Astragaloside IV attenuates penicillin-induced epilepsy via inhibiting activation of the MAPK signaling pathway

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Abstract. Astrocytes perform several functions in the brain and spinal cord. Penicillin is commonly used for establishment of experimental epilepsy models. Previous studies have demonstrated that astragaloside IV (3-o- β -d-xylopyranosyl-6-o- β -d-glucopyranosyl-cycloastragenol; AS-IV) has comprehensive pharmacological functions on the attenuation of inflammation. In the present study, primary astrocyte cell cultures were divided into three groups: Control group, penicillin (2,500 μ M) treatment group (epilepsy model), and penicillin+AS-IV (20, 40, 80 and 160 μ mol/l) treatment group. The expression levels of inflammatory factors, including interleukin-1 β and tumor necrosis factor- α , were determined in the groups using western blot and reverse transcription-quantitative polymerase chain reaction analyses. The levels of members of the phosphorylated-mitogen-activated protein kinase (p-MAPK) family, including p-c-Jun N-terminal kinase 1/2, p-extracellular signal-regulated protein kinase 1/2 and p-p38, were determined using western blot analysis. Cell viability of the astrocytes was detected using a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide assay and cell proliferation was evaluated using a Cell Counting Kit-8 assay. The results revealed that AS-IV significantly suppressed the expression of penicillin-induced inflammatory factors in the astrocytes at the transcriptional and translational levels, and occurred in a dose-dependent manner. The penicillin-induced increase in the protein levels of the p-MAPK family were notably decreased by AS-IV. In addition, the penicillin-induced downregulation of primary astrocyte viability/cell proliferation was

significantly reversed by the administration of AS-IV. From these results, it was concluded that AS-IV suppressed the penicillin-induced upregulation of inflammatory factors and p-MAPK in astrocytes, ultimately attenuating epilepsy.

Introduction

As a group of neurological diseases, epilepsy is characterized by epileptic seizures (1,2). Worldwide, ~1% (65,000,000 individuals) of the population suffer with epilepsy (3), with almost 80% of new cases occurring in developing countries (4). In 2013, the mortality rate was ~116,000, which was an increase from 112,000 in 1990 (5).

Astrocytes initiate, regulate and amplify immune-mediated mechanisms, and are associated with several diseases of the human central nervous system, including epilepsy (6,7). *In vitro* studies have documented the ability of astrocytes, particularly active astrocytes, to produce cytokines, including interleukin (IL)-1 β and tumor necrosis factor (TNF)- α , which are expressed at high levels in experimental and human epileptogenic brain tissues (8,9).

As a common antibiotic, penicillin functions as a chemical convulsant for the establishment of an experimental epilepsy model (10,11). At least 100-5,000 μ M penicillin is required to inhibit GABA (12).

It is understood that astragaloside IV (3-o- β -d-xylopyranosyl-6-o- β -d-glucopyranosyl-cycloastragenol; AS-IV), the primary pure saponin isolated from the root of *Astragalus membranaceus*, is an effective compound with distinct pharmacological effects, including anti-inflammatory effects (13,14). To the best of our knowledge, the protective effects of AS-IV on epilepsy remain to be elucidated, therefore, the present study aimed to investigate the effect of AS-IV in a primary astrocyte model of penicillin-induced epilepsy.

Materials and methods

Cell culture. Primary astrocytes were derived from 1-5 day postnatal Sprague-Dawley male rats (n=10). Neonatal rats were purchased from the Model Animal Research Center of Nanjing University. Briefly, neonatal rats were anesthetized and sacrificed by alcohol immersion. Following removal of the meninges and blood vessels, the cerebral cortices were collected and minced in 20 μ g/ml DNase and 0.3% bovine

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serum albumin (BSA)-containing medium (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The animal protocol was approved by Wuxi Laboratory Animal Management and the Animal Ethics Committee. All animal experiments were carried out in accordance with the ethical guidelines of the Ethics Committee of Jiangnan University (Wuxi, China).

