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BIOMEDICAL SCIENCES

Anti-inflammatory effects of vinpocetine in LPS-stimulated microglia via activation of AMPK

QIN ZHOU, DONGKAI GUO, XINJIA LI, YIXUAN WANG, XIAOLI YE, SUDONG XUE & XIAOYU WANG

Abstract: Microglia are the resident immune cells in the central nervous system (CNS), which play important roles in the repair of neuroinflammatory injury. The present study investigated the anti-neuroinflammatory effects of vinpocetine induced by lipopolysaccharide (LPS) in BV2 microglia. BV2 microglia were pretreated with vinpocetine, and then stimulated with LPS (100 ng/mL). The cytotoxicity of BV2 microglia was assessed by MTT assay. The expression levels of nitrite oxide were measured by Griess assay. Proinflammatory cytokines and mediators were determined by Western blot, ELISA, or quantitative real-time PCR. Vinpocetine significantly decreased the generation of nitric oxide-inducible nitric oxide synthase (iNOS), cyclooxygenase- (COX-) 2 in a dose-dependent manner. In addition, vinpocetine decreased the production of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), interleukin (IL)-6 and IL-1β. Furthermore, it was observed that phosphorylation levels of AMPK (Thr-172) decreased in LPS-stimulated BV2 microglia. Vinpocetine treatment increased AMPK phosphorylation in LPS-stimulated BV2 microglia. AMPK inhibition by siRNA blocked the anti-inflammatory effects of vinpocetine induced by LPS in BV2 microglia. The overall results demonstrate that vinpocetine has anti-inflammatory effects on LPS-stimulated BV2 microglia via inducing phosphorylation of AMPK, suggesting that vinpocetine is a potential therapeutic agent in neuroinflammatory injury.

Key words: Vinpocetine, BV2 microglia, LPS, neuroinflammation, AMPK.

INTRODUCTION

Microglia cells constitute 10-15% of the glial cell population in mammal adult brain, which originate as primitive macrophages from the embryonic yolk sac (Subhramanyam et al. 2019). Microglia are the primary immune cells in the central nervous system (CNS), which play a very important role in the internal immunity of the brain (Wolf et al. 2017, Colonna & Butovsky 2017). Microglia cells are the first line of defense against pathogens or injury in the brain. In response to brain injury, microglia cells can produce inflammatory factors such as iNOS, COX-2, Interleukin-1β (IL-1β), Interleukin-6 (IL-6) and TNF-α, which can cause damage to the surrounding neuron (Banati 2003).

Adenosine monophosphate-activated protein kinase (AMPK) is a highly conserved serine/threonine protein kinase, which is regulated by the AMP/ATP ratio to restore the energy balance (Garcia & Shaw 2017). AMPK is a key regulator of immunity and inflammation, which suppresses the expression of inflammatory genes through multiple downstream pathways such as nuclear factor kB (NF-kB) and toll-like receptors (Vaez et al. 2016). In recent years, the regulatory effect of AMPK on neuroinflammatory response has attracted much attention (Zheng et al. 2019, Harun-Or-Rashid & Inman 2018).

Vinpocetine is extracted from the periwinkle plant, Vinca minor, which has higher biological activity and less side effects than vincamine (Zhang et al. 2018c). Vinpocetine was widely used in many Asian and European countries for the treatment of stroke, Alzheimer's disease, and memory disturbances for more than 30 years (Bagoly et al. 2007). Previous studies indicated that vinpocetine is an inhibitor of phosphodiesterase 1 (PDE1), which can cause an increase in cAMP/cGMP and initiates gene expression associated with plasticity (Beavo 1995). Vinpocetine has a high affinity with translocated protein (TPSO), which is a biomarker of activated microglia, can inhibit microglia proliferation through the NF-κB /AP-1 pathway (Jeon et al. 2010). Vinpocetine inhibited the release of inflammatory factors stimulated by TNF-α through inhibiting the IKK/NF-κB pathway (Zhao et al. 2011). The present study firstly indicated that vinpocetine exerts anti-neuroinflammatory effects mainly by AMPK signaling pathway.

MATERIALS AND METHODS

Cell culture

The murine BV2 microglia were cultured in high-glucose DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin. The cells were cultured in a humid atmosphere with 5% CO_2 at 37° C.

