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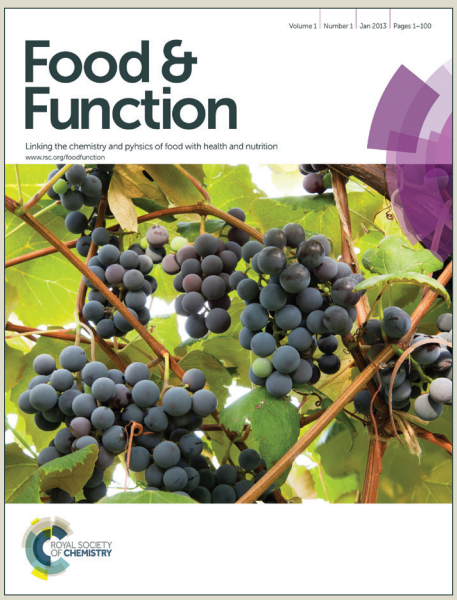

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PAPER

ARTICLE

Curcumenol isolated from *Curcuma zedoaria* suppresses Akt-mediated NF-κB activation and p38 MAPK signaling pathway in LPS-stimulated BV-2 microglial cells.

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Curcumenol, a sesquiterpene isolated from *Curcuma zedoaria* is known to possess a variety of health and medicinal values which includes neuroprotection, anti-inflammatory, anti-tumor and hepatoprotective activities. The current study aim is to investigate the modulatory effects of curcumenol towards the lipopolysaccharides (LPS)-induced inflammation in BV-2 microglia. Curcumenol markedly decreased LPS-induced production of nitric oxide (NO), pro-inflammatory cytokines [(IL-6) and (TNF-α)] and pro-inflammatory proteins expression, iNOS and COX-2. Moreover, curcumenol inhibited NF-κB activation by suppressing the nuclear translocation the NF-κB p65 subunit and blocking IκBα phosphorylation and degradation. Furthermore, an NF-κB inhibitor, ethyl 3,4-dihydroxycinnamate also known as caffeic acid ethyl ester (CAEE), attenuated LPS-stimulated iNOS and COX-2 expression, suggesting that NF-κB inhibition is a regulator in the expression of iNOS and COX-2 proteins. Further mechanistics study with Akt inhibitor, Triciribine hydrate (API-2), revealed curcumenol acted through Akt dependent NF-κB activation. Moreover, curcumenol inhibition on LPS-induced phosphorylation of p38 MAPK is confirmed by its inhibitor (SB 202190). These results indicate that curcumenol diminishes the proinflammatory mediators and the expression of the regulatory genes in LPS-stimulated BV-2 by inhibiting Akt-dependent NF-κB activation and downregulation of Akt and p38 MAPKs signaling.

Introduction

Microglia, the resident glial cells that compose 10% to 20% of the total brain cells and function as the immune cells of the central nervous system (CNS) that maintain cellular homeostasis and provide immune surveillance host defense under normal conditions besides playing an important role in neuroinflammation.[1] When the brain is exposed to injuries, infections or irritants, microglial cells are activated. It was well documented that microglial activation is closely related to the neurodegeneration CNS disorders such as Alzheimer's disease, Parkinson's disease (PD), multiple sclerosis, and Huntington's disease.[2-4] Activated microglia produce various neurotoxic factors including inflammatory mediators such as nitric oxide (NO) and prostaglandin E2 (PGE2), and pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, and IL-6, inducible NO synthase (iNOS) and cyclooxygenase (COX)-2. These microglial products are thought to be responsible for neuroglia-mediated neurotoxicity.[5, 6] Therefore, attenuation of neuroinflammatory events in microglia might be a promising strategy for preventing the progression of inflammatory-mediated neurodegenerative diseases.

Akt is a serine/threonine kinase that is recruited to the plasma membrane in cells when it is stimulated with various stimulants. Akt plays critical roles in multiple cellular processes, such as cell cycle regulation, cell survival, NF-κB activation and cell migration, depending on the nature of the stimuli. Findings have shown that phosphorylated Akt influences and promotes NF-κB activation, which is crucial in inflammatory processes.[7] On the other hand, strong evidence has shown that inflammation diseases, such as rheumatoid arthritis, Alzheimer's disease and inflammatory bowel disease, are all postulated to be regulated in part by the p38 MAPK pathway.[8] The activation of the p38 MAPK pathway is also linked to the production of pro-inflammatory cytokines playing a significant role in initiating and sustaining

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inflammation activities.[9, 10] Furthermore, NF-κB has been proposed as another signaling pathway that plays an important role to regulate microglia-mediated inflammation. The NF-κB subunits p50/p65 are normally bound to inhibitor of κBα (IκBα) and remain inactive in the cytoplasm. In response to inflammatory stimuli, IκBα is phosphorylated and degraded, allowing the p50/p65 subunit to translocate into the nucleus. A previous study showed that NF-κB is chronically active in regulating inflammatory genes, including iNOS, COX-2, and pro-inflammatory cytokines and chemokines.[11] In order to verify the pathways involved in the anti-inflammatory effect of curcumenol, each pathway was blocked with a specific inhibitor to observe the response of the cells towards the inhibition of these pathways as compared to that of curcumenol. Accordingly, CAEE, a potent and specific inhibitor of NF-κB and API-2, a highly selective cell-permeable nucleoside that inhibits the Akt signaling, were deployed for the verification of NF-κB and Akt signaling, respectively.[12, 13] On the other hand, SB 202190 is a well-known potent p38 MAPK inhibitor that specifically inhibits p38. These inhibitors were incorporated into respective studies by examining the production of inflammatory proteins, cytokines and mediators.

Curcuma zedoaria, also known as white turmeric, *Temu putih* or gajutsu, is a perennial rhizomatous herb that belongs to the Zingiberaceae family. It is used traditionally as spices, flavors in dishes and food preparations in postpartum confinement.[14, 15] This plant is also widely used by the people in Asia in the preparation of traditional medicine for the treatment of various ailments including menstrual disorder, dyspepsia, antiseptic, stomach diseases and vomiting.[16, 17] The anticancer and biological properties of *C. zedoaria* rhizomes have been extensively reported.[18, 19] Sesquiterpenes from *C. zedoaria* have been shown to possess anti-bacterial and anti-inflammatory effects in previous studies.[20, 21] A study has also shown that curcumenol may be safely used in the evaluation of potential pharmacokinetic drug interactions.[22] Our previous study demonstrated that curcumenol possessed the most potent neuroprotective effects among all the compounds isolated from *C.zedoaria* in NG108-15 cells when exposed to H_2O_2.[23] Curcumenol has also been shown to exhibit hepatoprotective properties.[24] To the best of our knowledge, no study of neuroinflammation using curcumenol has been performed to date. The present study proposes some insight of possible mechanisms involved in the suppression of inflammation by curcumenol against LPS-stimulated BV-2 microglia.

