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Anti-neuroinflammatory effects of grossamide from hemp seed via suppression of TLR-4-mediated NF-κB signaling pathways in lipopolysaccharide-stimulated BV2 microglia cells

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Abstract Grossamide, a representative lignanamide in hemp seed, has been reported to possess potential anti-inflammatory effects. However, the potential anti-neuroinflammatory effects and underlying mechanisms of action of grossamide are still unclear. Therefore, the present study investigated the possible effects and underlying mechanisms of grossamide against lipopolysaccharide (LPS)-induced inflammatory response in BV2 microglia cells. BV2 microglia cells were pre-treated with various concentrations of grossamide before being stimulated with LPS to induce inflammation. The levels of pro-inflammatory cytokines were determined using the enzyme-linked immunoassay (ELISA) and mRNA expression levels were measured by real-time PCR. The translocation of nuclear factor-kappa B (NF-κB) and contribution of TLR4-mediated NF-κB activation on inflammatory effects were evaluated by immunostaining and Western blot analysis. This study demonstrated that grossamide significantly inhibited the secretion of pro-inflammatory mediators such as interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α), and decreased the level of LPS-mediated IL-6 and TNF-α mRNA. In addition, it significantly reduced the phosphorylation levels of NF-κB subunit p65 in a concentration-dependent manner and suppressed translocation of NF-κB p65 into the nucleus. Furthermore, grossamide markedly attenuated the LPS-induced expression of Toll-like receptor 4 (TLR4) and myeloid differentiation factor 88 (MyD88). Taken together, these data suggest that grossamide could be a potential therapeutic candidate for inhibiting neuroinflammation in neurodegenerative diseases.

Keywords Hemp seed · Lignanamide · Grossamide · Anti-neuroinflammatory · BV2 microglia cells · NF-κB

Introduction

Microglia cells are the immune cells of the central nervous system and consequently play important roles in brain infections and inflammation [1] by phagocytosing and killing pathogens and regulating immune responses. However, in response to abnormal stimulation, such as endogenous proteins or chemical toxins, they become overactive and release inflammatory molecules that cause neuronal damage and neurodegenerative diseases (i.e., Alzheimer's disease, Parkinson's disease, multiple sclerosis, and other nervous system pathologies) [1–3]. Thus, inhibiting the overactivation of microglia cells might be a promising approach for the treatment of neurodegenerative diseases [4].

Lipopolysaccharide (LPS), a stimulator of microglia, is recognized by Toll-like receptor 4 (TLR4) on the surface of microglia and induces TLR4 activation, which subsequently recruits the adapter protein MyD88 resulting in activation of NF-κB and MAPK signaling pathways. Nuclear factor-kappa B (NF-κB), a major transcription factor in the inflammatory response, can enhance the

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expression of pro-inflammatory mediators and enzymes [5]. Previous studies have shown that the NF-κB signaling pathway can be activated via phosphorylation and degradation of IκBα. Consequently, p65 translocates to the nucleus to regulate the expression of related genes. Overactivation of the NF-κB signaling pathway causes the excessive production of pro-inflammatory cytokines including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) [3, 6]. Consequently, an array of microglia responses is triggered leading to the release of inflammatory mediators [7, 8].

Grossamide is a representative lignanamide that has been isolated from the herbal plant heep seed (fructus cannabis, the dried fruit of *Cannabis sativa* L.), an important medicinal plant that has been used in traditional Chinese medicine for thousands of years. Recent research has found that hemp seed has a broad pharmacological effect on cardiovascular system [9], central nervous system [10], and the immune system. Crude extracts of hemp seed or hemp seed meal showed antioxidant [11, 12], anti-aging [13, 14], anti-inflammatory [15, 16], and learning and memory improving activities [17, 18]. Previous research has shown that hemp seed is rich in lignanamides [19–23]. Some reports showed that lignanamides were good antioxidant and anti-inflammatory agents [24]. In our previous chemical study of hemp seed [23], we have found that grossamide is one of the main lignanamides of hemp seed. The anti-inflammatory activity of grossamide related to NO production had been reported [24, 25].

