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Sinapic Acid Attenuates the Neuroinflammatory Response by Targeting AKT and MAPK in LPS-Activated Microglial Models

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Abstract

Sinapic acid (SA) is a phenolic acid that is widely distributed in fruits and vegetables, which has various bioactivities, such as antidiabetic, anticancer and anti-inflammatory functions. Over-activated microglial is involved in the development progress of neurodegenerative diseases, such as Parkinson's disease and Alzheimer's disease. The objective of this study was to investigate the effect of SA in microglia neuroinflammation models. Our results demonstrated that SA inhibited secretion of the nitric oxide (NO) and interleukin (IL)-6, reduced the expression of inducible nitric oxide synthase (iNOS) and enhanced the release of IL-10 in a dose-dependent manner. Besides, our further investigation revealed that SA attenuated the phosphorylation of AKT and MAPK cascades in LPS-induced microglia. Consistently, oral administration of SA in mouse regulated the production of inflammation-related cytokines and also suppressed the phosphorylation of MAPK cascades and AKT in the mouse cerebral cortex. These results suggested that SA may be a possible therapy candidate for anti-inflammatory activity by targeting the AKT/MAPK signaling pathway.

Key Words: Sinapic acid, Neuroinflammation, BV-2 microglia, Anti-inflammation, MAPK/AKT signaling pathway

INTRODUCTION

Sinapic acid (SA) is a natural phenolic acid that is found in various edible, functional and pharmaceutical plants, including lemon, orange and various Brassica vegetables, which is common in the human diet (Hameed *et al*., 2016; Nguyen *et al*., 2021). Importantly, SA has reported to contain various bioactivities, including antidiabetic, anti-inflammatory, anticancer and anti-depression functions (Chen, 2016; Lee, 2018; Bin Jardan *et al*., 2020; Huang *et al*., 2020). SA has been declared to attenuate IL-1β secretion and suppress the NLRP3 inflammasome both in macrophages and in mouse lung tissue (Lee *et al*., 2021). In rat heart muscle, Doxorubicin-induced inflammation is mediated by SA via downregulation of NF-κB (Bin Jardan *et al*., 2020). In addition, SA derivatives in broccoli sprouts also interrupt the release of proinflammatory cytokines (TNF-α and IL-6) and increase the production of IL-10 from LPS-stimulated human NK cells (Olszewska *et al*., 2020). SA has been demonstrated to improve cognitive impairment in a scopolamine-induced mouse model and attenuate proinflammatory cytokine secretion in a dementia rat model (Verma *et al*., 2020). Interestingly, one study has differently reported that SA does not show anti-inflammatory activity when it is isolated from the roots of *Polygala arillata* (Xiang *et al*., 2019).

Neuroinflammation has long been recognized as a key pathophysiological process that is involved in various neurodegenerative diseases, such as Huntington's disease, Alzheimer's disease and multiple sclerosis. Microglial cells are elemental immune cells that sustain the health in the central neuron system by abolishing destructive stimuli or repairing impaired cells at the initial stage of neuroinflammation (Perry *et al*., 2010). Long-standing pathological stimulation motivates microglial cells to be overactivated, releasing a large number of pro-inflammatory factors and cytokines, which provoke the neuroinflammatory response and damage brain tissue (Saha and Pahan, 2006; Zhao *et al*., 2021). Recently, many studies have indicated that microglia-derived neuroinflammation plays an indicative role in the pathogenesis of neurodegenerative

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diseases (Streit *et al*., 2004; Krause and Muller, 2010). Therefore, inhibiting the overactivation of microglia may be beneficial to rescue or slow the progression of neuroinflammation and neurodegenerative conditions. During the development of neuroinflammation, immune system cells, including microglia, are activated through the recognition of a pathogen endotoxin by Toll-like receptors (TLRs) and then propagate antigen-induced signal transduction pathways to subsequently mobilize other downstream proteins, such as mitogen-activated protein kinases (MAPKs) and protein kinase B (AKT) (Chen *et al*., 2019). MAPK cascades, including c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK) and p38, are central intracellular mediators that are activated in response to lipopolysaccharide (LPS) stimulation, which is an endotoxin of the outer membrane of gram-negative bacteria. AKT, a serine/threonine kinase, is also considered one of the most important effector kinases downstream of PI3K and the main contributor of the PI3K/AKT signaling pathway (Tang *et al*., 2018). It has been indicated that these signaling pathways are involved in neuroinflammation by regulating the expression of inflammation-related cytokines and enzyme. Thus, targeting inflammation-related signaling pathways may be a beneficial way to slow the progression of neuroinflammation and neurodegenerative conditions.

Therefore, SA may have the potential to impede the key cycle of cytokine secretion, inflammation and neuro-damage, thereby providing a new therapeutically beneficial option for neuroinflammation. However, it remains unclear whether SA has an anti-inflammatory effect on LPS-induced microglial neuroinflammation. Thus, it is necessary not only to investigate whether SA has an anti-inflammatory effect in the CNS but also to clarify the potential underlying mechanisms.