Materials and Methods

Reagents and Antibodies

LPS, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dulbecco's modified Eagle's medium (DMEM), Caffeic acid ethyl ester (CAEE), triciribine hydrate, SB202190, fetal bovine serum (FBS) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St Louis, MO,USA). The 100 unit/mL streptomycin/penicillin and 50 µg/mL Amphotericin B were purchased from PAA Lab in Austria. The Total Nitric Oxide Assay Kit and RIPA buffer were purchased from Thermo Fisher Scientific in Pierce, USA. The Bradford colorimetric protein assay kit and Enhanced Chemiluminescence (ECL) detection system were purchased from Bio-Rad (CA, USA). The CBA kit was purchased from BD Biosciences (San Jose, CA, USA). The COX-2, β-actin, phosphorylated-p38, p38 MAPK, iNOS and horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). The Lamin B, Akt, phosphorylated Akt, NF-κB p65, phosphorylated IκBα, IκBα and TNF-α antibodies were purchased from Thermo Fisher Scientific in Pierce.

Cell culture

The BV-2 cell line was developed from mouse microglia in the laboratory of Dr. Blasi at the University of Perugia (Perugia, Italy).[25] The cells were supplied with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 unit/mL streptomycin/penicillin and 50 µg/mL Amphotericin B. The cells were cultured in a 5% CO_2 incubator at 37°C in a humidified atmosphere. The medium was changed every two days, and the cells were observed under the light microscope to ensure that they were free from any contamination. The cells were sub-cultured and maintained appropriately at 60-70% confluency.

Curcumenol and LPS treatment

Curcuma zedoaria rhizomes were collected from Tawamangu, Indonesia, and a voucher specimen (KL 5764) was deposited at the herbarium of the Department of Chemistry (Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia). Extraction and isolation was performed according to a previous report.[26] Curcumenol was dissolved in dimethyl sulfoxide (DMSO) at a concentration of < 0.5% before being added to the cells. LPS was dissolved at a concentration of 0.4 µg/mL in 10% DMEM medium. BV-2 cells were seeded into a sterile flat bottom 96-well plate. They were then allowed to adhere overnight. The cells were pretreated with various concentrations of curcumenol for 2 h followed by LPS exposure for 12 h to 24 h.

MTT Assay

The cells were grown to confluency, harvested and plated at a density of $2x10^4$ cells/well in a sterile 96-well plate. The cells were allowed to adhere overnight, and then, they were pretreated with serial concentrations of curcumenol for 2 h followed by an addition of LPS for the 24 h. Next, 20 µl of MTT (filter sterile, 5 mg/ml) was added in each well and incubated at 37°C in a 5% CO_2, 95% air for 4 hours. The media were then aspirated, and 150 µl dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. The absorbance was measured spectrophotometrically at 570 nm and 650 nm (reference length) with a microplate reader (ASYS Hitech UVM340). Cell viability was calculated based on the following formula:

% of viability = (absorbance of treated cells/ absorbance of untreated cells) x 100%.

Nitric Oxide Quantification Assay

The nitric oxide level in BV-2 cell culture supernatant was assessed using the Total Nitric Oxide Assay Kit according to manufacturer's protocol. Briefly, BV-2 cells were subjected to the designated treatment, and the supernatants were collected. Nitrate reductase was mixed with the supernatant followed by Griess reagent. The interaction of nitric oxide in a system was measured by the determination of total nitrate and nitrite concentrations in the sample. The absorbance was read using a microplate reader (ASYS Hitech UVM340) at an absorbance of 540 nm.

Western Blot

BV-2 cells were subjected to pretreatment with or without curcumenol (5, 10 and 20 µg/mL) for 2 h and prior to LPS exposure (0.4 µg/mL). The cells were harvested and lysed in ice-cold lysis RIPA buffer containing phosphatase and protease inhibitor. The protein content was measured using a Bradford colorimetric protein assay kit. Equal amounts of protein (25 µg) were separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane. After blocking with 5% bovine serum albumin, the membranes were probed with primary antibodies against COX-2, β-actin, lamin B, phospho-ERK, ERK, phospho-JNK, JNK, phospho-p38, p38 MAPK, iNOS, Akt, phospho Akt, NF-κB p65, IκBα or TNF-α overnight at 4°C. Horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG were used as secondary antibodies. An Enhanced Chemiluminescence (ECL) detection system was used to detect the protein bands on the membrane and analyzed using Bio-1D software.

Cytometric Cytokine Bead Array (CBA) Kit

Cytokine production (TNF-α and IL-6) was evaluated using the CBA kit. The assay was conducted according to the manufacturer's protocol. Briefly, following treatment, 50 µl of cell culture medium was mixed and stained with the cytokine capture beads with recombinant standards. The detection antibodies that conjugated with PE were then added to form sandwich complexes. The samples were then analyzed using a BD Accuri™ C6 flow cytometer, and the results were analyzed using FCAP Array™ software (Soft Flow, Inc. Hungary).

Data analysis

All of the experimental data are expressed as the means ± standard error (S.E.). Significant differences between groups were analyzed and calculated by one-way analysis of variance (ANOVA) from at least three independent experiments and followed by Dunnett's test. P *<0.05* was considered to be significantly different.

