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Original Article

Bavachin attenuates LPS-induced inflammatory response and inhibits the activation of NLRP3 inflammasome in macrophages

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ABSTRACT

Background: Bavachin is a natural product isolated from *Psoralea corylifolia* L. that has been applied as a traditional medicine in Asian countries. However, the anti-inflammatory effects of bavachin on LPS-induced inflammation and NLRP3 inflammasome activation by macrophages remain unclear.

Purpose: We investigated the anti-inflammatory effects of bavachin on LPS-activated murine macrophage cell line J774A.1 cells and murine peritoneal macrophages.

Methods: J774A.1 cells and murine peritoneal macrophages were pre-treated with bavachin following LPS treatment. The concentrations of NO, PGE_2, IL-6 and IL-12p40 in cell culture supernatant were analyzed. The expressions of iNOS, COX-2, mPGES-1 and MAPKs were analyzed using Western blotting, while NF-κB activity was detected using promoter reporter assay. To examine the activation of NLRP3 inflammasome, J774A.1 cells were incubated with LPS, and then treated with bavachin following treatment with ATP. The concentration of IL-1β in the cell culture supernatant was measured. The expressions of NLRP3, ASC, caspase-1 and IL-1β were analyzed using Western blotting. The formation of inflammasome complex was observed by immunofluorescence microscopy.

Results: Bavachin suppressed LPS-induced NO and PGE_2 production, and decreased iNOS and mPGES-1 expression. Bavachin also reduced LPS-induced IL-6 and IL-12p40 production and decreased the activation of MAPKs and NF-κB. Additionally, bavachin suppressed NLRP3 inflammasome-derived IL-1β secretion, decreased caspase-1 activation, repressed mature IL-1β expression, and inhibited inflammasome complex formation. Furthermore, bavachin also suppressed the production of NO, IL-6 and IL-12p40 by LPS-stimulated murine peritoneal macrophages.

Conclusion: Our experimental results indicated anti-inflammatory effects of bavachin exhibit attenuation of LPS-

Abbreviations: ASC, apoptosis-associated speck-like protein containing a caspase-activation and -recruitment domain; DAMPs, damage-associated molecular patterns; iNOS, inducible nitric oxide synthase; mPGES-1, microsomal prostaglandin E synthases 1; NLRP3, NLR-family pyrin domain-containing 3; NLRs, nucleotide-binding and oligomerization domain-like receptors; PAMP, pathogen-associated molecular patterns; PGE_2, prostaglandin E_2; PRRs, pattern recognition receptors; RIPA, radioimmunoprecipitation assay; SEAP, secrete embryonic alkaline phosphatase

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induced inflammation and inhibit activation of NLRP3 inflammasome in macrophages. These results suggest that bavachin might have potential in treating inflammatory and autoimmune diseases.

Introduction

Psoralea corylifolia L. is an important medicinal plant used in Asian countries as a kidney-tonifying herbal medicine for treating various diseases such as osteoporosis, leukoderma, and inflammatory skin diseases (Chopra et al., 2013). Many natural products of *Psoralea corylifolia* L. have been indicated to possess estrogenic, anti-oxidant, anti-diabetic, and anti-inflammatory effects (Alam et al., 2018; Chopra et al., 2013). Bavachin is a natural product that is isolated from the seed and fruit of *Psoralea corylifolia* L. (Yang et al., 2018; Zhang et al., 2016). Although the information about absorption and transformation of bavachin in human body remains unclear, it has been found that concentration of bavachin increased plasma after oral administration with *Psoralea corylifolia* L. in rats (Gao et al., 2016; Yang et al., 2018). Previous studies have found that bavachin protects against ovariectomy-induced bone loss in the rat animal model, and activates osteoblastic proliferation in primary human osteoblasts (Li et al., 2014; Weng et al., 2015). Especially, bavachin decreases the activation and phosphorylation of signal transducer and activator of transcription 3 in IL-6-stimulated hepatoma cells (Lee et al., 2012), inhibits NF-κB activity and chemokine production in IL-1β-stimulated chondrocyte CHON-002 cells, and blocks migration in IL-1β-stimulated monocyte THP-1 cells (Cheng et al., 2010). However, the anti-inflammatory effects of bavachin on lipopolysaccharide (LPS)-induced inflammation and the NLR-family pyrin domain-containing 3 (NLRP3) inflammasome activation-induced IL-1β productions by macrophages remain unclear.

The inflammatory response of innate immunity is crucial in dealing with microbial infection, tissue damage, stress and malfunction (Medzhitov, 2008). The major inflammatory response is initiated by pattern recognition receptors (PRRs) of immune cells to recognize molecules of microorganisms such as bacterial LPS. The molecules of microorganisms are called pathogen-associated molecular patterns (PAMPs), and endogenous molecules of necrotic cells are called damage-associated molecular patterns (DAMPs) (Takeuchi and Akira, 2010). Toll-like receptor 4 (TLR4) is an important PRR that recognizes bacterial LPS and transduces a variety of signaling pathways including MAPKs and NF-κB pathways to produce pro-inflammatory mediators such as nitric oxide (NO), prostaglandin E_2 (PGE_2) and pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6. The TLR4 signaling pathway leads to inflammation and is associated with pathogenesis of many inflammatory diseases such as atherosclerosis, diabetes and cancer (Drexler and Foxwell, 2010). Macrophages are considered to play an important role in modulating inflammatory response; therefore, activated macrophages are involved in the pathogenesis of multiple diseases (Wynn et al., 2013).

The MAPK subfamilies, JNK, ERK and p38 MAPK, are phosphorylated by TLR4 signaling pathways and activate several downstream transcription factors such as activator protein 1 and NF-κB (Guha and Mackman, 2001) that mediate the production of inducible enzymes such as inducible NOS (iNOS), cyclooxygenase-2 (COX-2) and microsomal prostaglandin E synthases 1 (mPGES-1) (Diaz-Munoz et al., 2010; Murakami and Ohigashi, 2007). NF-κB is considered as a critical contributor in transcription of pro-inflammatory mediators and cytokines. Activation of NF-κB is initiated by phosphorylation and degradation of IκBα, and NF-κB translocates into the nucleus. Especially, NF-κB is a central regulator of iNOS and COX-2 (Surh et al., 2001). Taken together, TLR4 regulates the activation of MAPKs, and their downstream transcription factors in turn produce pro-inflammatory mediators and cytokines.