In this study, we evaluated the anti-inflammatory effects of grossamide in the LPS-stimulated BV2 microglia cells. Our results show that grossamide exhibits anti-neuroinflammatory effects in BV2 microglia cells through suppression of TLR4-mediated NF-κB pathway.

Materials and methods

Cell line and reagents

Murine BV2 microglia cells were obtained from the China Infrastructure of Cell Line Resources (Beijing, China). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), and antibiotics were purchased from Macgene (Beijing, China). LPS (*E. coli* 0111:B4), DAPI, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St Louis, MO, USA).The microscope cover glasses were purchased from NEST (Wuxi, China). The antibodies against NF-κB p65 were obtained from Santa Cruz (Santa Cruz, CA, USA); the antibodies against IκBα, phospho-IκBα, and TLR4 were from Abcam (Cambridge, UK). Antibodies against MyD88, phospho-p65, and β-actin were obtained from cell signaling (Danvers, MA, USA). TNF-α and IL-6 enzyme-linked immunosorbent assay (ELISA) kits were purchased from Boster (Wuhan, China). Trizol reagent was purchased from Takara (Takara; Shiga, Japan); RT-PCR primers were obtained from Sangon Biotech (Shanghai, China); and horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from ZSGB-BIO (Beijing, China). Immobilon Western Chemiluminescent Substrate (ECL) was purchased from Millipore (Billerica, MA, USA), and other materials for Western blot analysis were purchased from Bio-Rad (Hercules, CA, USA).

Grossamide was isolated from hemp seed as previously described by us [23], with the purity of more than 98% according to the analysis using high-performance liquid chromatography (HPLC). Briefly, the air-dried hemp seed (5.7 kg) were crushed and defatted with petroleum ether before percolating with 75% EtOH. The 75% EtOH extract was successively partitioned with petroleum ether, ethyl acetate, and n-butanol. The ethyl acetate extract was subjected to reverse-phase column liquid chromatography (methanol/H_2O as eluent), MCI gel column chromatography (methanol/H_2O as eluent), and silica gel column chromatography (dichlormethan/methanol as eluent) successively, and then grossamide (22.94 mg) was crystallized from some fractions.

Cell culture

The murine BV-2 microglia cell line was maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin antibiotics, and incubated at 37 °C in a humidified atmosphere containing 5% CO_2.

Cell viability

Cell viability was measured using the MTT assay according to the manufacturer's instructions [26]. Briefly, 100 µL cells (8×10^4 cells/mL) were seeded into 96-well plates and incubated overnight. Then the cells were treated with different concentrations of grossamide (0, 10, 15, and 20 µM) for 1 h and co-cultured in the absence or presence of 100 ng/mL LPS for 24 h. Next, cells were incubated with 10 µL MTT solution (5 mg/mL) for 4 h. The formazan crystals were dissolved with 100 µL DMSO, and the absorbance was measured at 570 nm using a microplate reader (Bio-rad). Results are expressed as a percentage of the control.

ELISA

The production of TNF-α and IL-6 in the culture supernatants was evaluated using ELISA kits, as previously described [27]. Briefly, BV2 cells (8×10^4 cells/mL) were seeded in a 96-well plate and incubated overnight. Then,

the cells were treated with or without different concentrations of grossamide for 1 h, and co-cultured with LPS (100 ng/mL) for another 24 h. Cell-free supernatants were collected and stored at −20 °C before analysis. TNF-α and IL-6 levels were measured with ELISA kits according to the manufacturer's instructions (Boster), and the absorbance was read at 450 nm on a microplate spectrophotometer (Bio-rad). For TNF-α, the cell-free supernatants were diluted ten times before measurement, and for IL-6, the cell-free supernatants were diluted three times.

