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ARTICLE

Icariside II inhibits lipopolysaccharide-induced inflammation and amyloid production in rat astrocytes by regulating IKK/IκB/NF-κB/BACE1 signaling pathway

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β-amyloid (Aβ) is one of the inducing factors of astrocytes activation and neuroinflammation, and it is also a crucial factor for the development of Alzheimer's disease (AD). Icariside II (ICS II) is an active component isolated from a traditional Chinese herb *Epimedium*, which has shown to attnuate lipopolysaccharide (LPS)-induced neuroinflammation through regulation of NF-κB signaling pathway. In this study we investigated the effects of ICS II on LPS-induced astrocytes activation and Aβ accumulation. Primary rat astrocytes were pretreated with ICS II (5, 10, and 20 μM) or dexamethasone (DXMS, 1 μM) for 1 h, thereafter, treated with LPS for another 24 h. We found that ICS II pretreatment dose dependently mitigated the levels of tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) in the astrocytes. Moreover, ICS II not only exerted the inhibitory effect on LPS-induced IκB-α degradation and NF-κB activation, but also decreased the levels of $Aβ_{1-40}$, $Aβ_{1-42}$, amyloid precursor protein (APP) and beta secretase 1 (BACE1) in the astrocytes. Interestingly, molecular docking revealed that ICS II might directly bind to BACE1. It is concluded that ICS II has potential value as a new therapeutic agent to treat neuroinflammation-related diseases, such as AD.

Keywords: icariside II; dexamethasone; astrocytes; nuclear factor-kappa B; beta secretase 1; β-amyloid; neuroinflammation; Alzheimer's disease

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INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disease characterized by beta-amyloid (Aβ) peptide fibrils, which are extracellular depositions of a particular protein and are accompanied by extensive neuroinflammation [1–3]. A number of studies have reported that inflammation, which may precede amyloid deposition, exerts vital effects in the pathogenesis of AD [4, 5]. Moreover, inflammatory mediators increase the expression of amyloid precursor protein (APP) and the formation of Aβ and upregulate beta-secretase activity [6]. Therefore, antiinflammatory drugs may prevent or treat AD by inhibiting neuroinflammation, thereby reducing the production or deposition of Aβ [7–10]. However, there are still no ideal antiinflammatory drugs to prevent or treat AD.

Astrocytes and microglia are important components of homeostasis in the brain [11]. When the brain is exposed to undesirable environmental conditions, both astrocytes and microglia, which are crucial perpetrators of inflammation and potential neuronal dysfunction [12, 13], acquire special "response" or "activation" phenotypes [14, 15]. In AD, the interaction between microglia and astrocytes may be of great significance for the development of neurodegenerative disease. In particular, astrocytes represent the most plentiful cell type in the brain and play an imperative role in maintaining the homeostasis of the central nervous system [16–18]. Under physiological conditions, astrocytes play a role in supporting and separating nerve cells [19–22]. Nevertheless, under neuroinflammatory conditions, the excessive activation of astrocytes is involved in the inflammatory response through its ability to release multiple molecules and further lead to neurodegenerative diseases [23–25].

Epimedium is a traditional Chinese herb used for the treatment of cardiovascular diseases, osteoporosis, and sexual and neurological disorders [26]. Icariside II (ICS II) is known as one of the major active pharmaceutical ingredients of *Epimedium*, and it has been indicated to have an extensive range of pharmacological effects, including antiinflammatory [27, 28], anticancer [29, 30], antioxidative [31, 32], and antiaging activities [33]. Our previous study found that ICS II attenuates streptozotocin- or $Aβ_{25-35}$-induced cognitive deficits in rats by increasing the number of surviving neurons in the hippocampus [28] or inhibiting neuronal apoptosis and reducing PDE5 protein expression [34]. In our previous in vivo studies, we proved that ICS II exerts beneficial effects on lipopolysaccharide (LPS)-induced neuroinflammation by regulating the TLR4/MyD88/NF-κB signaling pathway in rats and inhibiting LPS-induced astrocyte overactivation [35]. However, whether ICS II can suppress neuroinflammatory responses in vitro

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remains unclear. Thus, in the current study, we investigated the effects of ICS II on lipopolysaccharide (LPS)-induced neuroinflammation in primary astrocytes and the underlying molecular mechanism.

MATERIALS AND METHODS

ICS II (purity ≥ 98%) was obtained from Nanjing Zelang Medical Technology Corporation Ltd (Nanjing, China). DMEM/F12 was obtained from HyClone (Logan, UT, USA), and fetal bovine serum (FBS) was purchased from Gibco (Thermo Fisher Scientific, MA, USA). Dexamethasone (DXMS) and LPS (L 2630) (*Escherichia coli* O111:B4) were purchased from Sigma-Aldrich (St Louis, MO, USA). DXMS and LPS were dissolved in normal saline at concentrations of 1 mM and 1 mg/mL, respectively, and ICS II was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM. Anticyclooxygenase-2 (COX-2) (#ab15191), anti-nitric oxide synthase (iNOS) (#ab15323), antinuclear factor-κB (NF-κB) (p65) (#ab16502), anti-p-NF-κB (p65) (#ab86299), anti-IκB-α (#ab7217), anti-IKK-α (#ab32041), anti-p-IKK-α (#ab38515), anti-IKK-β (#ab124957), anti-p-IKK-β (#ab59195), and anti-β-site APP cleaving enzyme (BACE1) (#ab108394) antibodies were purchased from Abcam (Cambridge, UK). Anti-APP (#AB60097b) was purchased from BBI Life Sciences Corporation (Shanghai, China). Tumor necrosis factor-alpha (TNF-α) enzyme-linked immunosorbent assay (ELISA) kits (an interleukin-1 beta (IL-1β) ELISA kit, an $A\beta_{1-40}$ ELISA assay kit (JL10226), and an $A\beta_{1-42}$ ELISA kit (JL10958)) were purchased from Shanghai Jianglai Biotechnology (Shanghai, China). A nitric oxide (NO) detection kit (S0021) was purchased from Beyotime Biotechnology (Shanghai, China).