On the other hand, another PRR, nucleotide-binding and oligomerization domain-like receptors (NLRs) and absent in melanoma 2 like receptors recruit inflammasomes to regulate pyroptosis and produce IL-1β and IL-18 (Lamkanfi and Dixit, 2011). Pyroptosis has been defined as programmed cell death induced in response to inflammation (Sharma and Kanneganti, 2016). Inflammasomes are involved in pathogenesis of multiple inflammatory diseases. Especially, NLRP3 inflammasome has been regarded as a regulator in development of atherosclerosis, diabetes and several immune-mediated diseases (Strowig et al., 2012). The NLRP3 inflammasome complex is composed of NLRP3, apoptosis-associated speck-like protein containing a caspase-activation and -recruitment domain (ASC), caspase-1, which activates by recognition of PAMPs such as influenza and fungi, and DAMPs such as monosodium urate crystals and ATP. The activation of NLRP3 inflammasome in macrophages is triggered by two steps (priming and activation). In the priming step, NLRP3 is produced by TLRs- or TNF-α receptor-mediated NF-κB. Concurrently, NF-κB also produces pro-IL-1β and pro-IL-18. NLRP3 is activated by PAMPs or DAMPs, and increases ASC oligomerization in the activation step. Subsequently, ASC converts caspase-1 into the active form, and activated caspase-1 cleaves the pro-IL-1β and pro-IL-18 (Kesavardhana and Kanneganti, 2017).

In the present study, we investigated the effects of bavachin on the production of pro-inflammatory mediators (NO and PGE_2) and cytokines (IL-6 and IL-12p40) by LPS-stimulated murine macrophage cell line J774A.1 cells. In addition, we also examined the effects of bavachin on the expression of iNOS, COX-2 and mPGES-1, and the activation of the MAPKs signaling pathway and NF-κB by LPS-stimulated J774A.1 cells. We also confirmed the effects of bavachin on NLRP3 inflammasome activation-induced IL-1β production and NLRP3 inflammasome complex (NLRP3, ASC and caspase-1) protein expression levels. Additionally, we examined the effects of bavachin on production of NO, IL-6 and IL-12p40 by LPS-stimulated murine peritoneal macrophages.

Material and methods

Ethics statement

The animal care and experimental protocols in this study complied with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and followed a protocol approved by the Committee on the Ethics of Animal Experiments of the Kaohsiung Medical University (Permit Number: 104,092)(2015).

Reagents

Bavachin (purity ≧ 98%, Supplementary Fig. 1) was obtained from ChemFaces (Wuhan, Hubei, China), and the results of the limulus amebocyte lysate single test (Associates of Cape Cod, Inc., Falmouth, MA, USA) confirmed that endotoxin levels in bavachin were less than 0.03 EU ml^{-1}. All cell culture reagents were obtained from Gibco-BRL (Life Technologies, Grand Island, NY, USA). Thioglycollate medium, PBS, LPS (from *E. coli* O111:B4), radioimmunoprecipitation assay (RIPA) buffer, protease inhibitor cocktail, phosphatase inhibitor cocktail, 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), trypan blue, Griess reagent, curcumin and dexamethasone were obtained from Sigma Aldrich (St. Louis, MO, USA). BCA protein assay reagent, ECL chemiluminescence substrate and stripping buffer were obtained from Thermo Scientific (Waltham, MA, USA). For Western blotting, rabbit antibodies against mouse NLRP3, phospho-JNK1/2, JNK1/2, phospho-ERK1/2, ERK1/2, phospho-p38 MAPK, and p38 MAPK were obtained from Cell Signaling (Farmingdale, NY, USA).

Mouse antibody against caspase-1 (p20) was obtained from Adipogen (San Diego, CA, USA). Mouse antibody against β-tubulin was obtained from Thermo Scientific (Waltham, MA, USA). Rabbit antibodies against mouse iNOS, COX-2, ASC, IL-1β and secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit antibody against mouse mPGES-1 and PGE_2 ELISA kit were obtained from Cayman Chemical Company (Ann Arbor, MI, USA). For immunofluorescence staining, mouse antibody against ASC and rabbit antibody against caspase-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Alexa Fluor® 488-cougugated anti-rabbit IgG and Alexa Fluor® 647-cougugated anti-mouse IgG were purchased from Jackson ImmunoResearch Labs Inc. (West Grove, PA, USA). Hoechst 33,342 used for nuclear staining was obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). IL-1β, IL-6 and IL-12p40 ELISA kits were obtained from eBioscience (San Diego, CA, USA).

Cell culture

Murine macrophage cell line J774A.1 cells were obtained from Bioresource Collection and Research Center (Food Industry Research and Development Institute, Hsinchu, Taiwan), and were cultured in RPMI-1640 medium supplemented with 10% (v/v) FBS (Gibco-BRL, Life Technologies, Grand Island, NY, USA) and antibiotics (100 U ml^{-1} penicillin and 100 U ml^{-1} streptomycin) and passaged every 2–3 days to maintain growth at 37 °C in a humidified incubator containing 5% CO_2. The detailed experimental procedure for isolation of murine peritoneal macrophages has been described previously (Hung et al., 2017). Briefly, the specific pathogen-free female C57BL/6 mice (6–8 week of age) were intraperitoneally injected with 1 ml sterile 3% thioglycollate medium and peritoneal macrophages were collected after 4 days. The peritoneal macrophages had 95% viability as judged by the trypan blue dye exclusion assay, and were re-suspended in RPMI-1640 medium. The cells were seeded in a 96-well plate at a concentration of 1×10^6 cells ml^{-1} to allow macrophage adherence by incubation for 3 h at 37 °C in a humidified incubator containing 5% CO_2.

NO determination and cell viability

J774A.1 cells and murine peritoneal macrophages were seeded in a 96-well plate at a concentration of 1×10^6 cells ml^{-1}. The cells were pre-treated with various concentrations of bavachin (0, 10, 20, 30 and 40 μM) for 1 h following treatment of LPS (1 μg ml^{-1}). After 24 h, the supernatant of cell culture was harvested and the concentration of NO was quantified using the Griess reagent according to the manufacturer's instructions (Sigma Aldrich, St. Louis, MO, USA). The cells were incubated with MTT (5 mg ml^{-1}) at 37 °C for 4 h, and then lysed with isopropanol (0.04 M HCl). Cell viability was assessed by measuring the OD at 570 nm using a microplate reader.

Cytokine measurement

J774A.1 cells and murine peritoneal macrophages were seeded in a 96-well plate at a concentration of 1×10^6 cells ml^{-1}, and were allowed to acclimatize overnight. The cells were pre-treated with various concentrations (0, 10, 20, 30 and 40 μM) of bavachin for 1 h following treatment of LPS (1 μg ml^{-1}) for 24 h. The cell culture supernatant was used for determination of PGE_2, IL-6 and IL-12p40 concentrations by ELISA according to the manufacturer's protocols (eBioscience, San Diego, CA, USA).

