



# Efonidipine Inhibits JNK and NF- $\kappa$ B Pathway to Attenuate Inflammation and Cell Migration Induced by Lipopolysaccharide in Microglial Cells

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## Abstract

Efonidipine, a calcium channel blocker, is widely used for the treatment of hypertension and cardiovascular diseases. In our preliminary study using structure-based virtual screening, efonidipine was identified as a potential inhibitor of c-Jun N-terminal kinase 3 (JNK3). Although its antihypertensive effect is widely known, the role of efonidipine in the central nervous system has remained elusive. The present study investigated the effects of efonidipine on the inflammation and cell migration induced by lipopolysaccharide (LPS) using murine BV2 and human HMC3 microglial cell lines and elucidated signaling molecules mediating its effects. We found that the phosphorylations of JNK and its downstream molecule c-Jun in LPS-treated BV2 cells were declined by efonidipine, confirming the finding from virtual screening. In addition, efonidipine inhibited the LPS-induced production of pro-inflammatory factors, including interleukin-1 $\beta$  (IL-1 $\beta$ ) and nitric oxide. Similarly, the IL-1 $\beta$  production in LPS-treated HMC3 cells was also inhibited by efonidipine. Efonidipine markedly impeded cell migration stimulated by LPS in both cells. Furthermore, it inhibited the phosphorylation of inhibitor kappa B, thereby suppressing nuclear translocation of nuclear factor- $\kappa$ B (NF- $\kappa$ B) in LPS-treated BV2 cells. Taken together, efonidipine exerts anti-inflammatory and anti-migratory effects in LPS-treated microglial cells through inhibition of the JNK/NF- $\kappa$ B pathway. These findings imply that efonidipine may be a potential candidate for drug repositioning, with beneficial impacts on brain disorders associated with neuroinflammation.

**Key Words:** Efonidipine, JNK, NF- $\kappa$ B, Neuroinflammation, Microglia, Virtual screening

## INTRODUCTION

Microglial cells are the principal resident immune cells in the brain, playing crucial roles in homeostasis and host-defense mechanisms against pathogens in the central nervous system (CNS) (El Khoury, 2010). These cells are also recognized as an essential component in numerous brain disorders, in which inflammatory processes are involved in the pathophysiology (Salter and Stevens, 2017). Neuroinflammation is an important feature of many neurodegenerative disorders, such as Alzheimer's disease (AD), Parkinson's disease (PD), and multiple sclerosis (Carson *et al.*, 2006). In the presence of abnormal and hazardous stimulations, the inflammatory responses in the CNS are mainly mediated by the activated microglia. Upon activation, microglia proliferate, migrate and

release a variety of pro-inflammatory cytokines, including interleukin (IL)-1 $\beta$ , IL-6, IL-18, and tumor necrosis factor-alpha (TNF- $\alpha$ ), chemokines, small-molecule messengers, including prostaglandins and nitric oxide (NO), and reactive oxygen species (Park *et al.*, 2011). These inflammatory factors trigger and subsequently exacerbate neuroinflammatory processes in the brain and attack vulnerable neurons, resulting in neuronal damage and death (Park *et al.*, 2011; Kempuraj *et al.*, 2016). Moreover, these mediators further attract microglial cells toward the sites of injury, leading to persistent inflammation during the initial stages of neurodegeneration (Dou *et al.*, 2012; Nguyen *et al.*, 2021a). Therefore, suppression of microglial activation would be beneficial to mitigate neuroinflammation in various brain disorders, including AD and PD.

The c-Jun N-terminal kinases (JNKs) are involved in a

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plethora of pathologies, such as inflammatory, oncological, and neurodegenerative diseases (Cui *et al.*, 2007). Three different isoforms (JNK1, JNK2, and JNK3) have been identified within this group, which are encoded by the closely related genes, *JNK1*, *JNK2*, and *JNK3*, respectively (Bogoyevitch, 2006). While JNK1 and JNK2 are widely expressed in all body tissues, JNK3 is selectively expressed in the CNS (Gupta *et al.*, 1996). Higher levels of phosphorylated JNK3 were observed in post-mortem brain samples of patients with AD (Zhu *et al.*, 2001). Subsequent studies also reported high expression and activation of JNK3 in the brain tissue and cerebrospinal fluid in AD patients (Gourmaud *et al.*, 2015; Wang *et al.*, 2020). The degree of JNK3 expression correlated with cognitive decline. The JNK3 activity is also involved in PD, which is characterized by the loss of dopaminergic neurons within the substantia nigra pars compacta (Pan *et al.*, 2007). JNK3 was the main active isoform of JNKs in dopaminergic neurons treated with rotenone, paraquat, or 6-hydroxydopamine, the neurotoxins used to induce cellular models of PD. Moreover, the ablation of the *JNK3* gene exhibited neuroprotective effects in wild-type mice treated with these neurotoxic agents (Pan *et al.*, 2007; Choi *et al.*, 2010). Thus, JNK3 may be an enticing target for these neurodegenerative brain diseases associated with neuroinflammation.

Drug repositioning (or repurposing) is the process of uncovering new indications outside the scope of the original medical use for existing drugs (Ashburn and Thor, 2004). This strategy is economically alluring in terms of the cost and time compared to the *de novo* drug discovery and development (Ashburn and Thor, 2004). Many small-molecule inhibitors of JNK3 have been identified through virtual screening (Dou *et al.*, 2019; Rajan and Ramanathan, 2020; Nguyen *et al.*, 2021a). However, the structure homology among kinases, particularly among the JNK isoforms, makes the development of a selective JNK3 inhibitor challenging (Resnick and Fennell, 2004). Herein, a structure-based virtual screening was performed to explore potential JNK3 inhibitors from the U.S. Food and Drug Administration (FDA)-approved drug library, using the three-dimensional (3D) structure of JNK3. Among the compounds exhibiting JNK3 inhibitory activities, efonidipine was selected for further investigation in the present study. Efonidipine is an antihypertensive and anti-anginal drug mainly through dual blockade of L- and T-type Ca<sup>2+</sup> channels (Tanaka and Shigenobu, 2002). Although the pharmacological actions of efonidipine in the cardiovascular system are well documented, its effect in the CNS through inhibition of JNK activity remains to be elucidated. Therefore, we aimed to determine the effects of efonidipine on inflammation and cell migration induced by lipopolysaccharide (LPS) using murine and human microglial cell lines. The underlying signaling mechanisms mediating the anti-inflammatory and anti-migratory effects of efonidipine were also investigated in this study.

