjugated donkey anti-rabbit IgG antibody. Cells were counterstained with DAPI and fluorescence images acquired with an EVOS® FLoid® cell imaging station (scale bar: 100 μm). (D) Thymoquinone inhibited acetyl-p65 protein expression in LPS-stimulated BV2 microglia. BV2 cells were treated with thymoquinone (2.5-10 μM) prior to LPS (100 ng/ml) for 1 h. Immunofluorescence experiments were carried out to detect p65 protein localisation using an anti-acetyl-p65 antibody and Alexa Fluor 488-conjugated donkey anti-rabbit IgG antibody. Cells were counterstained with DAPI and fluorescence images acquired with an EVOS® FLoid® cell imaging station (scale bar: 100 μm). (E) Thymoquinone inhibited DNA binding of NF-κB in LPS-stimulated BV2 microglia. Nuclear extracts from cells were added to 96-well plates to which an oligonucleotide containing the NF-κB consensus site (5'-GGGACTTTCC-3') has been immobilised, followed by addition of NF-κB and HRP-conjugated antibodies. Absorbance was read in a microplate reader. (Mean ± SEM; *P<0.05, **p<0.01; one-way ANOVA with post-hoc Newman-Keuls test). (F) Thymoquinone supressed NF-κB luciferase activity in BV2 cells transfected with pGL4.32 [luc2P/NF-κB-RE/Hygro] vector and stimulated with LPS (100 ng/ml) in the absence or presence of thymoquinone (2.5-10 μM) for 6 h. (Mean ± SEM; ***p<0.01; one-way ANOVA with post-hoc Newman-Keuls test).

Figure 12

Thymoquinone activates the Nrf2/ARE antioxidant mechanism in BV2 microglia. (A) BV2 cells were treated with vehicle or thymoquinone (2.5-10 μM) for 12 h. Nuclear extracts were analysed using immunoblotting for Nrf2 and lamin B. Representative blots and densitometric analyses of three independent experiments are shown (Mean ± SEM; *p<0.05, **p<0.01; one-way ANOVA with post-hoc Newman-Keuls

test). (B) BV2 cells were treated with vehicle or thymoquinone (2.5-10 µM) for 12 h. Immunofluorescence experiments were carried out to detect Nrf2 protein localisation in the nucleus using an anti-Nrf2 antibody and Alexa Fluor 488-conjugated donkey anti-rabbit IgG antibody. Cells were counterstained with DAPI and fluorescence images acquired with an EVOS® FLoid® cell imaging station (scale bar: 100 µm). (C) Thymoquinone increased binding of Nrf2 to ARE consensus sites in BV2 microglia. Nuclear extracts from cells were added to 96-well plates to which an oligonucleotide containing the ARE consensus site (5' GTCACAGTGACTCAGCAGAATCTG-3') has been immobilised, followed by addition of Nrf2 and HRP-conjugated antibodies. Absorbance was read in a microplate reader. (Mean ± SEM; **p<0.01, ***p<0.001; one-way ANOVA with post-hoc Newman-Keuls test). (D) Thymoquinone increased ARE luciferase activity in BV2 cells transfected with pGL4.37 [luc2P/ARE/Hygro] vector and treated with thymoquinone (2.5-10 µM) for 6 h. (Mean ± SEM; ***p<0.01; one-way ANOVA with post-hoc Newman-Keuls test). (E) Protein levels of HO-1 are increased by thymoquinone (2.5-10 µM) incubated with BV2 microglia for 12 h. Cell lysates were analysed using immunoblotting for HO-1 and actin. Representative blots and densitometric analyses of three independent experiments are shown (Mean ± SEM; *p<0.05, ***p<0.001; one-way ANOVA with post-hoc Newman-Keuls test). (F) Protein levels of NQO1 are increased by thymoquinone (2.5-10 µM) incubated with BV2 microglia for 12 h. Cell lysates were analysed using immunoblotting for NQO1 and actin. Representative blots and densitometric analyses of three independent experiments are shown (Mean ± SEM; **p<0.01, ***p<0.001; one-way ANOVA with post-hoc Newman-Keuls test).

Figure 13

Inhibition of neuroinflammation by thymoquinone is dependent on Nrf2. Control siRNA- and Nrf2 siRNA-transfected BV2 cells were pre-treated with thymoquinone (10 µM) prior to stimulation with LPS (100 ng/ml) for 24 h. Culture supernatants were analysed for TNFα (A), IL-6 (B), IL-1β (C), nitrite (D) and PGE_2 (E). In (F) nuclear extracts from cells were added to 96-well plates to which an oligonucleotide containing the NF-κB consensus site (5'-GGGACTTTCC-3') has been immobilised, followed by addition of NF-κB and HRP-conjugated antibodies. Absorbance was read in a microplate reader. (G) Western blot experiments on nuclear extracts to

determine knockout efficiency. (Mean ± SEM; **p<0.01, ***p<0.001 compared with LPS in control siRNA-transfected cells; #p<0.05, ###p<0.001 compared with thymoquinone + LPS treatment in control siRNA-transfected cells; one-way ANOVA with post-hoc Newman-Keuls test).

Figure 14

Inhibition of neuroinflammation by thymoquinone was abolished in the presence of trigonelline. BV2 cells were treated with trigonelline (100 µM), followed by thymoquinone (10 µM) and LPS (100 ng/ml) for 24 h. Culture supernatants were collected and analysed for TNFα (A), IL-6 (B), IL-1β (C), nitrite (D) and PGE_2 (E). In (F) nuclear extracts from cells were added to 96-well plates to which an oligonucleotide containing the NF-κB consensus site (5'-GGGACTTTCC-3') has been immobilised, followed by addition of NF-κB and HRP-conjugated antibodies. Absorbance was read in a microplate reader. (Mean ± SEM; **p<0.01, ***p<0.001 compared with LPS stimulation; #p<0.05, ##p<0.01, ###p<0.001 compared with thymoquinone + LPS treatment; one-way ANOVA with post-hoc Newman-Keuls test).