NF-κB promoter reporter assay

J-blue cells were J774A.1 cells that were stably transfected with pNiFty2-SEAP plasmids. The pNiFty2-SEAP plasmids carry a reporter gene induced by NF-κB, and secrete embryonic alkaline phosphatase (SEAP). J-blue cells were maintained in RPMI-1640 medium supplemented with Zeocin (200 μg ml^{-1}) (InvivoGen, San Diego, CA, USA) (Hua et al., 2015). Cells were seeded in a 96-well plate at a concentration of 1×10^6 cells ml^{-1}, allowed to acclimatize overnight, and were then pre-treated with different concentrations of bavachin (0, 20 and 40 μM) for 1 h following treatment of LPS (1 μg ml^{-1}) for 24 h. The 20 μl cell culture supernatant was mixed with 200 μl QUANTI-blue medium (InvivoGen, San Diego, CA, USA) and incubated at 37 °C for 45 min. SEAP activity was analyzed by estimating the OD at 655 nm using a microplate reader.

NLRP3 inflammasome experiment

J774A.1 cells were seeded in a 6-well plate at a concentration of 1×10^6 cells ml^{-1}, and were allowed to acclimatize overnight. The cells were then treated with LPS (1 μg ml^{-1}) for 5 h, and then with various concentrations (0, 10, 20, 30 and 40 μM) of bavachin for 1 h following treatment of ATP (5 mM) for 30 min. The cell culture supernatant was collected and the concentration of IL-1β determined by using ELISA according to the manufacturer's instructions (eBioscience, San Diego, CA, USA), and the cell lysate was used for analysis of NLRP3 inflammasome protein levels by Western blotting.

Western blotting

Cells were lysed by RIPA buffer with protease inhibitors and phosphatase inhibitors (Sigma Aldrich, St. Louis, MO, USA), and were centrifuged at 12,000 rpm for 10 min to isolate cell lysate samples. The protein concentration of cell lysate samples was quantified by BCA protein assay. Cell lysate samples were separated on 8–15% SDS-PAGE and were transferred onto polyvinylidene difluoride membranes. The protein expression levels were analyzed using antibodies against NLRP3, phospho-JNK1/2, JNK1/2, phospho-ERK1/2, ERK1/2, phospho-p38 MAPK, p38 MAPK, iNOS, COX-2, ASC, IL-1β, caspase-1 (p20), mPGES-1 and β-tubulin. The protein band signals were detected by ECL chemiluminescence substrate and quantified by Alphaview SA software (Alpha Innotech Corporation, San Leandro, CA, USA).

Immunofluorescence staining

The formation of inflammasome was detected by ASC/caspase-1 staining. J774A.1 cells were seeded on 12-mm glass coverslips in 24 well-plates overnight. Cells were primed with LPS (1 μg ml^{-1}), and treated with bavachin and ATP as described above. Cells were fixed with 4% paraformaldehyde and permeabilized using 0.2% Triton X-100 in PBS. Cells were incubated with anti-ASC and anti-caspase-1 primary antibodies overnight, washed with PBS to remove the excessive primary antibodies, and then incubated with fluorescent secondary antibodies. The cell nucleus was labeled with Hoechst 33,342. High magnification fluorescent images were taken using an inverted epi-fluorescent microscope (Nikon-Ti, Nikon, Japan) with 60x oil immersion objectives. The localization and expression of ASC and caspase-1 were processed using NIS-Elements software (Nikon, Japan) and ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

All data were expressed as means ± SD. Each value is the mean of three independent experiments. Statistical analysis was assessed via one-way ANOVA followed by Tukey post-hoc test by GraphPad Prism 5 (San Diego, CA, USA), and the significant difference was set at $^*p < 0.05$; $^{**}p < 0.01$.

Results

Bavachin suppresses the production of NO and PGE_2 by LPS-stimulated J774A.1 cells

Firstly, we examined the effect of bavachin on the cell viability of J774A.1 cells using MTT assay. The cells were pre-treated with various concentrations of bavachin (0–40 μM) for 1 h following treatment of LPS (1 μg ml^{-1}) for 24 h. As shown in Fig. 1A, no toxic effect was revealed when the J774A.1 cells were treated with bavachin ≦40 μM (Fig. 1A). To avoid the potential toxic effects of bavachin, the maximum concentration of bavachin used in the following experiments was 40 μM.

Since NO and PGE_2 are pivotal pro-inflammatory mediators in LPS-induced inflammation, we investigated the effects of bavachin on the pro-inflammatory mediators, NO and PGE_2, in LPS-stimulated J774A.1 cells. Cells were pre-treated with various concentrations of bavachin (0–40 μM) for 1 h following LPS treatment (1 μg ml^{-1}) for 24 h. Our experimental results showed that bavachin significantly suppressed the production of NO (EC_{50} = 28.0 μM) and PGE_2 (EC_{50} = 15.3 μM) by LPS-stimulated J774A.1 cells in a concentration-dependent manner (Fig. 1B and C). We further compared the anti-inflammatory effects of bavachin with two anti-inflammatory drugs, curcumin and dexamethasone (Patel and Bhutani, 2014). Our results showed that high-dose dexamethasone (≥ 30 μM) protected J774A.1 cells against LPS-induced cell death (Fig. 1D). In addition, bavachin, curcumin and dexamethasone significantly reduced the production of NO by LPS-stimulated J774A.1 cells (Fig. 1E).

Bavachin decreases the expression of iNOS and mPGES-1 by LPS-stimulated J774A.1 cells, but does not affect the expression of COX-2

The pro-inflammatory mediator NO is produced by inducible enzyme iNOS, and PGE_2 is synthesized by COX-2 and mPGES-1 in LPS-stimulated macrophages. Since bavachin inhibited LPS-induced NO and PGE_2 production, we further confirmed the effects of bavachin on the expression levels of iNOS, COX-2 and mPEGS-1 in LPS-stimulated J774A.1 cells. Cells were pre-treated with bavachin (0, 20 and 40 μM) for 1 h, and then treated with or without LPS (1 μg ml^{-1}) for 16 h. The expression levels of iNOS, COX-2 and mPGES-1 were analyzed by Western blotting. Our experimental results indicated that the expression levels of iNOS, COX-2 and mPGES-1 were dramatically increased by LPS stimulation. In addition, bavachin decreased the expression of iNOS and mPGES-1 in a concentration-dependent manner, but there was no effect on the expression of COX-2 (Fig. 2).

