

Anti-neuro-inflammatory Effects of the Bioactive Compound Capsaicin through the NF- κ B Signaling Pathway in LPS-Stimulated BV2 Microglial Cells

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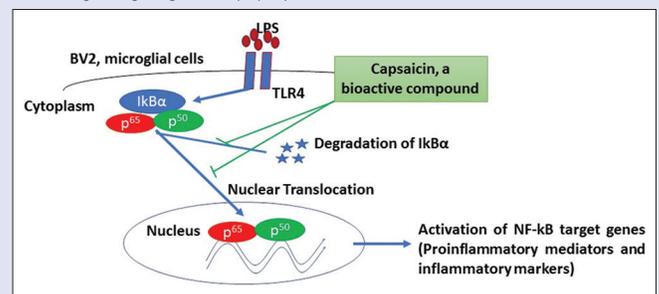
ABSTRACT

Background: Inflammation in the central nervous system, resulting from a loss of control involving a network of neuronal cells, is foremost contributors to the instigation and advancement of major neurodegenerative diseases. Therefore, therapeutic strategies should restore back to a well-controlled and finely-tuned balance of immune reactions, and protect neurons from inflammatory damage. **Objective:** The objective of this study is to evaluate the anti-neuroinflammatory potential of Capsaicin in lipopolysaccharide (LPS)-stimulated BV-2 microglial cells. **Materials and Methods:** In this present study, we selected Capsaicin and investigated through cell-based-assay systems through the various cellular techniques enzyme-linked immunosorbent, immunoblot and immunofluorescence assays to identify anti-inflammatory effects. **Results:** We found that capsaicin exhibited highly anti-inflammatory and neuroprotective effects in cell culture experiments, reduced nitric oxide, tumor necrosis factor- α , interleukin-1 β , and interleukin expression from activated BV-2 microglia cells dose-dependently. On the intracellular level, capsaicin inhibited I κ B-phosphorylation and subsequently nuclear Factor- κ B (NF- κ B)-translocation in microglia cells. Further, capsaicin blocked the protein expressions of inducible nitric oxide synthase and cyclooxygenase-2. Further, capsaicin inhibits the increased production of pro-inflammatory responses in LPS-stimulated BV-2 cells by suppressing NF- κ B activation. **Conclusion:** The significant inhibition of neuroinflammatory responses in stimulated microglial cells together indicate that capsaicin is a potential therapeutic agent and could possibly be used in the development of novel drug for the prevention and treatment of neuroinflammatory diseases.

Key words: Capsaicin, cyclooxygenase-2, natural products, neuroinflammation, nuclear factor- κ B molecules, pro-inflammatory cytokines

SUMMARY

- Capsaicin, a bioactive compound effectively inhibits the increased production of numerous pro-inflammatory mediators and suppresses the expression of NF- κ B signaling targets in lipopolysaccharide-stimulated BV-2 cells.



Abbreviations used: CNS: Central Nervous System; iNOS: Inducible nitric oxide synthase; COX-2: Cyclooxygenase; NF- κ B: Nuclear factor kappa-light-chain-enhancer of activated B cells; LPS: Lipopolysaccharide; PGE2: Prostaglandin E2; NO: Nitric oxide; IL6: Interleukin; IL-1 β -interleukin-1 β ; TNF- α : Tumor necrosis factor- α

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INTRODUCTION

Inflammation is an adaptive physiological response induced by exterior challenge or cellular injury that leads to the release of a complex array of inflammatory moderators to restore tissue structure and functions.^[1] Withal, protracted inflammation can be detrimental, contributing to the pathogenesis of many diseases such as cancer, diabetes, arthritis, and atherosclerosis.^[2] The term “neuroinflammation” has gained immense attention in recent years after it has been implicated in chronic unremitting neurodegenerative diseases such as Alzheimer’s, Parkinson’s disease, and multiple sclerosis.^[3] All the neurological afflictions from traumatic brain injuries and nervous system infections to chronic neurodegenerative diseases are implicated with neuroinflammations. This new understanding has come from rapid advances in the field of glial, immune cells resident of the central nervous system (CNS). In the midst of the discrete cell types associated with inflammation-mediated neurodegeneration, microglia have been implicated as vital components of the immunological insult to neurons.^[4]

