Acta Pharmacol Sin 2007 Oct; 28 (10): 1645–1651

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Full-length article

Curcumin attenuates the release of pro-inflammatory cytokines in lipopolysaccharide-stimulated BV2 microglia[1]

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Key words

curcumin; inducible nitric oxide synthase; cyclooxygenase-2; pro-inflammatory cytokines; NF-κB

[1]This study was supported by a grant of the Korea Health 21 R&D Project, Ministry of Health and Welfare, Korea (No A062406).
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Received 2007-03-25
Accepted 2007-04-23

doi: 10.1111/j.1745-7254.2007.00651.x

Abstract

Aim: Pro-inflammatory mediators, such as prostaglandin E_2 (PGE_2) and nitric oxide (NO), and pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, and TNF-α, play pivotal roles in brain injuries. The anti-inflammatory properties are known to be associated with significant reductions in pro-inflammatory mediators in brain injuries. In the present study we investigate whether the effects of curcumin on the production of pro-inflammatory mediators in lipopolysaccharide (LPS)-stimulated BV2 microglia. **Methods:** Curcumin were administered and their effects on LPS-induced pro-inflammatory mediators were monitored by Western blotting and RT-PCR. **Result:** Curcumin significantly inhibited the release of NO, PGE_2, and pro-inflammatory cytokines in a dose-dependent manner. Curcumin also attenuated the expressions of inducible NO synthase and cyclooxygenase-2 mRNA and protein levels. Moreover, curcumin suppressed NF-κB activation via the translocation of p65 into the nucleus. Our data also indicate that curcumin exerts anti-inflammatory properties by suppressing the transcription of proinflammatory cytokine genes through the NF-κB signaling pathway. **Conclusion:** Anti-inflammatory properties of curcumin may be useful for treating the inflammatory and deleterious effects of microglial activation in response to LPS stimulation.

Introduction

Microglia have been suggested to play a role in host defense and tissue repair in the central nervous system (CNS)[1]. The activation of microglia is also observed in brain injuries and is induced after exposure to lipopolysaccharides (LPS), interferon-γ, or β-amyloid[2,3]. Once chronically activated, microglia can produce a variety of proinflammatory mediators and potentially neurotoxic compounds. These mediators include proinflammatory cytokines [eg, interleukin (IL)-1β, IL-6, and TNF-α], reactive oxygen, nitric oxide (NO) and prostaglandin E_2 (PGE_2), which are thought to be responsible for brain injuries and diseases including trauma, ischemia, Alzheimer's disease and neural death[4]. Therefore, it is not surprising that LPS was reported to increase the expressions of NO, PGE_2, and pro-inflammatory cytokines in microglial cells after CNS injury[5]. Among these mediators, PGE_2 and NO are the products of the inducible isoforms of cyclooxygenase (COX)-2 and inducible NO synthase (iNOS) enzymes, respectively[6]. The overexpres-sion of pro-inflammatory cytokines has emerged as an important determinant of the cytotoxicity associated with inflammation in pathologies, such as Alzheimer's disease, cerebral ischemia and multiple sclerosis[7]. Thus, the inhibition of these pro-inflammatory mediators offers a potentially effective therapeutic approach to mitigate the progression of neurodegenerative diseases.

Curcumin, the active component in turmeric, has been shown to possess anti-inflammatory, antioxidant, and anti-tumor activities[8–10]. Our previous study has also shown that curcumin significantly ameliorates LPS-induced functional maturation of dendritic cells. This function involves the inhibition of NF-κB inhibition by blocking IκB degradation[11]. Recent evidence suggests that the anti-inflam-

matory effects of curcumin in BV2 microglia are closely related to the suppression of COX-2 expression through the inhibition of the NF-κB and AP-1 pathways[12]. Other reports have also ascribed curcumin's neuroprotective effect against ischemia-induced neuronal damage to its antioxidant capacity, which would reduce oxidative stress and inhibit the activation of signaling cascades that lead to apoptotic cell death[13]. Curcumin has been strongly implicated in the expression of NF-κB, however the expression of NF-κB-related genes and proteins is largely unknown in microglia.