Astrocyte culture and drug treatment

Sprague-Dawley rats (250 ± 50 g) were housed under a 12-h-light/dark cycle at a humidity of 50% ± 5% and a temperature of 24 °C. All animals were given free access to water and food. Animal experiments were performed in compliance with the State Committee of Science and Technology of the People's Republic of China Order No. 2 of November 14, 1988, and the protocol was approved by the Experimental Animal Ethics Committee of the Zunyi Medical University. Primary astrocytes were extracted from

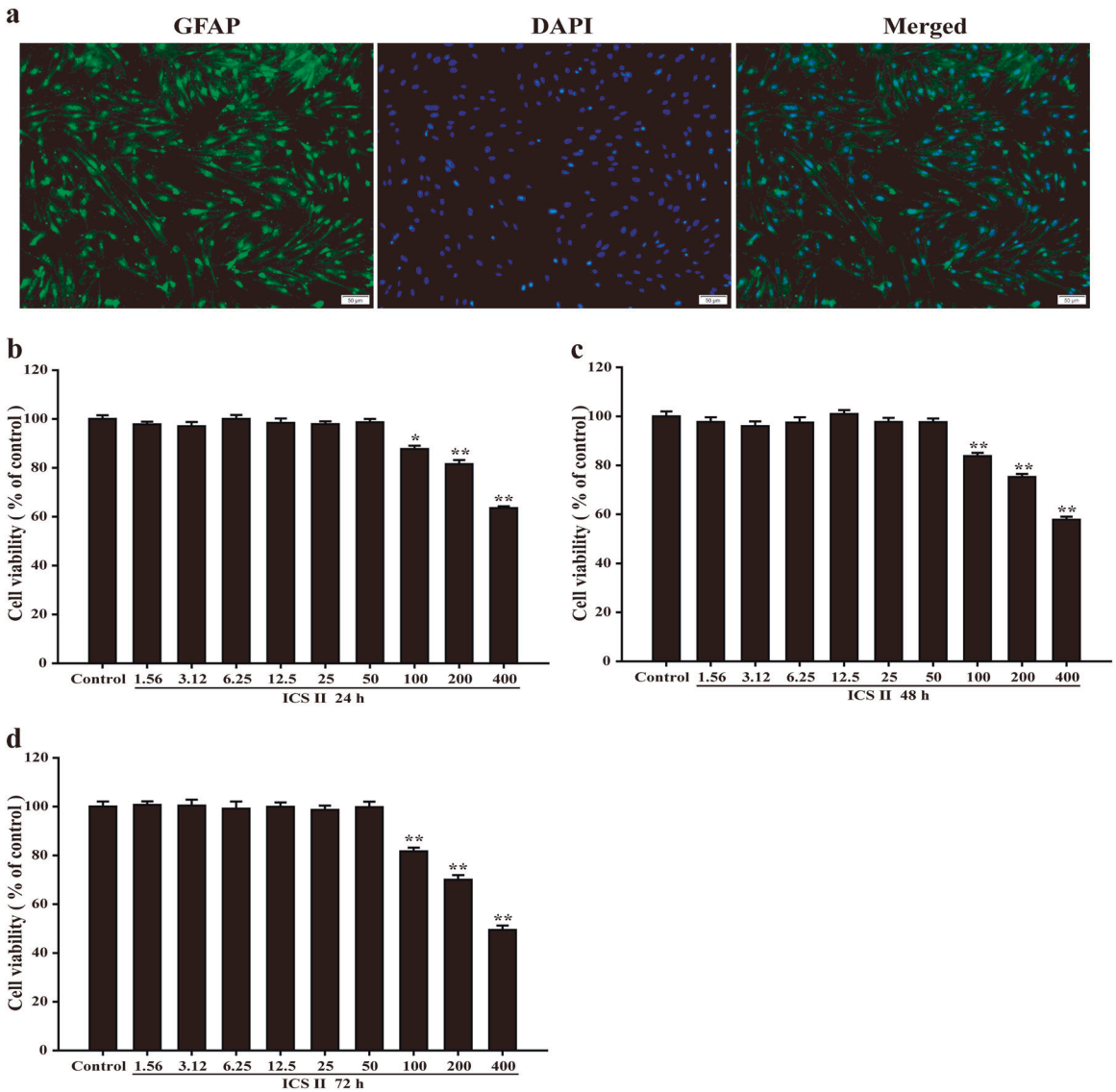

Fig. 1 Identification of astrocytes and the determination of the safe concentration range of ICS II. **a** Identification of cellular immunofluorescence (scale bar = 50 μm, x200). **b** The safe concentration range of ICS II within 24 h ($n = 5$). **c** The safe concentration range of ICS II within 48 h ($n = 5$). **d** The safe concentration range of ICS II within 72 h ($n = 5$). $^{*}P < 0.05$, $^{**}P < 0.01$ versus control group