MATERIAL AND METHODS

Chemicals and reagent

Antibodies (iNOS (2982), COX-2 (12282), phospho-AKT(9271), ERK (9102), phosphor-ERK (9101), JNK (9252), phosphor-JNK (9251), p38 (9212) and phosphor-p38 (9211) primary antibodies as well as an anti-rabbit immunoglobulin G (IgG) secondary antibody) were purchased from Cell Signaling Technology (Boston, MA, USA). Fetal bovine serum (FBS) was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Penicillin/streptomycin (P/S) and Dulbecco's modified Eagle's medium (DMEM/F12) were purchased from Gibco (Grand Island, NY, USA). LPS originating from Escherichia coli O55:B5, dexamethasone (DEX, purity>98%) and SA (Fig. 1A, purity>98%) were obtained from Sigma–Aldrich (St. Louis, MO, USA).

Cell culture

The BV-2 mouse microglial cell line was obtained from Prof. Lee Sung Jung's research team at the School of Dentistry, Seoul National University (Seoul, Korea). And the cell was cultured in Dulbecco's modified Eagle medium (DMEM; Gibco, Invitrogen, Grand Island, NY, USA) added with 10% fetal bovine serum (FBS; Gibco, Invitrogen) and 1% penicillin/

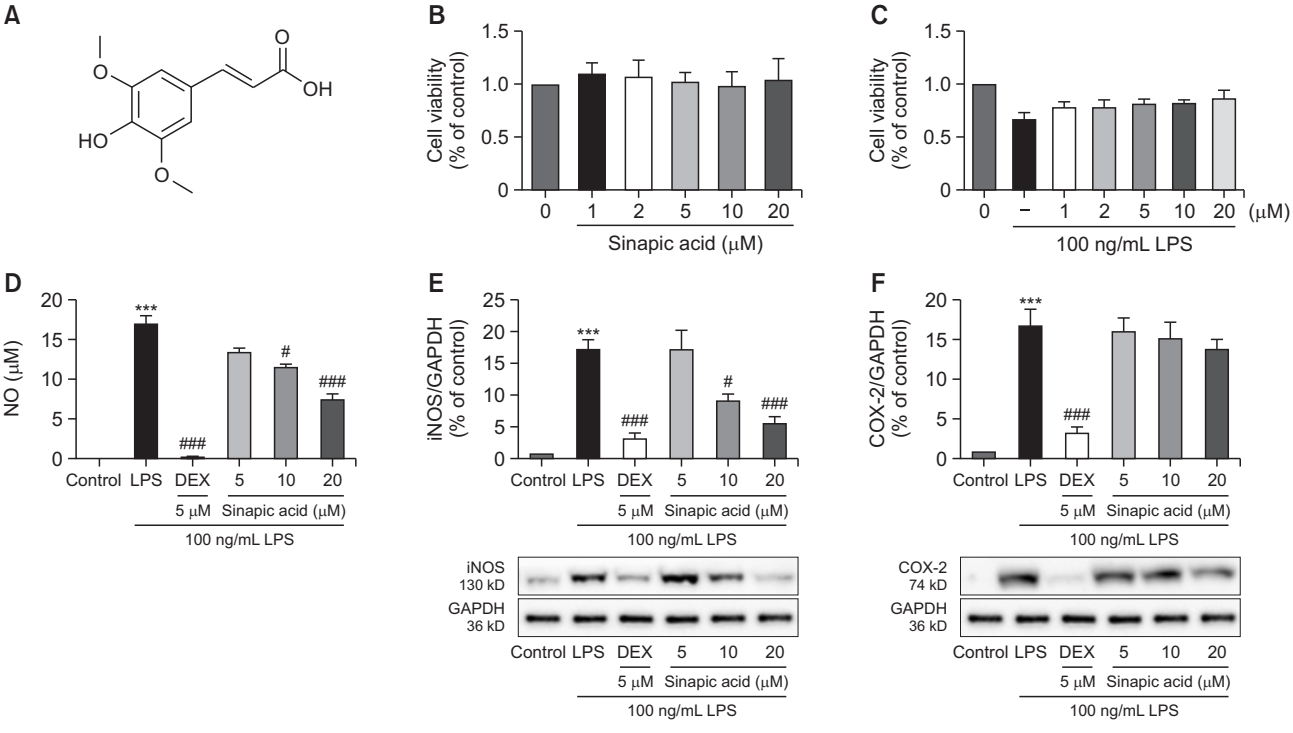

Fig. 1. Effect of SA on nitric oxide (NO) and iNOS/COX-2 production in LPS-stimulated BV-2 cells. Chemical structure of SA (A). MTT assay was measured to perform the cell viability with LPS (B) or without LPS (C). NO levels in the cell supernatant were measured by Griess reagent colorimetric reaction (D) . Respective Western blots presenting the expression level of the iNOS (E) & COX-2 (F). DEX was used as positive control. The Control added with 0.025% DMSO solvent for 12 h. Experimental results were presented as mean (± SEM, n=3). ***p<0.001, the significant difference compared with the control. Compared with LPS group, $^{\#}p$<0.05, $^{\#\#\#}p$<0.001.

streptomycin (P/S; Gibco, Invitrogen) at 37°C under a humidified atmosphere containing 5% CO_2.