Real-time PCR

BV2 microglia cells (2×10^6 cells/mL) were plated in 6-well culture plates overnight and pre-treated with various concentrations of grossamide for 1 h, after which they were co-cultured with LPS (100 ng/mL) for another 6 h. Total RNA was extracted using Trizol (Takara) and evaluated at 260 and 280 nm. RNA (1 μg) was reverse-transcribed using the PrimeScript TMRT reagent kit (Takara) according to the manufacturer's instructions. cDNA was used for real-time PCR with SYBR Primix Ex Taq™ (Takara). The real-time PCR reactions were performed for 40 cycles in 10 μL reaction volumes. Samples were incubated at 95 °C for 15 s, 53 °C for 15 s, and at 72 °C for 20 s. The relative amounts of mRNA were calculated with the comparative CT method. GAPDH was used as the internal control. The primer sequences were: 5′-ATGAGCACAGAAAGC ATGATC-3′ and 5′-TACAGGCTTGTCACTCGAATT-3′ forward and reverse primers for TNF-α, 5′-CCACTTCAC AAGTCGGAGGC-3′ and 5′-CCAGCTTATCTGTTAGGA GA-3′ forward and reverse primers for IL-6, 5′-GCA GTG GCA AAG TGG AGA TTG-3′ and 5′-TGC AGG ATG CAT TGC TGA CA-3′ forward and reverse primers for GAPDH.

Western blot analysis

The BV2 cells were harvested and washed with PBS two times. Then, the cells were lysed on ice for 30 min using RIPA buffer (Beyotime Biotechnology, China), followed by centrifugation at 14,000×*g* for 10 min at 4 °C. The protein concentrations were determined using the BCA Protein Assay Kit according to the manufacturer's instructions (Beyotime Biotechnology, China). Protein extracts were mixed with the sample loading dye and heated at 95 °C for 5 min. Samples were separated on 8–12% sodium dodecyl sulfate polyacrylamide gels and electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Germany). Then, the PVDF membranes were blocked in blocking buffer (1X TBS, 0.1% Tween-20, 5% nonfat dry milk) for 4 h and incubated at 4 °C overnight with primary antibodies against IκBα, p-IκBα (1:1000, Abcam), p65 (1:1000, Santa Cruz), phospho-p65 (1:1000, Cell Signaling), TLR4 (1:500, Abcam), MyD44 (1:500, Cell Signaling), and β-actin (1:2000, Cell Signaling). After washing, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (1:5000, ZSGB-BIO) for another 1 h at room temperature. Then, the blots were washed in TBST (Tris-buffered saline, 0.1% Tween 20) 4 times and developed using an enhanced chemiluminescence detection kit (Millipore). Optical density analysis of signals was performed using Image Lab software.

Immunofluorescence assay

To determine the intracellular localization of the p65 subunit of NF-κB, BV2 cells (5×10^5 cells/well) were cultured overnight in 24-well plates containing sterilized microscope cover glasses. Then, the cells were pre-treated with grossamide for 1 h and treated with LPS for 1 h, fixed in 4% paraformaldehyde for 10 min, and washed with PBS for 5 min. Next, cells were permeabilized with 1% Triton X-100 in PBS for 10 min and then incubated with 5% BSA for 1 h at room temperature. Treated cells were stained with NF-κB p65 primary antibody (1:200, Santa Cruz) overnight at 4 °C. The cells were washed with PBS for 5 min, incubated with an Alexa Fluor 594-labeled antibody (1:100, ZSGB-BIO) for 1 h at room temperature, and washed again with PBS containing 0.02% Tween-20 for 5 min. The nuclei were stained with 2.5 μg/mL DAPI (Sigma) for 10 min at room temperature and then washed. Finally, all of the images were captured using a confocal fluorescence microscope (Carl Zeiss, Göttingen, Germany), and all images were observed at ×63 Oil Mirror.

Statistical analysis

Experiments were repeated 3–5 times. All of the data are expressed as the mean ± standard deviation (SD). The difference between treated and control cells was analyzed by one-way analysis of variance (ANOVA) using Graphpad Prism v5.0 software (GraphPad, La Jolla, CA, USA). *p* values less than 0.05 were considered statistically significant.