Bavachin reduces the production of IL-6 and IL-12p40 by LPS-stimulated J774A.1 cells

The structures of IL-6 and IL-12p40 are similar, and included in the IL-6/IL-12 family of cytokines. Both IL-6 and IL-12p40 connect innate and adaptive immune responses crucially (Hasegawa et al., 2016), and are associated with several inflammatory diseases (Zundler and Neurath, 2015). To examine whether bavachin exerts inhibitory effects on LPS-induced IL-6 and IL-12p40 production, J774A.1 cells were pre-treated with different concentrations of bavachin (0–40 μM) for 1 h, and then treated with LPS (1 μg ml^{-1}) for 24 h. As shown in Fig. 3A and B,

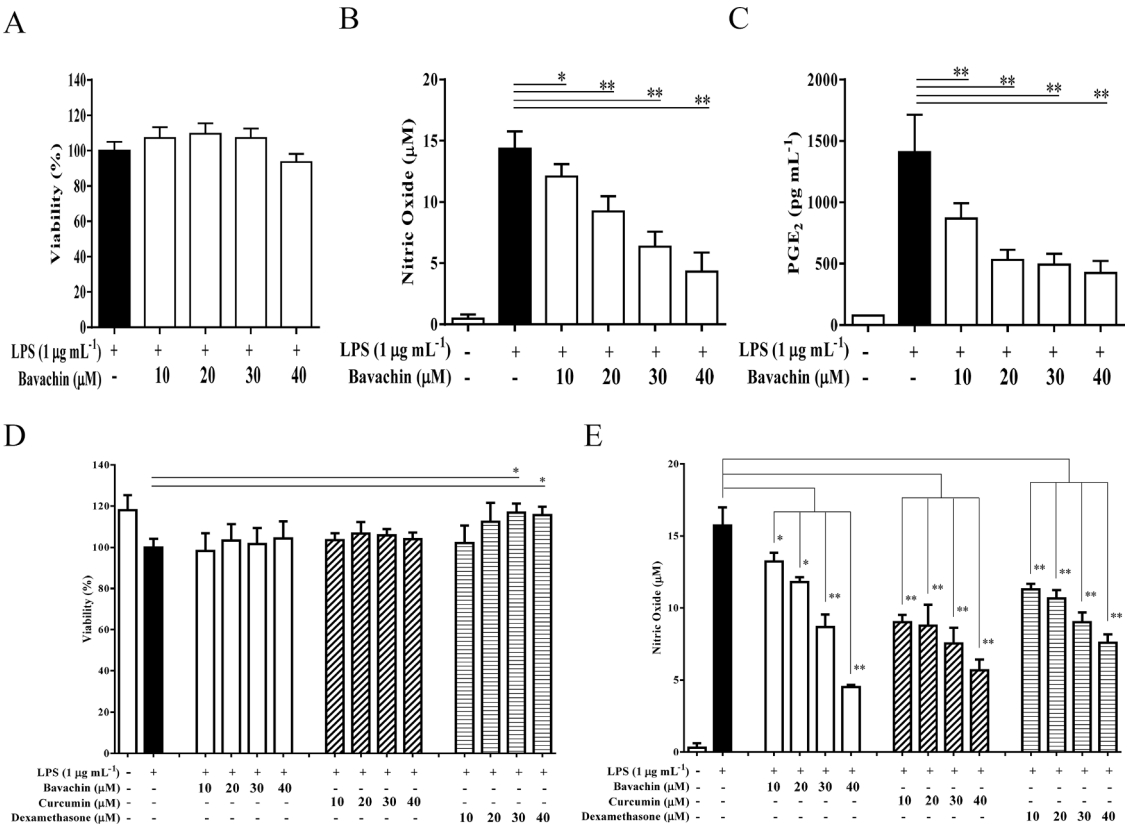

Fig. 1. The effects of bavachin on the cell viability and the production of pro-inflammatory mediators, NO and PGE_2, by LPS-stimulated J774A.1 cells. Cells were pre-treated with various concentrations of bavachin (0–40 μM) for 1 h following treatment of LPS (1 μg ml^{-1}) for 24 h. (A) The cell viability was confirmed by the MTT assay. (B) The concentration of NO in the cell culture supernatant was examined using Griess reagent assay. (C) The concentration of PGE_2 in the cell culture supernatant was detected using ELISA. To compare the effects of bavachin with curcumin and dexamethasone on the cell viability and the production of NO, J774A.1 cells were pre-treated with various concentrations (0–40 μM) of bavachin, curcumin or dexamethasone for 1 h following treatment of LPS (1 μg ml^{-1}) for 24 h. (D) The cell viability was confirmed by the MTT assay. (E) The concentration of NO in the cell culture supernatant was examined using Griess reagent assay. The data are presented as means ± SD of three independent experiments. Statistical significance was represented as follows: $*p < 0.05$ and $**p < 0.01$.