Cell viability assay

A colorimetric assay was performed to detect cell metabolic ability according to a previously reported protocol (Zhao *et al*., 2019). BV-2 microglial cells were cultured with DMEM/F12 medium (Gibco) in 96-well plates (1×10^4 cells/well) for 24 h. Then, Cells were treated for 12 h with various concentrations of SA in the presence or absence of 100 ng/mL LPS. After incubation for 12 h, 10 μL of EZ-Cytox reagent (Daeil Lab Co., Ltd., Seoul, Korea) was added to each well followed by incubation for 30 min at 37°C. The absorbance of each reaction product was then measured using a microplate reader (Infinite 1000, Tecan Trading AG, Männedorf, Switzerland) at a wavelength of 450 nm. The results are presented as a percentage of the MTT absorbance of the control cells.

Measurement of nitric oxide

Cells were cultured in 6-well plates (5×10^5 cells/well) for 24 h. Cells were then pretreated with 25, 50 and 100 μg/mL SA for 1 h and then treated with 100 ng/mL LPS for 12 h. The medium was collected and centrifuged at 13,000 rpm to remove dead cells. The supernatant medium was collected and mixed with an equal volume (50 μL) of Griess reagents (1% sulfanilamide and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride in 2.5% H_3PO_4) for 10 min followed by incubation at room temperature. The positive control group was treated with DEX (5 μM). The nitric oxide concentration was measured by a sodium nitrite standard curve at 540 nm using a microplate reader (Multiskan SkyHigh Microplate Reader, Thermo Fisher Scientific, Lafayette, CA, USA).

Animal experiments

Male ICR mice (aged 8 weeks and weighing 26 ± 2 g) were purchased from Orient Bio, Inc., Seongnam, Korea. Animals were kept in the housing facility for one week to adapt to the environment before the experiment and were maintained at 24 ± 1°C under a 16/8 h light/dark cycle with food and water provided *ad libitum*. Five groups of animals were maintained in different cages with 6 mice per cage. The normal control group and LPS-injected only group were administered PBS orally. The positive control group was administered DEX via gavage needle (2.5 mg/kg/day) orally. The other two groups were administered SA (dissolved in PBS) orally via gavage needle (10 and 20 mg/kg/day). The oral administration continued for 8 days. Three days before sampling the LPS-injected group, the positive control group and the two SA-administered groups were intraperitoneal (i.p.) injected with LPS (2.5 mg/kg/day) after 30 min of oral gavage treatment. Animals were anesthetized by diethyl ether and sacrificed after the last LPS stimulation for 3 h. Brain tissue samples were collected and stored at –80°C until further analysis.

Total protein isolation from BV-2 cells

Total protein was isolated according to a previous study (Zhao *et al*., 2019). Briefly, cells were washed 3 times with ice-cold PBS and centrifuged at 13,000 rpm for 5 min to remove dead cells. After discarding the supernatant, the collected cells were lysed with lysis buffer (Cell Signaling Technology), containing protease inhibitor cocktail (PIC, Roche, Penzberg, Germany) and phenylmethylsulfonyl fluoride (PMSF), for 20 min on ice. The lysates were centrifuged (13,000 rpm) for 20 min. Protein quantification was conducted with the Bradford reagent (Bio–Rad, Hercules, CA, USA), and the BSA standard (0-20 mg/mL) was prepared as a standard curve. Western blot samples were prepared with lysate and an equal volume of 2× NuPAGE LDS sample buffer (Thermo Fisher Scientific) with 10% 2-mercaptoethanol. After denaturized at 99°C, the samples were stored at –80°C until further analysis.

Total protein isolation from the mouse brain

For the preparation of mouse brain samples as described previously (Zhao *et al*., 2019), the mouse cerebral cortex was collected, stored at –80°C and then homogenized with PRO-PREP protein extraction buffer (iNtRON, Seongnam, Korea) supplemented with 1× PIC set III (Sigma–Aldrich). The homogenates were then centrifuged (13,000 rpm) for 20 min at 4°C. Protein quantification assays were performed as described for total protein samples.

Western blot analysis

Total protein samples (20 μg) were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with 10% acrylamide/bis gels and electrotransferred to Immobilon-P polyvinylidene difluoride (PVDF) membranes (Merck KGaA, Darmstadt, Germany). The membranes were then blocked with 5% BSA in Tris-buffered saline with Tween (TBST) and incubated overnight at 4°C with specific primary antibodies (1:1000). After washing with TBST, the membranes were incubated with secondary HRP-conjugated IgG (1:2000) at room temperature for 1 h and determined using Super-Signal™ West Femto Chemiluminescent Substrate (Thermo Fisher Scientific). Band signal densitometry analysis was performed with the LAS4000 system (Fujifilm, Tokyo, Japan), and the intensities of the proteins were quantified by Multi Gauge V3.0 software (Fujifilm).