Results

Curcumenol inhibited LPS-induced NO, IL-6 and TNF-α production in BV-2 cells

In the CNS, pro-inflammatory mediators and cytokines are products from activated microglia that have been triggered by stimulants and are involved in the exacerbation of brain inflammation. The presence of curcumenol (Figure 1a) at varying concentrations (2.5-20µM) increased the LPS-stimulated BV-2 cells viability and did not exhibit any signs of toxicity at the selected concentrations of up to 20 µM (Figure 1b). Based on these results, concentrations of up to 20 µM were selected for further investigations. To evaluate the effects of curcumenol on pro-inflammatory mediators, we determined the NO, IL-6 and TNF-α production in LPS-stimulated BV-2 cells after pretreatment with curcumenol using the Total Nitric Oxide Assay Kit and Cytokine Bead Array (CBA) assay. NO production was significantly increased following stimulation with LPS compared to the untreated control group, whereas pretreatment with curcumenol (5-20 µM) significantly attenuated the production of NO in a dose dependent manner (Figure 1c). Further, the dual parametric dot plot obtained from CBA assay (Figure 1d), demonstrated a marked increase in the levels of both IL-6 and TNF-α as evidenced by a right shift of the log fluorescence intensity upon stimulation with LPS. However, pretreatment with curcumenol significantly reduced the production of these two cytokines in LPS-induced BV-2 cells. Thus, these results indicated that curcumenol suppressed the production of pro-inflammatory mediators, NO, IL-6 and TNF-α, which are crucial in the inflammatory process.

Suppression of iNOS and COX-2 protein expression by curcumenol

To determine the protein expression of iNOS and COX-2, BV-2 cells were stimulated with LPS in the presence of various concentrations of curcumenol. As shown in Figure 2, stimulation with LPS increased iNOS protein expression in BV-2 cells. However, pretreatment with curcumenol significantly attenuated LPS-induced iNOS protein expression. These data indicate that curcumenol suppresses LPS-induced NO production by inhibiting iNOS expression in microglial cells. COX-2 protein expression level was also elevated in LPS-stimulated BV-2 cells. However, pretreatment with curcumenol (5-20 µM) significantly and concentration-dependently reduced this increase in LPS-stimulated BV-2 cells.

Curcumenol attenuated LPS-Induced NF-κB activation in BV-2 cells

NF-κB is an important factor in the transcriptional regulation of pro-inflammatory mediators including iNOS, COX-2 and cytokines. Accumulating evidences indicate that NF-κB is one of the principal regulators in regulating the expression of pro-inflammatory factors, including iNOS and cytokines. To further verify the mechanism by which curcumenol inhibits the expression of pro-inflammatory genes, we next investigated the effect of curcumenol on NF-κB signaling by western blot analysis. Since NF-κB plays an essential role in the regulation of inflammatory mediators, the phosphorylation levels of IκBα and p65 were analyzed. NF-κB is inactive in the cytosol by binding to IκB and becomes active through degradation of