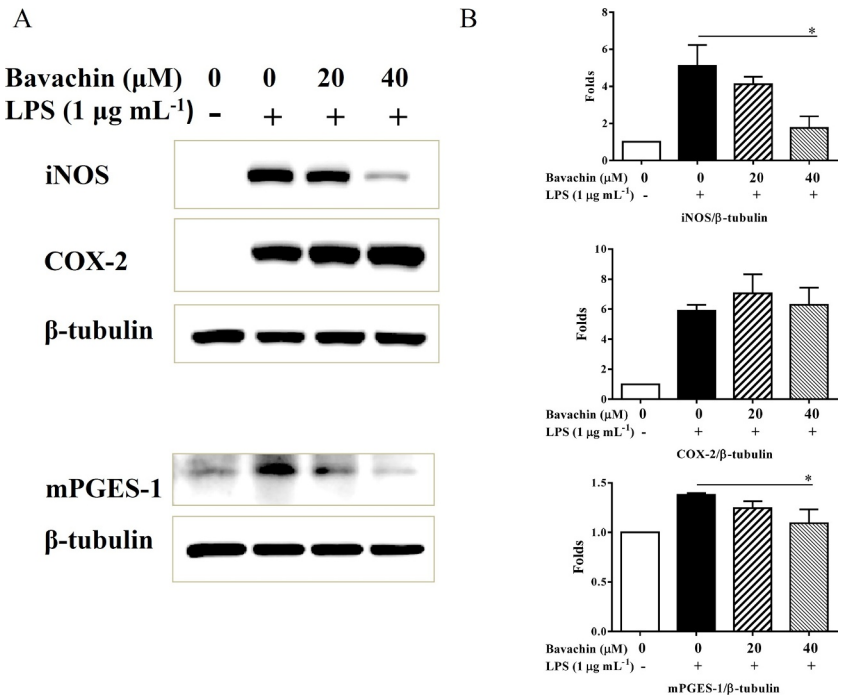

Fig. 2. The effects of bavachin on the expression of iNOS, COX-2 and mPGES-1 by LPS-stimulated J774A.1 cells. Cells were pre-treated with different concentrations of bavachin (0, 20 and 40 μM) for 1 h following treatment of LPS (1 μg ml^{-1}). After 16 h, cell lysates were collected and the expressions of iNOS, COX-2, mPEGS-1 and β-tubulin analyzed using Western blotting. (A) The representative of Western blotting results was obtained in three separate experiments. (B) The intensities of bands were quantified from three separate experiments and normalized to untreated samples. The data are presented as means ± SD of three independent experiments. Statistical significance was represented as follows: *$p < 0.05$.

bavachin reduced the production of IL-6 and IL-12p40 production significantly at 40 μM.

Bavachin decreases the phosphorylation of JNK 1/2 and ERK 1/2 in LPS-stimulated J774A.1 cells, but does not affect the phosphorylation of p38 MAPK

MAPKs are key regulators in initiating the activation of transcription factor and producing the pro-inflammatory mediators and cytokines in LPS-induced inflammation. We investigated the effect of bavachin on LPS-induced MAPKs activation. Cells were pre-treated with bavachin at different concentrations (0, 20 and 40 μM) for 1 h in the absence or presence of LPS treatment (1 μg ml^{-1}) for 1 h. As shown in Fig. 4A and B, the phosphorylation levels of JNK 1/2, ERK 1/2 and p38 MAPK were dramatically increased following LPS stimulation. Moreover, bavachin dramatically reduced LPS-induced phosphorylation of JNK 1/2 and ERK 1/2, but not p38 MAPK (Fig. 4A and B).

Bavachin inhibits the activation of NF-κB in LPS-stimulated J-blue cells

NF-κB crucially contributes to transcription of iNOS, COX-2 and mPGES-1, and produces pro-inflammatory mediators such as NO and PGE_2. We investigated the effect of bavachin on NF-κB activity by detection of SEAP activity. J-blue cells were pre-treated with bavachin at various concentrations (0, 20 and 40 μM) for 1 h in the absence or presence of LPS treatment (1 μg ml^{-1}) for 24 h. As shown in Fig. 4C, the NF-κB activity was approximately increased 3-fold following LPS stimulation. In addition, bavachin significantly suppressed the activity of NF-κB at both 20 μM and 40 μM concentrations by LPS-stimulated J-blue cells.

Bavachin suppresses NLRP3 inflammasome-derived caspase-1 activation and IL-1β production and inhibits the formation of inflammasome complex

The NLRP3 inflammasome has been considered as a critical regulator of IL-1β production, and contributes to pathogenesis of several

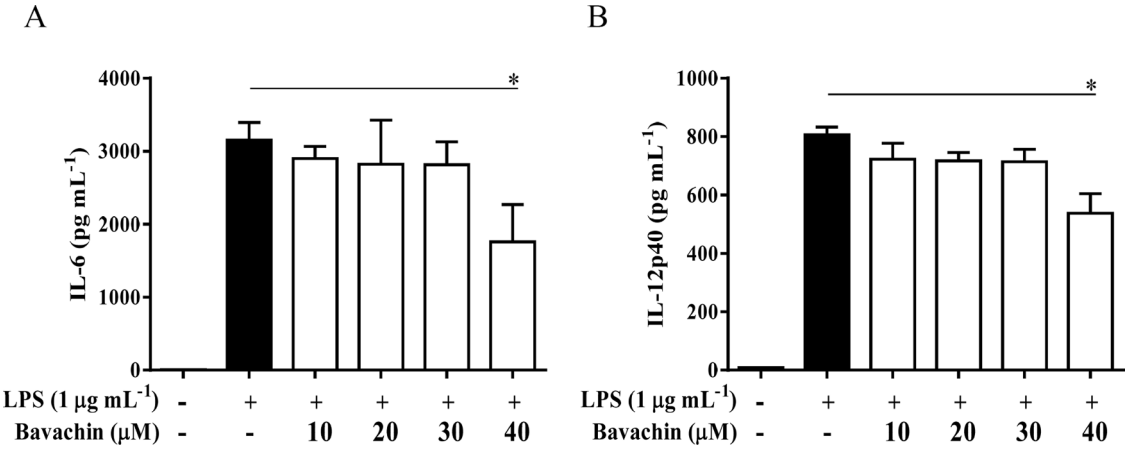

Fig. 3. The effects of bavachin on the production of pro-inflammatory cytokines, IL-6 and IL-12p40, by LPS-stimulated J774A.1 cells. Cells were pre-treated with various concentrations of bavachin (0–40 μM) for 1 h following treatment of LPS (1 μg ml^{-1}) for 24 h. Cell culture supernatant was collected. The concentrations of (A) IL-6 and (B) IL-12p40 in the cell culture supernatant were measured by ELISA. The data are presented as means ± SD of three independent experiments. Statistical significance was represented as follows: *$p < 0.05$.