## MATERIALS AND METHODS

### Chemicals and reagents

Efonidipine was purchased from Selleckchem (Houston, TX, USA). SP600125 was purchased from Calbiochem (Darmstadt, Germany). LPS (*Escherichia coli*, serotype O111:B4), dimethyl sulfoxide (DMSO), and anti- $\beta$ -actin and anti-lamin B1 antibodies were obtained from Sigma-Aldrich (St. Louis, MO,

USA). 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) and Alexa Fluor 488-conjugated anti-mouse immunoglobulin G were purchased from Thermo Fisher Scientific (Rockford, IL, USA). Fluorescent mounting medium was obtained from Dako (Carpinteria, CA, USA). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), and antibiotic-antimycotic reagent were obtained from Corning Life Sciences (Corning, NY, USA). The anti-phospho-inhibitory kappa B $\alpha$  (I $\kappa$ B $\alpha$ ) antibody was obtained from Abcam (Cambridge, MA, USA). Primary antibodies specifically recognizing phospho-JNK, JNK3, phospho-c-Jun, c-Jun, inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2), nuclear factor-kappa B (NF- $\kappa$ B) p65, and I $\kappa$ B $\alpha$ , and horseradish peroxidase (HRP)-conjugated secondary anti-rabbit and anti-mouse antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA).

### Virtual screening

Virtual screening and molecular docking simulation were accomplished as described previously (Nguyen *et al.*, 2021a). Briefly, the 3D structure of JNK3 in complex with an inhibitor (Protein Data Bank ID: 4KKH) was used as a receptor, and the FDA-approved drug library consisting of 2,580 compounds was screened for ligands in the docking simulation by using the software AutoDock Vina (Trott and Olson, 2010). The ligand-binding site was set at the ATP-binding site. The docking simulation was performed according to the manual of the software.

### In vitro JNK3 activity assay

The inhibitory effect of efonidipine (10  $\mu$ M) on JNK3 activity was evaluated by the JNK3 ADP-Glo™ assay (Promega, Madison, WI, USA), as described previously (Nguyen *et al.*, 2021a). SP600125, a specific JNK inhibitor, was used as a positive control in this assay.

### Cell culture and treatment

BV2 and HMC3 cells were maintained in DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic agents at 37°C in a humidified incubator under an atmosphere of 95% O<sub>2</sub> and 5% CO<sub>2</sub>, as previously described (Bui *et al.*, 2020; Prakash *et al.*, 2021). Efonidipine was dissolved in DMSO to 10 mM as a stock solution, and serial dilutions were performed to prepare desired concentrations. Cells were treated for 24 h with LPS (1  $\mu$ g/mL for BV2 cells and 0.1  $\mu$ g/mL for HMC3 cells) and a series of concentrations of efonidipine. Cells in the control group were treated with serum-free media and 0.3% DMSO.

### Western blotting

BV2 cells were seeded on 35 mm culture dishes (1 $\times$ 10<sup>6</sup> cells/dish) and co-treated with LPS (1  $\mu$ g/mL) and various concentrations of efonidipine for 24 h. After the desired treatment, cells were lysed in the lysis buffer, as reported previously (Bui *et al.*, 2020). To determine the effect of efonidipine on the NF- $\kappa$ B nuclear translocation, BV2 cells were seeded on 60 mm culture dishes (2.5 $\times$ 10<sup>6</sup> cells/dish) and treated with LPS and various concentrations of efonidipine. Following treatment, cytosolic and nuclear fractions were separated using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific). The cell lysates were kept at -80°C until use. The protein concentrations were measured using a Bio-Rad DC Protein Assay kit (Bio-Rad, Hercules, CA, USA). Immu-

noblotting experiments with equal amounts of proteins were conducted, as reported previously (Bui *et al.*, 2020). Briefly, proteins were resolved by SDS-PAGE electrophoresis and transferred to PVDF membranes (Merck Millipore Ltd., Billerica, MA, USA). Afterward, the membranes were blocked with 5% skim milk (BD Biosciences, San Jose, CA, USA) in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 1 h at room temperature to limit non-specific bindings. The membranes were then incubated with primary antibodies at 4°C overnight. After washing with TBS-T, the membranes were incubated with HRP-conjugated secondary antibodies for 2 h at room temperature. Finally, the blots were visualized by a Bio-Rad ChemiDoc XRS imaging system using enhanced chemiluminescence reagents (Bio-Rad).

### Measurements of IL-6, IL-1 $\beta$ , and TNF- $\alpha$

The levels of IL-6, IL-1 $\beta$ , and TNF- $\alpha$  in the culture media or cell lysates were determined using enzyme-linked immunosorbent assay (ELISA) kits (Koma Biotech, Seoul, Korea), as previously described (Bui *et al.*, 2020). Briefly, BV2 cells were seeded in 24-well plates ( $2.5 \times 10^5$  cells/well) and treated for 24 h with LPS (1  $\mu\text{g}/\text{mL}$ ) in the presence or absence of efonidipine at the indicated concentrations. Following treatment, the media were cautiously collected and centrifuged at 1,500 rpm at 4°C for 10 min, and the supernatants were stored at  $-80^\circ\text{C}$  until use. HMC3 cells were seeded in 60 mm culture dishes ( $2.5 \times 10^5$  cells/dish) and treated with LPS (0.1  $\mu\text{g}/\text{mL}$ ) and the indicated concentrations of efonidipine. After 24 h of treatment, cells were lysed in the lysis buffer, centrifuged at 14,000 rpm at 4°C for 30 min, and the supernatants were stored at  $-80^\circ\text{C}$  until use. The absorbance was read at 450 nm on a microplate reader (SpectraMax M2e, Molecular Devices, Sunnyvale, CA, USA). The concentrations of IL-6, IL-1 $\beta$ , and TNF- $\alpha$  were calculated from the respective standard curves generated simultaneously.