Microglia occupies approximately 12% of cell population in the CNS, and it is defined as cerebral macrophages. They are brain-specific macrophages that provide healthy tissue, trophic support, and maintains homeostasis of the CNS.^[5] The brain can be insulted by a variety of factors, including inflammation, excitotoxicity, and oxidative stress. In the event of brain damage, microglia cells acting as a first-line defense in neuronal injury, undergo dramatic morphological changes

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from resting ramified cells to activated amoeboid microglia.^[6] Under these conditions, activated microglia secrete various neurotoxic and excess pro-inflammatory mediators including prostaglandin E2, nitric oxide (NO), interleukin-(IL-6), IL-1 β , tumor necrosis factor- α (TNF- α), and superoxide anions causing demyelination and subsequent neurodegeneration.^[7] Therefore, modulation of neuroinflammation corollary may delay or halt neurodegenerative disease progression before irreversible damage and clinical symptoms occur.

In general, nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin, ketoprofen, and cyclooxygenase (COX)-2 inhibitors are utilized to treat neurodegenerative diseases. Treatment with NSAIDs however, is accompanied by side effects such as dizziness, headache, gastrointestinal discomfort, and dyspepsia.^[8] Thus, this hitch heightens the interest in the rapid discovery of new therapeutic strategies targeting the neuroinflammation. In recent years, many medicine-based plants and their active constituents have been subjected to extensive research as alternative candidates for therapeutic purposes. Medicinal plants have a reputation for being safe, inexpensive, and readily available. In addition, it is well documented that their bioactive components and associated molecules possess neurotrophic and neuroprotective abilities, which can be valuable in averting microglial activation, successively ameliorating microglia-associated neurodegenerative and neuroinflammatory diseases.^[9,10]

Chili peppers (*Capsicum*), is one of the most commonly used spices in the world as additives in cooking because of their unique pungency, aroma, and color. Capsaicin (8-methyl-N-vanillyl-6-nonenamide), is the active compound and a type of alkaloid found primarily in hot chili pepper, which is responsible for their characteristic taste.^[11] Large consumption of capsaicin known to promote pain relief and these properties attracts the attention of researchers to study the pain-related events using capsaicin.^[12] Recent progress indicates that the headway of neuroinflammation which is accountable for engendering and nourishing the sensitization of nociceptive neurons leads to chronic pain.^[13] This opens the door to potentially exciting therapeutic and diagnostic advances of capsaicin in the field of neurological disorders. It also points to a paradigm shift in focusing on brain abnormalities in relation to chronic pain. To the best of our knowledge, no previous reports have described the anti-inflammatory activity of capsaicin. Hence, in this study, we sought to determine whether capsaicin possesses anti-neuro-inflammatory activity in lipopolysaccharide (LPS)-stimulated BV-2 microglial cells.

MATERIALS AND METHODS

Cell culture and sample treatment

Murine BV-2 microglial cells (ATCC, USA) were maintained in DMEM supplemented with 10% FBS, penicillin and streptomycin sulfate (1%) in a humidified atmosphere of 5% CO₂. Cells pretreated with various concentrations of capsaicin (Sigma-Aldrich, St. Louis, USA) for 2 h were stimulated with LPS (1 μ g/mL) (Sigma-Aldrich, St. Louis, USA) for the indicated times.

Cytotoxicity assay

Cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (Sigma-Aldrich, St. Louis, USA). Cells were inoculated at a density of 1 \times 10⁴ cells into 96-well plates and cultured at 37°C for 24 h. Cells were then treated in the presence or absence of capsaicin at various concentrations for 24 h. 5 μ L of MTT was added to each well then incubated for additional 4 h in the dark at 37°C. The medium was then aspirated from the wells and the purple formazan product obtained was dissolved in dimethyl sulfoxide (Sigma-Aldrich, St. Louis, USA). The plates were analyzed at 570 nm using a microplate reader.