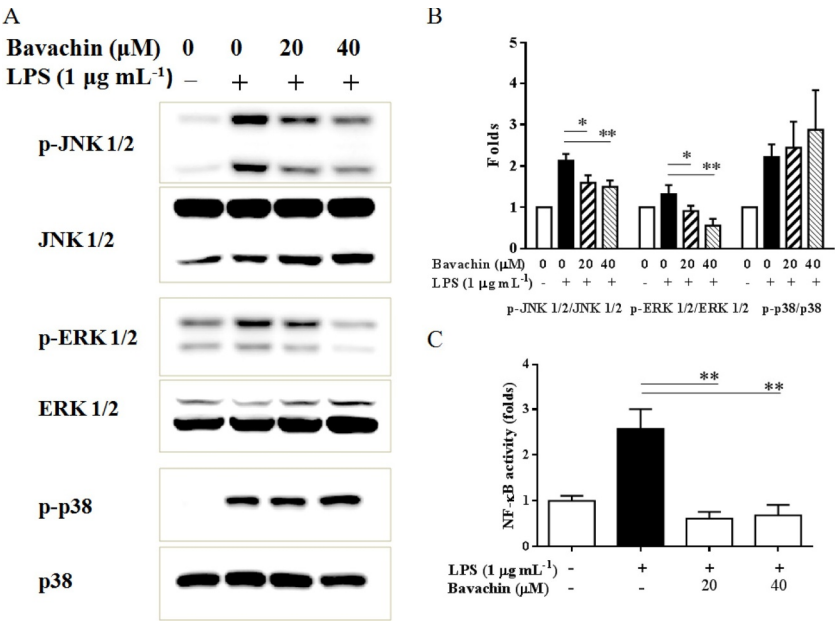

Fig. 4. The effects of bavachin on the activation of MAPKs by LPS-stimulated J774A.1 cells, and the activity of NF-κB by LPS-stimulated J-blue cells. J774A.1 cells were pre-treated with various concentrations of bavachin (0, 20 and 40 μM) for 1 h following treatment of LPS (1 μg ml^{-1}) for 1 h. (A) Cell lysates were collected and the expression levels of p-JNK 1/2, JNK 1/2, p-ERK 1/2, ERK 1/2, p-p38 MAPK and p38 MAPK were detected using Western blotting. The representative of Western blotting results was obtained in three separate experiments. (B) The intensities of bands were quantified from three separate experiments. The relative fold of phosphorylation activity was normalized to that of the unphosphorylated form and compared to untreated samples. (C) J-blue cells were pre-treated with various concentrations of bavachin (0, 20 and 40 μM) for 1 h following treatment of LPS (1 μg ml^{-1}) for 24 h. Cell culture supernatant was collected for SEAP activity assay. The data are presented as means ± SD of three independent experiments. Statistical significance was represented as follows: $*p < 0.05$ and $**p < 0.01$.

immune-mediated diseases (Strowig et al., 2012). We confirmed the effects of bavachin on NLRP3 inflammasome-derived IL-1β production and NLRP3 inflammasome complex formation. Cells were incubated in the absence or presence of LPS treatment (1 μg ml^{-1}) for 5 h, and then treated with various concentrations of bavachin (0–40 μM) for 1 h following treatment of ATP (5 mM) for 30 min. Our experimental results indicated that the production of IL-1β (EC_{50} = 21.5 μM) was dramatically increased by ATP-activated NLRP3 inflammasome. Bavachin significantly suppressed the production of IL-1β by NLRP3 inflammasome in a concentration-dependent manner (Fig. 5A). Additionally, the activity form of caspase-1 p20 protein expression level was increased by ATP-activated NLRP3 inflammasome (Fig. 5B). Our experimental results showed that bavachin inhibited LPS/ATP-induced inflammasome activation through attenuating the expression of caspase-1 p20 and mature IL-1β protein in a concentration-dependent manner (Fig. 5B). As shown in Fig. 5C, LPS/ATP treatment facilitated the inflammasome complex formation in J774A.1 cells. Bavachin obviously repressed the formation of inflammasome complex compared with the LPS/ATP group (Fig. 5C). In addition, we also compared the effect of bavachin on NLRP3 inflammasome-derived IL-1β production with curcumin and dexamethasone. Our results indicated that bavachin and curcumin had more efficiency in inhibiting NLRP3 inflammasome-derived IL-1β production than did dexamethasone (Fig. 5D).

Bavachin suppresses the production of NO, IL-6 and IL-12p40 in LPS-stimulated murine peritoneal macrophages

The above results suggest that bavachin exerts anti-inflammatory effects in LPS-stimulated J774A.1 cells. We further confirmed whether bavachin affected the production of NO, IL-6 and IL-12p40 by using murine peritoneal macrophages. We also investigated the effects of bavachin in LPS-stimulated murine peritoneal macrophages. Thioglycollate-elicited peritoneal macrophages were pre-treated with various concentrations of bavachin (0–40 μM) for 1 h in the absence or presence of LPS treatment (1 μg ml^{-1}) for 24 h. To avoid the toxic effect of bavachin in murine peritoneal macrophages, cells were treated with various concentrations of bavachin for 24 h. The cell viability was assessed by MTT assay. Our experimental results showed there was no toxic effect on the cell viability when cells were treated with bavachin at ≦ 40 μM (Fig. 6A). Subsequently, we investigated the effect of bavachin on the production of NO by LPS-stimulated murine peritoneal macrophages. Cells were pre-treated with various concentrations of bavachin (0–40 μM) for 1 h following LPS treatment (1 μg ml^{-1}) for 24 h. Our results demonstrated that bavachin significantly suppressed the production of NO (EC_{50} = 35.2 μM) by LPS-stimulated murine peritoneal macrophages in a concentration-dependent manner (Fig. 6B). Moreover, bavachin inhibited the production of IL-6 (EC_{50} = 25.7 μM) and IL-12p40 (EC_{50} = 24.7 μM) by LPS-stimulated murine peritoneal macrophages in a concentration-dependent manner (Fig. 6C and D).

Discussion

Macrophages activated by TLR4 and other pattern recognition receptors are known as several of the major contributors in regulating inflammatory response. Uncontrolled inflammatory response triggered by activated macrophages leads to overproduction of pro-inflammatory mediators and cytokines, which causes the pathogenesis of many inflammatory diseases. Hence, dysregulation of pro-inflammatory secretion by activated macrophages is considered a therapeutic target for preventing or treating inflammatory-related diseases (Drexler and Foxwell, 2010; Wynn et al., 2013). In this study, our experimental results indicated that bavachin suppressed LPS-induced pro-inflammatory mediator NO and PGE_2 production via the inhibitory effects on iNOS and mPGES-1. Additionally, bavachin reduced LPS-induced pro-inflammatory cytokine IL-6 and IL-12p40 production, and downregulated MAPKs and NF-κB activation. On the other hand, our experimental results indicated that bavachin inhibited NLRP3 inflammasome activation-induced IL-1β production and decreased caspase-1 activation and mature IL-1β protein expression level. Furthermore, bavachin also suppressed the production of NO, IL-6 and IL-12p40 by LPS-stimulated murine peritoneal macrophages.

NO is an important pro-inflammatory mediator against infectious microorganisms by oxidative stress. NO is produced from L-Arginine by iNOS, and is oxidized to cytotoxic peroxynitrite. Large amounts of NO and peroxynitrite lead to tissue damage in several inflammatory diseases such as atherosclerosis and inflammatory bowel disease (Olson and van der Vliet, 2011). Our results indicated that bavachin suppressed LPS-induced NO production by both J774A.1 cells and murine peritoneal macrophages. Additionally, our experimental results also demonstrated that bavachin reduced the expression of iNOS by LPS-stimulated J774A.1 cells.