### Measurements of NO

Nitrite concentration was measured using the Griess reagent (Promega), as described previously (Bui *et al.*, 2020). Briefly, BV2 cells seeded in 24-well plates ( $2.5 \times 10^5$  cells/well) were treated with LPS (1  $\mu\text{g}/\text{mL}$ ) and the indicated concentrations of efonidipine for 24 h. After the desired treatment, the culture media were collected and centrifuged at 1,500 rpm at 4°C for 10 min. The supernatants were stored at  $-80^\circ\text{C}$  until use. The absorbance was read at 540 nm on a microplate reader (SpectraMax M2e, Molecular Devices). The NO concentration was calculated from the respective standard curve generated simultaneously.

### Cell migration assays

**Transwell migration assay:** BV2 cells ( $1 \times 10^5$  cells/well) and HMC3 cells ( $5 \times 10^5$  cells/well) were seeded in the upper chamber of a Costar transwell plate (Corning Life Sciences) and cultured for 24 h in the incubator. A solution containing LPS with or without efonidipine at the indicated concentrations was applied to the bottom wells, and the transwell migration assay was performed, as described previously (Bui *et al.*, 2020). Briefly, cells were fixed with 4% paraformaldehyde for 20 min, permeabilized with 100% methanol for 10 min (BV2 cells) or 20 min (HMC3 cells), and subsequently stained with 0.5% crystal violet for 10 min. Non-migrated cells were gently removed with a cotton swab. The migrated cells were cap-

tured with a phase-contrast microscope (Nikon Instruments, Inc., Melville, NY, USA) and counted from at least four random fields in each well. Cell migration was presented as a percentage of the control-treated cells.

**Wound healing assay:** BV2 cells ( $5 \times 10^5$  cells/well) or HMC3 cells ( $1 \times 10^6$  cells/well) were seeded in 24-well plates and incubated at 37°C to reach 80-90% confluence. The cells were then scratched with a sterile scratcher (SPL, Pocheon, Korea) and treated with LPS and efonidipine at the indicated concentrations. Cell migration was determined, as previously described, by measuring the relative distance migrated by cells over 24 h using ImageJ software (Bui *et al.*, 2020). Images at 0 and 24 h were collected by a phase-contrast microscope (Nikon Instruments, Inc.). Cell migration was presented as a percentage of the control-treated cells.

### Immunocytochemistry

To examine the effect of efonidipine on the LPS-induced nuclear translocation of NF- $\kappa\text{B}$ , immunocytochemistry was conducted following the procedures reported previously (Bui *et al.*, 2020). In brief, BV2 cells were seeded at a density of  $2.5 \times 10^4$  cells/well on the coverslips placed on each well of the 24-well culture plates. The cells were incubated at 37°C and treated with efonidipine (10  $\mu\text{M}$ ) in the presence of LPS (1  $\mu\text{g}/\text{mL}$ ) for 24 h. The cells were then fixed with 4% paraformaldehyde for 15 min, permeabilized with 1% Triton X-100 for 5 min, and blocked with 5% goat serum for 30 min. Subsequently, the cells were incubated with the primary antibody recognizing NF- $\kappa\text{B}$  p65 in the blocking solution at 4°C overnight. After gentle washing, the cells were incubated with Alexa Fluor 488-conjugated secondary antibody and DAPI (for counterstaining) at room temperature in the dark for 1 h. The coverslips were then mounted with fluorescent mounting medium (Dako) and analyzed using a confocal microscope (Nikon Instruments, Inc.).

### Statistical analysis

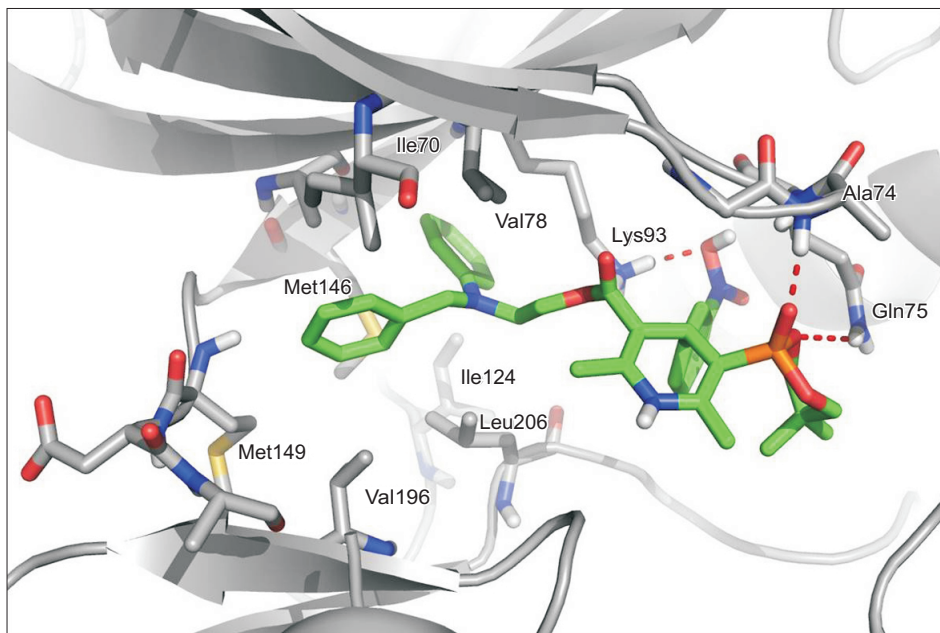
All data were presented as the mean  $\pm$  SEM from at least three independent experiments. Statistical significance between the experimental groups was analyzed by one-way analysis of variance (ANOVA) using Sigma Plot 12.5 software (Systat Software, Inc., San Jose, CA, USA). A *p* value less than 0.05 was considered statistically significant.