Nitric oxide assay

The production of NO was assayed by measuring the levels of nitrite in the culture supernatant using colorimetric assay with Griess reagent. Briefly, BV-2 cells (1 \times 10⁶ cells/ml) were seeded in 6-well plates in 2 mL complete culture medium and incubated for 24 h, at 37°C. This followed by replacing the old culture media with the different concentrations of capsaicin and then stimulated with LPS (1 μ g/ml) after 2 h. 100 μ L of culture supernatant reacted with an equal volume of Griess reagent (0.1% naphthylethylenediamine and 1% sulfanilamide in 5% H₃PO₄) in 96 well plates at room temperature in the dark for 10 min. Nitrite concentrations in the culture supernatant were determined by using standard solutions of sodium nitrite (Sigma-Aldrich, St. Louis, USA). The absorbance was read at 540 nm using a microplate reader.

Enzyme-linked immunosorbent assay

Cells (1 \times 10⁶ cells/well) were pretreated with capsaicin for 2 h before LPS treatment for 24 h. After treatment with LPS, culture media were collected. Levels of TNF- α , IL-1 β , and IL-6 (R and D systems, USA) in culture media from the treatment were quantitatively determined by enzyme-linked immunosorbent assay according to the manufacturer's instructions.

Immunofluorescence analysis

BV-2 cells were seeded in a chamber slide at the concentration of 5 \times 10³ and allowed to plate overnight. Next, the cells were treated with capsaicin 2 h before LPS treatment for 24 h. Furthermore, the cells were treated with LPS (1 μ g/mL); then, the slides were washed with ice-cold phosphate-buffered saline (PBS), and the cells were fixed with ice-cold methanol: acetone for 20 min at -20°C (1:1). The slides were then blocked with 5% BSA in Tris-buffered saline-Tween-20 for 90 min. The sections were then immunostained with mouse monoclonal immunoglobulin G to mouse p65-nuclear factor- κ B (NF- κ B) (Abcam, USA), diluted 1:200 with 5% BSA in TBS, and incubated overnight at 4°C. After washing the sections three times with TBS, the slides were incubated with their corresponding secondary antibodies, DyLight™ 550 (Thermo Scientific, MA, USA), diluted 1:500 with TBS, and incubated in the dark for 60 min at room temperature. The sections were then washed with TBS and incubated with counterstain Hoechst to stain the cell nuclei. The slides were coverslipped and visualized under an Olympus FS X100 fluorescent microscope (Tokyo, Japan).

Isolation of nuclear fraction and cytosolic fraction

After all the treatments, the BV-2 cells washed in PBS and resuspended in 1 ml of solution A (250 mM sucrose, 1.5 mM MgCl₂, 20 mM HEPES, 10 mM KCl, 1 mM EDTA, pH 8.0, 1 mM EGTA and 1 \times protease inhibitor cocktail; ~300 mOsm) with gently tapping and pipetting. Then the samples were homogenized, and the lysates were centrifuged at 720 g for 8 min at 4°C. The supernatant (cytosolic fraction) was transferred into a new 1.5 ml tube and the remaining cell pellets were washed twice with 0.5 ml solution A, and resuspended in 50 μ L of solution B (20 mM HEPES, pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, pH 8.0, 0.5 mM PMSF, 0.5 mM DTT, 25% glycerol, and 1 \times protease inhibitor cocktail; ~3860 mOsm). Samples were kept on ice for 1 h and centrifuged at 9300 g for 10 min at 4°C. The supernatant for nuclear fraction was taken and kept at -80°C until use.