Results

Effects of grossamide and LPS on viability of BV2 microglia cells

MTT assay showed that grossamide itself had no obvious cytotoxicity at 10, 15, and 25 μM in the BV2 microglial cells after 24 or 48 h treatments (data not shown). To evaluate the cytotoxic effects of grossamide and LPS on BV2 microglia, the cells were treated with grossamide with

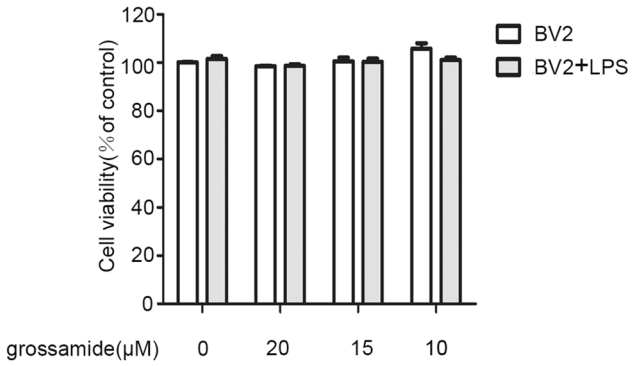

Fig. 1 Effects of grossamide (0, 10, 15, and 20 μM) on viability of BV2 microglia cells. Cell viability was determined using the MTT assay. Cells were treated with grossamide with or without LPS (100 ng/mL) for 24 h. The results are presented as mean ± SD from three independent experiments. *$p < 0.05$ and **$p < 0.01$ versus cells without treatment

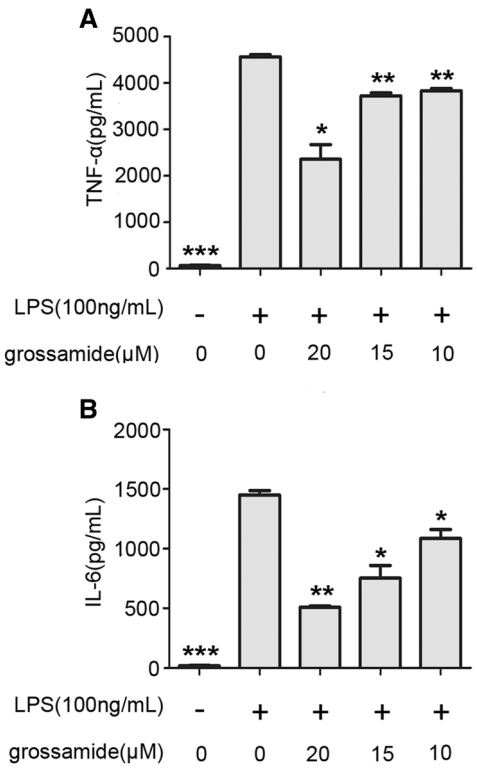

Fig. 2 Effects of grossamide on TNF-α and IL-6 release from LPS-stimulated BV2 microglia cells. BV2 microglia cells were pre-treated with grossamide for 1 h prior to incubation with LPS (100 ng/mL) for another 24 h. The culture supernatants were harvested, and secretion of TNF-α (**a**) and IL-6 (**b**) was measured by ELISA. The data are presented as mean ± SD from at least three independent experiments. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ versus cells treated with LPS

or without LPS (100 ng/mL) for 24 h. We have found that grossamide had no significant effects on cell viability at concentrations up to 20 μM, and 100 ng/mL LPS had no obvious cytotoxic effects (Fig. 1). Thus, 100 ng/mL LPS and concentrations of 10, 15, and 20 μM grossamide were used for the subsequent experiments.

Effects of grossamide on the release of pro-inflammatory cytokines from LPS-stimulated BV2 microglia cells

TNF-α and IL-6 are representative pro-inflammatory cytokines produced by microglia cells [28]. As shown in Fig. 2, LPS significantly increased TNF-α and IL-6 release in the absence of grossamide. However, pre-treatment of cells with grossamide followed by stimulation with LPS led to a significant and dose-dependent inhibition of TNF-α and IL-6 release. These results suggest that grossamide downregulates LPS-mediated production of inflammatory molecules.