## RESULTS

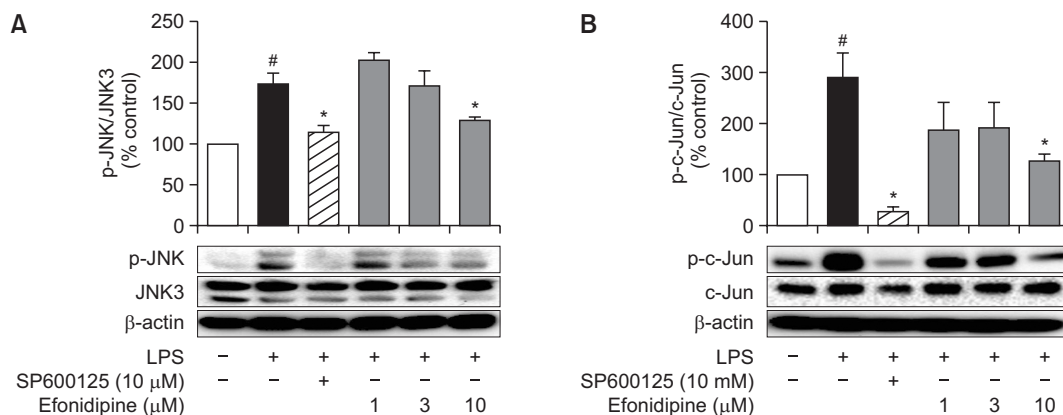
### The docking pose of efonidipine in JNK3 and its *in vitro* effect on JNK3 activity

The docking simulation of efonidipine to JNK3 is illustrated in Fig. 1. The simulation score was  $-10.9$  kcal/mol, suggesting a high-affinity binding of the compound to JNK3. According to the lowest energy structure of efonidipine binding to JNK3, two phenyl rings of the compound interact with several hydrophobic residues of JNK3, such as Ile70, Val78, Ile124, Met146, Met149, Val196, and Leu206. A hydrogen bond between the nitril moiety of efonidipine and the sidechain  $-\text{NH}$  of Lys93 and two hydrogen bonds between the phosphate of the compound and the amide of Ala74 or the sidechain of Gln75 were found in the simulated structure (the red broken lines in Fig. 1). In the proposed model, efonidipine occupied the ATP-binding site of JNK3 and competed with ATP.

Following the docking simulation study, an *in vitro* JNK3 ac-



**Fig. 1.** The lowest energy structure of efonidipine bound to JNK3. JNK3 is displayed as a gray cartoon and efonidipine as green sticks. The hydrogen bonds are expressed as red broken lines, and the residues in close contact with efonidipine are highlighted as gray sticks. The proposed model was produced using the PyMOL Molecular Graphics System (Version 2.1.0; Schrödinger, LLC., New York, NY, USA).



**Fig. 2.** Effect of efonidipine on the phosphorylation of JNK and c-Jun in LPS-treated BV2 cells. Cells were co-treated with LPS (1 μg/mL) and the indicated concentrations of efonidipine or SP600125 (a JNK inhibitor, 10 μM) for 24 h. The phosphorylation levels of p-JNK/JNK3 (A) and p-c-Jun/c-Jun (B) were measured by western blotting analysis, as described in Materials and methods. β-Actin was used as a loading control. Representative blots are shown. The data are presented as the mean ± SEM of at least three independent experiments (#*p*<0.05 vs. vehicle-treated control cells; \**p*<0.05 vs. LPS-treated cells). LPS: lipopolysaccharide, p-JNK: phosphorylated JNK, p-c-Jun: phosphorylated c-Jun.

tivity assay was performed. We found that efonidipine inhibited the JNK3 activity by more than 50% at 10 μM concentration, confirming its docked pose to JNK3 with the lowest energy. By contrast, SP600125 showed more potent inhibition than efonidipine, inhibiting the JNK3 activity by more than 90% at the same concentration (data not shown).

**Effect of efonidipine on the LPS-induced phosphorylation of JNK and c-Jun in BV2 cells**

To verify the results from the virtual screening and *in vitro* JNK3 activity assay, the effect of efonidipine on JNK phosphorylation was assessed in LPS-treated BV2 cells by west-

ern blotting analysis using anti-phospho-JNK antibody. As shown in Fig. 2A, LPS treatment significantly enhanced the phosphorylation of JNK in BV2 cells. Similarly, LPS also increased the phosphorylation of c-Jun, the downstream molecule of JNK (Fig. 2B). The LPS-induced phosphorylations of both JNK and c-Jun were markedly inhibited by efonidipine at the concentration of 10 μM (Fig. 2A, 2B). SP600125 also exhibited comparable inhibition of the LPS-induced phosphorylations of JNK and c-Jun (Fig. 2).



**Effect of efonidipine on the LPS-induced production of pro-inflammatory mediators in BV2 and HMC3 cells**

Activation of microglial cells by various neurodegenerative stimuli enhances the release of NO and pro-inflammatory cytokines, such as IL-1 $\beta$ , IL-6, and TNF- $\alpha$  (DiSabato *et al.*, 2016). It has been demonstrated that JNKs mediate essential pro-inflammatory functions in microglia (Waetzig *et al.*, 2005; Bui *et al.*, 2020). Therefore, inhibition of JNK activity could be an effective strategy to interrupt the release of these pro-inflammatory mediators (Bui *et al.*, 2020; Do *et al.*, 2020; Nguyen *et al.*, 2021a). Efonidipine inhibited the LPS-induced JNK phosphorylation in BV2 cells (Fig. 2A). Therefore, we proceeded to examine its effect on the production of pro-inflammatory mediators. LPS treatment dramatically increased the release of IL-1 $\beta$  and NO in BV2 cells. The enhanced productions of these mediators were inhibited by efonidipine (Fig. 3A, 3B). However, although the levels of IL-6 and TNF- $\alpha$  were also exceedingly increased in the LPS-treated BV2 cells, these levels were not significantly attenuated by efonidipine at the concentrations tested in this study (data not shown).

We also examined the inhibitory effect of efonidipine on the production of IL-1 $\beta$  in HMC3 cells, a human microglial cell line, to confirm its anti-inflammatory action. As observed in BV2

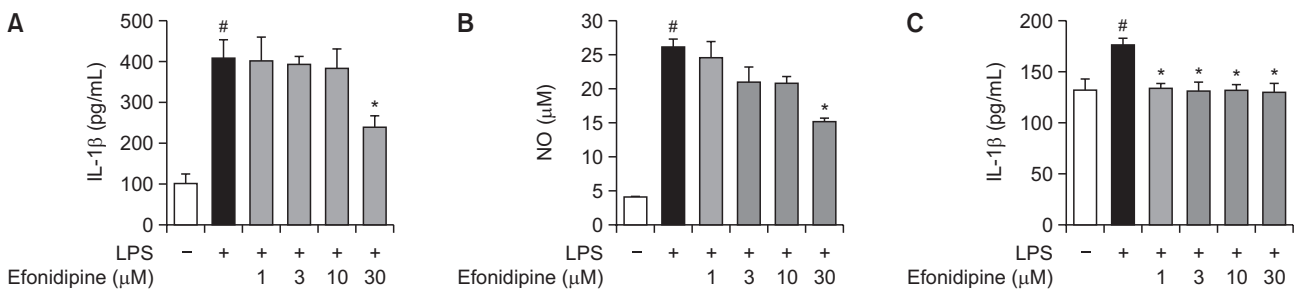
cells, efonidipine markedly inhibited the LPS-induced production of this cytokine in HMC3 cells at concentrations of 1  $\mu$ M and above (Fig. 3C).