Reagents and chemicals

LPS was obtained from Sigma Aldrich (St. Louis, MO). Vinpocetine and compound C were purchased from Selleck Chemicals (Houston, TX, USA).

MTT assay

Cell viability was assessed by MTT reagent. MTT assays were performed according to the manufacturer's protocol from the Cell Growth Determination Kit MTT Based (Sigma, St. Louis, MO, USA). BV2 microglia were seeded in triplicate at a density of 2×10^4 cells/well on a 96-well plate. After the supernatant were removed, the cells were incubated with MTT solution (0.5 mg/mL) at 37 °C for 4 h in an incubator. Then, the MTT solution was discarded, and an equal volume of DMSO was added to dissolve the formazan. The absorbance was measured at a wave length of 570 nm.

Nitrite quantifications

NO production was estimated by Griess reagent (1% sulfanilamide, 0.1% naphthylethylene, 2% phosphoric acid). BV2 microglia were seeded in triplicate at a density of 2×10^4 cells/well on a 96-well plate. Briefly, 50 ul of BV2 microglia culture medium was transferred into a new 96-well plate, and mixed with 50 ul of Griess reagent. Then, the mixture was placed at room temperature for 10 min. The absorbance was measured at a wave length of 540 nm. Eight known concentrations of sodium nitrite (0–150 uM) were used to draw the standard curve.

Knockdown of AMPK in BV2 microglia

AMPK siRNA and nonsense siRNA duplexes were synthesized by Genepharm (Suzhou, China). AMPK sense strand (5'-GAGAAGCAGAAGCACGACGTT-3') and nonsense siRNA (5'-UUCUCCGAACGUGUCACGUTT-3') were previously reported (Zhou et al. 2014). BV2 cells seeded on 6-well plates were transiently transfected using Lipofectamine RNA iMAX Reagent (Invitrogen, Waltham, MA, USA) in Opti-MEM (GIBCO, Waltham, MA, USA) according to the manufacturer's instructions. The cells were cultured for 48 h and then analyzed.

Isolation of rna and quantitative real-time PCR

RT-PCR was performed to analyze the mRNA levels of iNOS, COX-2, TNF-α, IL-6 and IL-1β in BV2 microglia, cells were collected 6 hours after LPS stimulation as described (Gan et al. 2015). Total RNAs were isolated with RNAiso Plus (Takara, Dalian, China). Total RNA (1 μg) was reverse-transcribed into cDNA using oligo (dT), according to the manufacturer's instructions (Takara, Dalian, China). Quantitative PCR was performed using the following specific primers:

iNOS, forward primer: 5'-CAGGAGGAGAGAGATCCGATTTA-3', reverse primer: 5'- GCATTAGCATGGAAGCAAAGA-3';

COX-2, forward primer: 5'-CAGGCTGAACTTCGAAACA-3', reverse primer: 5'-GCTCACGAGGCCACTGATACCTA-3';

TNF-α, forward primer: 5'-CATCTTCTCAAAATTCGAGTGACAA-3', reverse primer: 5'- TGGGAGTAGACAAGGTACAACCC-3';

IL-1β, forward primer: 5'-GCAACTGTTCCTGAACTC-3', reverse primer: 5'-CTCGGAGCCTGTAGTGCA-3';

IL-6, forward primer: 5'-GAGGATACCACTCCCAACAGACC-3', reverse primer: 5'-AAGTGCATCATCGTTGTTCATACA-3';

GAPDH, forward primer: 5'-TGTGTCCGTCGTGGATCTGA-3', reverse primer: 5'- TTGCTGTTGAAGTCGCAGGAG-3'.

Quantitative real-time PCR was performed using SYBR Premix Ex Taq (TaKaRa, China). The $2^{-\Delta\Delta CT}$ formula was used for calculations of the relative quantification. The target gene expressions were normalized to GAPDH and expressed as the fold change relative to the expression level of the target gene in the control group.