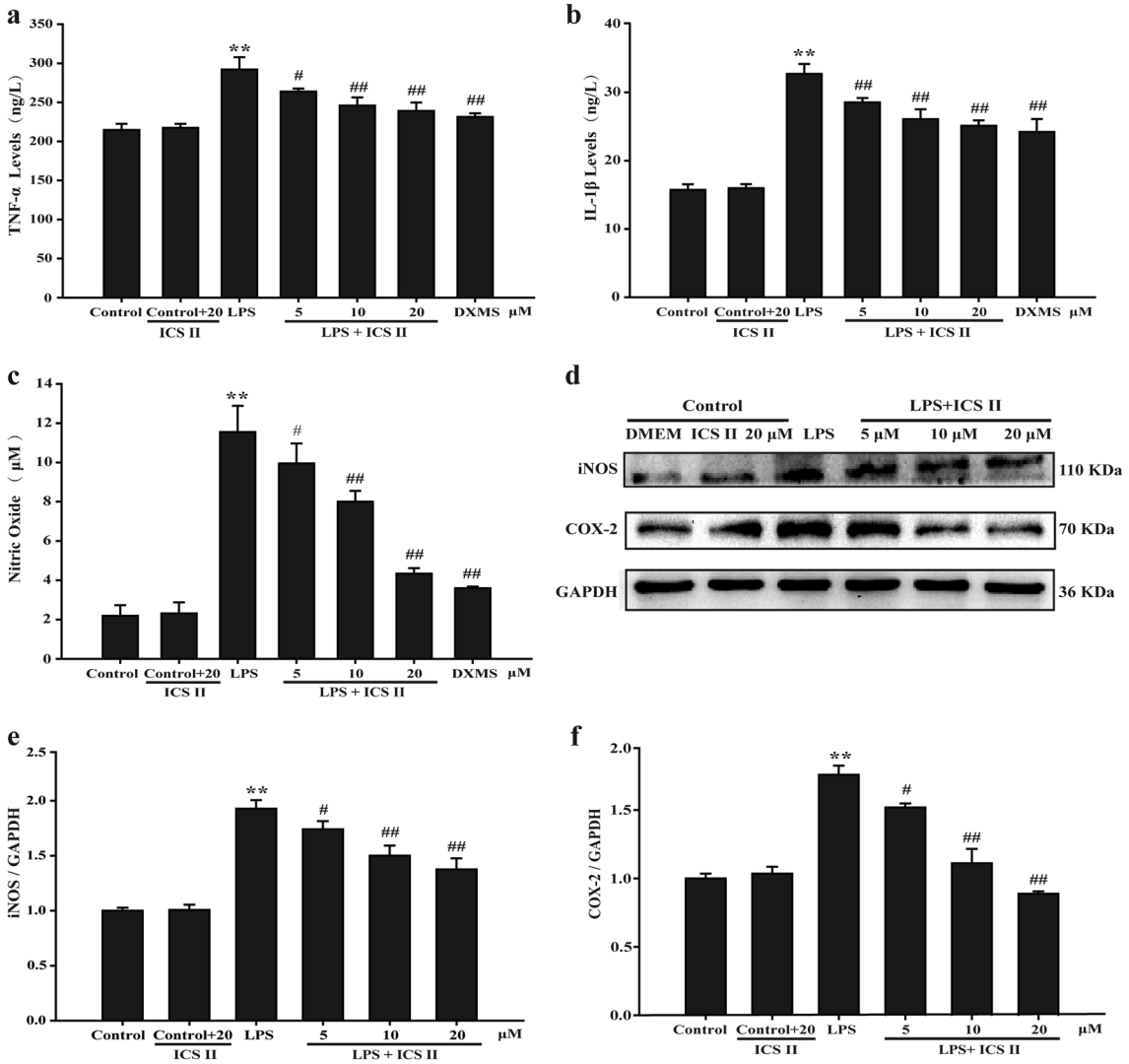

Fig. 2 ICS II suppressed inflammatory factors in astrocytes after LPS insult. Astrocytes were pretreated with or without ICS II (5, 10, and 20 μM) or DXMS (1 μM) for 1 h followed by LPS (1 μg/mL) for another 24 h. **a** TNF-α level ($n = 5$). **b** IL-1β level ($n = 5$). **c** Production of NO ($n = 5$). **d** Representative Western blot bands for iNOS and COX-2 proteins. **e** Quantitation of iNOS protein ($n = 3$). **f** Quantitation of COX-2 protein ($n = 3$). $^{**}P < 0.01$ versus control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus LPS group

neonatal rat brains as previously described [36]. Primary astrocytes were isolated from the cerebral cortex of 24-h-old neonatal rats. Briefly, suckling rats were repeatedly disinfected with 75% alcohol three times, and then the brains were removed, and the brain tissues were separated. Then, the cerebral cortex was collected under low temperatures, and the meninges and blood vessels were slightly removed. The cerebral cortex was fully dissociated by the addition of 10 mL of trypsin, the flask was treated with polylysine, and the suspension was spread in a culture flask at a cell density of 3×10^5 cells/mL. The differential adherence method was used to remove other types of cells in the tissue, and then astrocytes were cultured in DMEM/F12 containing 10% FBS. The DMEM/F12 was changed every 3 days until the 13th day. The primary cells were shaken at 37 °C at a constant temperature to remove other glial cells, such as microglia and oligodendrocytes. Thereafter, the astrocytes were fixed with 4% paraformaldehyde, incubated with an anti-GFAP antibody (1:500, Abcam), and visualized using an Alexa Fluor-conjugated secondary antibody (1:1000, Abcam). More than 95% of the cultured astrocytes were identified by GFAP staining, and the astrocytes were pretreated with different concentrations of ICS II or DXMS (1 μM) for 1 h. Thereafter, they were treated with LPS for another 24 h. Since DXMS is an effective and classical antiinflammatory drug, it was selected as a positive control drug to evaluate the antiinflammatory effect of ICS II, and 1 μM was chosen as the desired concentration of DXMS according to a previous study [37].