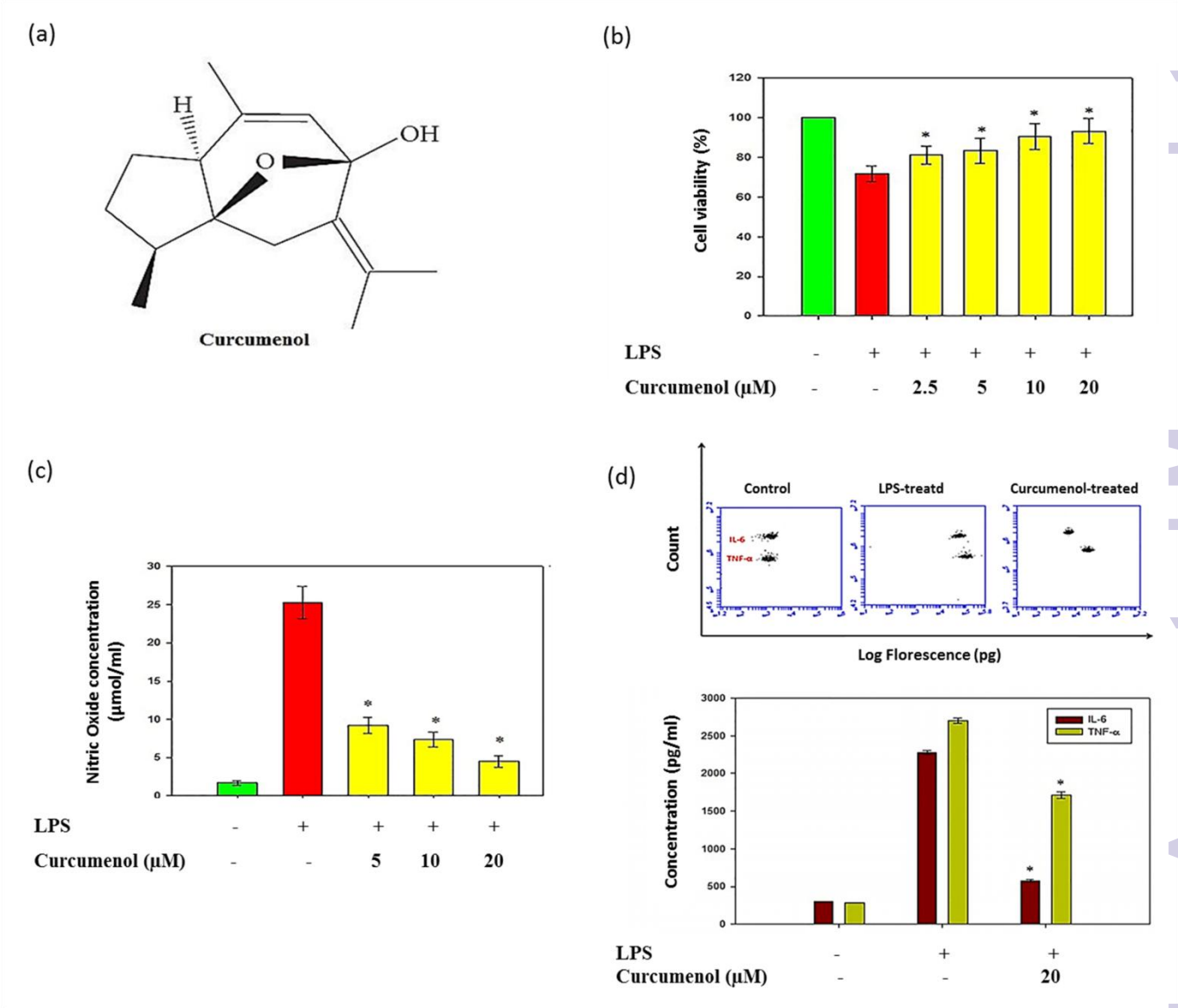

Figure 1. (a) Chemical structure of curcumenol and effects of curcumenol on the LPS-induced on the (b) cell viability measured using MTT assay (c) NO determined using Total Nitric Oxide assay kit and (d) TNF-α and IL-6 production in BV-2 cells measured using the cytokine cytometric bead array (CBA) assay: the dual parametric dot plot and the graph of concentration of cytokines against a concentration of curcumenol at 20 μM. Cells were pretreated with curcumenol (2.5-20 μM) for 2 h followed by LPS (0.4 μg/mL) for 12 - 24 h. Cell viability was determined by an MTT assay. NO production in cells was measured using the Total Nitric Oxide Assay kit. The production of inflammatory cytokines was performed according to the manufacturer's protocol (BD Biosciences). The data were then acquired using the BD Accuri™ C6 Flow Cytometer and were then analyzed by using the FCAP Array™ software. The data are presented as the means ± SE from at least 3 independent experiments. *P < 0.05, compared with the LPS-treated group

IKKα/β and the subsequent nuclear translocation of NF-κB proceeded by inflammatory factors.[27] We next investigated the effect of curcumenol on the phosphorylation of IκBα. Upon LPS stimulation, IκBα phosphorylation was greatly increased in BV-2 cells (Figure 3b). However, this increase was attenuated significantly when the cells were pretreated with curcumenol. Therefore, this study suggested that curcumenol suppression of NF-κB activation was caused by blocking the phosphorylation of the IκBα. In addition, we performed a western blot analysis to investigate the functional effect of NF-κB activity using a specific NF-κB inhibitor, caffeic acid ethyl ester (CAEE). Similar to curcumenol, CAEE significantly decreased the LPS-induced expression of iNOS, COX-2 and TNF-α proteins (Figure 3c).

Curcumenol inhibited Akt-dependent NF-κB activity

The Akt pathway is closely involved in the regulation of NF-κB activity, thus, we next confirmed the relationship between Akt and NF-κB activation. The marked increase in phosphorylated Akt when induced by LPS was significantly decreased following pretreatment with curcumenol (Figure 4a). In addition, a specific inhibitor of Akt activity, triciribine hydrate (API-2) completely abrogated the LPS-induced NF-κB activity as evidenced by the significant decrease of IκBα phosphorylation

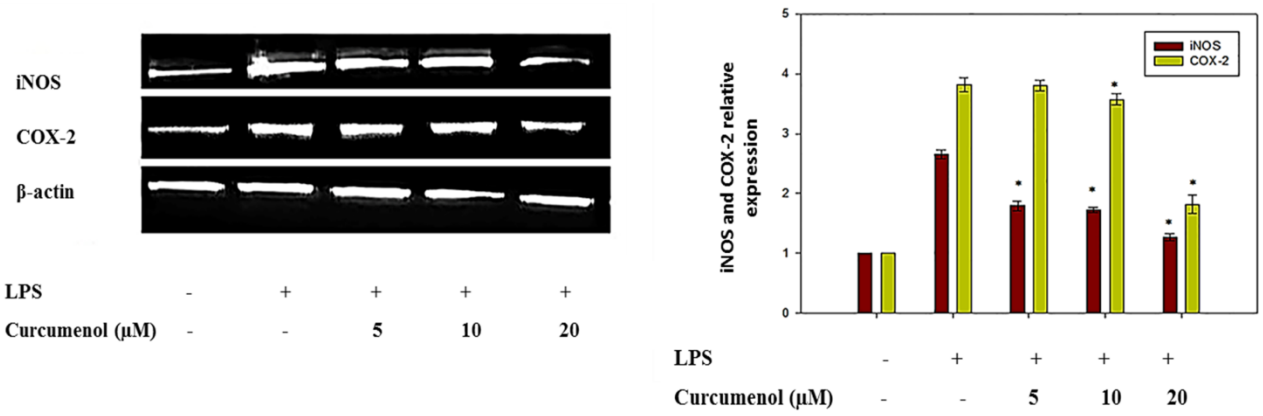

Figure 2. Effects of curcumenol on LPS-induced iNOS and COX-2 production in BV-2 cells. BV-2 cells were pretreated with curcumenol (5-20 μM) for 2 h followed by LPS (0.4 μg/mL) for 24 h. β-actin was used as housekeeping protein. The data are presented as the means ± SE from at least 3 independent experiments. *P < 0.05, lower compared with the LPS-treated group.