PGE_2 is a pivotal pro-inflammatory mediator to cause edema, fever and pain of inflammatory response, and increases vascular permeability

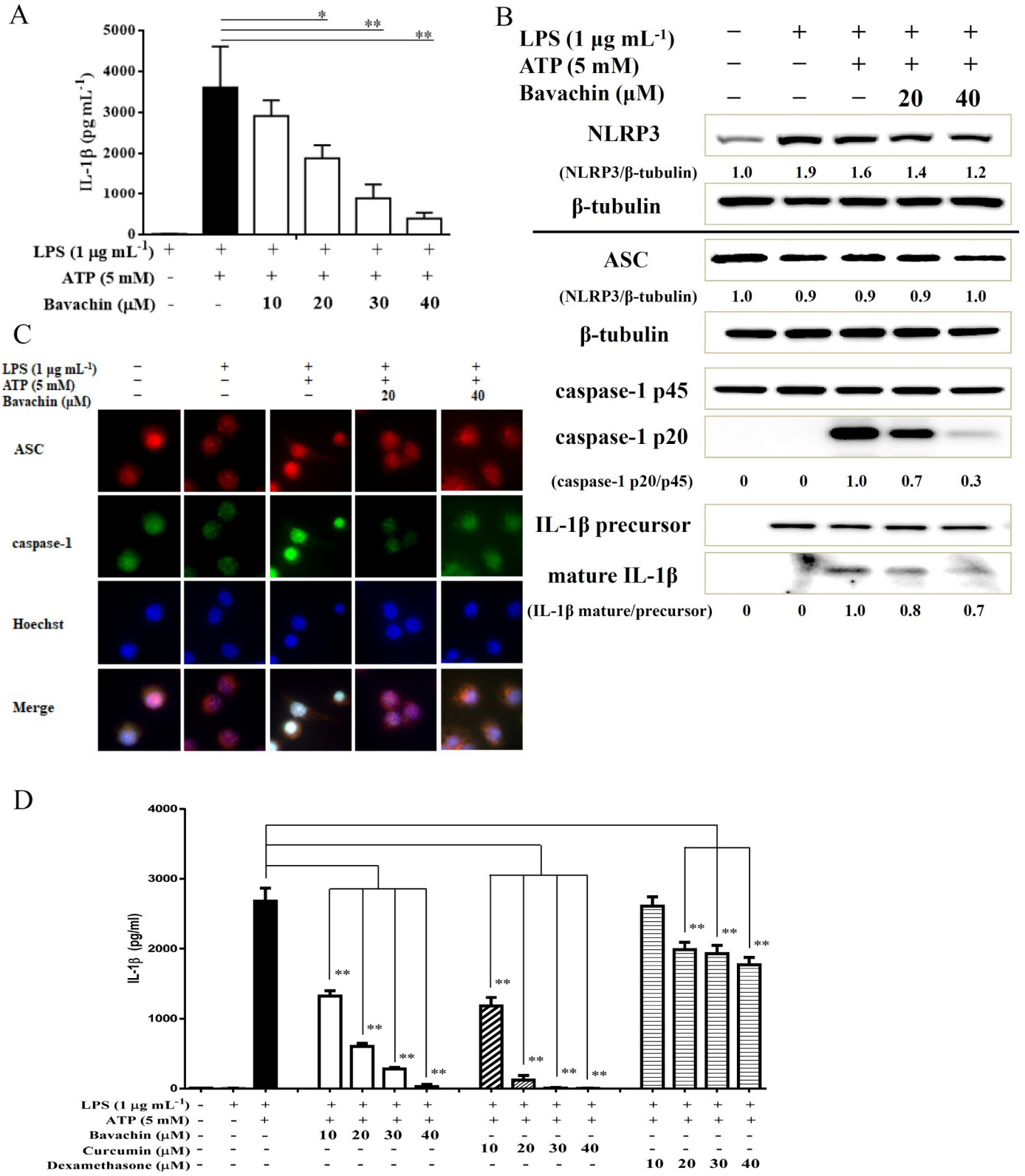

Fig. 5. The effects of bavachin on the production of IL-β and the expression of NLRP3 inflammasome complex. J774A.1 cells were treated with or without LPS (1 μg ml^{-1}) for 5 h, and then treated with various concentrations of bavachin (0–40 μM) for 1 h following treatment of ATP (5 mM) for 30 min. (A) The concentration of IL-1β in the cell culture supernatant was measured by ELISA. The data are presented as means ± SD of three independent experiments. Statistical significance was represented as follows: *$p < 0.05$ and **$p < 0.01$. (B) The cell lysates were collected and the expression levels of NLRP3, ASC, capase-1, IL-1β and β-tubulin were analyzed using Western blotting. Images are representative of three individual experiments. (C) Immunofluorescence staining images of J774A.1 under different experimental conditions. Caspase-1 was colored with green, ASC with red, and Hoechst 33,342 with blue. Images are representative of three individual experiments. (D) To compare the effects of bavachin with curcumin and dexamethasone on NLRP3 inflammasome-derived IL-1β production by J774A.1 cells, cells were treated with or without LPS (1 μg ml^{-1}) for 5 h, and then treated with various concentrations (0–40 μM) of bavachin, curcumin or dexamethasone for 1 h following treatment of ATP (5 mM) for 30 min. The concentration of IL-1β in the cell culture supernatant was measured by ELISA. The data are presented as means ± SD of three independent experiments. Statistical significance was represented as follows: *$p < 0.05$ and **$p < 0.01$.