Cytokine ELISA analyses

Cytokine (IL-6 and IL-10) levels were estimated in both cell supernatants and mouse serum by using ELISA kits (IL-6: #M6000B, IL-10: #M1000B, R&D Systems Inc., Minneapolis, MN, USA) following the manufacturer's protocols. For IL-6 analyze, cell supernatant was diluted for 1000 times via DMEM-F12 and serum was diluted for 10 times via PBS. For IL-10, both the cell supernatant and mouse serum were not diluted. Briefly, samples were pipetted into a micro-plate pre-coated with capture antibodies and incubated at room temperature for 2 h. After washing 5 times with wash buffer, HRP conjugate antibodies were added and incubated for 2 h at room temperature. Then substrate solution was added and incubated the plates at room temperature protecting from light for 30 min. The reaction was stopped by the stop solution and the optical density of each well was measured at 450 nm with a microplate reader (Multiskan SkyHigh Microplate reader, Thermo Fisher Scientific).

Statistics

Data are expressed as the mean ± SEM of each independent replication. For comparison of three or more replications, data were analyzed by one-way analysis of variance (ANOVA) with Dunnett's post-hoc test. A value of $p<0.05$ was considered statistically significant. Statistical tests were performed using GraphPad Prism 5.0 (GraphPad Software, San Diego,

CA, USA).

RESULTS

Effect of SA on cell viability, NO and iNOS/COX-2 production in LPS-stimulated BV-2 cells

To elucidate the potential protective effect of sinapic acid on BV-2 cells, an MTT assay was performed. As shown in Fig. 1B and 1C, the cell viability of BV-2 cells was not strongly affected in the presence of sinapic acid, and cytotoxicity was not significantly changed when the concentration of SA was treated with 100 ng/mL LPS. The anti-inflammatory effects of SA were also evaluated by the production of NO. As depicted in Fig. 1D, treatment with LPS significantly stimulated the production of NO in the supernatant of the BV-2 cell culture medium, and the expression of iNOS was significantly decreased in the SA-treated group compared to the LPS groups (Fig. 1E). In addition, there was no significant reduction in COX-2 expression after exposure to SA and LPS (Fig. 1F). Therefore, these results indicated that SA participates in the anti-inflammatory response in microglial cells.

Effect of SA on the production of inflammatory cytokines in LPS-stimulated microglial cells

To determine the effects of SA on the release of pro-inflammatory and anti-inflammatory cytokines, the secretion of IL-6 and IL-10 was detected after LPS stimulation. As shown in Fig. 2A, pre-treatment of BV-2 microglia with SA (5, 10 and 20 μM) facilitated a reduction in IL-6 level, compared with LPS group. Moreover, SA increased the production of IL-10 compared to cells induced by LPS, even more significant than DEX-treated cells (Fig. 2B). These results demonstrated that SA pretreatment significantly suppresses the LPS-induced inflammatory response by regulating the secretion of inflammation-related cytokines.

SA suppresses MAPK phosphorylation in LPS-stimulated BV-2 microglial cells

The MAPK signaling pathway is involved in the regulation of many inflammatory responses, including LPS-induced microglial activation. To investigate the participation of SA in the molecular mechanism of the LPS-induced inflammatory response, JNK, p38 and ERK protein phosphorylation was detected in LPS-treated BV-2 cells. As shown in Fig. 3A-3C, LPS treatment mobilized a significant increase in the phosphorylation of JNK, p38 and ERK in BV-2 cells. However, SA treatment significantly attenuated the LPS-stimulated activation of JNK, p38 and ERK. And treatment of 20 μM SA can more significantly inhibit the p38 phosphorylation, compared to DEX (5 μM)-treated cell (Fig. 3B). These data suggested that SA may attenuate proinflammatory factors by inhibiting the MAPK signaling cascade.

SA suppresses AKT phosphorylation in LPS-stimulated BV-2 microglial cells

As illustrated in Fig. 3D, SA significantly decreased the phosphorylation of AKT under LPS stimulation, indicating that SA may suppress the activation of the AKT signaling pathway. To further investigate whether SA suppresses the AKT signaling pathway, pre-treatment with the AKT1/2 kinase inhibitor (A6730) and AKT enhancer (SC79) was performed in the LPS-induced BV-2 cell. The AKT inhibitor reduced not only NO production but also the expression levels of iNOS, while co-treatment with SA and the AKT inhibitor further suppressed NO production (Fig. 4A, 4B). The SC79 had a significant effect on the phosphorylation levels of AKT (Fig. 4C). However, co-treatment with SC79 and SA rescued this trend and attenuated the phosphorylation levels of AKT. Therefore, these results indicated that SA may protect microglia against the LPS-induced inflammatory response by inhibiting AKT activation.

SA suppresses IL-6 and enhances IL-10 secretion in LPS-induced mouse serum

Systemic LPS injection modulates inflammatory cytokine production in the serum and brain. To further determine the effect of SA on the inflammatory response, LPS-stimulated mouse model was performed. As shown in Fig. 5A, systemic LPS injection significantly increased IL-6 levels in the serum, but this trend was reversed in SA-administrated group. Moreover, SA administration induced a further increase in IL-10 in the serum of LPS-treated mice, revealing that the anti-inflammatory response is activated by SA (Fig. 5B). Identical with the *in vitro* result (Fig. 2B), this increase was also stronger than the DEX-administrated mouse. These data suggested that SA systemically regulates the LPS-mediated inflammatory response.