**Effect of efonidipine on the LPS-induced expression of iNOS and COX-2 in BV2 cells**

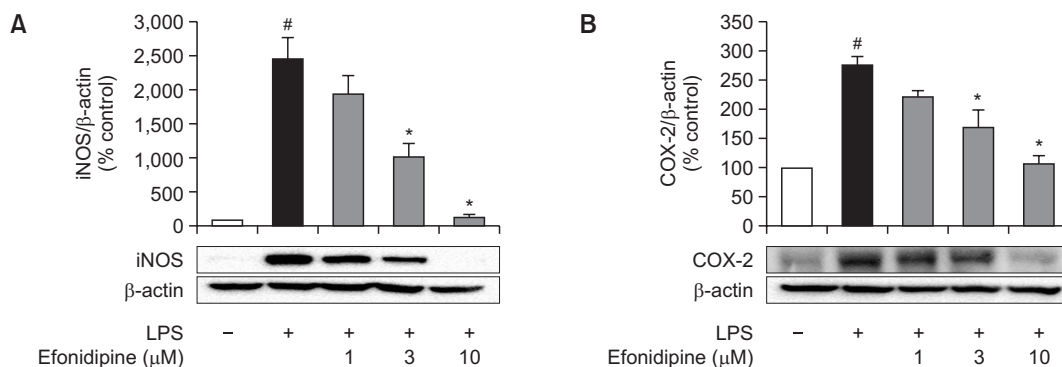
iNOS and COX-2 are two inducible pro-inflammatory effector enzymes. Their expressions are upregulated upon inflammation and known to contribute to neurodegeneration (Heneka and Feinstein, 2001). In the present study, western blotting analysis was performed to evaluate the effect of efonidipine on iNOS and COX-2 expression. Our results revealed that LPS treatment markedly augmented the protein levels of iNOS and COX-2 in BV2 cells. Simultaneous treatment with LPS and efonidipine at 10  $\mu$ M significantly decreased the LPS-induced iNOS and COX-2 expression (Fig. 4). These results suggest that the inhibition of pro-inflammatory mediators by efonidipine may be attributed to the reduced expression of iNOS and COX-2 in LPS-stimulated BV2 cells.

**Effect of efonidipine on the LPS-stimulated migration in BV2 and HMC3 cells**

Cell migration plays a central role in many physiological and



**Fig. 3.** Effect of efonidipine on the production of pro-inflammatory mediators in LPS-stimulated BV2 and HMC3 cells. (A, B) BV2 cells were co-treated with LPS (1  $\mu$ g/mL) and the indicated concentrations of efonidipine for 24 h. The concentrations of IL-1 $\beta$  (A) and NO (B) in the culture media were determined using a commercial ELISA kit and Griess reaction, respectively, as described in the Materials and methods. (C) HMC3 cells were co-treated with LPS (0.1  $\mu$ g/mL) and the indicated concentrations of efonidipine for 24 h, and the concentration of IL-1 $\beta$  in the cell lysate was determined using a commercial ELISA kit, as described. The absorbance was measured using a microplate reader. The data are presented as the mean  $\pm$  SEM of at least three independent experiments (<sup>#</sup> $p$ <0.05 vs. vehicle-treated control cells; <sup>\*</sup> $p$ <0.05 vs. LPS-treated cells). LPS: lipopolysaccharide.



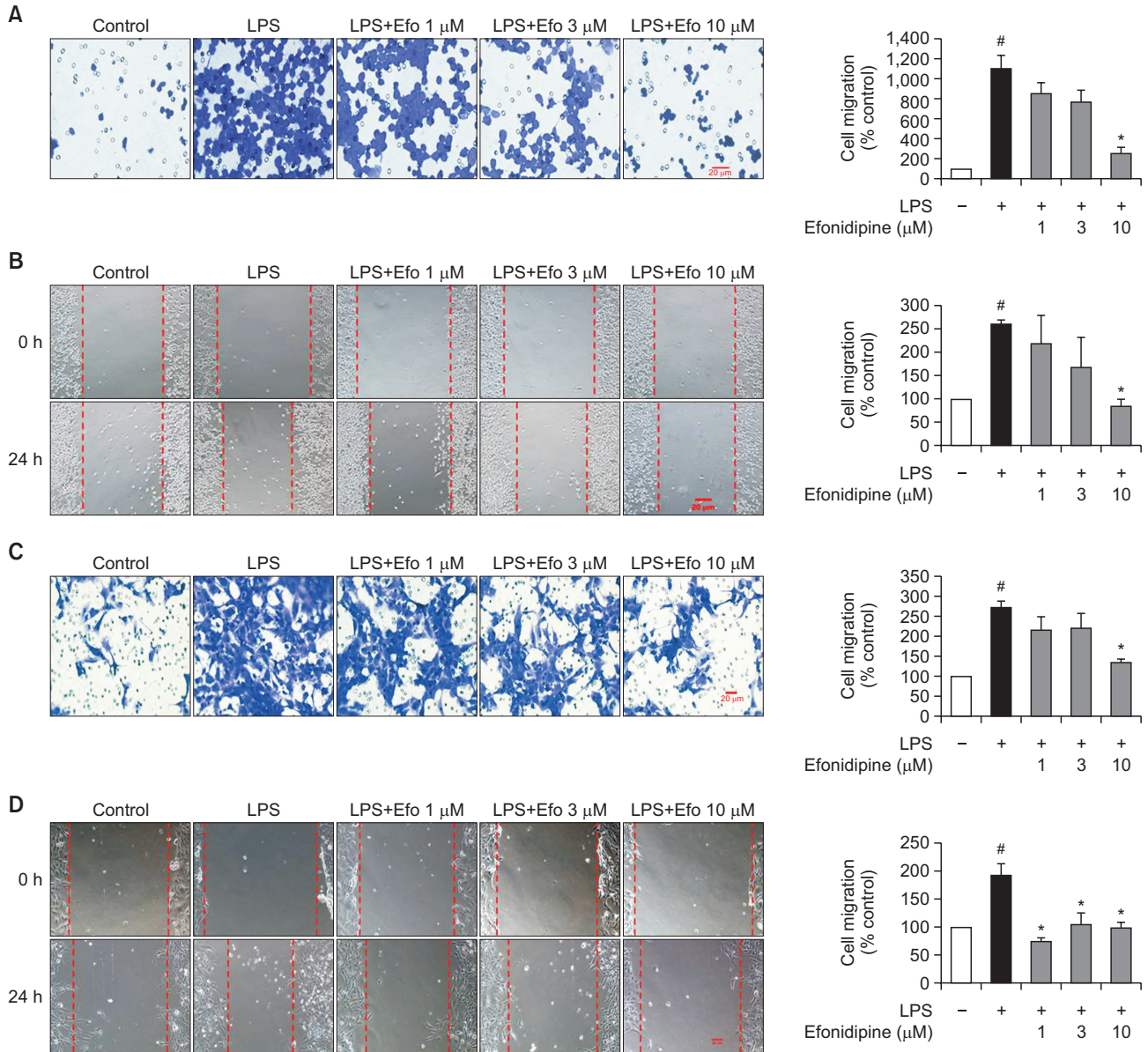
**Fig. 4.** Effect of efonidipine on the LPS-induced expression of iNOS and COX-2 in BV2 cells. Cells were co-treated with LPS (1  $\mu$ g/mL) and the indicated concentrations of efonidipine for 24 h. The expression levels of iNOS (A) and COX-2 (B) were measured by western blotting analysis, as described in Materials and methods.  $\beta$ -Actin was used as a loading control. Representative blots are shown. The data are presented as the mean  $\pm$  SEM of at least three independent experiments (<sup>#</sup> $p$ <0.05 vs. vehicle-treated control cells; <sup>\*</sup> $p$ <0.05 vs. LPS-treated cells). LPS: lipopolysaccharide, iNOS: inducible nitric oxide synthase, COX-2: cyclooxygenase-2.