Measurement of cell viability

Cell viability was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay as described in our previous study [38]. In brief, astrocytes were seeded in a 96-well plate at 1×10^5 cells/well and treated as described above. Then, 20 μL of MTT (5 mg/mL) was added to FBS-free medium and cultured for another 4 h. The MTT was removed, and the cells were lysed with 150 μL of DMSO in each well. The optical density was measured at 490 nm using a microplate reader. The results of the treatments were expressed as a percentage of the control.

ELISA assay

In brief, astrocytes were inoculated into 96-well plates at a density of 1×10^5 cells/well and pretreated with ICS II (5, 10, 20 μM) for 1 h. LPS (1 mg/mL) and ICS II were added to the plates for 24 h.

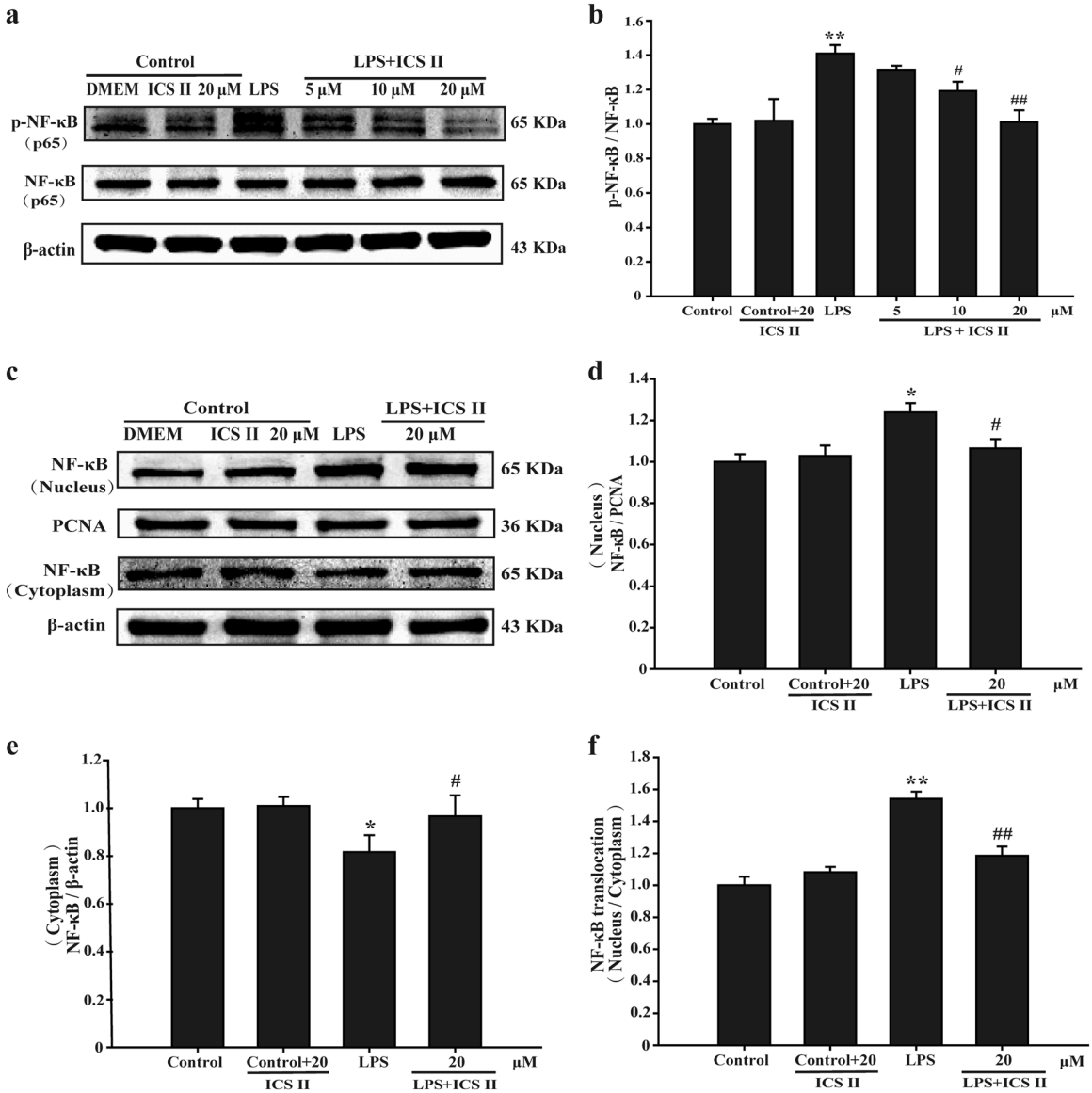

Fig. 3 ICS II inhibited the LPS-induced phosphorylation of NF-κB in astrocytes. **a** Representative Western blot bands for p-NF-κB and NF-κB. **b** Quantitation of p-NF-κB levels ($n = 3$). **c** Representative Western blot bands for NF-κB (nuclear) and NF-κB (cytoplasmic) proteins. **d** Quantitation of NF-κB (nucleus) protein ($n = 3$). **e** Quantitation of NF-κB (cytoplasmic) protein ($n = 3$). **f** Quantitation of NF-κB (nuclear)/NF-κB (cytoplasmic) protein ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ versus control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus LPS group

Thereafter, the culture medium was collected and centrifuged for 10 min at 16 000 × *g*. The supernatants were then collected and used to measure TNF-α, IL-1β, $A\beta_{1-40}$, and $A\beta_{1-42}$ levels using ELISA kits. All data were obtained from at least three independent experiments.