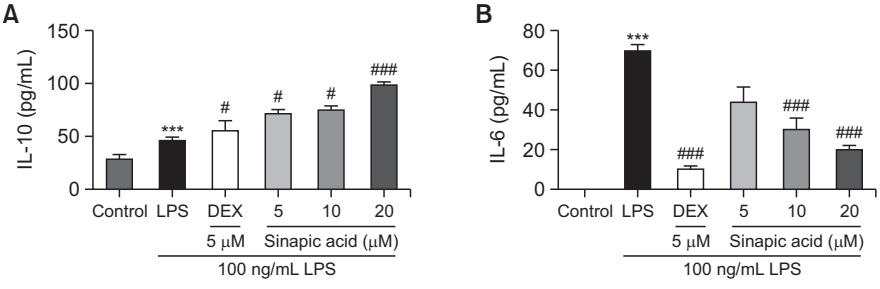

Fig. 2. Effect of SA on the production of inflammatory cytokine production in LPS-stimulated microglial cells. Cells (5×10^5 cells/well) were cultured in 6-well culture dishes at 37°C in presence of 5% CO2. BV-2 Cells were pretreated with the indicated concentrations (5, 10 and 20 μM) of SA for 1 h and then stimulated with 100 ng/mL LPS for 24 h. The Elisa were performed to measure IL-6 (A) & Il-10 (B) secretions as the manufacture protocol. DEX was used as positive control. Data are detected as mean (± SEM, n=3). ***p<0.001, the significant difference compared with the control. Compared with LPS group, $^{\#}p$<0.05, $^{\#\#\#}p$<0.001.

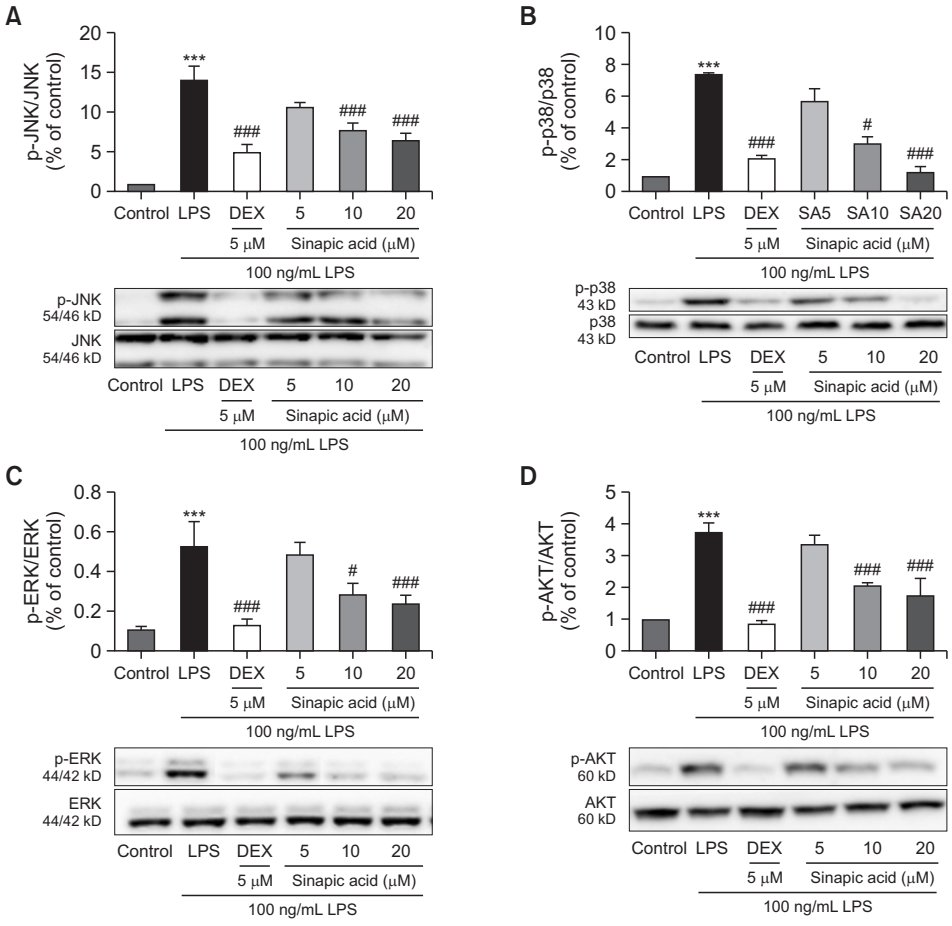

Fig. 3. SA suppressed MAPK phosphorylation in LPS-stimulated BV-2 Microglia cell. The phosphorylation of JNK (A), p38 MAP kinases (B) and ERK (C), as well as on AKT (D) were measured by western blot. DEX was used as positive control. Data are detected as mean (± SEM, n=3). ***p<0.001, the significant difference compared with the control. Compared with LPS group, $^{\#}p$<0.05, $^{\#\#\#}p$<0.001.