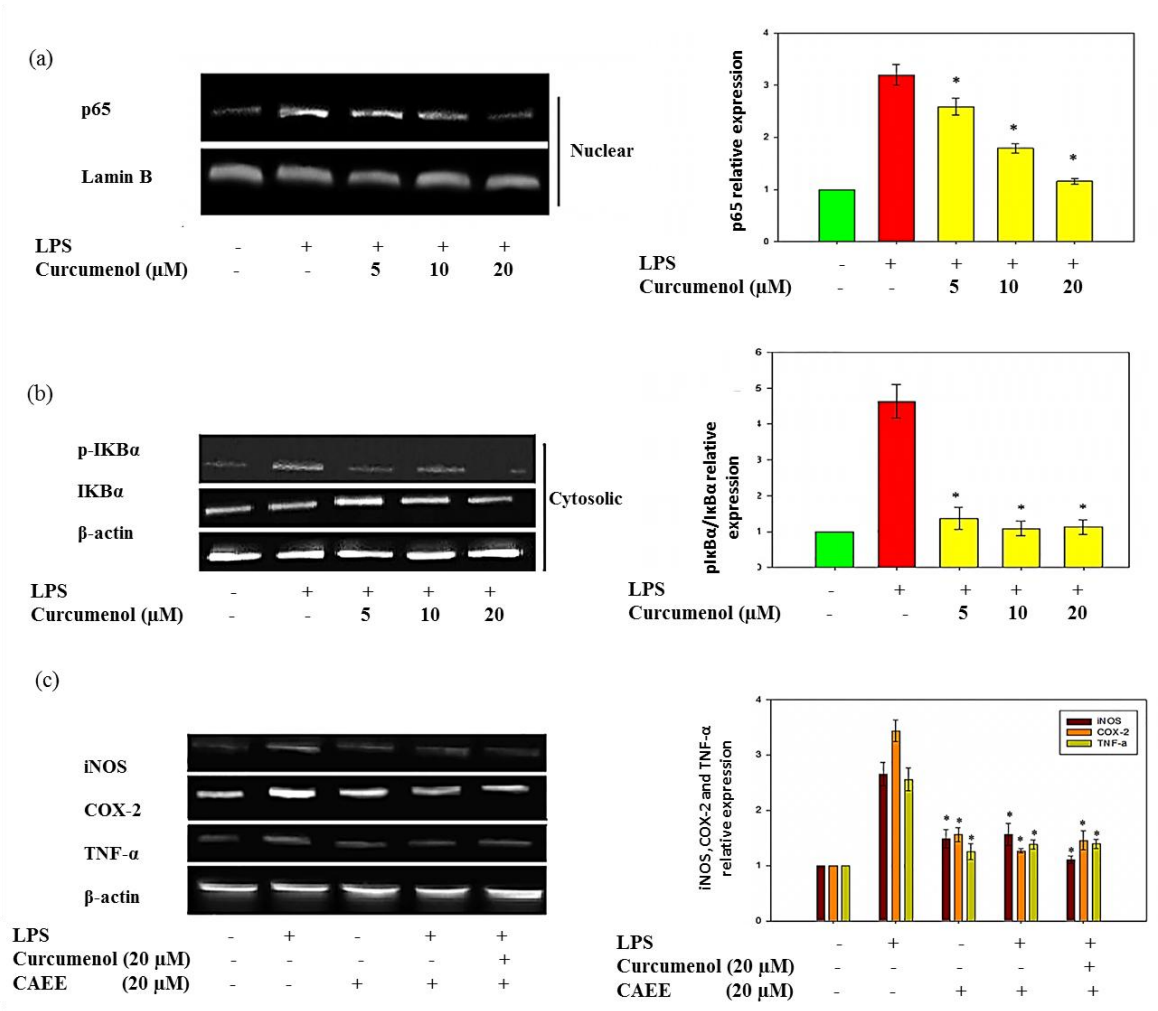

Figure 3. Effects of curcumenol on LPS-induced (a) translocation of p65 and (b) phosphorylation of IκBα. (c) Regulatory effects of the NF-κB inhibitor (CAEE) on the LPS-induced iNOS, COX 2 and TNF-α expressions. BV-2 cells were treated with or without CAEE (10 μM) for 1 h followed by curcumenol (20 μM) for 2 h before stimulation with LPS (0.4 μg/mL) for 12-24h. Relative protein expressions were analyzed by Western blot analysis. β-actin and Lamin B were used as housekeeping proteins. The data are expressed as the means ± SE from at least 3 independent experiments. *P < 0.05, compared with the LPS-treated group

(Figure 4b), and the nuclear translocation of the NF-κB p65 subunit (Figure 4c), suggesting that curcumenol can suppress the LPS-induced NF-κB activity by inhibiting the phosphorylation of Akt.

Curcumenol mediated inhibition of LPS-induced p38 MAPK pathway

Along with NF-κB, MAPKs have been shown to play an important role in signaling pathways that regulate pro-inflammatory cytokines, iNOS, and COX-2 expression in microglia cell.[28, 29] Thus, the effect of curcumenol on the production of phosphorylated MAPKs was determined using western blot analysis. LPS stimulation resulted in a significant increase of the phosphorylated JNK, ERK and p38 MAPKs in LPS-treated cells compared with the untreated control cells. However, pretreatment with curcumenol in BV-2 cells significantly reduced the phosphorylation of p38 MAPK but not the JNK and ERK phosphorylation in LPS-induced BV-2 cells (Figure 5a, b, c, d). To investigate the molecular mechanism underlying the curcumenol-mediated inhibition of iNOS and COX-2 expression, western blot analysis was performed to quantify the p38 MAPK phosphorylation. Interestingly, a specific p38 MAPK inhibitor, SB 202190, caused a significant decrease in the LPS-induced expression of iNOS, COX-2 and TNF-α expression (Figure 5e). These findings suggested that the inhibition of LPS-induced expression of iNOS, COX-2 and TNF-α by curcumenol is at least partially dependent on the p38 MAPK pathway. Taken together, these results indicated that inhibitory effects of curcumenol on pro-inflammatory mediators and cytokines were mediated by inhibition of NF-κB and the MAPK signaling pathway in LPS-stimulated microglia. Therefore, inhibitors of these inflammatory molecules have been considered as potential anti-inflammatory drug for the alleviation of neurodegenerative diseases caused by the activation of microglia. In the present study, we demonstrated that curcumenol regulates the neuroinflammatory events in LPS-stimulated BV-2 microglial cells in multiple aspects