Measurement of nitric oxide (NO)

Astrocytes were inoculated in 96-well plates at a density of 1×10^5 cells/well and pretreated with ICS II (5, 10, 20 μM) for 1 h. LPS (1 mg/mL) and ICS II were added to the plates for 24 h. The accumulation of nitrite in the supernatant was evaluated using the Griess reaction. Each 50 μL of supernatant was reacted with an equal volume of Griess reagent and cultured for 15 min at room temperature. The absorbance was detected in a microplate absorbance reader at a wavelength of 540 nm, and a series of known concentrations of sodium nitrite was utilized as a standard.

Western blot analysis

Western blot analysis was performed as described in our previous study [38]. Briefly, astrocytes were treated as mentioned above, homogenized with protein extraction solution, and lysed for 40 min on ice. The lysate was centrifuged for 15 min at 14 000 × *g*. Equal amounts of protein (30 μg) were separated on a 10% SDS-polyacrylamide gel, transferred onto a polyvinylidene difluoride membrane and incubated with the following specific antibodies: anti-iNOS (1:1000), anti-COX-2 (1:2000), anti-p65 (1:2000), anti-p-p65 (1:1000), IκB-α (1:2000), anti-IKK-α (1:2000), anti-p-IKK-α (1:1000), anti-IKK-β (1:2000), anti-p-IKK-β (1:1000), anti-APP (1:1000), anti-BACE1 (1:1000), and anti-β-actin (1:2000). Then, the blots were subjected to the corresponding horseradish peroxidase-conjugated anti-rabbit or mouse immunoglobulin G. Thereafter, immunoreactive proteins were measured using an enhanced chemiluminescence Western blotting detection system.

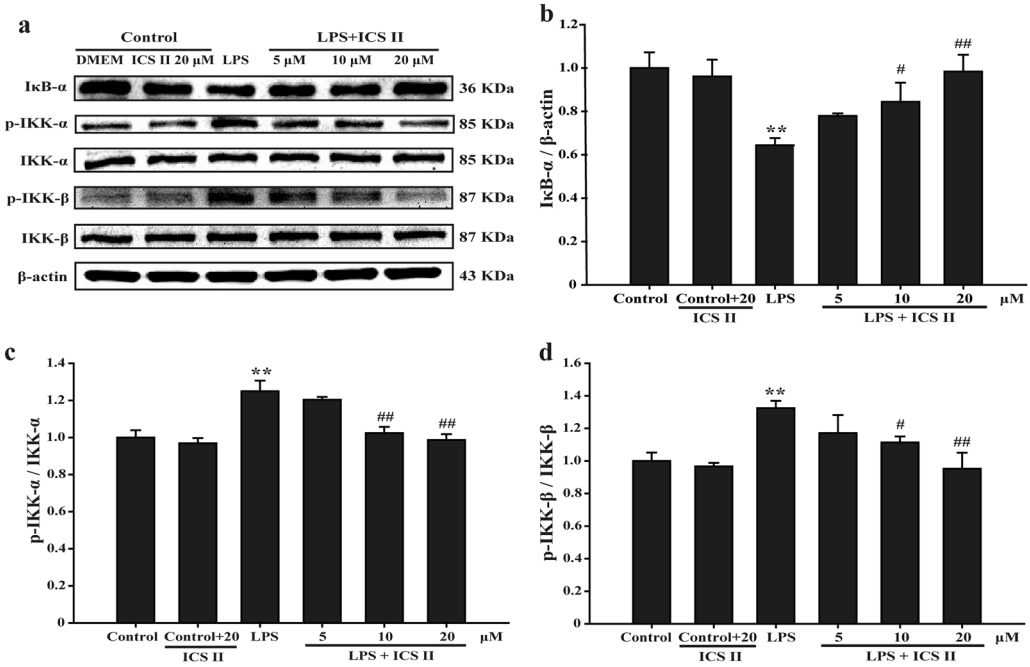

Fig. 4 ICS II suppressed the LPS-induced activation of the NF-κB pathway in astrocytes. **a** Representative Western blot bands for IκB-α, p-IKK-α, IKK-α, p-IKK-β, and IKK-β proteins. **b** Quantitation of IκB-α protein ($n = 3$). **c** Quantitation of p-IKK-α/IKK-α protein ($n = 3$). **d** Quantitation of p-IKK-β/IKK-β protein ($n = 3$). $^{**}P < 0.01$ versus control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus LPS group

Molecular docking

Molecular docking was performed using Autodock 4.2 and AutodockTools software to observe the affinity of ICS II and BACE1. The pdb format file for the BACE1 protein was obtained from the Protein Data Bank (http://www.rcsb.org) database with a PDB ID of 2ZHV. The molecular docking of the ICS II and BACE1 proteins was performed using Autodock 4.2.

Statistical analysis

All data were analyzed by SPSS 17.0 statistics software and are expressed as the mean ± SD. The data were analyzed using one-way ANOVA followed by the post hoc least significant difference. All data were obtained from at least three independent experiments. $P < 0.05$ was considered significant.

RESULTS

Immunofluorescence identification of astrocytes

We extracted the cultured cells and identified them by staining for GFAP, which is a specific marker of astrocytes [39]. The results showed that astrocytes made up 95% of the cultured cells (Fig. 1a). Furthermore, the results showed that ICS II (1.56, 3.12, 6.25, 12.5, 25, or 50 μM) for 24, 48, or 72 h had no effect on the astrocytes; however, ICS II (100, 200, or 400 μM) for 24, 48, or 72 h was cytotoxic to the astrocytes. Since the concentration of DMSO used to dissolve ICS II in the experiment did not exceed 0.1%, it was assumed that the concentration of DMSO itself was not toxic to the cells and that the toxicity to the cells therefore resulted from the high concentration of ICS II. Thus, a concentration of ICS below 50 μM was considered to be the safe concentration range (Fig. 1b–d). Thereafter, 5, 10, and 20 μM ICS II were used in subsequent experiments, and DXMS (1 μM) was used as a positive control agent.