Immunoblot assay

Cells obtained from treatment were washed twice with cold PBS and lysed using RIPA buffer for immunoblot analyses. Cell lysates

were normalized for protein content using BCA reagent. An equal amount of cellular proteins was loaded on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions for separation. The separated protein was then transferred to polyvinylidene difluoride (PVDF; GE Healthcare) membranes for 1 h. The membrane undergoes washing with PBS Tween-20, followed by blocking step for minimum 1 h with blocking solution (5% of BSA in PBS containing 1% Tween-20 [PBST]) at room temperature. Membranes were incubated overnight with specific primary antibodies of p65-NF- κ B, p-p65-NF- κ B, p-I κ B- α (Cell Signaling Technology, USA), inducible nitric oxide synthase (iNOS), COX-2, and β -actin (Santa Cruz Biotechnology, USA) at 4°C overnight. The membrane was then exposed to secondary antibodies (Santa Cruz Biotechnology, USA) coupled to horseradish peroxidase for 2 h and washed PBST. The bands were visualized using chemiluminescence system (Chemi Doc, Bio-Rad, USA) and the bands followed by analyzed using ImageJ software (NIH, USA).

Statistical analyses

Data were expressed as means \pm standard deviation of at least three independent experiments unless otherwise indicated. The data were processed by the statistical analysis software SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). Hypothesis testing methods included one-way analysis of variance followed by the least significant difference test; $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Capsaicin does not significantly affect the cell viability of BV-2 cells

To determine the cytotoxic effects and effective dose of capsaicin, BV-2 microglial cells were treated with increasing concentrations (25–500 μ g/mL) for 24 h, and the cell viability was quantified by the conversion of yellow MTT into formazan crystals by MTT assay. Cell viability remained above 90% as determined by the MTT assay; thus, no significant cytotoxicity was observed up to 500 μ g/mL [Figure 1]. Based on these preliminary data, minimum nontoxic doses, i.e., 5 and 100 μ g/ml were used for all subsequent assays. In addition, the concentration of LPS (1 μ g/ml) used to induce inflammation.

Capsaicin suppresses nitric oxide production in lipopolysaccharide-stimulated BV-2 cells

The low-dose anti-inflammatory potency of capsaicin on the production of NO in BV-2 cell was studied by pretreating BV-2 cells with Capsaicin

for 2 h before LPS stimulation for 24 h. In BV-2 cells, LPS markedly induced NO production as detected by the accumulation of nitrite in the culture medium after 24 h. In the presence of capsaicin, however, there was a dose-dependent inhibition of NO production to almost control levels at the highest concentration of Capsaicin, 100 μ g/mL. These effects of Capsaicin were not due to cytotoxicity since capsaicin at these concentrations did not show any significant reduction in cell viability [Figure 2].

Capsaicin inhibits lipopolysaccharide-induced production of tumor necrosis factor- α , interleukin, and interleukin-1 β in BV-2 cells

The suppression of pro-inflammatory cytokines such as TNF- α , IL-6, and IL-1 β by capsaicin in BV-2 cells was investigated. For this study, capsaicin pre-incubated BV-2 microglia were challenged with LPS for 24 h, and the cytokine levels in the culture supernatants were evaluated. As indicated in Figure 3a-c, the production of TNF- α , IL-6, and IL-1 β induced by LPS treatment was significantly decreased by the treatment with capsaicin in a dose-dependent manner, further justifying the anti-inflammatory activity of capsaicin in a cell system.

Capsaicin inhibits the expression of inflammatory markers, inducible nitric oxide synthase, cyclooxygenase-2, and Nuclear Factor- κ B