In this study, we investigated curcumin's effects on the regulation of iNOS, NO, COX-2, PGE_2, and pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) in LPS-stimulated BV2 microglia. Curcumin significantly inhibited the inflammatory mediators in LPS-stimulated BV2 microglia. Moreover, the biological effects of curcumin may involve the inhibition of the NF-κB pathway. The present study's results may provide critical information that will contribute to the therapeutic use of curcumin in the prevention of microglial activation.

Materials and methods

Materials Curcumin, LPS, Tween-20 and *p*-nitrophenyl phosphate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St Louis, MO, USA). Rabbit anti-human iNOS, COX-2, p65, and nucleolin polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody against β-actin was also from Sigma. Peroxidase-labeled goat antirabbit immunoglobulin was purchased from Amersham (Arlington Heights, IL, USA). Dulbecco's modified Eagle's medium (DMEM)–containing *L*-arginine (200 mg/L)–fetal bovine serum (FBS), and other tissue culture reagents were purchased from Gibco (Grand Island, NY, USA). Other chemicals were purchased from Sigma.

Cell culture The BV2, immortalized murine microglial cell line was provided by Dr E J CHOI (Korea University, South Korea). The BV2 cells were constructed by infecting primary microglia with a v-raf/v-myc oncogene-carrying retrovirus (J2). The murine BV2 microglia were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin, and maintained in a humidified incubator with 5% CO_2. In all the experiments, the cells were pretreated for 60 min in the presence of the indicated concentrations of curcumin before the addition of LPS (0.5 μg/mL) in serum-free DMEM.

Cytotoxicity assay Cell viability was evaluated by the MTT reduction assay. In brief, cells (5×10^5 cells/mL) were seeded and treated with various reagents for the indicated time periods. After various treatments, the medium was removed and the cells were incubated with 0.5 mg/mL of MTT solution. After incubation for 3 h at 37 °C and in 5% CO_2, the supernatant was removed and the formation of formazan crystals was measured at 540 nm with a microplate reader.

Isolation of total RNA and RT-PCR Total RNA was isolated according to the manufacturer's instructions. RNA (3 μg) was reverse-transcribed using M-MLV reverse transcriptase (Promega, Madison, WI, USA). Single-stranded cDNA was amplified by PCR with primers for iNOS, COX-2, GAPDH, IL-1β, IL-6, and TNF-α, respectively. The primer sequences for these cDNA have been described in previous studies[14,15]. The following PCR conditions were applied. GAPDH: 18 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, and extension at 72 °C for 30 s; iNOS, COX-2, IL-1β, IL-6, and TNF-α: 25 cycles of denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, and extension at 72 °C for 30 s. The GAPDH was used as an internal control to evaluate the relative expressions of COX-2 and iNOS, IL-1β, IL-6, and TNF-α.

Western blot analysis The cells were washed with phosphate-buffered solution three times, incubated at 4 °C, and lysed for 30 min in lysis buffer (1% Triton X-100, 1% deoxycholate, and 0.1% NaN_3). The lysates were then centrifuged at 12 000×*g* at 4 °C. The supernatants were collected for further analysis. Equal amounts of protein were separated electrophoretically using 10% SDS-PAGE and then the gel was transferred to 0.45 μm polyvinylidene fluoride (PVDF: Millipore, Bedford, MA, USA). The membranes were soaked in blocking buffer (5% skimmed milk), incubated overnight with primary antibodies, incubated with horseradish peroxidase conjugated antibodies, and then detected by the enhanced chemiluminescence detection system according to the recommended procedure (Amersham, USA). In a parallel experiment, the nuclear protein was prepared using lysis buffer [10 mmol/L Tris-Cl (pH 7.4), 3 mmol/L $CaCl_2$, 2 mmol/L $MgCl_2$, 0.5 mmol/L dithiothreitol, 0.5 mmol/L phenylmethylsulfonyl fluoride, and protease inhibitors] for 15 min at 4 °C. After centrifugation and washing of the nuclei pellet, ice-cold hypertonic extraction buffer [20 mmol/L HEPES (pH 7.1), 25% glycerol, 420 mmol/L NaCl, 0.2 mmol/L EDTA, 1.5 mmol/L $MgCl_2$, 0.5 mmol/L dithiothreitol, and protease inhibitors] was added and the samples were incubated at 4 °C for 30 min with constant shaking. The nuclear protein extracts were isolated by centrifugation at 12 000×*g* for 30 min.