Effect of ICS II on the LPS-induced production of TNF-α and IL-1β

The effect of ICS II on the LPS-induced production of TNF-α and IL-1β in astrocytes was determined using an ELISA assay. The results showed that ICS II (5, 10, or 20 μM) slightly mitigated the production of TNF-α (Fig. 2a) and IL-1β (Fig. 2b). Following LPS stimulation, the production of TNF-α and IL-1β was substantially elevated compared with that in the control group, while ICS II markedly alleviated LPS-induced TNF-α and IL-1β overproduction in a concentration-dependent manner.

Effect of ICS II on LPS-induced NO production and iNOS and COX-2 expression in astrocytes

The effect of ICS II on LPS-induced NO production and iNOS and COX-2 expression in astrocytes was determined using the Griess reaction and Western blot analysis, respectively. The results showed that ICS II decreased LPS-induced NO production in astrocytes (Fig. 2c). Moreover, iNOS and COX-2 expression levels were dramatically increased after LPS insult. However, ICS II (5, 10, and 20 μM) induced a concentration-dependent decrease in the expression of iNOS and COX-2 in astrocytes compared with that in the LPS group (Fig. 2d–f).

Effect of ICS II on LPS-induced NF-κB (p65) translocation and the degradation of IKK-α, IKK-β, and IκB-α

The results suggested that ICS II not only suppressed the LPS-induced phosphorylation of p65 (Fig. 3a, b) but also prevented the nuclear translocation of p65 (Fig. 3c–f). Furthermore, ICS II also suppressed the LPS-induced degradation of IκB-α (Fig. 4a, b), IKK-α, and IKK-β (Fig. 4c, d). These results indicate that ICS II mitigates the LPS-induced activation of NF-κB via inhibiting IκB-α phosphorylation and the translocation of p65 to the nucleus.

Effect of ICS II on LPS-induced amyloidogenesis in astrocytes

The effect of inflammation on amyloid formation in vitro was also investigated because neuroinflammation can cause amyloid production, whereas the aberrant activation of astrocytes is a major source of neuroinflammation. Astrocytes provide both mechanical and metabolic support to neurons, regulating the environment in which they function. To determine the relationship between neuroinflammation and amyloidogenesis, we investigated whether the antiinflammatory effect of ICS II can result in antiamyloidogenesis. As shown in Fig. 5a, b, when the

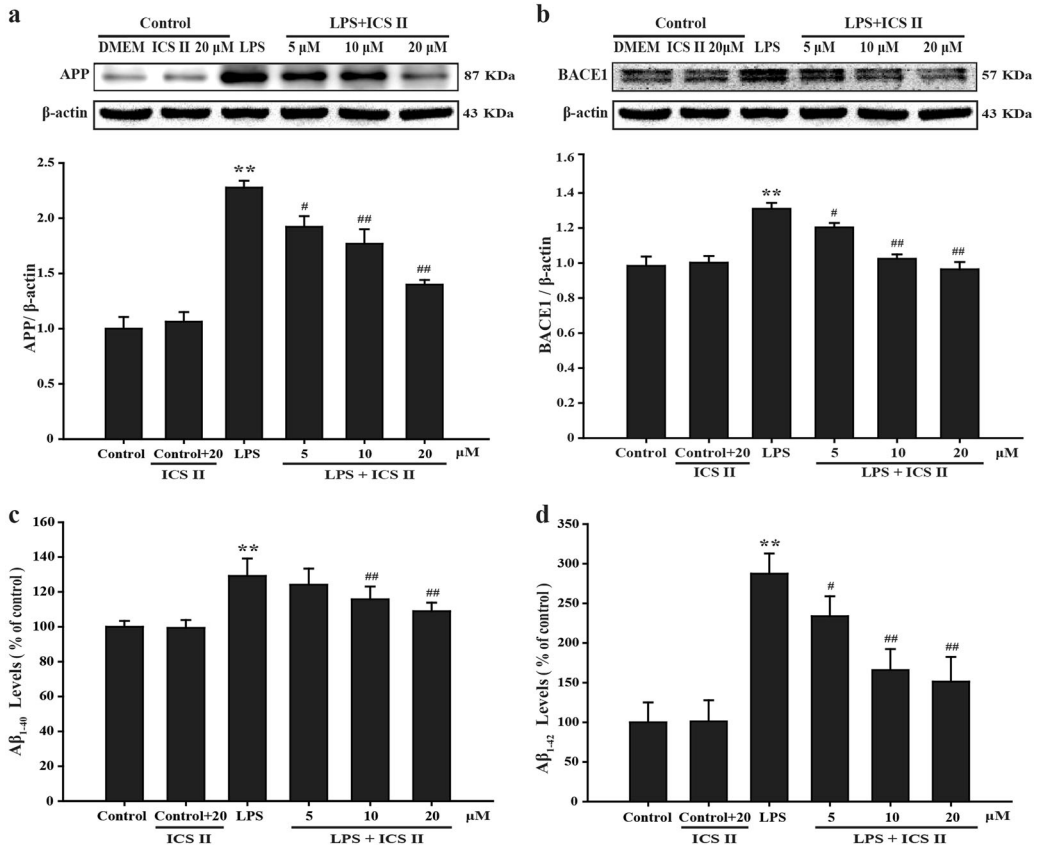

Fig. 5 Effects of ICS II on the expression of APP and BACE1 and the levels of $A\beta_{1-40}$ and $A\beta_{1-42}$. **a** Quantitation of APP expression ($n = 3$). **b** Quantitation of BACE1 expression ($n = 3$). **c** $A\beta_{1-40}$ level ($n = 5$). **d** $A\beta_{1-42}$ level ($n = 5$). $^{**}P < 0.01$ versus control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus LPS group

cells were unstimulated, they expressed low protein levels of APP and BACE1, whereas the protein expression of APP and BACE1 increased in response to LPS after 24 h. In addition, ICS II also decreased LPS-induced $A\beta_{1-40}$ and $A\beta_{1-42}$ secretion into the culture media of astrocytes (Fig. 5c, d). In astrocytes, we also found that ICS II inhibited the LPS-induced expression of APP and BACE1, as well as $A\beta_{1-40}$ and $A\beta_{1-42}$ levels in a concentration-dependent manner. These results further indicate that the amyloidogenic pathway can be promoted by neuroinflammatory stimulation and that the antiinflammatory effect of ICS II can result in antiamyloidogenesis.