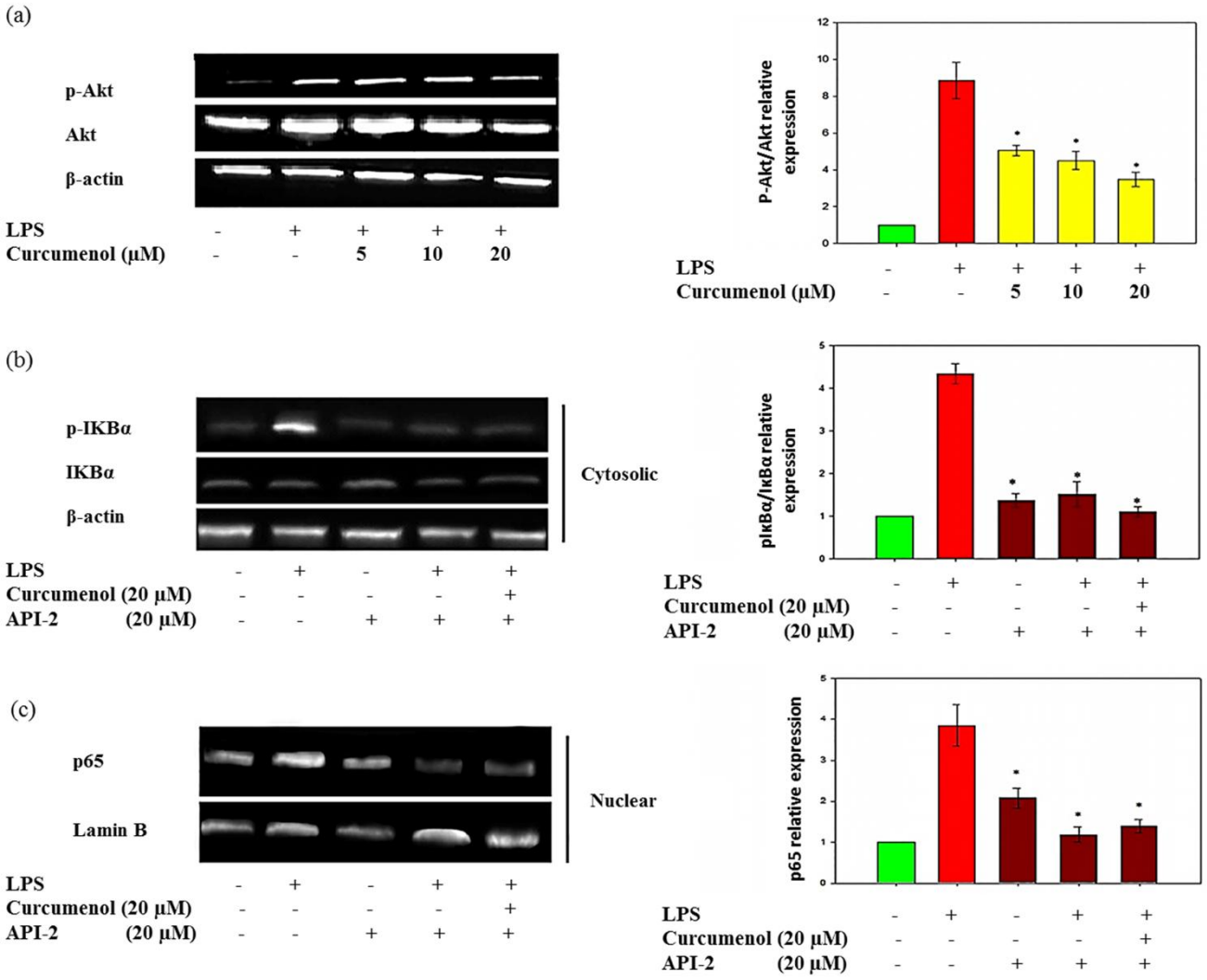

Figure 4. Effects curcumenol on LPS-induced (a) phosphorylation of Akt (b) Akt inhibitor (API-2) on LPS-induced phosphorylation of IκBα production and (c) phosphorylation of the NF-κB p65 subunit production in BV-2 cells. BV-2 cells were treated with API-2 (15 μM) for 1 h followed by curcumenol (20 μM) for 2 h before stimulation with LPS (0.4 μg/mL) for 12h. Relative protein expressions were analyzed by Western blot analysis. β-actin and Lamin B were used as housekeeping proteins. The data are expressed as the means ± SE from at least 3 independent experiments. *$P < 0.05$, compared with the LPS-treated group.