Nitrite assays (Griess assay) The NO levels in the culture supernatants were measured by a Griess reaction. The

cells were seeded in 24-well plates (5×10^5 cells/mL) and stimulated for 24 h with LPS. After LPS stimulation, 100 μL conditioned culture medium from each sample was mixed with the same volume of the Griess reagent (1% sulfanilamide/0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride/2.5% H_3PO_4). The NO concentration was determined by measuring the absorbance at 540 nm with a 96-well microplate spectrophotometer (Labsystems Inc, Franklin, MA, USA). The nitrite concentration was calculated with reference to a standard curve of sodium nitrite generated by known concentrations.

Measurement of PGE_2 TheBV2 cells were subcultured in 6-well plates (5×10^5 cells /mL) and incubated with curcumin (0, 5, 10, and 20 μmol/L, respectively) in the presence or absence of LPS (0.5 μg/mL) for 24 h. One hundred microliters of culture-medium supernatant was collected for the determination of the PGE_2 concentration by ELISA kit (Cayman, MI, USA).

ELISA The BV2 cells were subcultured in 6-well plates (5×10^5 cells /mL) and incubated with curcumin (0, 5, 10, and 20 μmol/L) in the presence or absence of LPS (0.5 μg/mL) for 24 h. The cell-free supernatant was collected at 24 h stimulation with LPS, and IL-1β, IL-6, and TNF-α were measured by ELISA kits (R&D, Minneapolis, MN, USA) according to the manufacturer's instructions. The absorbance at 450 nm was determined using a microplate reader. The minimum detection levels were as follows: 10 ng/mL for IL-1β, 15.6 ng/mL for IL-6, and 15.6 pg/mL for TNF-α.

NF-κB luciferase assay A total of 1×10^6 BV2 cells were transfected with 2 μg NF-κB-luciferase reporter plasmids (BD Sciences, San Jose, CA, USA) using Lipofectamine according to the manufacturer's protocol (Gibco, USA). After incubating with DNA–Lipofectamine mixtures, the cells were pre-incubated in the presence or absence of curcumin before being stimulated with LPS for 6 h. After stimulation, the cells were washed, lysed, and assayed for luciferase activity according to the manufacturer's instructions (Luciferase Assay System kit; Promega) with a microplate luminometer LB96V (Perkin–Elmer, Foster City, CA, USA).

Statistical analysis Data were presented as the mean±SD of at least 3 separate experiments. Comparisons between 2 groups were analyzed using Student's *t*-test. *P*-values less than 0.05 were considered to be statistically significant.

Results

Curcumin decreases NO and PGE_2 production in LPS-stimulated BV2 microglia in a dose-dependent manner To evaluate the anti-inflammatory effects of curcumin on LPS-stimulated BV2 microglia, cell culture media were collected, and NO and PGE_2 levels were measured in LPS-stimulated BV2 microglia. The amount of NO significantly increased from 7.5±3.3 μmol/L to 33.9 ± 4.2 μmol/L 24 h after LPS stimulation (Figure 1). Curcumin suppressed NO production in LPS-stimulated BV2 microglia to 21.2±3.4 μmol/L and 12.3±3.8 μmol/L at a concentration of 10 and 20 μmol/L, respectively. To investigate whether curcumin inhibits PGE_2 production, the cells were also pretreated with curcumin for 1 h and then stimulated with 0.5 μg/mL LPS for 24 h. While the unstimulated cells produced low levels of PGE_2, the LPS-stimulated cells exhibited an 8-fold increase in their PGE_2 expression (Figure 1B). Curcumin inhibited LPS-mediated PGE_2 production in a dose-dependent manner. These