Effects of grossamide on TNF-α and IL-6 mRNA expression in BV2 microglia cells

To determine if treatment with grossamide suppressed the LPS-mediated pro-inflammatory process, RT-PCR was used to quantify the mRNA levels of TNF-α and IL-6. As shown in Fig. 3, grossamide inhibited the mRNA levels of TNF-α and IL-6 in a dose-dependent manner.

Effects of grossamide on NF-κB activation in BV2 microglia cells

NF-κB plays a major role in controlling a number of genes involved in inflammatory responses [29]. In the active state, NF-κB is released from IκBα through phosphorylation and degradation of IκBα, followed by translocation into the nucleus and regulation of pro-inflammatory gene expression [30]. To determine the anti-inflammatory mechanism underlying the effects of grossamide in BV2 microglia cells, LPS-stimulated activation of the NF-κB pathway was evaluated by Western blot analysis. Upon LPS stimulation, the phosphorylation and degradation of IκBα resulted in activation of NF-κB and subsequent translocation into the nucleus. As shown in Fig. 4a, pre-treatment of cells with grossamide inhibited LPS-induced phosphorylation of IκBα and significantly reduced phosphorylation of NF-κB p65 levels. To further confirm the effects of grossamide on NF-κB, we explored whether grossamide prevented LPS-induced NF-κB p65 nuclear localization. Immunofluorescence images of NF-κB p65 showed that grossamide

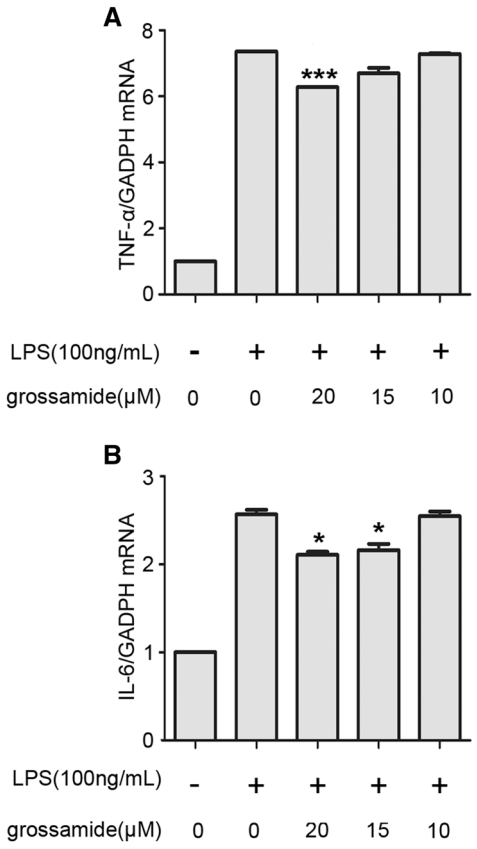

Fig. 3 Effects of grossamide on TNF-α and IL-6 mRNA levels in LPS-stimulated BV2 microglia cells. BV2 microglia cells were pre-treated with grossamide for 1 h prior to incubation with LPS (100 ng/mL) for 6 h. The mRNA expression levels of TNF-α (**a**) and IL-6 (**b**) were measured with RT-PCR. The data are presented as mean ± SD from at least three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ versus cells treated with LPS

markedly blocked the nuclear localization of NF-κB p65 (Fig. 4d).

Effects of grossamide on TLR4 and MyD88 expression in BV2 microglia cells stimulated with LPS

TLR4 regulates the expression of LPS-induced inflammatory mediators through the activation of NF-κB [31]. Therefore, the effects of grossamide on LPS-induced TLR4 expression were examined. As shown in Fig. 5a, in LPS-stimulated microglia cells, pre-treatment with grossamide dose-dependently decreased the expression of TLR4. The interaction of MyD88 and TLR4 is critical for TLR4 to stimulate downstream signaling pathways and activate inflammatory responses. Therefore, the effects of grossamide with MyD88 were also evaluated. As shown in Fig. 5a, after treatment of BV2 microglia cells with LPS (100 ng/mL), MyD88 protein levels gradually increased, but were suppressed upon treatment with grossamide. These data suggest that TLR4 and MyD88 proteins are involved in the anti-neuroinflammatory effects of grossamide in LPS-stimulated BV2 microglia cells.