Western blot analysis

Cells were lysed in 1×SDS lysis buffer (150 mmol/L NaCl, 25 mmol/L Tris-HCl, pH 7.6, 1% sodium deoxycholate, and 1% NP-40) in the presence of a protease inhibitor cocktail. Nearly 20 mg of cell lysate was isolated with SDS-PAGE and then transferred onto a PVDF membrane (Millipore, Billerica, MA, USA). The PVDF membranes were then blocked with 10% skimmed milk for 30 min. Immunoblot analysis was performed with the following primary antibodies: monoclonal mouse anti-iNOS (1:1000; Abcam, Cambridge, UK); polyclonal rabbit anti-mouse COX-2 (1:800; Abcam, Cambridge, MA); anti-AMPK, anti-p-AMPK (Thr172) antibodies (Cell Signaling Technology, Danvers, MA, USA); and anti-GAPDH antibody (1:1000; Santa Cruz, CA, USA) overnight at 4 °C. The secondary antibodies, horseradish peroxidase (HRP)-conjugated sheep anti-mouse or anti-rabbit antibodies (Thermo Fisher, Waltham, MA, USA) were incubated for 2 h at room temperature. The proteins were visualized using an ECL detection kit (Thermo Fisher, Waltham, MA, USA). Densitometry was quantified with Image J software.

Enzyme-linked immunosorbent assay (ELISA)

BV2 microglia were seeded in 96-well plates at the density of 2×10^4 cells/well. Cells were pretreated with or without Vinpocetine for 12 h and then cultured for 12 h in the absence or presence of LPS. The supernatants were collected for detections of IL-1β-IL-6 and TNF-α using commercial ELISA kits (BOSTER, Wuhan, China) per the manufacturer's instructions. A microplate reader (Infinite M200 PRO, Tecan, Switzerland) was used to assess the OD value.

Statistical analysis

The values are presented as the mean ± SEM. One-way ANOVAs followed by Bonferroni tests were utilized for multiple-group comparisons. Student's t-test was used for comparisons between two groups. A P-value of < 0.05 was considered statistically significant.

RESULTS

Vinpocetine reduces the generation of NO in LPS-stimulated BV2 microglia

We first investigated the effect of vinpocetine on cell viability and NO generation. BV2 microglia were cultured in the presence of various concentrations of vinpocetine (0, 1, 5, 10, or 50 μM) for 24 h. We found that vinpocetine did not influence the viability of BV2 microglia up to 50 μM (Figure 1a). Subsequently, the Griess assay was used to investigate the effect of vinpocetine on NO released in the culture medium. BV2 microglia were pretreated with different doses of vinpocetine (0-50 μM) for 12 h and then stimulated with lipopolysaccharide (LPS) (100 ng/mL) for 6 h, a well-established activator of microglia/macrophages. We found that vinpocetine significantly decreased LPS-induced NO generation in a dose dependent manner (Figure 1b).

Effects of vinpocetine on the protein expression of pro-inflammatory mediators in activated BV2 microglia

Given the strong inhibitory effects of vinpocetine on Nitrite generation in LPS-induced BV2 microglia, we further investigate the role of vinpocetine on microglia activation. BV2 microglia were pretreated with vinpocetine at the concentrations from 1 to 50 μM, and then stimulated with LPS (100 ng/mL) for 24 h. Interestingly, pretreatment of BV2 cells with vinpocetine significantly inhibited LPS-induced iNOS and COX-2 protein expression levels in a dose-dependent manner (Figure 2a). In view of the significant anti-inflammatory effects and non-cytotoxicity for vinpocetine at a concentration of 50 μM, we chose this concentration for the following study. We next detect the expression levels of TNF-α, IL-6 and IL-1β protein by an enzyme-linked immunosorbent assay (ELISA). Consistently, TNF-α, IL-6 and IL-1β were significantly blunted by Vinpocetine treatment at the protein level (Figure 2b-d).

Effects of vinpocetine on the mRNA expressin of pro-inflammatory mediators in activated BV2 microglia

Since we found that vinpocetine was able to inhibit the expression of pro-inflammatory factors in protein levels, we wonder whether LPS-induced pro-inflammatory factors could be transcriptionally repressed by vinpocetine. We further access the inhibitory effects of vinpocetine in LPS-treated BV2 microglia. The mRNA expression levels of pro-inflammatory cytokines were evaluated by qRT-PCR analysis. In the vinpocetine pretreatment group, iNOS, COX-2, TNF-α, IL-6 and IL-1β mRNA levels were significantly reduced (Figure 3a-e), suggesting that vinpocetine influences iNOS, COX-2, TNF-α, IL-6 and IL-1β at the transcriptional level.