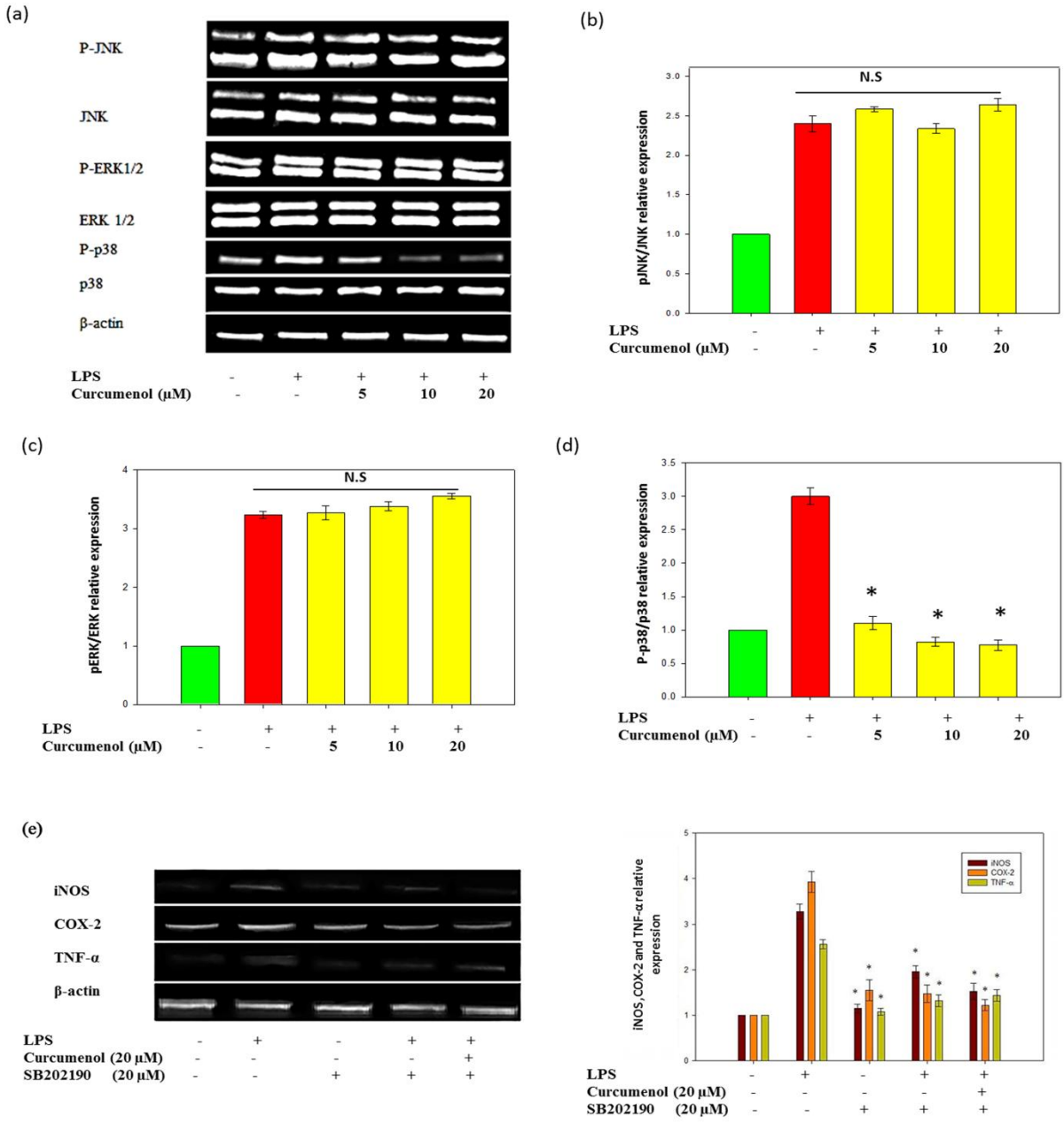

Figure 5. Effects of curcumenol on LPS-induced (a) phosphorylation of MAPKs (b) phosphorylation of JNK (c) phosphorylation of ERK (d) phosphorylation of p38 and (e) regulatory effects of p38 MAPK inhibitor (SB 202190) on the LPS-induced iNOS, COX -2 and TNF-α expressions in BV-2 cells. BV-2 cells were pretreated with or without SB 202190 (10 μM) for 1 h followed by curcumenol (5-20 μM) for 2 h before stimulation with LPS (0.4 μg/mL) for 12-24h. Cell lysates were extracted, and relative protein expression was analyzed b Western blot. β-actin was used as housekeeping protein. The data are presented as the means ± SE from at least 3 independent experiments. *$P < 0.05$, compared with the LPS treated group.

DISCUSSION

Curcumenol treatment of LPS-stimulated BV-2 cells has resulted in significant attenuation of the expression of iNOS and COX-2 proteins accompanied by the inhibition of the release of the LPS-induced pro-inflammatory mediators (NO) and cytokines (including TNF-α, IL-6). These inhibitory effects were related to the suppression of Akt-mediated NF-κB activation and MAPK signaling pathways (Figure 6). Therefore, the inhibition of pro-inflammatory molecules by curcumenol, as demonstrated in this study, could be beneficial in the treatment of neurodegenerative diseases.

Microglia are activated by the broad spectrum of stimuli such as lipopolysaccharide (LPS), interferon-γ, or β-amyloid.[30, 31] LPS is an endotoxin consisting of a lipid-carbohydrate component from the outer membrane of gram-negative bacteria, such as E. coli and is a common toxin used to investigate the impact of inflammation on neuronal death and microglial cells[32]. In the present study, LPS was used to

stimulate microglia as this is the most widely used and characterized method as LPS mimics many inflammatory effects implicated in the cells for inducing the release of pro-inflammatory molecules, and the reduction of LPS stimulated pro-inflammatory molecule release effectively represents the benchmark procedure for evaluating the anti-inflammatory potency of putative anti-inflammatory compounds.[33]

Several studies have reported that iNOS and COX-2 are induced in various types of CNS diseases.[34, 35] NO is a major inflammatory mediator and excessive NO production occurs in both acute and chronic inflammation.[36] Furthermore, NO and iNOS levels are reportedly corresponding to the degree of inflammation. Similarly, another well-known inflammatory mediator, PGE2, which is generated by COX-2 from arachidonic acid, contributes to the evolution of many chronic inflammatory diseases.[37] Thus, inhibition of the production of these mediators has been targeted for therapeutic anti-inflammatory drugs. The present findings demonstrated that treatment of LPS-stimulated BV-2 cells with curcumenol significantly inhibited the production of NO. In connection with the inhibitory effects on NO, we determined its correlation with iNOS expression in LPS-stimulated BV-2 cells. As a result, iNOS protein expression was suppressed by curcumenol treatment at 5, 10 and 20 μM. Additionally, pretreatment with curcumenol attenuated the expression of COX-2 protein in LPS-stimulated BV-2 cells. These results suggested that a significant decrease in NO release by curcumenol is associated with suppression of the upstream iNOS gene expression. Therefore, any substance that can attenuate the expression of iNOS and COX-2 proteins could be beneficial for preventing and delaying the progression of neurodegenerative disease.

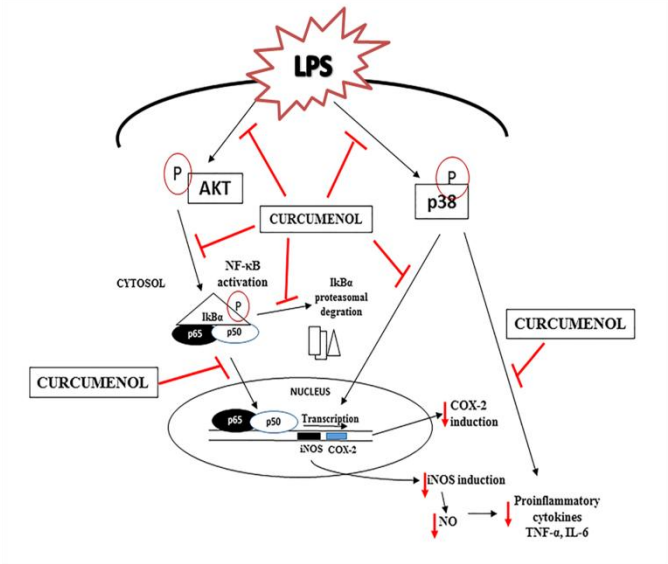

Figure 6. Proposed schematic mechanisms of curcumenol in inhibiting inflammation in BV-2 microglial cells. Curcumenol suppresses the LPS-induced production of pro-inflammatory cytokines and proteins via attenuation of Akt-mediated NF-κB activation and p38 MAPK signaling pathway.

Pro-inflammatory cytokines, including TNF-α, IL-1β and IL-6 are proteins known to regulate immunity and inflammation. Activated microglia produce increased levels of these pro-inflammatory cytokines, which play central roles in inflammatory neuronal damage.[38] Overproduction of these pro-inflammatory cytokines, in turn, activates microglia and lead to neuronal cell deaths, resulting in the pathogenesis of several neurodegenerative disorders.[39] Thus, naturally derived compounds that are able to inhibit the production of pro-inflammatory cytokines and mediators may potentially be alternative anti-inflammatory agents. The inhibitory effects of phytochemicals on the production of pro-inflammatory cytokines have been intensively studied for developing anti-inflammatory agents in preventing inflammatory diseases.[10, 40] In the present study, we have demonstrated that curcumenol remarkably suppressed the production of TNF-α and IL-6 in LPS-stimulated BV-2 cells (Figure 1d). Thus, the present findings may further support the potential of curcumenol as a neuroprotective agent by reducing inflammation.