Discussion

This study showed that pre-treatment with grossamide decreased the release of the pro-inflammatory cytokines, including IL-6 and TNF-α, and significantly inhibited inflammatory processes and associated signaling molecules including phospho-IκBα and phospho-NF-κB p65 proteins. In addition, grossamide decreased TLR4 and MyD88 protein expression in LPS-stimulated BV2 microglia cells. These data suggest that grossamide exhibits anti-neuroinflammatory effects in LPS-stimulated BV2 microglia cells.

Previous studies have reported that hemp seed has been used as a medicine in China for at least 3000 years due to its anti-aging and inflammatory effects [32–34]. Recent phytochemical studies isolated active components, including cannabinoids and lignanamides, which are attractive candidates for the treatment of neurodegenerative diseases [21, 35–38]. Grossamide is a lignanamide isolated from hemp seed, which has been shown to inhibit nitric oxide production in LPS-stimulated RAW 264.7 macrophages [25]. However, its anti-neuroinflammatory effects are not fully explored. Hence, as an example of lignanamides in hemp seed, we investigated the anti-inflammatory effects and underlying molecular mechanisms of grossamide in LPS-stimulated BV2 microglia cells.

LPS-activated microglia cells secrete pro-inflammatory cytokines including TNF-α, IL-1β, and IL-6, all of which play major roles in the inflammation-associated diseases [39]. Microglia cells release pro-inflammatory cytokines to promote neuronal cell damage. TNF-α, a crucial mediator of the inflammatory response, activates the expression of chemokines, thereby affecting biological functions [40]. IL-6 is also an important pro-inflammatory cytokine in the immune response [41]. In this study, we found that LPS highly stimulated the release of both TNF-α and IL-6, and this release was inhibited by grossamide in a dose-dependent manner. In addition, grossamide suppressed the mRNA levels of TNF-α and IL-6 in a dose-dependent manner. These results demonstrate that grossamide decreases LPS-induced gene expression and protein production and release of IL-6 and TNF-α in BV2 microglia cells in a concentration-dependent manner.

A number of reports have indicated that NF-κB activity can control the immune and inflammatory responses, and regulate the expression of inflammation-associated genes such as iNOS, COX-2, TNF-α, IL-6, and IL-1β [42]. Under inactive conditions, NF-κB is located in the cytoplasm and is associated with the inhibitory protein