The intracellular mechanism, potentially responsible for the inhibitory effects of capsaicin on iNOS, COX-2, and p65-NF- κ B was examined by quantitative protein expression. As shown in Figure 4a, LPS markedly increased the phosphorylation of I κ B- α and NF- κ B p65 subunit compared with those in nonstimulated BV-2 cells suggesting the activation of p65-NF- κ B. However, the pretreatment with capsaicin effectively reduced the expression of p-I κ B- α and NF- κ B p65 in LPS-stimulated BV-2 cells. We wanted to check the expression of both NF- κ B and phosphorylated NF- κ B in the nuclear fraction of BV-2 cells treated with LPS and Capsaicin [Figure 4c]. It was observed that the expressions of both p65-NF- κ B and p-p65-NF- κ B were increased in LPS-induced BV-2 cells. In contrast, capsaicin dose-dependently decreased the expressions of p65-NF- κ B and p-p65-NF- κ B. Further, we analyzed the effect of capsaicin on the localization of p65-NF- κ B in LPS-induced BV-2 cells [Figure 4b]. It was noted that LPS administration resulted

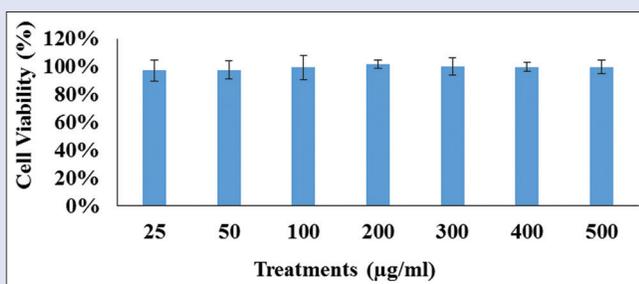


Figure 1: Effect of capsaicin on cell viability in lipopolysaccharide-stimulated BV-2 cells. Cells pretreated with various concentrations of capsaicin for 2 h were stimulated with lipopolysaccharide (1 μ g/mL) for 24 h. Cell viability was analyzed by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay. All data are presented as means \pm standard deviations of three independent experiments

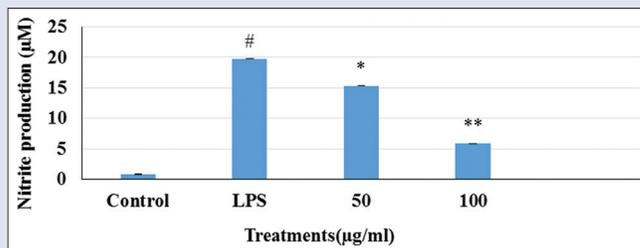


Figure 2: Effect of capsaicin on the inhibition of nitric oxide production in lipopolysaccharide-stimulated BV-2 cells. Cells pretreated with various concentrations of capsaicin for 2 h were stimulated with lipopolysaccharide (1 μ g/mL) for 24 h. The culture media of the treated cells were used to measure the amount of nitrite to evaluate nitric oxide level. Relative data represent the mean values of three independent experiments \pm standard deviation. * $P < 0.001$, lipopolysaccharide-treated group versus control; ** $P < 0.01$ and * $P < 0.05$, significant difference from the lipopolysaccharide-treated group