SA suppresses the MAPK and AKT signaling pathways in the LPS-induced mouse cerebral cortex

The *in vitro* data revealed that SA mediated LPS-treated microglial activation by attenuating the AKT and MAPK pathways. Thus, we further investigated whether SA is involved in these inflammation-related signaling pathways in LPS-injected mouse brains. Western blot results indicated that LPS treatment increased the expression of p-ERK (Fig. 6A), p-JNK (Fig. 6B), p-p38 (Fig. 6C) and p-AKT (Fig. 6D). In contrast, SA administration significantly reduced MAPK and AKT phosphorylation in cerebral cortex tissue compared to the LPS group.

DISCUSSION

Chronic inflammation is a central process involved in many metabolic disorders, including neurodegenerative (Alzheimer and Parkinson diseases) and autoimmune diseases (Margină *et al*., 2020). Dietary components have the ability to affect the immune response through the modulation of gut bacteria metabolism or impacting immune cells through the brain-blood barrier (Galland, 2010; Leigh and Morris, 2020). Thus, the constituents of daily diets, including vegetables and crops, have been focused on in recent days. SA is an interesting and widely present hydroxycinnamic acid, especially in the *Brassicaceae* family (Nguyen *et al*., 2021). In recent years, SA and some of its derivatives (sinapine and sinapoyl esters) have been studied due their various biological characteristics, including antioxidant and anti-inflammatory activities (Chen, 2016). This study investigated the effects of SA on inflammatory factors secretion through the AKT/MAPK signaling pathway and underlying mechanism in LPS-induced BV-2 microglia model and *in vivo* mouse model. In this study, we found that LPS-induced excessive proinflammatory factor release, such as intercellular NO and iNOS production, was inhibited by SA in LPS-treated microglia. Consistently, SA protected the mice against an acute inflammatory response and decreased proinflammatory cytokines secretion in serum, such as IL-6, after LPS administration. Our results revealed that SA treatment suppressed inflammatory response by modulating the phosphorylation of AKT, leading to the suppression of MAPK phosphorylation under LPS-induced inflammatory challenge. Additionally, SA administration also suppressed the AKT/MAPK activation and the LPS-induced inflammatory response *in vivo*.

Neuroinflammation is a complicated process derived from the initial inflammatory responses regulated by inflammatory

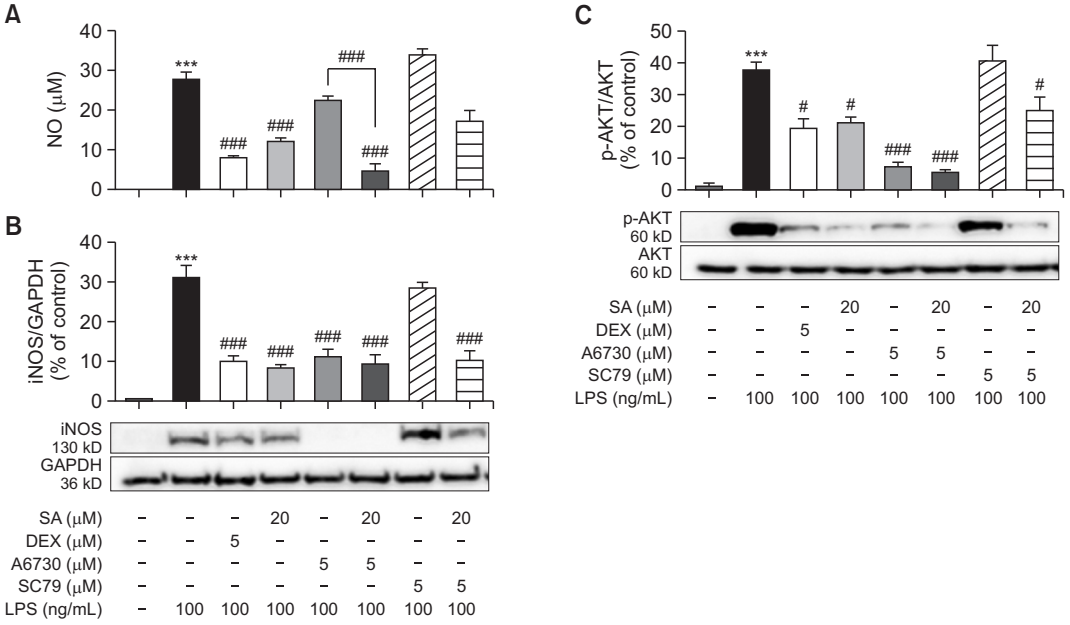

Fig. 4. SA suppressed AKT phosphorylation in LPS-stimulated BV-2 Microglia cell. NO production (A) and the expression of AKT (B), iNOS (C) and was detected after co-treatment with SA, the specific inhibitor AKT1/2 kinase inhibitor and specific enhancer SC79. DEX was used as positive control. Data are detected as mean (± SEM, n=3). ***p<0.001, the significant difference compared with the control. Compared with LPS group, $^{\#}p$<0.05, $^{\#\#\#}p$<0.001.