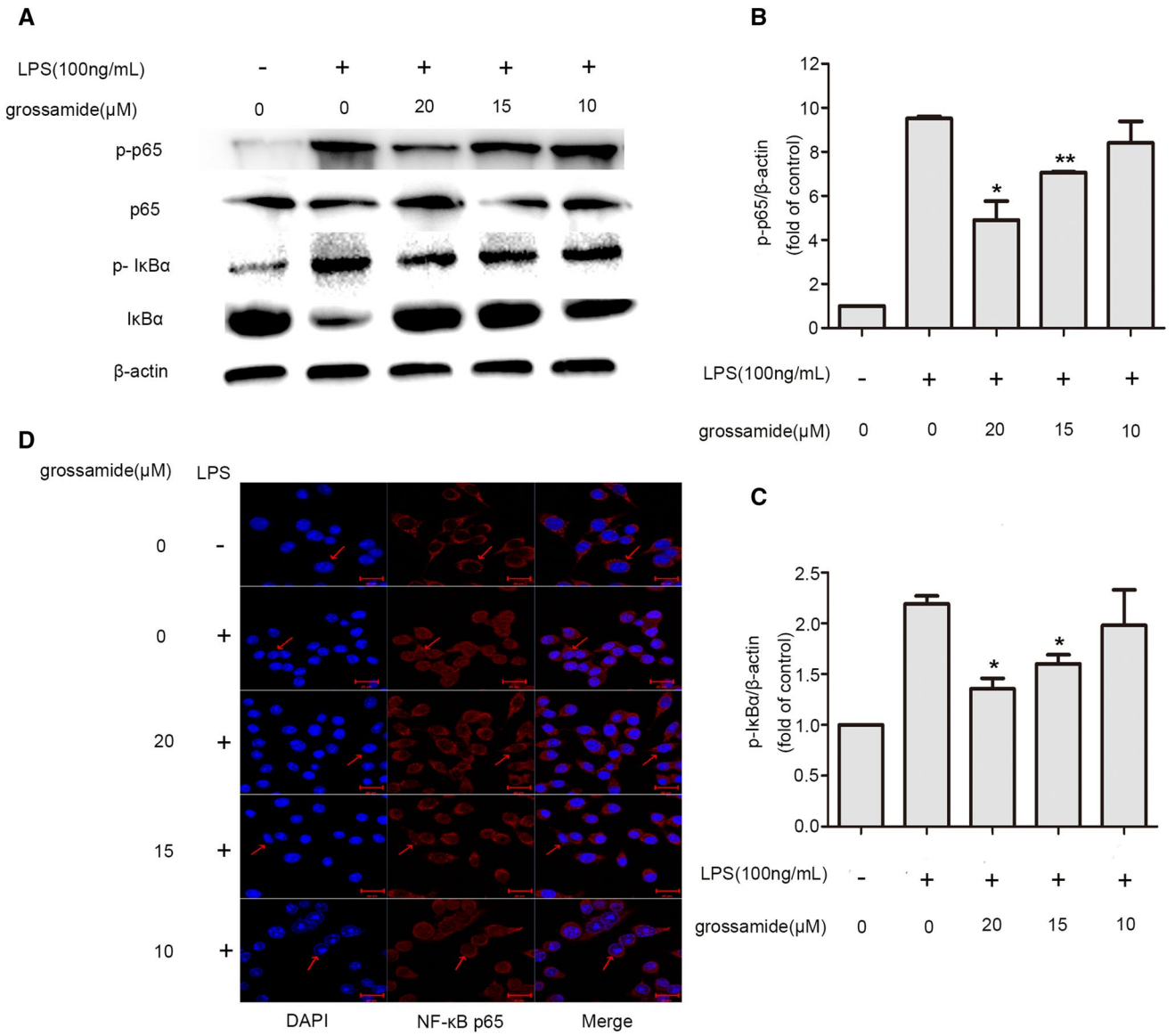

Fig. 4 Effects of grossamide on the NF-κB pathway in LPS-stimulated BV2 microglia cells. BV2 microglia cells were pre-treated with grossamide for 1 h followed by LPS (100 ng/mL) stimulation for 1 h. **a** Protein samples were evaluated by Western blot analysis. Phospho-IκBα and phospho-p65 proteins were analyzed. β-actin was used as the internal control for normalization. The *bar chart* shows the quantitative evaluation of phospho-p65 (**b**) and phospho-IκBα (**c**) bands by densitometry. **d** Confocal microscopic images of immunofluorescent staining of NF-κB p65 in BV2 microglia cells (magnification = ×63, *scale bar* = 20 μm, *red arrows* labeled co-localization of the representative cell). The data are presented as mean ± SD from at least three independent experiments. $*p < 0.05$ and $**p < 0.01$ versus cells treated with LPS

IκBα. In response to LPS stimuli, IκBα is phosphorylated and degraded, and activated NF-κB then translocates into the nucleus [43–45]. This study found that grossamide inhibited the phosphorylation of IκBα and NF-κB p65. In addition, grossamide suppressed NF-κB p65 protein translocation to the nucleus. Thus, the decreased production of pro-inflammatory molecules by grossamide is probably due to the suppressed activation of the NF-κB signaling pathway. LPS binds to TLR4 on the surface of microglia cells, resulting in the overexpression of pro-inflammatory genes and oversecretion of pro-inflammatory molecules by NF-κB activation [31, 46]. Here, grossamide suppressed the protein expression of TLR4 and MyD88, which probably resulted in the decreased production of pro-inflammatory molecules. Thus, it appears that grossamide suppressed the NF-κB signaling pathway by downregulating the protein expression of TLR4 and MyD88.