AMPK mediates the anti-inflammatory effects of vinpocetine in BV2 microglia

AMPK activation is directly correlated to the phosphorylation state at Thr 172 on the AMPKα subunit (Garcia & Shaw 2017). To evaluate vinpocetine effects on microglial AMPK activation, we examined whether vinpocetine enhanced AMPK phosphorylation (Thr 172) levels in BV2 microglia. We found that LPS significantly decreased AMPK activation (phosphorylation) in BV2 cells after LPS stimulation. In the presence of LPS stimulation, vinpocetine remarkably enhanced AMPK activation (Figure 4). These data provide evidence that vinpocetine inhibit the activation of microglia via the phosporylation of AMPK.

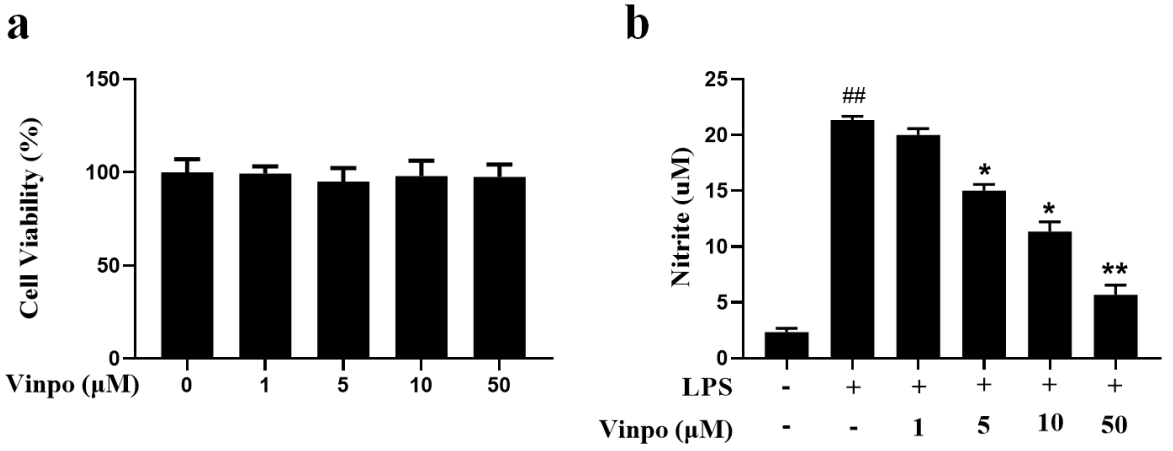

Figure 1. Vinpocetine suppressed LPS-induced NO generation in BV2 microglia. (a) BV2 cells were treated with various concentrations of vinpocetine (0-50uM), cell viability was determined by MTT assay after 24 h. (b) BV2 cells were pretreated with different doses of vinpocetine (0-50 μM) for 12 h before exposed to LPS (100 ng/mL) for 6 h, Nitrite generation was measured by Griess reaction. Data are representative of three independent experiments. ##P < 0.01, compared with the DMSO treated group; *P < 0.05 and **P < 0.01 compared with the 100 ng/mL LPS-treated group.