NF-κB has been extensively reported to play a critical role in the regulation of inflammatory responses.[41, 42] The molecular mechanisms of NF-κB activation have been well studied, and they involve the activation of a cascade of cytoplasmic proteins and the nuclear translocation of the NF-κB p65 subunit.[43] Inactive NF-κB dimers are sequestered in the cytoplasm in association with IκB proteins. The phosphorylation of IκB by IκB kinases targets it for ubiquitination and its subsequent proteasomal degradation. This leads to the release and translocation of NF-κB dimers to the nucleus. In the nucleus, NF-κB binds to the specific DNA sequence in the promoter region of target genes and modulates their transcription. In line with this notion, we found that curcumenol suppressed NF-κB activation by inhibiting LPS-induced IκBα phosphorylation and degradation, thus blocking the nuclear translocation of the p65 subunit. NF-κB is a key transcription factor that is triggered by several cellular signal transduction pathways in association with the expression of pro-inflammatory cytokines and enzymes, such as iNOS, COX-2, IL-6 and TNF-α, together with the regulation of cell survival.[44] In support of these findings, we observed a marked increase in TNF-α, iNOS and COX-2 protein expression concomitantly with the activation of the NF-κB pathway in LPS-stimulated BV-2 microglia. Curcumenol, however, downregulates the expression levels of iNOS and COX-2, suggesting that curcumenol suppresses the expression of pro-inflammatory mediators by attenuating the NF-κB activation in LPS-stimulated microglia. Further, a specific NF-κB inhibitor, ethyl 3,4-dihydroxycinnamate, was shown to inhibit the LPS-induced expression of iNOS and COX-2 proteins, indicating that NF-κB is the primary effector molecule involved in the regulation of iNOS and COX-2 in curcumenol-treated BV-2 cells.

Additionally, LPS-induced NF-κB activation has been well reported to be directly regulated via the phosphorylation of Akt, which is the primary upstream molecule of NF-κB.[45] The involvement of the Akt pathway has also been shown in the expression of inflammatory mediators in microglia through

activation of NF-κB and IκB degradation in microglia.[46] Akt has been shown to mediate the degradation of IκB through the activation of the IκB kinase and phosphorylation of IκB, which eventually leads to the activation of NF-κB. Therefore, blocking NF-κB transcriptional activity and Akt activation via phosphorylation have been identified as an important target for the treatment of inflammatory diseases. Thus, Akt activation in BV-2 cells could be linked to LPS-induced NF-κB activation resulting in increased inflammatory cytokine release. In the present study, we found that curcumenol treatment of BV-2 cells significantly attenuated the phosphorylation and degradation of IκB in the cytosol. This consequently inhibited the translocation of the NF-κB p65 subunit, which is normally translocated to the nucleus after LPS stimulation. Concurrently, the increase in phosphorylation of Akt normally observed following exposure to LPS was also markedly inhibited by curcumenol. To further substantiate the link between the Akt signaling pathway and the subsequent inactivation of NF-κB in BV-2 cells, we demonstrated that LPS-induced NF-κB translocation activity was tightly regulated by a specific Akt inhibitor, API-2, consistent with the effects of curcumenol. Cumulatively, these data have indicated that curcumenol ameliorates the LPS-induced inflammatory response by inhibiting the Akt signaling pathway and subsequently inactivating NF-κB in BV-2 cells.

In addition to NF-κB, the MAPK pathway is the other major signaling pathway involved in neuroinflammation, especially the p38 pathway.[47] MAPKs are critical regulators of pro-inflammatory cytokines during inflammation and play an important role in initiating and sustaining inflammatory reactions.[48] Upon activation, MAPKs modulate the functional responses of cells through the phosphorylation of transcription factors and the activation of other kinases.[49] The MAPKs, such as p38, ERK, and JNK, are also known to regulate inflammatory protein expression, including iNOS, COX-2, TNF-α and cytokines, via control of NF-κB activation in microglial cells[10, 50], indicating that MAPKs function as important targets for anti-inflammatory molecules. The current findings indicated that curcumenol treatment significantly inhibited LPS-induced phosphorylation of p38 MAPK but not JNK and ERK. Furthermore, the current results also demonstrated that suppression of p38 phosphorylation decreased production of iNOS, COX-2 and TNF-α in LPS-stimulated BV-2 cells. In accordance with previous reports, the abrogation of iNOS, COX-2 and TNF-α production by curcumenol in LPS-stimulated BV-2 cells may be attributed to its effect in blocking p38 phosphorylation in inflammatory conditions. To further substantiate the involvement of MAPK pathway, we demonstrated that a specific p38 inhibitor, SB202190, significantly suppressed LPS-induced pro-inflammatory protein expression in BV-2 cells. These findings indicated that curcumenol may block the inflammatory mediator production by inhibiting p38 MAPK signaling pathway.

Conclusions

The present findings demonstrated that curcumenol, a compound isolated from *Curcuma zedoaria*, suppressed LPS-induced microglial inflammatory responses by diminishing pro-inflammatory mediators and cytokines, including NO, IL-6, and TNF-α. Curcumenol also suppressed the expression of the regulatory proteins, iNOS and COX-2, in LPS-stimulated BV-2 microglial cells by inhibiting Akt-dependent NF-κB activation and suppression of p38 MAPK signaling pathway. Taken together, these finding provide evidence that curcumenol possesses potential anti-inflammatory activities.

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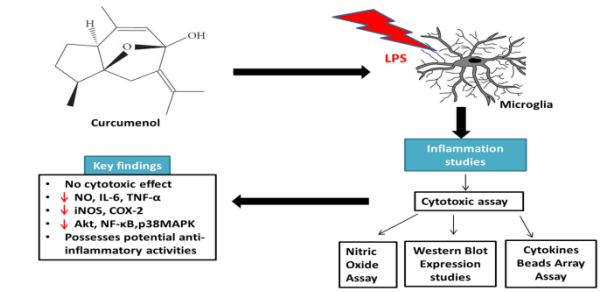
Table of content



Curcumenol attenuates the inflammatory responses induced by LPS in BV-2 microglial cells.