pathophysiological processes, including immune defense and wound healing. Under pathological conditions, such as neurodegenerative disorders, activated microglia migrate to the injury site (Kettenmann *et al.*, 2011). Thus, we proceeded to examine whether efonidipine could suppress the LPS-stimulated migration of BV2 cells. Wound healing and transwell migration assays indicated that LPS dramatically stimulated cell migration, which was remarkably abolished by efonidipine at the concentration of 10  $\mu\text{M}$  (Fig. 5A, 5B). Similarly, LPS treatment also induced cell migration in HMC3 cells, while simultaneous treatment with LPS and efonidipine significantly inhibited the

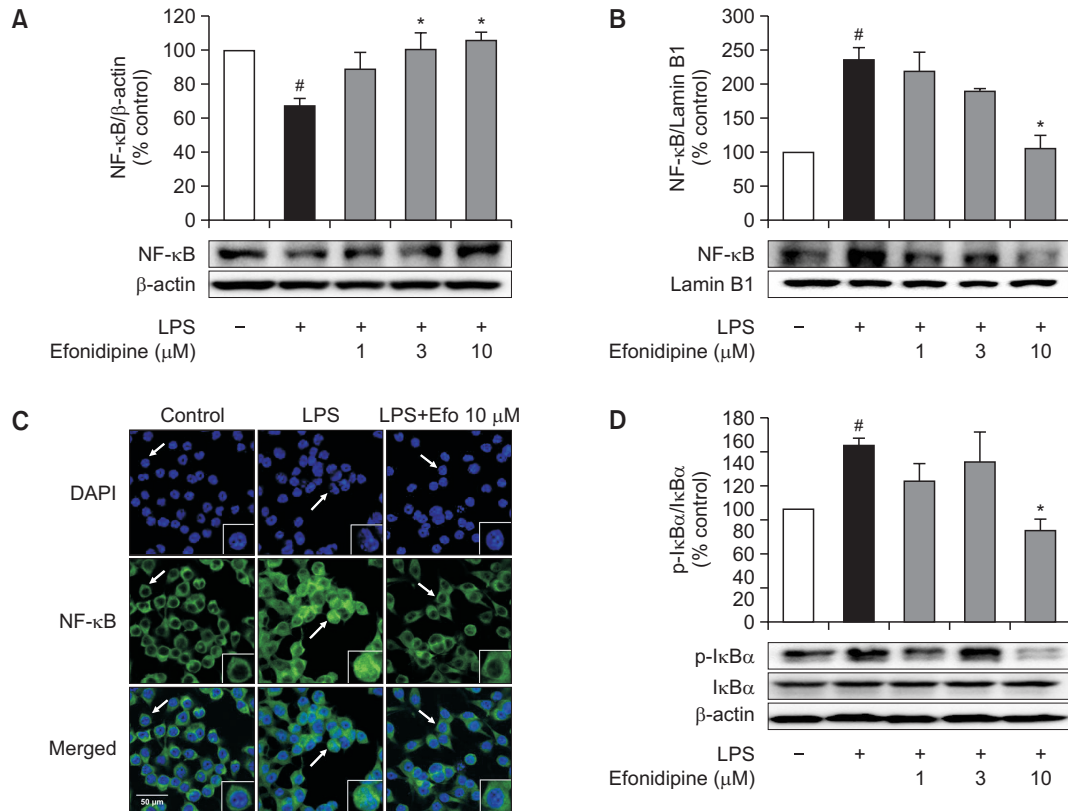
LPS-stimulated migration of HMC3 cells (Fig. 5C, 5D).