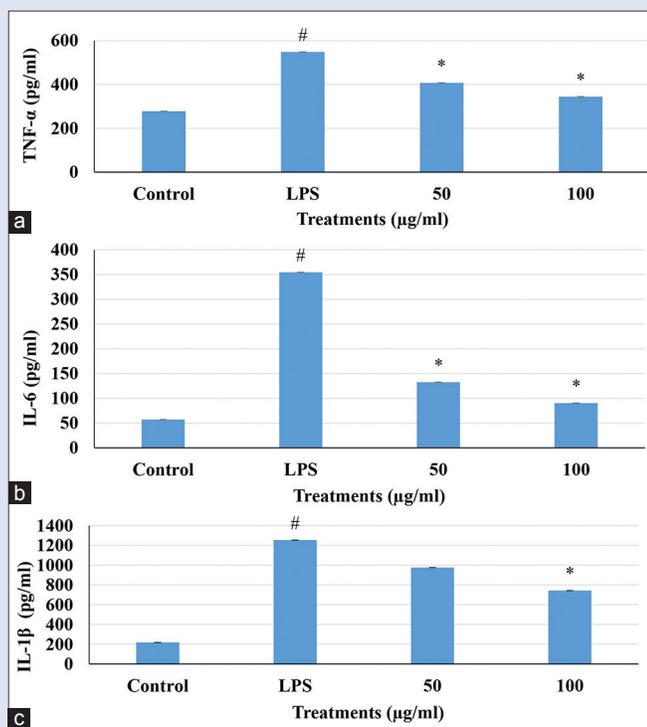


Figure 3: Effect of capsaicin on the secretion of pro-inflammatory cytokines, (a) tumor necrosis factor- α , (b) interleukin-6, and (c) interleukin-1 β in lipopolysaccharide-stimulated BV-2 cells. Cells pretreated with various concentrations of capsaicin were stimulated with lipopolysaccharide (1 μ g/mL) for 24 h. Tumor necrosis factor- α , interleukin-6 and interleukin-1 β in the culture media were measured by enzyme-linked immunosorbent. Relative data represent the mean values of three independent experiments. [#] $P < 0.001$, lipopolysaccharide-treated group versus control; ^{*} $P < 0.05$, significant difference from the lipopolysaccharide-treated group

in the nuclear accumulation of p65-NF- κ B. In contrast, capsaicin at dose-dependently reduced the nuclear translocation of p65-NF- κ B. Since the expressions, pro-inflammatory mediators are known to be modulated by NF- κ B, inhibition of iNOS, and COX-2 protein expression was evidenced in parallel to the suppression of NF- κ B by capsaicin in dose-dependent manner.

DISCUSSION

One of the perilous players in brain inflammation is the uncontrolled activation of microglia which is dispersed ubiquitously in the neuronal system, and that contributes to neuro-inflammatory-mediated neuro-degeneration. Elevated release and activation of microglial is the starting point for a wide array of potentially neurotoxic molecules including pro-inflammatory cytokines and neurotoxic reactive oxygen species.^[14] It is decisive to prevent the activation of the microglial cells so that various neuroinflammatory diseases can be prevented. Many lines of evidence have indicated that in the culture system, microglial cells can be stimulated by LPS, triggering the production of inflammatory mediators and cytokines. Therefore, LPS-induced inflammatory mediators *in vitro* were one of the key tools to evaluate anti-neuroinflammatory agents.^[15] Numerous studies demonstrated that LPS-activated microglial cells can be effectively prevented by bioactive compounds that have been isolated from natural resources.^[16-18] Thus, the present study was due to the anti-inflammatory potential the capsaicin protects the murine BV-2 microglial cells from the LPS.

Intercussion in molecular mechanism of inflammatory microglia activation is measured to be a very hopeful therapeutic strategy for treating neurodegenerative disease.^[19] The major extracellular signal transduction pathway stimulated by inflammatory mediators is NF- κ B.^[20] In most cell types, NF- κ B is located in the cytosol as an inactive complex bound to inhibitory kappa B (I κ B) family and is activated by diverse external stimuli including inflammatory cytokines; bacterial components (LPS), and viral infection.^[21] LPS stimulates the phosphorylation, ubiquitination, and proteasomal degradation of I κ B, resulting in the translocation of p65-NF- κ B into the nucleus by dissociation of the NF- κ B-I κ B- α complex and subsequently assists the transcription of several genes involved in inflammation, such as COX-2 and iNOS.^[22] Stimulated microglial cells also secrete TNF- α and interleukins.^[23] We demonstrated in this study that Capsaicin inhibited LPS-induced NO release, iNOS, COX-2, TNF- α , IL-1 β , and IL-6 expression and that the anti-inflammatory effects of Capsaicin were exerted by the regulation of NF- κ B signaling pathways.