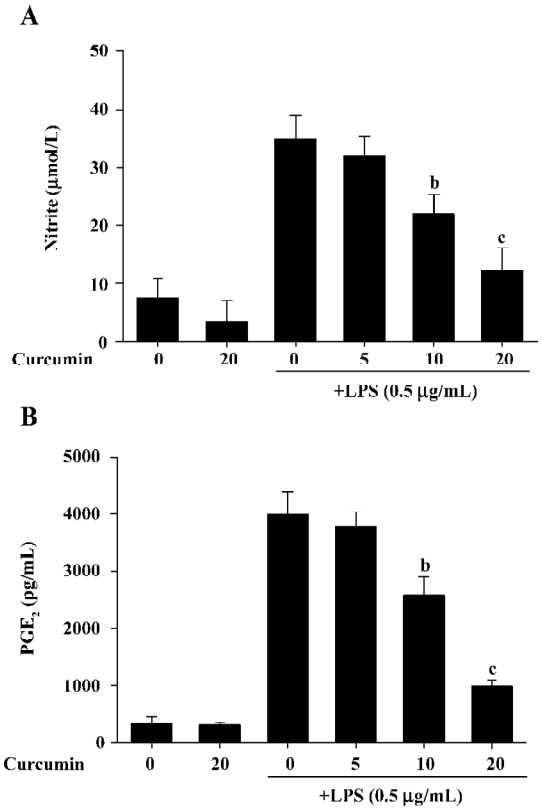

Figure 1. Effects of curcumin in LPS-induced NO (A) and PGE_2 (B) production in BV2 microglia. (A) cells were treated with LPS (0.5 μg/mL) in the absence or presence of curcumin at different concentrations (0, 5, 10, and 20 μmol/L, respectively) for 24 h. Control (0) values were obtained in the absence of LPS or curcumin. Amounts of NO were determined using the Griess reagent and a standard curve created using $NaNO_2$ in culture medium. (B) levels of PGE_2 were detected using a specific enzyme immunoassay according to the manufacturer's instructions. Each value indicates the mean±SD and is representative of results obtained from 3 independent experiments. $^bP<0.05$, $^cP<0.01$ *vs* cells treated with LPS in the absence of curcumin.

results indicate that curcumin significantly inhibited NO and PGE_2 production in BV2 microglia following LPS stimulation.

It is possible that the observed inhibition of NO and PGE_2 production was due to curcumin's cytotoxicity in LPS-stimulated BV2 microglia. In order to exclude this possibility, we performed MTT assays in BV2 microglia treated with curcumin for 24 h. The results demonstrated that curcumin treatment did not increase the MTT activity, even at a concentration of 20 μmol/L (Figure 2). These data confirmed that the inhibition of NO and PGE_2 production in LPS-stimulated BV2 microglia was not due to a cytotoxic action of curcumin.

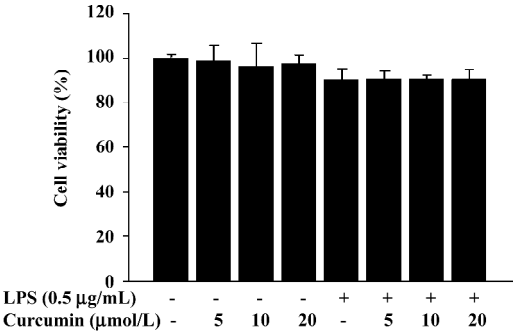

Figure 2. Effect of curcumin on the cell viability of BV2 microglia. Cells were treated with curcumin in the presence or absence of LPS (0.5 μg/mL) for 24 h. Cell viability was assessed by MTT reduction assays and the results were expressed as percentage of surviving cells over control cells (no addition of curcumin). Each value indicates the mean±SD and is representative of results obtained from 3 independent experiments. $^bP<0.05$ *vs* cells treated with LPS in the absence of curcumin.

Curcumin attenuates the expressions of LPS-stimulated iNOS and the COX-2 protein and mRNA We further examined whether curcumin's inhibitory effect on NO and PGE_2 production was associated with decreased iNOS and COX-2 expressions in LPS-stimulated BV2 microglia. A Western blot analysis revealed that iNOS and COX-2 protein levels were undetectable in unstimulated BV2 microglia. LPS treatment significantly increased iNOS and COX-2 protein levels; however, these expressions were markedly attenuated in BV2 microglia pretreated with curcumin (Figure 3A). Consistent with these results, the RT-PCR analysis showed that iNOS and COX-2 mRNA transcription also decreased following curcumin pretreatment (Figure 3B). These results showed that curcumin significantly suppressed the expressions of iNOS and COX-2, which was upregulated by LPS treatment through transcriptional inhibition.