**Effect of efonidipine on the LPS-induced activation of NF- $\kappa\text{B}$  in BV2 cells**

Activation of NF- $\kappa\text{B}$  in glial cells plays a major role in the neuroinflammatory processes of many neurodegenerative diseases (Shabab *et al.*, 2017). Therefore, we determined whether efonidipine impacted the LPS-induced nuclear translocation of NF- $\kappa\text{B}$  in BV2 cells. Our western blotting study showed that LPS treatment significantly augmented the translocation of the NF- $\kappa\text{B}$  p65 subunit to the nucleus. The LPS-induced



**Fig. 5.** Effect of efonidipine on the cell migration in LPS-stimulated BV2 and HMC3 cells. (A, B) BV2 cells were co-treated with LPS (1  $\mu\text{g}/\text{mL}$ ) and the indicated concentrations of efonidipine for 24 h. After treatment, the degrees of cell migration were determined by transwell migration (A) and wound healing assays (B), as described in the Materials and methods. (C, D) HMC3 cells were co-treated with LPS (0.1  $\mu\text{g}/\text{mL}$ ) and the indicated concentrations of efonidipine for 24 h. The effect of efonidipine on the migration of HMC3 cells was assessed by transwell migration (C) and wound healing assays (D). The data are presented as the mean  $\pm$  SEM of at least three independent experiments (<sup>#</sup> $p < 0.05$  vs. vehicle-treated control cells; <sup>\*</sup> $p < 0.05$  vs. LPS-treated cells). LPS: lipopolysaccharide, Efo: Efonidipine.



**Fig. 6.** Effect of efonidipine on the nuclear translocation of NF-κB in LPS-treated BV2 cells. (A, B) Cells were co-treated with LPS (1 μg/mL) and the indicated concentrations of efonidipine for 24 h. The expression levels of cytosolic NF-κB (A) and nuclear NF-κB (B) were measured by western blotting analysis, as described in the Materials and methods. β-Actin and lamin B1 were used as loading controls for the cytosolic and nuclear fractions, respectively. Representative blots are shown. The data are presented as the mean ± SEM of at least three independent experiments ( $^{\#}p < 0.05$  vs. vehicle-treated control cells;  $^*p < 0.05$  vs. LPS-treated cells). (C) Cells were co-treated with LPS (1 μg/mL) and efonidipine (10 μM) for 24 h, and immunocytochemical analysis was carried out using DAPI and anti-NF-κB p65 antibodies, as described in the Materials and methods. Representative immunofluorescence images are shown. The arrow in each image indicates the magnified cell shown in the inset. Scale bar, 50 μm. (D) Cells were co-treated with LPS (1 μg/mL) and the indicated concentrations of efonidipine for 24 h, and western blotting analysis of the cell lysates was conducted to determine the level of phospho-IκBα. β-Actin was used as an internal control. Representative blots are shown. LPS: lipopolysaccharide, Efo: Efonidipine, IκBα: inhibitory kappa Bα, NF-κB: nuclear factor-kappa B.

NF-κB translocation was dramatically inhibited by efonidipine (Fig. 6A, 6B). These findings were further confirmed by the immunocytochemical study (Fig. 6C). The NF-κB p65, localized in the cytoplasm of non-activated control cells, moved to the nucleus upon LPS treatment. Again, efonidipine markedly blocked the LPS-induced nuclear translocation of NF-κB (Fig. 6C). The activity of NF-κB is regulated by the phosphorylation of an inhibitory molecule, IκBα (Shabab *et al.*, 2017). To investigate the effect of efonidipine on the phosphorylation of IκBα, we performed immunoblotting analyses. Our results showed that the increased phosphorylation of IκBα by LPS was significantly reversed by efonidipine at the concentration of 10 μM (Fig. 6D). These data demonstrate that efonidipine suppresses the LPS-induced nuclear translocation of NF-κB through inhibition of IκBα phosphorylation.

## DISCUSSION

Drug repositioning is attracting worldwide attention in pharmaceutical research due to several interrelated advantages,

such as simplification of the regulatory procedures for introducing previously approved drug(s) on the market (Jourdan *et al.*, 2020), faster development times, and reduced costs and risks (Ashburn and Thor, 2004). In the present study, we used computational docking techniques to explore potential JNK3 inhibitors screened from the FDA-approved drug library. Efonidipine was among the top 50 compounds with high binding affinities chosen from a list of 2,580 compounds. Based on the docking simulation (Fig. 1), efonidipine binds to the ATP-binding site of JNK3 and thereby inhibits the activity of JNK3 in an ATP-competitive manner. The inhibition of JNK phosphorylation by efonidipine was identified in BV2 cells treated with LPS (Fig. 2A), verifying the findings from virtual screening. The phosphorylation of c-Jun, a downstream molecule of JNK, was also subsequently inhibited by efonidipine in LPS-treated BV2 cells (Fig. 2B).

Efonidipine is a dihydropyridine Ca<sup>2+</sup> channel blocker with the ability of dual blockade of L- and T-type Ca<sup>2+</sup> channels (Tanaka and Shigenobu, 2002). Notably, T-type Ca<sup>2+</sup> channels are necessary for subthalamic burst firing. It has been reported that pharmacological blockade of T-type Ca<sup>2+</sup> chan-



nels by efonidipine reduces motor deficits in a rat model of PD (Tai *et al.*, 2011). In addition, previous studies reported that the L-type Ca<sup>2+</sup> channel blockers nicardipine and nimodipine exerted anti-inflammatory effects in microglia (Li *et al.*, 2009; Huang *et al.*, 2014). However, the anti-inflammatory effect of efonidipine and its action mechanism remained unclear. In the present study, we examined whether efonidipine regulated neuroinflammatory responses in microglia and elucidated the signaling molecules mediating its effect.

JNKs are involved in physiological processes, modulation of inflammation, and stress responses. Elevated phosphorylation of JNK3 and c-Jun, together with increased production of pro-inflammatory cytokines, were observed in the hippocampus of the chronic social defeat stress mouse model (Deng *et al.*, 2018). Moreover, JNK3 inhibitors restored antioxidant enzyme activity and reduced oxidative stress-induced JNK phosphorylation and pro-inflammatory mediators in rats with transient middle cerebral artery occlusion (Zulfiqar *et al.*, 2020). These findings strongly suggest that targeting JNK3 can be a favorable strategy to alleviate the progression of neuroinflammation associated with various brain disorders.

Inflammation is the process by which the human body attempts to counteract potentially injurious pathogens by promoting the release of a plethora of cell types and mediators (Haddad, 2002). Various stimuli, such as LPS endotoxin derived from Gram-negative bacteria, induce host cells to secrete pro-inflammatory mediators in order to recruit and activate neighboring cells necessary for eliciting an immune response (Boraschi *et al.*, 1998). Therefore, the production of pro-inflammatory cytokines is a useful indicator of inflammation. In the present study, efonidipine reduced the production of IL-1 $\beta$  in LPS-stimulated BV2 cells (Fig. 3A), but not the levels of TNF- $\alpha$  and IL-6 (data not shown). Our results suggest that the anti-inflammatory effect of efonidipine may be mediated mostly by IL-1 $\beta$  other than TNF- $\alpha$  and IL-6. IL-1 $\beta$  is produced as an inactive precursor, termed pro-IL-1 $\beta$ , in response to infectious pathogens (Lopez-Castejon and Brough, 2011). Following caspase-1-dependent processing of pro-IL-1 $\beta$ , mature IL-1 $\beta$  is rapidly secreted from the cell (Brough and Rothwell, 2007).