The tissues were digested in 0.25% trypsin/EDTA solution for 30 min at 37°C. The tissues were centrifuged (300 x g, 5 min at room temperature) and digested in 0.25% trypsin/EDTA solution for 30 min at 37°C. The suspension was filtered through a 70 µm nylon filter, pelleted by centrifugation (600 x g, 5 min at room temperature) to remove trypsin, and then suspended in 10% (v/v) fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in Dulbecco's modified Eagle's medium/F12 (DMEM/F12; Gibco; Thermo Fisher Scientific, Inc.) containing a penicillin and streptomycin antibiotic mixture. The mixture was transferred to flasks and incubated under conditions of 37°C, 5% CO₂ and 90% relative humidity. When the cells reached confluence, the flasks were gently shaken to remove microglia and oligodendrocytes. Following shaking, the astrocytes were rinsed with phosphate-buffered saline (PBS) three times and trypsinized, followed by physical loosening of the astrocytes. The medium was removed and the astrocytes were placed in new flasks for culture in medium (DMEM/F12, 15% FBS, L-glutamine and 500 ng/ml insulin) until they were confluent. The cells were trypsinized for subsequent experiments.

For primary astrocyte culture, 2,500 µM (500 IU) of penicillin was used (15). Different concentrations of AST-IV (20, 40, 80 and 160 µmol/l) were administered 2 h prior to penicillin treatment. At 12 h following treatment with penicillin, the primary astrocytes were used to perform a series of experiments.

Western blot analysis. To determine temporal expression profiles of β-actin (1:1,000, #3700, Cell Signaling Technology, Inc., Danvers, MA, USA), IL-1β (1:1,000, #5204, Cell Signaling Technology, Inc.), TNF-α (1:1,000, #11948, Cell Signaling Technology) and phosphorylated extracellular signal-regulated kinase (p-ERK, 1:1,000, #8544, Cell Signaling Technology, Inc.)/c-Jun N-terminal kinase (p-JNK, 1:2,000, #9255, Cell Signaling Technology, Inc.)/p-P38 (1:1,000, #4511, Cell Signaling Technology, Inc.) mitogen-activated protein kinases (MAPKs), the astrocyte extract lysates were collected and analyzed. Briefly, the samples were washed with PBS rapidly and homogenized in RIPA lysis buffer containing a cocktail of protease inhibitors and phosphatase inhibitors (Roche Diagnostics, Nanjing, China). Protein concentration was determined and quantified using the bicinchoninic acid (Sigma-Aldrich) method. The sample lysates (10 µg) were transferred and collected carefully for separation by 8% SDS-PAGE, following which they were electrotransferred onto polyvinylidene fluoride membranes (EMD Millipore, Bedford, MA, USA). The membranes were blocked in 5% BSA for 1 h at room temperature, and incubated overnight at 4°C with primary antibodies, respectively. Following washing with TBST, the membranes were incubated with corresponding HRP-conjugated secondary antibodies (RPN4301, GE Healthcare Life Sciences, Little

Chalfont, UK) for 1 h at room temperature. β-actin was used as a loading control.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted by Trizol (Roche Diagnostics) method. RNA was reverse transcribed into cDNA using reverse transcriptase, in accordance with the manufacturer's protocol (Takara Biotechnology, Co., Ltd., Dalian, China). The sample used for RT-qPCR analysis comprised 2 µl cDNA, 5 µl 2X mix (Bio-Rad Laboratories, Inc., Hercules, CA, USA), 0.5 µl forward primer, 0.5 µl reverse primer and 2 µl nanopure water to a final volume of 10 µl. The conditions for amplification were as follows: 10 sec at 95°C for denaturation, 60 sec at 60°C for annealing and extension, and 40 cycles for all primers. Experiments were performed in replicate. GAPDH was used as an endogenous control to normalize differences in the quantity of total RNA from each sample. The primer sequences were as follows: IL-1β, forward 5'-TGAAATGCCACCTTTTGACAG-3' and reverse 5'-CCACAGCCACAATGAGTGATAC-3'; TNF-α, forward 5'-CCTGTCTCTTCTACCCAACC-3' and reverse 5'-GCAGGAGTGTCCGTGTCTTC-3'; GAPDH, forward 5'-CTTTGGTATCGTGGAAGGACTC-3' and reverse 5'-GTAGAGGCAGGGATGATGTTCT-3'. The comparative C_q (2^{-ΔΔC_q}) method was used to analyze the relative expression levels (16).

3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay. Cell viability was measured using an MTT assay. The cells (~200 µl) at a concentration of 1x10⁴/ml were seeded into 96-well plates. Following incubation for 24 h, 20 µl of 5 mg/ml MTT solution was added to each well and the plate was incubated at 37°C for 4 h. Subsequently, the medium was aspirated, the wells were washed with PBS and allowed to dry for ~4 h, following which 150 µl DMSO was added to each well. The microtitre plate was placed on a shaker in order to dissolve the dye and absorbance was read at 450 nm using a Bio-Rad iMark plate reader (Bio-Rad Laboratories, Inc.).