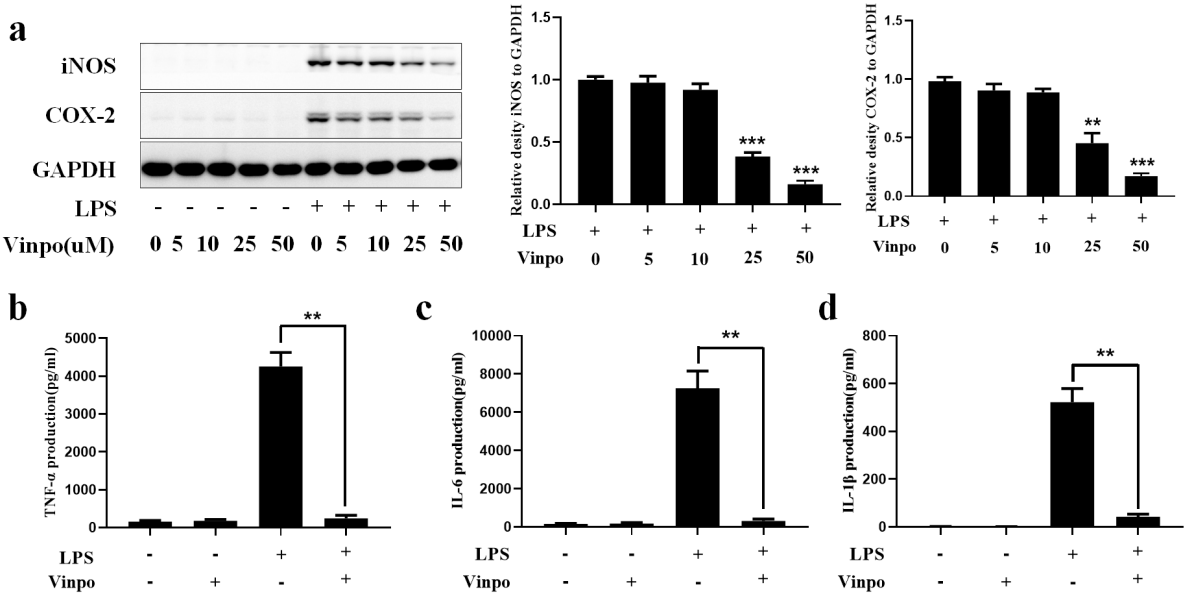

Figure 2. Vinpocetine suppressed the protein expression levels of the pro-inflammatory mediators in BV2 microglia stimulated with LPS. (a) BV2 cells were pretreated with different doses of vinpocetine (0-50 μM) for 12 h and then exposed to LPS (100 ng/mL) for 24 h. Cell lysates were analyzed by Western blot for iNOS, COX-2 and GAPDH. The relative band intensity of iNOS and COX-2 to GAPDH were analyzed. (b-d) Supernatants were measured for the release of TNF-α, IL-6 and IL-1β by ELISA assays. Data are representative of three independent experiments. **P < 0.01, ***P < 0.001 compared between the indicated groups.