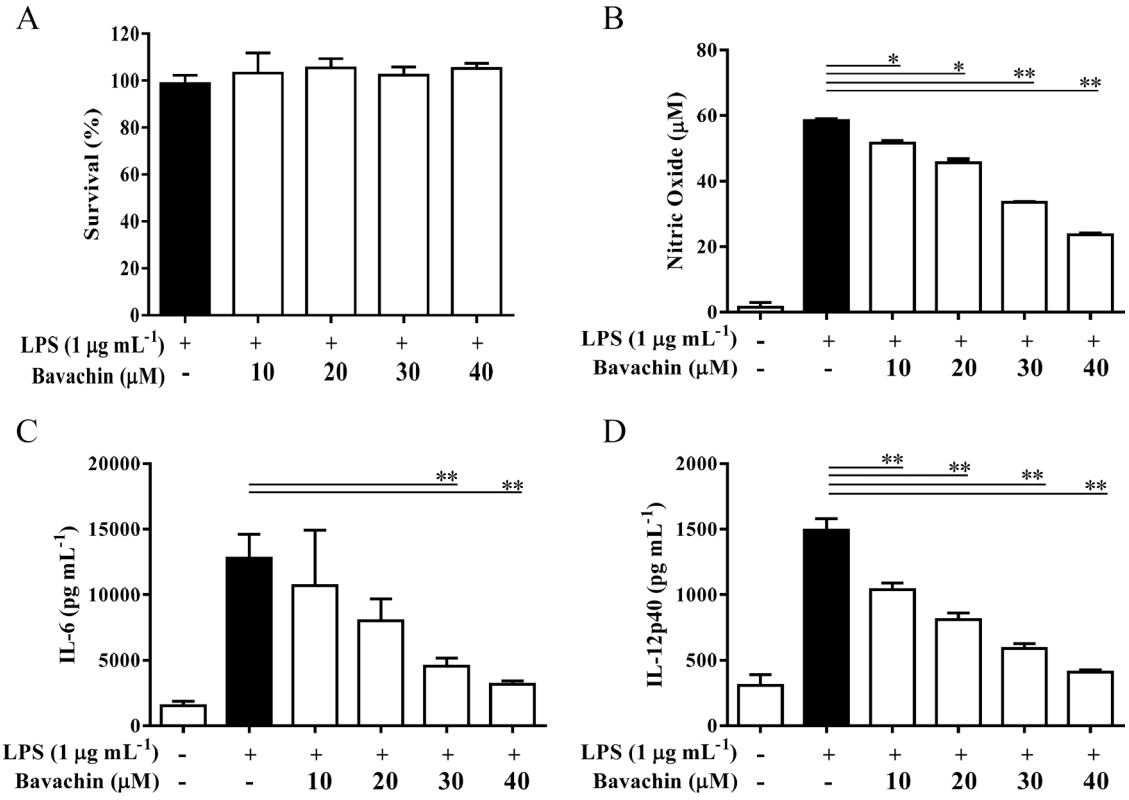

Fig. 6. The effects of bavachin on the production of NO, IL-6 and IL-12p40 by LPS-stimulated murine peritoneal macrophages. Thioglycollate-elicited macrophages were pre-treated with various concentrations of bavachin (0 ~ 40 μM) for 1 h following treatment of LPS (1 μg ml^{-1}) for 24 h. (A) The cell viability was analyzed by the MTT assay. (B) The concentration of NO in the cell culture supernatant was measured using Griess reagent assay. The concentrations of (C) IL-6 and (D) IL-12p40 in the cell culture supernatant were measured by ELISA. The data are presented as means ± SD of three independent experiments. Statistical significance was represented as follows: *$p < 0.05$ and **$p < 0.01$.

and immune cell infiltration. The synthesis of PGE_2 divides into two steps. First, COX-2 catalyzes arachidonic acid to PGH_2. Second, mPGES-1 converts PGH_2 to PGE_2 (Kawahara et al., 2015). Our results demonstrated that bavachin inhibited LPS-induced PGE_2 production and mPGES-1 expression by J774A.1 cells, but did not affect the expression of COX-2. These results suggest that bavachin can inhibit the production of PGE_2 via inhibiting mPGES-1, not COX-2. It has been demonstrated that mPGES-1 inhibitor exerts a lower risk of cardiovascular adverse effects through reducing vascular tone in contrast to COX-2 inhibitor (Ozen et al., 2017).

IL-6 is a pivotal pro-inflammatory cytokines that is produced by LPS-activated macrophages (Guha and Mackman, 2001). Dysregulation of IL-6 is associated with several inflammatory diseases such as rheumatoid arthritis and asthma (Gabay, 2006; Rincon and Irvin, 2012). IL-12 is classified as part of the IL-6/IL-12 family since the structure of IL-12 is similar to the structure of IL-6 (Hasegawa et al., 2016). The role of IL-12 has been considered as a key immunoregulatory cytokine that contributes to T-cell differentiation and coordinates innate and adaptive immune systems (Zundler and Neurath, 2015). Our results indicated that bavachin reduced LPS-induced IL-6 and IL-12p40 production in J774A.1 cells and murine peritoneal macrophages. These results suggest that bavachin might have benefit in attenuating LPS-induced inflammatory responses.

MAPKs critically contribute to initiate the activation of transcription factor and produce pro-inflammatory mediators and cytokines, and are considered as therapeutic targets of inflammatory diseases (Arthur and Ley, 2013). Our results demonstrated that bavachin suppressed LPS-induced activation of JNK 1/2 and ERK 1/2, but did not inhibit LPS-induced activation of p38 MAPK. In addition, NF-κB is a critical transcription factor of iNOS, COX-2 and pro-inflammatory cytokines (Surh et al., 2001). Our results demonstrated that bavachin attenuated the activity of NF-κB by LPS-stimulated J-blue cells. These results suggest that bavachin might reduce LPS-induced production of pro-inflammatory mediators (NO and PGE_2) and cytokines (IL-6 and IL-12p40) via inhibiting NF-κB activation.

NLRP3 inflammasome is a pivotal contributor to induce IL-1β, and is involved in regulating the development of several immune-related diseases critically (Sharma and Kanneganti, 2016). Hence, NLRP3 inflammasome is considered as a therapeutic target of immune-mediated diseases. Thus, the NLRP3 inflammasome inhibitor might have therapeutic potential for treatment of inflammation-related diseases. Our results demonstrated that bavachin attenuated NLRP3 inflammasome-derived caspase-1 activation and IL-1β production as well as inhibiting the formation of inflammasome complex.

Previous studies indicated that curcumin, a major bioactive component found in the roots of turmeric, had anti-inflammatory activities in inhibiting LPS-induced inflammatory response (Abe et al., 1999) and attenuating the activation of NLRP3 inflammasome (Yin et al., 2018). We compared the anti-inflammatory effects of bavachin with curcumin and dexamethasone on LPS-induced NO production and NLRP3 inflammasome-derived IL-1β production in J774A.1 cells. Our results demonstrated that bavachin, curcumin and dexamethasone all had quite an effect on the inhibition of LPS-induced NO production, although for NLRP3 inflammasome-derived IL-1β production, bavachin and curcumin had more efficacy than did dexamethasone.

Conclusion

In the present study, we firstly demonstrated the anti-inflammatory effects of bavachin by attenuating LPS-induced inflammation and inhibiting the activation of NLRP3 inflammasome in macrophages. Our results suggest bavachin, a natural compound isolated from *Psoralea*

corylifolia L., might have benefit for treating inflammatory-related diseases.

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Conflict of interest

The authors declare no conflict of interest.

Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.phymed.2018.12.008.

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