ICS II binds and inhibits BACE1

Interestingly, mock molecular docking was used to verify whether ICS II binds to BACE1 protein, and the results showed that the binding energy of ICS II and BACE1 was −5.78 kcal/mol, which confirmed that ICS II can bind to BACE1, as the standard threshold for a molecule to bind to a protein is thought to be greater than or equal to −5 kcal/mol (Fig. 6a, b). We further investigated the supposed binding modes and interactions within the amino acid pocket, including Gly13, Gly230, Gly11, Thr232, Tyr71, Gly74, Lys107, Asp106, and Phe108 (Fig. 6c, d). Taken together, these findings show that ICS II may directly affect BACE1 and thus exert neuroprotective effects.

DISCUSSION

The present study revealed that (1) ICS II protects against LPS-induced inflammation in primary-cultured astrocytes; (2) the inhibitory effect of ICS II is due to regulation of the IKK/IκB/NF-κB signaling pathway; and (3) ICS II decreases $A\beta_{1-40}$ and $A\beta_{1-42}$ levels by downregulating APP and BACE1 expression (Fig. 7).

LPS is an effective component of gram-negative bacilli endotoxin, which can cause a series of inflammatory reactions in the body and is widely applied to establish animal or cellular inflammatory models. Moreover, astrocytes represent the most abundant cell type in the central nervous system, and they exert a variety of physiological functions through close association with neurons and other brain structures. Similar to microglia, the immune and inflammatory properties of astrocytes, which can promote the secretion of various neuroinflammatory factors, such as TNF-α and IL-1β, are activated by LPS. Accumulating evidence has demonstrated that the production of multiple neuroinflammatory factors, including TNF-α, IL-1β, and iNOS, can activate the NF-κB pathway, resulting in neuroinflammation [40]. Moreover, the excessive production of neuroinflammatory factors leads to neuronal damage through the activation of glial cells. Notably, astrocytes play a key role in central nervous system inflammation by producing cytokines, such as TNF-α, IL-1β, and NO, leading to neuronal injury [41]. Therefore, the suppression of astrocyte activation may be an effective treatment for neuroinflammation-related diseases [42]. Our findings showed that LPS induces an increase in proinflammatory factors, which is consistent with the results of previous studies [43, 44]. ICS II significantly inhibited the LPS-induced accumulation of TNF-α, IL-1β, NO, iNOS, and COX-2 in primary culture astrocytes. In addition, these effects were not due to the cytotoxic effect of ICS II, as evidenced by the observation that there was no effect on astrocytes at concentrations up to 50 μM. Moreover, ICS II also inhibited iNOS and COX-2 protein

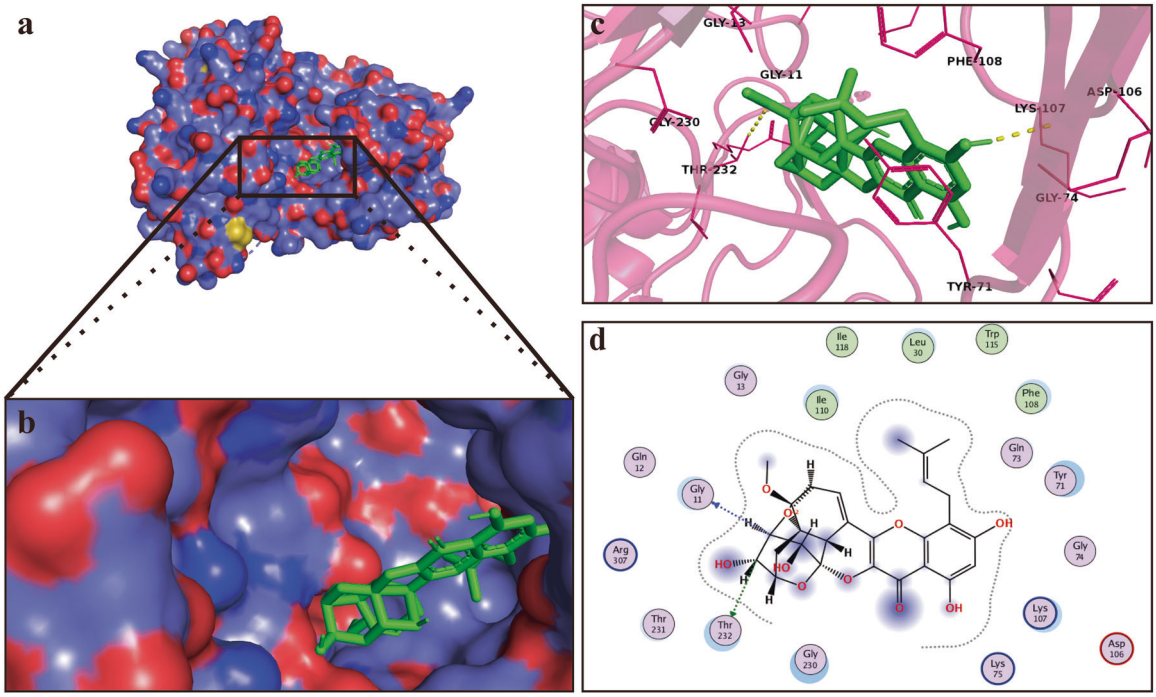

Fig. 6 ICS II binds and inhibits BACE1. **a** Visual of the binding sites between ICS II and BACE1. **b** Visual of the binding surface between ICS II and BACE1. **c** Crystal structure of ICS II (green) displaying BACE1 (yellow and purple) bound to the docking pocket. **d** Amino acid residues