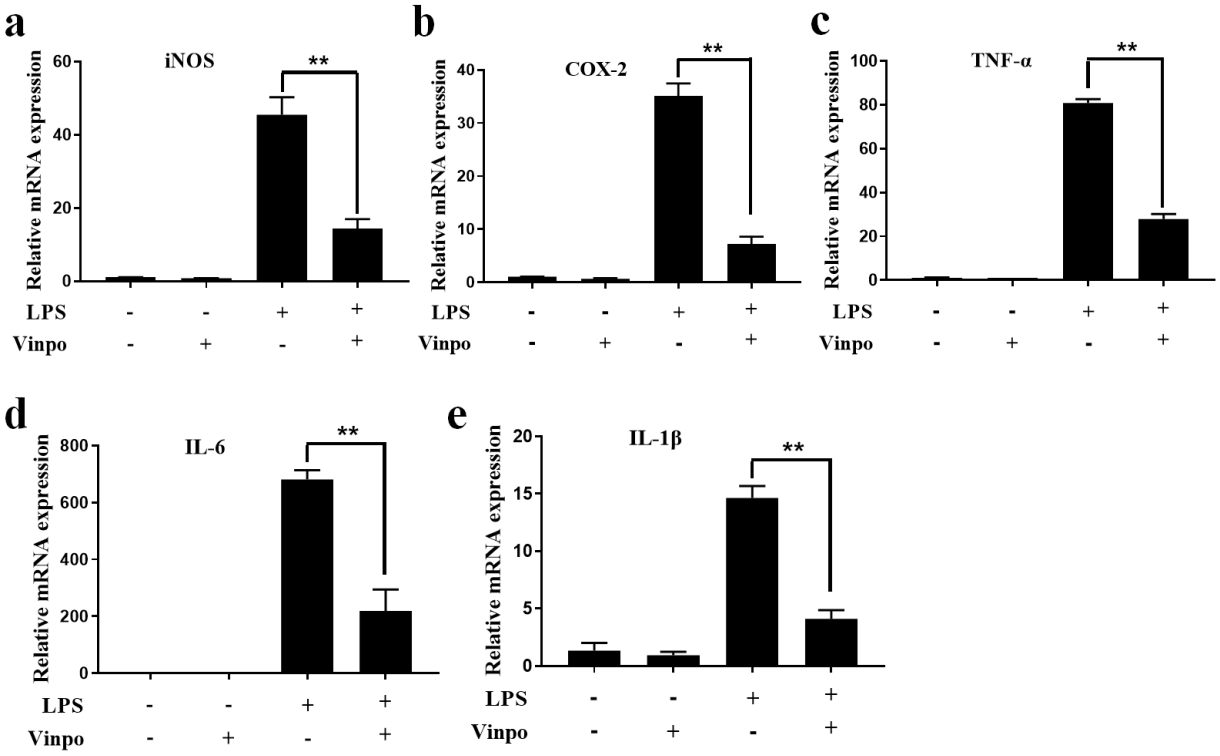

Figure 3. Vinpocetine suppressed the mRNA expression levels of the pro-inflammatory mediators in BV2 microglia stimulated with LPS. Vinpocetine suppresses the transcription and release of proinflammatory factor. BV2 cells were pretreated with vinpocetine (50 μM) for 12h and then exposed to LPS (100 ng/mL) for 6 h. Cell lysates were collected to measure iNOS (a), COX-2 (b), TNF-α (c), IL-6 (d) and IL-1β (e) mRNA levels by qRT-PCR assays. Data are representative of three independent experiments. **P < 0.01 compared between the indicated groups.

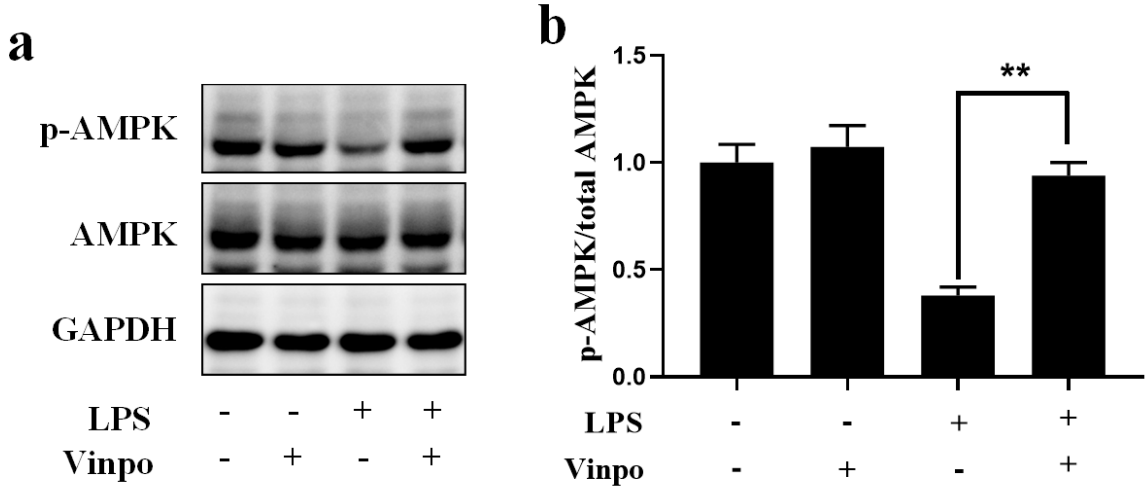

Figure 4. Vinpocetine suppressed LPS-induced inflammatory cytokine release in BV2 microglia via AMPK phosphorylation. (a) BV2 microglia were incubated with vinpocetine (50 μM) for 23 h and then exposed to LPS (100 ng/mL) for 1 h. The phosphorylation of AMPK and total AMPK were determined by western blot analysis. GAPDH was used as a loading control. (b) The relative band intensity phosphorylated AMPK to total AMPK were analyzed. Data are representative of three independent experiments. **P < 0.01 compared between the indicated groups.