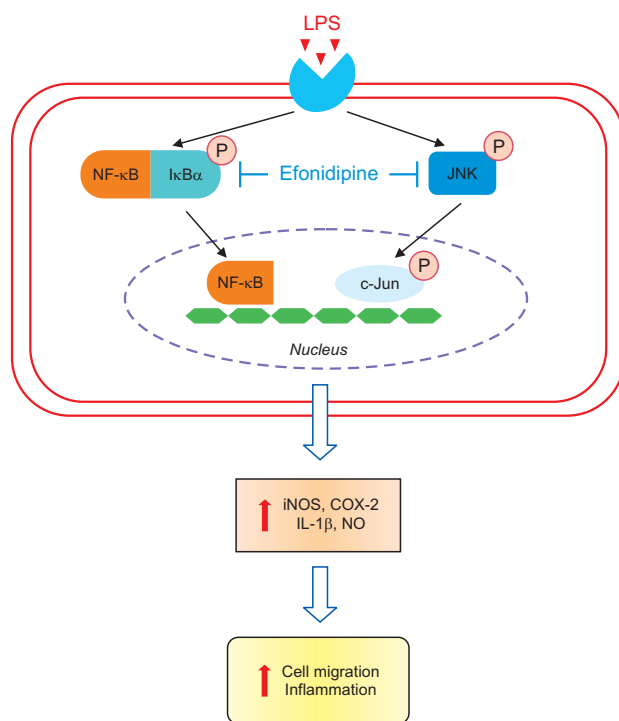
The present study used LPS-treated BV2 microglial cells as a cellular model to investigate the effect of efonidipine. LPS, a bacteria-derived endotoxin, is a potent immune stimulant and has been considered the gold standard for inducing microglial activation (Kloss *et al.*, 2001). Therefore, it is a well-characterized model commonly used to evaluate the anti-neuroinflammatory effects of potential candidates (Bui *et al.*, 2020; Do *et al.*, 2020; Nguyen *et al.*, 2021b). However, it should be noted that rodent microglia display important biochemical and pharmacological differences compared to human microglia (Smith and Dragunow, 2014). Due to the limitation and difficulties of obtaining primary sources of human microglia, several human microglial cell lines, such as HMO6 and HMC3 cells, have been established (Nagai *et al.*, 2001). The HMC3 cell line has been deemed suitable to examine anti-inflammatory properties (Lepiarz and Olajide, 2019), so we used this cell line to show and confirm the inhibitory action of efonidipine against the LPS-induced IL-1 $\beta$  production (Fig. 3C).

Efonidipine suppressed LPS-stimulated expression of iNOS and COX-2 in BV2 cells (Fig. 4). iNOS expression is induced in macrophages and microglia during inflammation but not in healthy states (Dawson and Dawson, 2018). Excessive

NO formation due to increased iNOS expression promotes the inflammatory response (Saini and Singh, 2019). The LPS-induced NO production was reduced by efonidipine in this study (Fig. 3B). COX-2 catalyzes the formation of prostaglandins, a group of hormone-like substances that participate in various body functions (Soufli *et al.*, 2016). Similar to iNOS, the excessive activation of COX-2 can lead to neuronal apoptosis and neurodegeneration (Liang *et al.*, 2007). Therefore, inhibition of iNOS and COX-2 expression by efonidipine may be beneficial in attenuating neuroinflammation and neurodegeneration.

Microglia are highly motile cells that play a crucial role in the active immune defense in the brain. Attracted by factors released from injured cells, microglia are activated and moved toward damaged sites (Dou *et al.*, 2012). Our wound healing and transwell migration assays confirmed that efonidipine dramatically reduced LPS-stimulated cell migration in BV2 and HMC3 cells (Fig. 5). Such findings strongly suggest that efonidipine abolishes the LPS-stimulated microglial cell migration, and thereby it may reduce neuroinflammatory processes.

NF- $\kappa$ B is known to be involved in the regulation of inflammatory processes in glial cells (Okun *et al.*, 2009). I $\kappa$ B is a natural biological inhibitor of NF- $\kappa$ B (Singh *et al.*, 2020). Due to their interaction with the I $\kappa$ B inhibitory family, NF- $\kappa$ B dimers are in a passive form in the cytoplasm. Upon disruption of the interaction between I $\kappa$ B $\alpha$  and NF- $\kappa$ B through phosphorylation of I $\kappa$ B $\alpha$ , the liberated NF- $\kappa$ B dimer is translocated to the nucleus (Shabab *et al.*, 2017). Our western blot and immunocytochemistry data showed that LPS-treated cells signifi-



**Fig. 7.** Schematic diagram demonstrating the anti-inflammatory and anti-migratory effects of efonidipine and the underlying mechanisms. LPS: lipopolysaccharide, I $\kappa$ B $\alpha$ : inhibitory kappa B $\alpha$ , NF- $\kappa$ B: nuclear factor-kappa B, JNK: c-Jun N-terminal kinase, iNOS: inducible nitric oxide synthase, COX-2: cyclooxygenase-2, IL-1 $\beta$ : interleukin-1 $\beta$ , NO: nitric oxide.



cantly enhanced the nuclear translocation of the NF- $\kappa$ B p65 subunit, which was dramatically inhibited by efonidipine (Fig. 6) through inhibition of  $\text{I}\kappa\text{B}\alpha$  phosphorylation in LPS-treated BV2 cells.

Taken together, our findings indicate that efonidipine inhibits pro-inflammatory mediators and cell migration in LPS-treated microglial cells through inhibition of the NF- $\kappa$ B/JNK pathway. A schematic diagram illustrating the anti-inflammatory and anti-migratory actions of efonidipine and the underlying mechanisms of action is provided (Fig. 7). Based on our findings in this study, efonidipine may be a potential candidate for drug repositioning, with beneficial impacts on brain disorders associated with neuroinflammation and microglial activation.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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