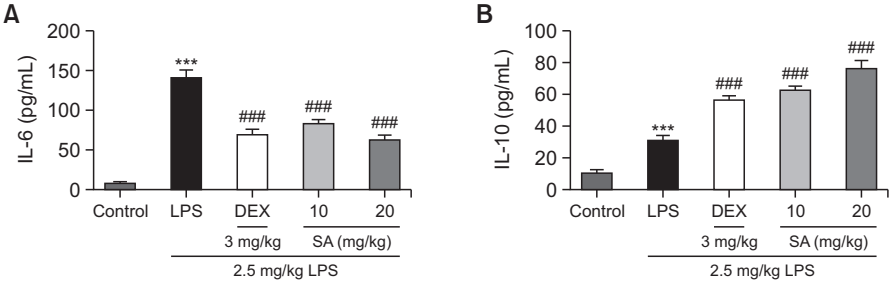

Fig. 5. SA suppressed IL-6 and enhanced IL-10 secretion in LPS-induced mouse serum. IL-6 (A) and IL-10 (B) levels in the mouse serum were detected by ELISA. DEX (3 mg/kg, oral gavage) was used as positive control. Data are detected as mean (± SEM, n=5). ***p<0.001, the significant difference compared with the control. Compared with LPS group, $^{\#\#\#}p$<0.001.

mediators, including nitric oxide (NO) (Aktan, 2004). Nitric oxide eliminates microorganisms and has a protective effect on specific tissues, but it also induces cytotoxicity in these tissues at high concentrations (Maksoud *et al*., 2019). Lipopolysaccharide (LPS) induces systemic inflammation by boosting immune system cells and expanding the secretion of inflammatory mediators by regulating microglial activation (Calabrese *et al*., 2007; Xue *et al*., 2018). The production of NO is regulated by the nitric oxide synthase enzyme family, in which iNOS is mainly involved (Singh *et al*., 2019). Increased levels of NO have also been reported to connect neuroinflammation with brain dysfunctions and diseases, such as learning and memory impairments(Picón-Pagès *et al*., 2019). IL-6 is one of the main pro-inflammatory cytokines produced by activated microglia (West *et al*., 2019). It involved in initiating and regulating the cytokine cascade during an inflammatory response, promote the microglia rresopnses and contributing to the neurodegenerative disease. IL-10 is well-known anti-inflammatory cytokines that induce microglia alter into anti-inflammatory state *in vitro* (Cherry *et al*., 2014). In our study, SA inhibited the production of IL-6 and enhanced the IL-10 in LPS-activated BV2 microglia. Besides, in mouse model, the administration of SA also suppressed the release of IL-6 and enhanced the IL-10 secretion. The present study demonstrated that SA treatment inhibits NO and inflammation related cytokine production and downregulates iNOS protein expression in BV-2 microglial cells, thereby suggesting the potential protective effect of SA against LPS-induced inflammatory stimuli.

The production of iNOS in LPS-induced microglial cells is modulated by MAPKs (Chen *et al*., 2019; Rai *et al*., 2019), which regulate important cellular processes in immune responses (Zhang and Liu, 2002). And several studies have suggested that MAPKs constitute a key step in the process of neuroinflammation and may be a potential target for pharmacological treatment (Kim and Choi, 2015; Rai *et al*., 2019). In the present study, we found reduced p-ERK, p-JNK and p-p38