The importance of AMPK activation in the process of vinpocetine anti-inflammatory effects

To investigate whether vinpocetine inhibited inflammation via activating AMPK, we investigated whether AMPK inhibition blocked the anti-inflammatory effects of vinpocetine induced by LPS in BV2 microglia. AMPK siRNA transfection (Figure 5a) significantly decreased AMPK expression as compared with nonsense siRNA (NC) transfection group. Interestingly, vinpocetine significantly decreased the LPS-induced iNOS protein expression, whereas this inhibition effect was reversed by AMPK siRNA (Figure 5b). Consistently, TNF-α was significantly blunted by AMPK siRNA at the protein level (Figure 5c). Collectively, our data demonstrated that AMPK activation played a key role in this inhibitory effect of vinpocetine against LPS-induced inflammatory response.

DISCUSSION

Microglia cells contribute to CNS development and help to maintain tissue homeostasis by supporting neuronal survival, cell death and synaptogenesis. Excessive activation of microglia leads to neuronal death, brain injury, and the release of a variety of neuroinflammation factors such as NO, iNOS, COX-2, TNF-α, IL-6, and IL-1β (Zhang et al. 2018c, Voet et al. 2019). It has been reported that overexpression of iNOS and COX-2 in microglia aggravating neurodegenerative diseases, while inhibition of iNOS and COX-2 expression was benefit (Wang et al. 2018, Lee et al. 2019). Lipopolysaccharides (LPS) is isolated from the membrane of Gram-negative bacteria, which act as prototypical endotoxins to induce inflammatory responses (Choi et al. 2017). Therefore, LPS has been commonly used to conduct an in vitro experimental model of microglia activation (Ni et al. 2015, Cunningham

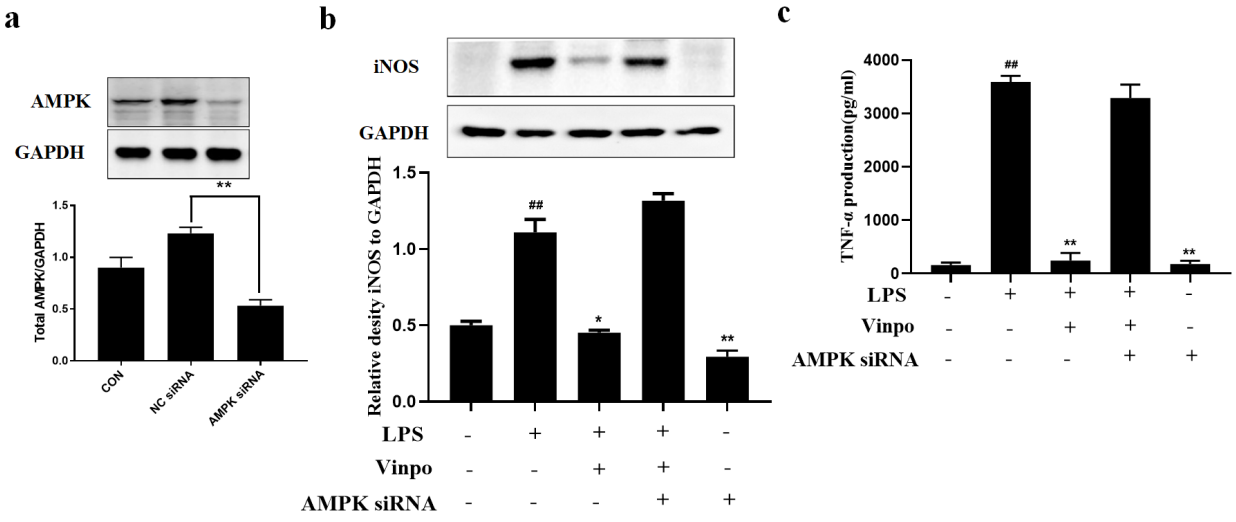

Figure 5. Effect of AMPK activation in the process of anti-inflammatory effects of vinpocetine induced by LPS in BV2 microglia. (a) AMPK siRNA significantly reduced AMPK protein expression in BV2 microglia. Data are representative of three independent experiments. **P < 0.01 compared with indicated groups. (b) Western blot measurement of iNOS protein expression in BV2 microglia. Data are representative of three independent experiments. (c) Supernatants were measured for the release of TNF-α by ELISA assays. Data are representative of three independent experiments. ##P < 0.01, compared with control group, *P < 0.05 and **P < 0.01 compared with LPS groups.

2013). The reduction of inflammatory mediators by modulation of the activation of microglia cells is important for the treatment of neuroinflammation.