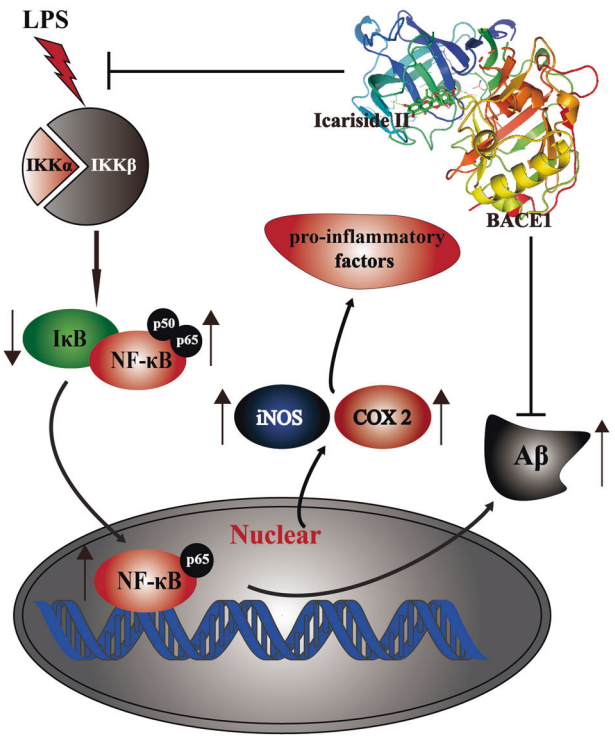

Fig. 7 A schematic showing the underlying mechanisms by which ICS II protects primary-cultured astrocytes against LPS-induced inflammation. ICS II inhibits proinflammatory mediators via regulating the IKK/IκB/NF-κB signaling pathway

expression in astrocytes. These findings indicate that ICS II exerts inhibitory effects on inflammation.

Notably, NF-κB not only regulates a variety of inflammatory factors, such as IL-1β and TNF-α but also plays a key role in mediating COX-2 and iNOS expression [45]. NF-κB (p50/p65) heterodimers exist in the cytoplasm of resting cells. However, under stimulation by LPS, the phosphorylation of IKK was induced, which subsequently phosphorylates the IκB protein, resulting in the release of NF-κB (p50/p65) heterodimers. Then, NF-κB-p65 translocates from the cytoplasm to the nucleus, thereby promoting the release of proinflammatory factors, including TNF-α and IL-6 [43, 44]. Consistent with these studies, the present study showed that LPS induces the phosphorylation of NF-κB-p65, IκB, and IKK. However, ICS II abolished these changes, suggesting that ICS II exerts potent antiinflammatory effects, at least partly through modulating the IKK/IκB/NF-κB pathway.

APP is a membrane-intrinsic protein expressed in a variety of tissues and is closely related to AD. APP can be cleaved by α, β, and γ proteases, and the continuous action of β-protease and γ-protease can cause APP to be cleaved to produce Aβ [46]. However, Aβ can cause the formation of senile plaques in the brain and the apoptosis of neuronal cells, thereby causing AD. Most importantly, mounting evidence has shown that neuroinflammation is related to the accumulation of Aβ in the brains of AD patients [47, 48]. In addition, APP is first cleaved by β-secretase at its β-cleavage site and is then proteolyzed by the second enzyme, γ-secretase, to produce $A\beta_{1-42}$ and $A\beta_{1-40}$. In particular, the $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides are involved in the amyloidogenic pathway; $A\beta_{1-40}$ is the most plentiful species, and $A\beta_{1-42}$ shows the strongest neurotoxicity [49, 50]. Of note, BACE1 is an important rate-limiting enzyme in the APP–Aβ pathway and plays an important role in the production of Aβ. BACE1 was overexpressed in the brains of animal models of AD [51]. In contrast, in the brains of BACE1 knockout mice, the levels of Aβ and sAPPβ are decreased [52]. In addition, activated astrocytes that release mediators can induce APP and BACE1 expression, thereby stimulating Aβ production [53–56]. Notably, NF-κB mediates the accumulation of APP and the expression of BACE1 to increase Aβ production [57, 58]. In this study, LPS not only augmented the production of $A\beta_{1-40}$ and $A\beta_{1-42}$ but also upregulated APP and BACE1, and these findings are consistent with those of previous

studies [36, 59]. However, ICS II reversed these changes, revealing that ICS II exerts inhibitory effects on neuroinflammation via suppressing the NF-κB/BACE1 signaling pathway, and this was also evidenced by molecular docking.

CONCLUSION

In conclusion, the current study revealed that ICS II exerts inhibitory effects on LPS-induced inflammation in astrocytes through the IKK/IκB/NF-κB/BACE1 signaling pathway, and thus ICS II may be a promising therapeutic agent for neuroinflammatory diseases, including AD.

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AUTHOR CONTRIBUTIONS

QHG and JSS designed the experimental protocols. YZ carried out all the studies except the Western blot analysis, which was performed by CL, LHL, and YD. YZ wrote the manuscript with help from QHG, CYY, and JMG.

ADDITIONAL INFORMATION

Competing interests: The authors declare no competing interests.

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