One of the most toxic substances released in large quantities by activated microglial cells is NO. NO is mainly produced by enzymatic action of iNOS and cautiously regulated to maintain homeostasis. Many studies have revealed that peculiarly high levels of NO are found in numerous types of brain injuries and neurodegenerative diseases under the influence by enhanced expression of iNOS.^[18,24] Treatment with inhibitors of iNOS marks in neuroprotection against LPS-induced neurotoxicity, signifying that NO is an imperative arbitrator in neurotoxicity.^[25] Our findings indicated that capsaicin inhibits the production of NO in LPS-stimulated BV-2 cells. Other molecules that are secreted by stimulated microglial cells include TNF- α and interleukins, both of which can cause neuronal cell death both directly and indirectly via the induction of NO. In agreement with the previous reports on microglial activation, we observed that pro-inflammatory cytokines such as TNF- α , IL-1 β , and IL-6 were enhanced by LPS-stimulation in BV-2 microglial cells.^[26] Withal, capsaicin inhibited the LPS-stimulated increase of the pro-inflammatory cytokines TNF- α , IL-1 β and IL-6. According to the study of Amor *et al.*,^[27] similar results regarding the anti-inflammatory action of Capsaicin were shown. These results suggested that capsaicin may have a protective effect against the neuroinflammatory response in neurodegenerative diseases.

The key inflammatory signaling mediator NF- κ B was known to be involved in neuroinflammation. The promoters of the inflammatory transcription genes iNOS and COX-2 contain NF- κ B binding regions, and the expression of these enzymes can be regulated by the activation of NF- κ B.^[28] We investigated the repressive effect of capsaicin on NF- κ B activation in BV-2 cells. Capsaicin inhibited LPS-induced I κ B- α phosphorylation and reversed LPS-mediated I κ B- α degradation and translocation of the NF- κ B p65 subunit, suggesting that transcriptional down-regulation of the iNOS, COX-2 and pro-inflammatory cytokines expression by capsaicin primarily results from inactivation of NF- κ B in LPS-stimulated microglia cells. NF- κ B thus appears to be an important target transcription factor mediating the anti-inflammatory effects of Capsaicin. Our study led to the identification of capsaicin as an inflammation-controlling, neuroprotective, and neurogenesis-promoting agent. However, further identification of the direct target molecules of capsaicin is required to support its use as an anti-neuro-inflammatory agent against the neurodegenerative disorders.

CONCLUSION

Capsaicin, a bioactive compound inhibits the secretion of inflammatory mediators such as NO and pro-inflammatory cytokines, including TNF- α , IL-1 β , and IL-6, in LPS-stimulated BV-2 microglia. Furthermore, the inhibitory effect of capsaicin was allied with the inactivation of the