Vinpocetine was firstly developed and marketed in Hungary, and has been extensively used in many countries for the treatment of cerebrovascular disorders for over 30 years (Zhang et al. 2018c). Due to little side effects and toxicity for long-term use at therapeutic doses, vinpocetine has attracted considerable attention to study its therapeutic mechanisms and pharmacological targets. In the present study, we provided convincing evidences that vinpocetine plays an anti-inflammatory role in LPS-stimulated BV2 microglia cells and identified a potential molecular mechanism. Our results indicated that pre-treatment with vinpocetine suppressed inflammatory reactions via inhibition of inflammatory mediators NO, iNOS, COX-2, TNF-α, IL-6, and IL-1β in LPS-induced BV2 microglia activation. Moreover, vinpocetine regulated LPS-induced phosphorylation of the AMPK pathway. Taken together, our results suggested that vinpocetine may be a potential candidate for the treatment of neuroinflammatory diseases.

Adenosine monophosphate-activated protein kinase (AMPK) is a highly conserved serine/threonine protein kinase that consists of three subunits: a catalytic α subunit and regulatory β and γ subunits (Peixoto et al. 2017). Recently, increasing evidences shown that AMPK activation suppressed neuroinflammation (Zhang et al. 2018b, Zhou et al. 2014). Our results reveal that vinpocetine treatment increased the expression of phosphorylated AMPKα in LPS-induced BV2 microglia. Vinpocetine has earlier been reported to produce anti-inflammatory effect via NF-κB pathway (Jeon et al. 2010, Zhang et al. 2018a). As is reported, NF-kB pathway was modulated by upstream AMPK pathway that suppress the expression of inflammatory genes (Jones et al. 2017). Activation of AMPK and its downstream protein NF-κB are involved in controlling neuroinflammation. However, the role of AMPK in inflammatory in BV2 microglia is still unclear. To our knowledge, this is the first study showing that vinpocetine activates AMPK phosphorylation in BV2 microglia.

Taken together, the present findings demonstrate that vinpocetine treatment can inhibit microglia-mediated inflammation, and the anti-inflammatory mechanism was associated with the activation of AMPK signaling pathway. This finding may provide an alternative therapeutic strategy to neuroinflammation-related diseases.

In summary, our results shown that vinpocetine significantly attenuated the expression of inflammatory mediators induced by LPS in BV2 microglia, including NO, iNOS, COX-2, TNF-α, IL-6, and IL-1β. In addition, the anti-inflammatory activity of vinpocetine was mediated via phosphorylation of AMPK signal pathway.

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QIN ZHOU[1#]
https://orcid.org/0000-0002-0448-8360

DONGKAI GUO[2, 3#]
https://orcid.org/0000-0001-6736-5344

XINJIA LI[1]
https://orcid.org/0000-0003-3703-1006

YIXUAN WANG[1]
https://orcid.org/0000-0002-3360-3508

XIAOLI YE[1]
https://orcid.org/0000-0003-3741-5341

SUDONG XUE[1]
https://orcid.org/0000-0002-7512-0272

XIAOYU WANG[1]
https://orcid.org/0000-0003-3817-1991

[1]Department of Pharmacy, The Affiliated Suzhou Hospital of Nanjing Medical University, Suzhou Municipal Hospital, Suzhou, Jiangsu, 215002, P.R. China

[2]Department of pharmacy, The Affiliated Suzhou Science and Technology Town Hospital of Nanjing Medical University, Suzhou, Jiangsu, 215153, P.R. China

[3]Jiangsu Key Laboratory of Neuropsychiatric Diseases Research and College of Pharmaceutical Sciences, Soochow University, Suzhou, Jiangsu, 215021, P.R. China

Corresponding to: **Xiaoyu Wang**
E-mail: cpuwxy@126.com

[#]These authors contributed equally to this work.

Author contributions

Qin Zhou, Dongkai Guo and Xiaoyu Wang designed the study. Xinjia Li and Yixuan Wang collected the data and participated in the experiments. XiaoLi Ye analyzed the data. Dongkai Guo and Xiaoyu Wang wrote the manuscript. Sudong Xue revised the manuscript. All authors approved the final version of the manuscript.