Curcumin inhibits the production of pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α at transcriptional

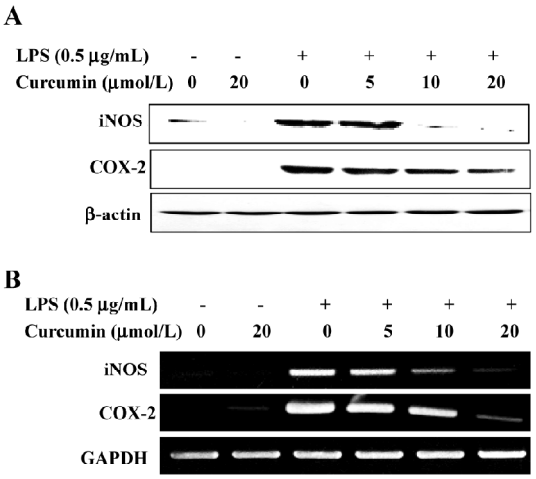

Figure 3. Effects of curcumin on LPS-induced iNOS and COX-2 protein (A) and mRNA (B) expressions in BV2 microglia. (A) lysates were prepared from untreated control cells and cells treated for 6 h with LPS (0.5 μg/mL) alone or with different concentrations (0, 5, 10, and 20 μmol/L, respectively) of curcumin. All lanes contained 30 μg/mL of total protein. A Western blot representative of 3 separate experiments is shown. (B) Total RNA was prepared for the RT-PCR analysis of iNOS and COX-2 gene expressions from LPS-stimulated BV2 microglia. β-Actin and GAPDH were used as internal controls for the Western blot analysis and RT-PCR assays, respectively. Experiment was repeated 3 times and similar results were obtained.

levels We next investigated curcumin's effect on the expression of pro-inflammatory cytokines, such as IL-1β, IL-6 and TNF-α. BV2 microglia were incubated with curcumin (0, 5, 10, and 20 μmol/L) in the presence or absence of LPS (0.5 μg/mL). After treatment with LPS for 24 h, the levels of the cytokines in the culture media were measured by ELISA. Curcumin had no effect on the production of IL-1β, IL-6, and TNF-α in normal BV2 microglia. However, the levels of IL-1β, IL-6, and TNF-α levels increased in the culture media containing LPS-stimulated BV2 microglia (Figure 4A). Pretreatment with curcumin resulted in a dose-dependent decrease in cytokine production. We next determined whether curcumin could regulate the transcription of IL-1β, IL-6, and TNF-α mRNA. IL-1β, IL-6, and TNF-α mRNA were not detectable under normal culture conditions; however, RT–PCR assays revealed that these cytokines were significantly upregulated following stimulation with LPS (0.5 μg/mL) for 6 h (Figure 4B). Pretreatment with curcumin for 1 h attenuated the upregulation of these cytokines in a dose-dependent manner. These results suggested that curcumin acted principally by preventing the accumulation of proinflammatory cytokines by inhibiting the transcription of these genes.