Cell Counting Kit-8 (CCK-8) assay. The numbers of cells were measured using a CCK-8 kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). The cells (~5x10³) cells were seeded into 96-well plates. Into each well, 10 µl CCK-8 solution was added and the cells were incubated at 37°C for 2 h. The absorbance was read at 450 nm using a Bio-Rad iMark plate reader.

Statistical analysis. Differences among groups were analyzed using two-way analysis of variance followed by Bonferroni post hoc tests. Data were analyzed by SPSS software version 19.0 (IBM SPSS, Armonk, NY, USA) and presented as mean ± standard error of the mean. P<0.05 was considered to indicate a statistically significant difference.

Results

AS-IV significantly suppresses the translation of penicillin-induced inflammatory factors of primary astrocytes in a dose-dependent manner. Penicillin upregulated the protein levels of TNF-α and IL-1β, whereas AS-IV (20, 40, 80 and 160 µmol/l) dose-dependently suppressed the

penicillin-induced upregulation in inflammatory factors (Fig. 1).

AS-IV significantly suppresses the transcription of penicillin-induced inflammatory factors of primary astrocytes in a dose-dependent manner. The changes in the transcription of inflammatory factors were consistent with those at the translational level. Only the higher doses of AS-IV (40, 80 and 160 $\mu\text{mol/l}$) notably reduced the penicillin-induced upregulation of inflammatory factors (Fig. 2A and B).

Penicillin-induced protein levels of the p-MAPK family are decreased by AS-IV. AS-IV selectively reduced the penicillin-induced p-JNK and p-p38 MAPKs, but not p-ERK (Fig. 3).

Penicillin-induced downregulation of primary astrocyte viability is significantly increased following administration of AS-IV. The MTT assay revealed that, compared with the control group, the viability of the primary astrocytes in the penicillin-induced group was markedly downregulated. In addition, administration with the higher doses of AS-IV (40, 80 and 160 $\mu\text{mol/l}$) significantly increased cell viability (Fig. 4).

Penicillin-induced downregulation of primary astrocyte numbers is significantly elevated following administration of AS-IV. The results of the CCK8 assay revealed that, compared with the control group, the number of primary astrocytes in the penicillin-induced group was markedly reduced. The administration of higher doses of AS-IV (40, 80 and 160 $\mu\text{mol/l}$) significantly reversed the penicillin-induced reduction in cell number (Fig. 5).

Discussion

As one of the most common serious neurological disorders (17), epilepsy is characterized by epileptic seizures (1,2) and becomes more common as people age (18,19). However, the exact mechanism underlying epilepsy remains to be elucidated (19). In normal conditions, brain electrical activity is modulated by various factors within and around neurons. Factors within neurons include the type, number and distribution of ion channels, and changes in receptor and gene expression (21). Factors around the neurons include ion concentrations, synaptic plasticity and the regulation of transmitter breakdown by glial cells (21,22).

Astrocytes initiate, regulate and amplify immune-mediated mechanisms associated with epilepsy (6,7). *In vitro* studies have confirmed the ability of active astrocytes to produce cytokines, including IL-1 β and TNF- α , which are expressed at high levels in experimental and human epileptogenic brain tissues (8,9). Reactive astrogliosis was found to be a pathological hallmark of medically refractory epilepsy (23). Increasing evidence supports the hypothesis that activation of the innate immune response is involved in experimental and human epilepsy, and in the critical association of the inflammatory process in the etiopathogenesis of seizures (8,24). Vezzani *et al* further examined the role of inflammation in epilepsy (25). The aim of the present study was to identify a

Figure 1. AS-IV dose-dependently suppresses penicillin-induced inflammatory factor upregulation at the protein level. Penicillin upregulated the protein levels of TNF- α and IL-1 β . AS-IV (20, 40, 80 and 160 $\mu\text{mol/l}$) dose-dependently suppressed the penicillin-induced increase in inflammatory factors. AS-IV, astragaloside IV; TNF-tumor necrosis factor- α ; IL-1 β , interleukin-1 β .

novel method to attenuate epilepsy via inhibiting the inflammatory pathway.

AS-IV is an effective compound with distinct pharmacological anti-inflammatory effects (13,14), however, the role of AS-IV in epilepsy remains to be fully elucidated. Penicillin has been demonstrated to function as a convulsant for the establishment of experimental epilepsy models (10,11). The present study examined the effect of AS-IV against penicillin-induced epilepsy in primary astrocytes.