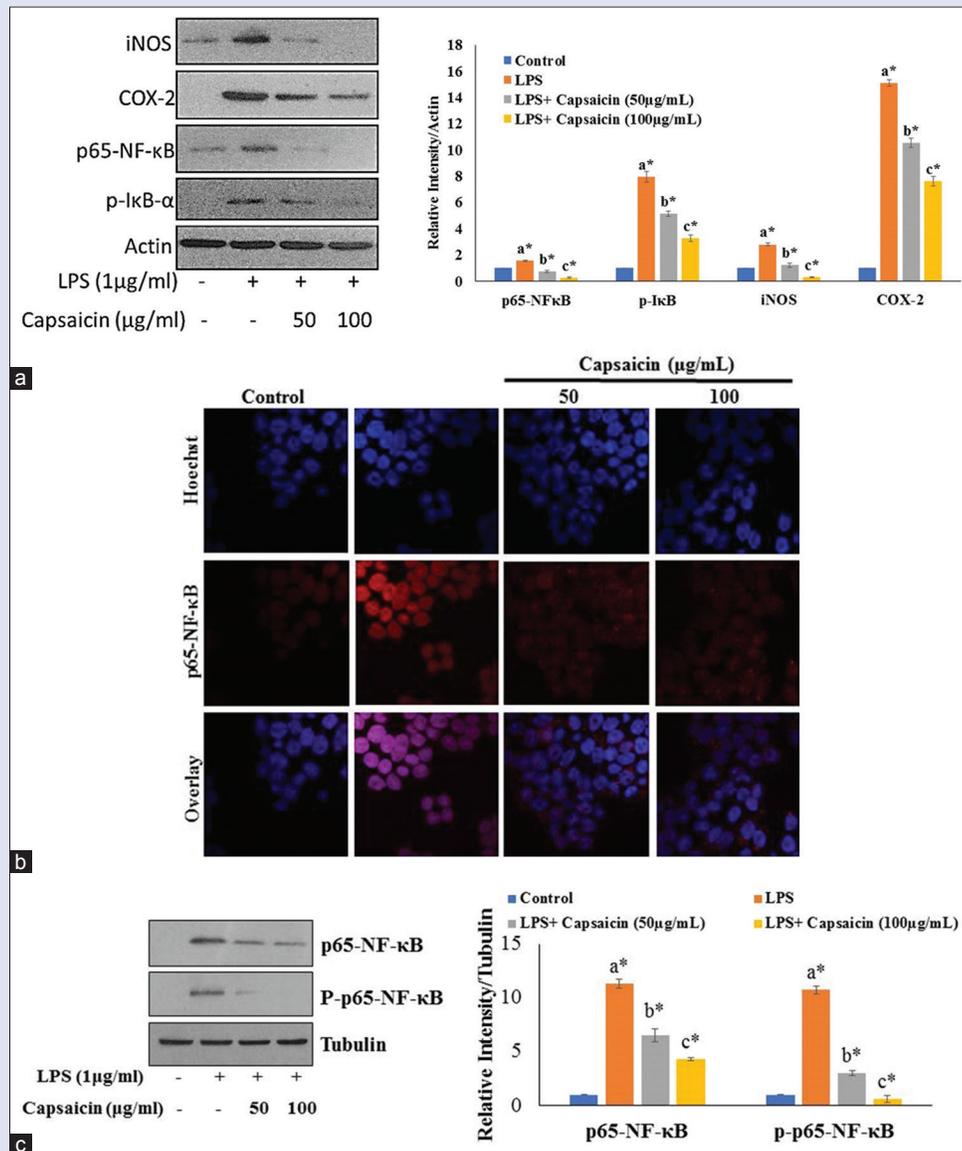


Figure 4: The effects of capsaicin on the protein expression of p65-nuclear factor-κB, p-IκB, inducible nitric oxide synthase, and cyclooxygenase-2 in BV-2 microglial cells stimulated with lipopolysaccharide. (a) Cells were pre-treated for 2 h with the indicated concentrations of capsaicin and then stimulated for 24 h with lipopolysaccharide (1 μg/mL). Western blot analyses were performed, and representative blots of three independent experiments are shown. Band intensity was normalized to β-actin; the values are presented below each band and the quantification of the respective blots by ImageJ software (NIH, USA). Relative data represent the mean values of three experiments ± standard deviation. Comparisons: ^aControl versus lipopolysaccharide; ^blipopolysaccharide versus lipopolysaccharide + Capsaicin (50 μg/mL); ^clipopolysaccharide versus Capsaicin (100 μg/mL), **P* < 0.05. (b) Effect of capsaicin on immunofluorescence analysis of p65-nuclear factor-κB in lipopolysaccharide-activated BV-2 cells. The images were taken at × 40 magnification (Scale bar 50 μM). (c) The expressions of p65-nuclear factor-κB and p-p65-nuclear factor-κB were studied in the nuclear fraction of BV-2 cells. Tubulin expression was studied as internal control. Relative data represent the mean values of three experiments ± standard deviation. Comparisons: ^aControl versus LPS; ^blipopolysaccharide versus lipopolysaccharide + Capsaicin (50 μg/mL); ^clipopolysaccharide versus Capsaicin (100 μg/mL), **P* < 0.05

NF-κB pathway through reduced IκB-α phosphorylation. However, the *in vitro* nature of our study limits conclusions regarding capsaicin effects on the *in vivo* neuro-inflammatory response. Further studies are required to investigate the anti-inflammatory and neuroprotective effects of capsaicin in animal models of neurodegenerative disease.

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Conflicts of interest

There are no conflicts of interest.

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