Inhibitory effects of curcumin are mediated by NF-κB

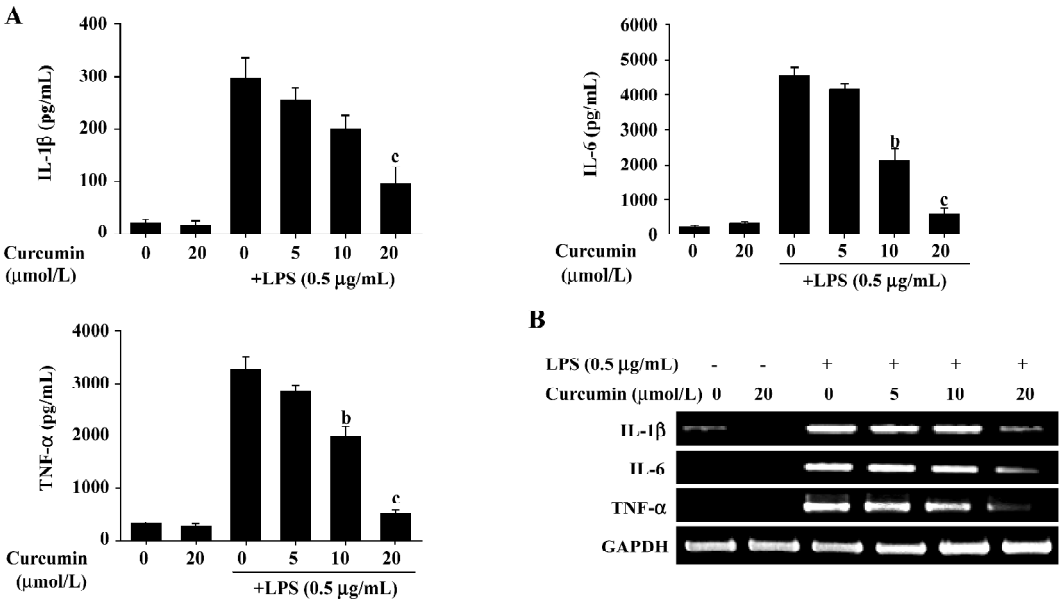

Figure 4. Effect of curcumin on proinflammatory cytokines in LPS-stimulated microglia. Cells were treated with the indicated doses of curcumin for 1 h before LPS treatment (0.5 μg/mL) and total RNA. Supernatants were isolated at 6 or 24 h after LPS treatment, respectively. (A) after incubation for 24 h, the supernatants were taken, and the amounts of IL-β, IL-6, and TNF-α were measured. (B) after incubation for 6 h, the levels of IL-1β, IL-6, and TNF-α mRNA were determined by RT–PCR. Each value indicates the mean±SD and is representative of results obtained from 3 independent experiments. $^bP<0.05$, $^cP<0.01$ *vs* cells treated with LPS in the absence of curcumin.

suppression in LPS-stimulated BV2 microglia Because the activation of NF-κB by LPS can induce the expression of proinflammatory mediators[16,17], we next investigated curcumin's effects on NF-κB activity using luciferase assays and Western blot analysis. BV2 microglia were pretreated with curcumin for 1 h and then stimulated with LPS for 6 h. The expression of a NF-κB reporter construct was measured by relative luciferase activity. As shown in Figure 5A, LPS significantly enhanced NF-κB activity. This activity was significantly inhibited following a 1 h pretreatment with 10 or 20 μmol/L curcumin. Curcumin's effect on LPS-induced NF-κB p65 translocation into the nucleus was also investigated. Significant NF-κB p65 proteins were localized within the nucleus at 15 and 30 min after LPS treatment (Figure 5B). Curcumin pretreatment significantly attenuated the observed nuclear translocation (Figure 5). These results indicated that curcumin's inhibition of NF-κB nuclear translocation may be the mechanism responsible for the suppression of NO, PGE_2, and pro-inflammatory cytokines in LPS-stimulated BV2 microglia.

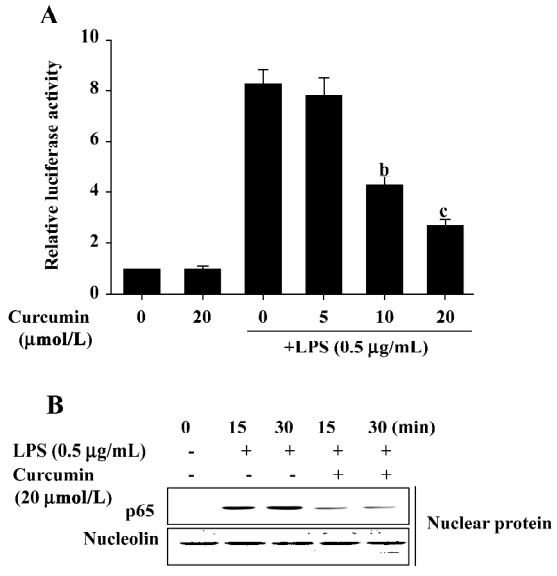

Figure 5. Effect of curcumin on NF-κB activity in LPS-stimulated BV2 microglia. (A) transfected cells were pretreated with curcumin at 0, 5, 10, and 20 μmol/L for 1 h and then stimulated with LPS for 6 h. NF-κB activity was expressed as relative luciferase activity. (B) cells were treated with the indicated doses of curcumin 1 h before LPS treatment (0.5 μg/mL) for the indicated times. Total nuclear protein was subjected to 10% SDS-PAGE followed by Western blotting using anti-NF-κB p65 antibody. Each value indicates the mean±SD and is representative of results obtained from 3 independent experiments. $^bP<0.05$, $^cP<0.01$ *vs* cells treated with LPS in the absence of curcumin.