In the present study, penicillin was used to induce epilepsy in Primary astrocytes from SD rats, and the protein and mRNA levels of TNF- α and IL-1 β were examined in different groups to investigate whether the model was successfully established. The results revealed that penicillin upregulated the protein and mRNA levels of TNF- α and IL-1 β , which were consistent with the results of previous studies showing the upregulation of pro-inflammatory cytokines (25,26). The present study then examined whether AS-IV (20, 40, 80 and 160 $\mu\text{mol/l}$) had effects on the levels of inflammatory factors using western blot and RT-qPCR analyses. The results revealed that AS-IV dose-dependently suppressed the penicillin-induced increase in inflammatory factors. These results suggested that the effects of AS-IV on ameliorating epilepsy were dependent on the reduced release of inflammatory factors from the cultured cells. These findings were consistent with previous studies. For example, it has been reported that transgenic mice with low-moderate overexpression of TNF- α in astrocytes exhibit reduced susceptibility to seizures (27), and mice lacking caspase-1, which is the biosynthetic enzyme of IL-1 β , are unable to release the biologically active form of IL-1 β and also exhibit reduced seizure susceptibility (28). However, which proteins are involved in this process remain to be elucidated, and the present study performed further experiments to investigate this.

TNF- α is a cytokine released from activated astrocytes and microglia, and is closely associated with IL-1 β . In the hippocampus, IL-1 β affects synaptic transmission, and inhibits long-term potentiation via the activation of JNK and p38 MAPK (29,30). Therefore, the present study performed western blot analysis to examine the levels of p-MAPK in different groups. The results revealed that AS-IV decreased the penicillin-induced upregulation of p-p38

Figure 2. AS-IV dose-dependently suppresses penicillin-induced inflammatory factor elevation at the mRNA level. Penicillin upregulated the mRNA levels of (A) TNF- α and (B) IL-1 β . Only higher doses of AS-IV (40, 80 and 160 μ mol/l) notably reduced the penicillin-induced upregulation in inflammatory factors (Fig. 2B). * P <0.01 penicillin group, vs. control group; ** P <0.01 AS-IV+penicillin group, vs. penicillin group. AS-IV, astragaloside IV; TNF-tumor necrosis factor- α ; IL-1 β , interleukin-1 β ; qPCR, quantitative polymerase chain reaction.

Figure 3. Penicillin-induced elevation in protein levels of the p-MAPK family are decreased by AS-IV. AS-IV selectively reduced the penicillin-induced upregulation of p-JNK and p-p38 MAPK, but not p-ERK. AS-IV, astragaloside IV; p-, phosphorylated; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase.

Figure 4. Penicillin-induced downregulation of primary astrocyte viability is significantly elevated by administration of AS-IV. Compared with the control group, cell viability of primary astrocytes in the penicillin group was downregulated. Higher doses of AS-IV (40, 80 and 160 μ mol/l) rescued cell viability. * P <0.01 penicillin group, vs. control group; # P <0.01 AS-IV+penicillin group, vs. penicillin group. AS-IV, astragaloside IV.

and p-JNK, which was in line with the results of previous studies (29,30).

Taken together, the above results led to the conclusion that AS-IV suppressed penicillin-induced inflammatory factor

Figure 5. Penicillin-induced downregulation in primary astrocyte number is significantly elevated by administration of AS-IV. Compared with the control group, the number of primary astrocytes in the penicillin group was reduced. Higher doses of AS-IV (40, 80 and 160 μ mol/l) significantly reversed the penicillin-induced decrease in cell number. * P <0.01 penicillin group, vs. control group; # P <0.01 AS-IV+penicillin group, vs. penicillin group. AS-IV, astragaloside IV.

release, and suppressed activation of the p-p38 and p-JNK MAPK signaling pathway.

It has been reported that cytokines have the ability to contribute to excitotoxic and apoptotic neuronal death (31), indicating the possibility of generating seizure-mediated neuronal damage. Therefore, the present study examined the effects of AS-IV on Primary astrocytes from SD rats. An MTT assay was performed, which revealed that the higher doses of AS-IV improved the viability of the primary astrocytes from SD rats. The effects of AS-IV on the proliferation of primary astrocytes were also determined using a CCK8 assay, the results of which were consistent with those of cell viability. Taken together, the results of the present study demonstrated that AS-IV suppressed the penicillin-induced release of inflammatory factors and suppressed activation of the MAPK signaling pathway, ultimately attenuating epilepsy. These findings provide a basis for further investigations of the therapeutic role of AS-IV in epilepsy via targeting astrocytes.

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