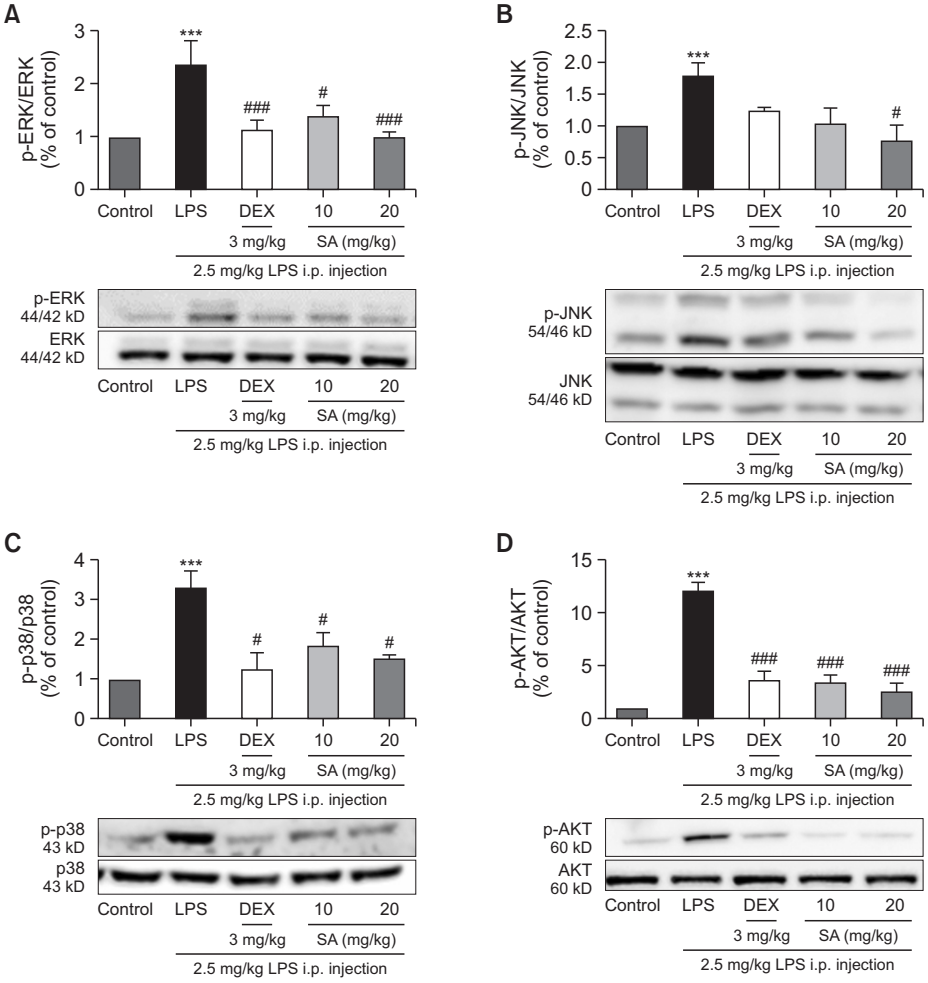

Fig. 6. SA suppressed MAPK & AKT signaling pathway in LPS-induced mouse cerebral cortex. Protein level of p-ERK (A), p-JNK (B), p-p38 (C), p-AKT (D) in mouse cerebral cortex were detected. DEX (3 mg/kg, oral gavage) was used as positive control. Five independent experiments were performed. Data are detected as mean (± SEM, n=5). ***p<0.001, the significant difference compared with the control. Compared with LPS group, $^{\#}p$<0.05, $^{\#\#\#}p$<0.001.

in response to SA treatment, which suggested suppression of MAPK phosphorylation during LPS-induced upregulated inflammation. The MAPK pathway is activated by TLR4/MyD88 and modulates the expression of inflammatory mediators, including iNOS, IL-6 and IL-10 (Arthur and Ley, 2013). Huang *et al*. (2018) reported that SA inhibits IL-1β-induced inflammation via blockade of IL-1β-induced MAPK signaling activation in rat chondrocytes. The present study found that LPS activates the MAPK signaling pathway and that treatment with SA significantly moderates the phosphorylation of JNK, ERK and p38.

AKT is a serine kinase that plays the essential role in the PI3K/AKT signaling pathway. AKT is activated during all aspects of the development of neuroinflammation. Previous studies have revealed that the PI3K/AKT signaling pathway alters LPS-induced COX-2 and iNOS expression in BV-2 cells (Zhao *et al*., 2021). Activated AKT releases NF-κB to translocate to the nucleus, which triggers stimulation of inflammatory target genes and promotes the proinflammatory response (Zhu *et al*., 2021). In addition, other studies have demonstrated that treatment with SA attenuates the NF-κB signaling pathway both *in vivo* and *in vitro* (Bin Jardan *et al*., 2020; Verma *et al*., 2020). Besides, the mechanism that controls cytokines like IL-10 or IL-6 production in immune cells in response to external stimuli has been shown to involve MAPKs such as ERK and p38 (Zhang *et al*., 2019) and the AKT signal pathway (Zhang *et al*., 2016). However, the relationship between AKT phosphorylation and SA requires further exploration. In the present study, western blot analysis indicated that the AKT activation induced by LPS was attenuated by SA in a dose-dependent manner in BV-2 microglia. For further confirmation, an AKT kinase inhibitor (A6730) and AKT agonist (SC79) were applied to investigate the relationship between the AKT signaling pathway and SA. These data suggested that the AKT signaling pathway plays a major role in the anti-inflammatory activity of SA *in vitro*, contributing to further understanding of the mechanism of SA bioactivity in the anti-inflammation model.

An LPS-injected *in vivo* mouse model was also used to explore the effect of SA. After LPS administration, a systematic inflammatory response generates and releases proinflammatory cytokines. As proinflammatory cytokines are generated, glial activation from the brain shifts to counter the impacts of attacking pathogens by upregulating IL-10 (Becher *et al*.,

2017). Also, SA attenuated IL-1β secretion in LPS-induced inflammation in mouse model (Lee *et al*., 2021), where IL-1β might further modulate the release of IL-10 (Coeffier *et al*., 2003). The release of proinflammatory cytokines and their expression in the mouse cerebral tissue demonstrated that anti-inflammatory activity can be detected under both LPS-induced systemic inflammatory and neuroinflammatory conditions. Besides, SA also inhibited the phosphorylation of LPS-stimulated AKT and MAPK in the mouse cerebral cortex, which were consistent with the *in vitro* effect, indicating that SA represses the inflammatory response of microglia by inhibiting AKT/MAPK signaling pathways.

In conclusion, our investigation revealed that SA is a potential suppressor of the neuroinflammatory pathway. Overall, both *in vivo* and *in vitro* findings showed that SA may protect against LPS-induced inflammation via the MAPK and AKT signaling pathways, thus acting as an active compound. Therefore, these results indicated that SA may be a possible therapeutic candidate against neurodegenerative diseases related to inflammation.

CONFLICT OF INTEREST

I declare that the authors have no competing interests, or other interests that might be perceived to influence the results and/or discussion reported in this paper.

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