Discussion

The present results showed that more than 10 μmol/L of curcumin significantly inhibited the production of NO and PGE_2 in LPS-stimulated BV2 microglia. These inhibitory

effects of curcumin were accompanied by the decreases in the expression of iNOS and COX-2 at the transcriptional levels. The magnitude of this inhibition was directly proportional to the concentration of curcumin added to the culture media. In addition, curcumin significantly attenuated the transcription of proinflammatory cytokines, such as IL-1β, IL-6, and TNF-α. Furthermore, this suppression was associated with a decrease in NF-κB transcriptional activity.

Several lines of evidence have shown that the expression of COX-2 and iNOS, the key enzymes for NO and PGE_2 production, is upregulated in activated glial cells[18]. Additionally, cytokines such as IL-1β, IL-6, and TNF-α, are known to promote pro-inflammatory responses, both *in vitro* and *in vivo*[19,20]. As previous studies have shown curcumin suppresses LPS-induced COX-2 expression in BV2 microlgial cells[12] and NO production in primary rat microglia[21]. Although previous data also have suggested that brain injuries stimulate the expression of pro-inflammatory cytokines, NO, and PGE_2 in microglia, curcumin's effects on LPS-stimulated microglia have yet to be elucidated. Like the previous data, the present study revealed that curcumin treatment significantly inhibited NO and PGE_2 production in LPS-stimulated BV2 microglia. Curcumin's inhibitory effects further attenuated the expression of iNOS and COX-2 mRNA/protein levels, indicating that curcumin influenced the transcriptional level. Our data first indicate that curcumin suppresses the production of pro-inflammatory cytokines, including IL-1β, IL-6, and TNF-α. These results indicated that approximately more than 10 μmol/L curcumin may be a promising candidate for mitigating LPS-stimulated microglial activation.

NF-κB is known to play a critical role in the regulation of cell survival genes and to coordinate the expression of pro-inflammatory enzymes and cytokines, such as iNOS, COX-2, IL-1β, IL-6, and TNF-α[22–24]. Furthermore, blocking the transcriptional activity of NF-κB in the microglial nucleus can suppress the expression of iNOS, COX-2, and pro-inflammatory cytokines[25,26]. Previous studies have revealed that curcumin inhibits NF-κB and AP-1, and reduces the production of iNOS, COX-2, and TNF-α in human cancer cells[27,28]. Because the expression of these pro-inflammatory mediators is known to be modulated by NF-κB, we used luciferase reporter assays and investigated p65 nuclear translocation assays to elucidate whether curcumin inhibits NF-κB activity by using p65 translocation activity. The present study demonstrated that curcumin's inhibition of NF-κB activation is responsible for the inhibition of pro-inflammatory mediators. The present study also indicated that the amount of p65 translocated in the nucleus was significantly attenuated in BV2 microglia by pretreatment with curcumin. Our findings suggested that curcumin's transcriptional downregulation of pro-inflammatory mediators was due to the inhibition of the NF-κB signaling pathway.

Some reports have shown that curcumin inhibits cytokine-mediated NF-κB activation by blocking IκB kinase activity in intestinal epithelial and mouse fibroblast cells and that it also attenuates phorbol ester-induced c-Jun/AP-1 activation[29–31]. Curcumin also suppresses Janus kinase-signal transducers and activator of transcription inflammatory signaling by activating Src homology 2 domaining tyrosine phosphatase 2 in brain microglia[32]. A recent study has shown that curcumin reduced oxidative damage and amyloid pathology in an Alzheimer's transgenic mouse[33,34]. Recent data also have suggested that curcumin may effectively modulate mitogen-activated protein kinases (MAPK) to regulate anti-inflammatory effects in stimulated microglia[21]. Therefore, future experiments are necessary to confirm whether curcumin tightly regulates the expression of other nuclear transcriptional factors and MAPK to induce anti-inflammatory effects in LPS-stimulated microglia.

In conclusion, the present study demonstrates that curcumin exhibits anti-inflammatory activity by suppressing the release of proinflammatory mediators in LPS-stimulated BV2 microglia. Curcumin significantly inhibited the release of NO, PGE2, IL-1β, IL-6 and TNF-α in a dose-dependent manner. Curcumin's anti-inflammatory properties are mediated by the downregulation of NF-κB in LPS-stimulated BV2 microglia. These findings enhance curcumin's importance as a compound for treating LPS-mediated sepsis syndrome and inflammatory disease.

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