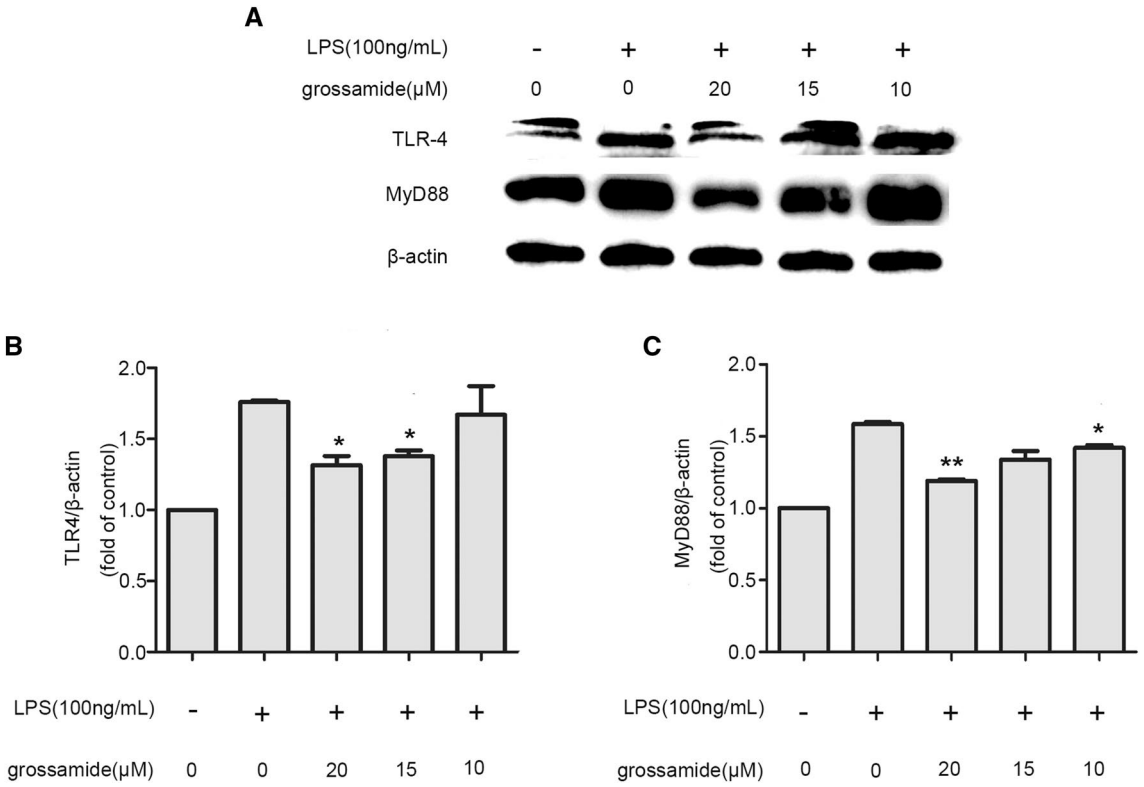

Fig. 5 Effects of grossamide on TLR4 and MyD88 protein expression in LPS-stimulated BV2 microglia cells. Cells were pre-incubated with grossamide for 1 h and/or incubated with LPS (100 ng/mL) for 24 h. **a** Total protein lysates were evaluated by Western blot analysis for TLR4 and MyD88 protein expression. β-actin was used as the internal control for normalization. The *bar chart* shows the quantitative evaluation of TLR4 (**b**) and MyD88 (**c**) bands by densitometry. The data are presented as mean ± SD from at least three independent experiments. $*p < 0.05$ and $**p < 0.01$ versus cells treated with LPS

Conclusions

The present study showed that grossamide significantly reduced the production of pro-inflammatory mediators, and these effects were due to inhibition of the TLR4-mediated NF-κB signaling pathway. These results suggest that grossamide may be a potent natural anti-neuroinflammatory agent. Based on the anti-neuroinflammatory effects of grossamide, future studies should determine if other chemical compounds from hemp seed also have anti-inflammatory effects.

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Compliance with ethical standards

Conflict of interest The authors declare no competing financial interest.

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