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Original Article

Silibinin inhibits inflammation and apoptosis in a rat model of temporal lobe epilepsy

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Abstract: Inflammation and apoptosis in the hippocampus are closely related to temporal lobe epilepsy (TLE). Silibinin has shown significant anti-inflammatory and anti-apoptotic effects in other diseases including hepatitis, cerebral ischemia, and neuroinflammatory disease. However, the biological effects of silibinin in brain during epilepsy remain unclear. Our study aimed to investigate the protective effects of silibinin in rats after status epilepticus (SE) and its potential mechanisms of protection. Silibinin was intragastrically administered to rats 30 minutes before induction of SE and the animals were then given a gavage of silibinin daily before sacrificing. Our data showed that silibinin inhibited overexpression of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), caspase-3, cleaved caspase-3, and hypoxia inducible factor-1α (HIF-1α) in the hippocampus at both 24 hours and 72 hours after SE induction. In addition, silibinin significantly reduced apoptotic cell death and neuronal loss in the CA1 region of the hippocampus. Overall, our study shows that silibinin has anti-inflammatory and neuroprotective effects during epileptogenesis and we conclude that its effects may result from the inhibition of HIF-1α signaling.

Keywords: Silibinin, epilepsy, inflammation, apoptosis, hypoxia inducible factor-1α

Introduction

Epilepsy is a progressive neurological disorder characterized by unpredictable aberrant electrical activity. Approximately 20-30% of epileptic patients may develop refractory epilepsy with drug resistance [1] and most instances of refractory epilepsy are temporal lobe epilepsy (TLE) caused by hippocampal sclerosis. TLE is one of the most common forms of epilepsy and is associated with inflammation, neuronal apoptosis, and neuronal loss in the hippocampus [2-4]. Recent studies have demonstrated that inflammation in the brain may be both a cause and a result of epileptic seizures [5-7]. Tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) are representative pro-inflammatory cytokines (PICs) that have been extensively studied in the brain. Their expression in the hippocampus has been shown to increase rapidly after status epilepticus (SE) [8]. PICs contribute to increasing neuronal excitotoxicity which induces neuronal death and reduces seizure threshold in acute and chronic seizure models [6, 9, 10].

Seizures can result in ischemia and brain hypoxia [11], especially in the hippocampus, and can induce overexpression of hypoxia inducible factor-1α (HIF-1α) [12]. As a key endogenous signal factor, HIF-1α activates a series of downstream target genes such as multidrug resistance gene 1 (MDR1), vascular endothelial growth factor (VEGF), glucose transporters, and erythropoietin (EPO) [13-15]. Recent studies have suggested that HIF-1α modulates pro-inflammatory cytokine TNF-α and regulates hippocampal apoptosis after SE [16, 17].

Silibinin, the major bioactive ingredient of silymarin, is a flavonoid compound extracted from *Silybum marianum*. This herb extract is considered to be safe and non-toxic, even at high doses, and has been widely used as a hepatoprotective drug for more than 2,000 years [18]. Silibinin has been widely used as a dietary supplement because of its therapeutic effects on hepatitis and liver cirrhosis through its anti-inflammatory and anti-apoptotic activities [19]. Moreover, recent studies have shown that silib-

inin exerts remarkable anticancer efficacy in various cancers such as prostate, cervical, colorectal, and hepatocellular carcinomas by inhibiting HIF-1α signaling [20-23]. Silibinin also exhibits neuroprotective and anti-apoptotic properties in cerebral ischemia [24] and can reduce neuro-inflammatory injury that is induced by lipopolysaccharides [25]. Moreover, silibinin inhibits microglia activation and attenuates neuro-inflammatory reactions in Alzheimer's disease (AD) animal models [26]. However, the biological effects of silibinin on inflammation and apoptosis in the brain in TLE models are still unclear. In this study, we assessed the neuroprotective effects of silibinin using a rat epilepsy model.

Materials and methods

Animals

Male Sprague Dawley (SD) rats (210-240 g, n= 102) were obtained from the Hubei Research Center of Laboratory Animals (Wuhan, Hubei province, China). All animals were maintained under controlled conditions with appropriate temperature (23 ± 2°C) and humidity (60 ± 5%) regulations and free access to food and water. Animal procedures were conducted in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals.

Establishment of epilepsy model

The lithium-pilocarpine model of epilepsy is one of the most common forms of the TLE model. To induce SE in rats, the experimental animals first received intraperitoneal injections of lithium chloride (130 mg/kg, Sigma, St. Louis, MO, USA). Twenty hours later, scopolamine methylbromide (1 mg/kg, Sigma) was intraperitoneally administered to inhibit cholinergic effects 30 minutes before the intraperitoneal injection of pilocarpine (30 mg/kg, Sigma). After pilocarpine administration, rats developed convulsive seizures within 1 hour and only the animals with class V or IV seizures were used for subsequent experiments, in accordance with Racine's scale [27]. One hour after the onset of epilepsy, a 10% chloral hydrate solution (3 ml/kg, i.p.) was administered to terminate the seizures.

Groups and silibinin treatments

The SD rats were randomly divided into a control group (n=19), a SE group (n=43), and a silibinin-treated group (n=40). As previously mentioned, rats in the SE and silibinin-treated groups were induced with status epilepticus. Rats in the control group received the same dose of saline at the same time point. Silibinin (Tasly Pharmaceuticals, Tianjin, China) was suspended in 0.9% NaCl and intragastrically administered to rats in the silibinin-treated group at 100 mg/kg [24, 25] 30 minutes before pilocarpine injection. The animals were then given a gavage of silibinin daily after SE induction (day 0) with doses of 100 mg/kg from day 1 to day 3 and 50 mg/kg from day 4 to day 13. Animals in the silibinin-treated group were anesthetized and sacrificed at 24 hours, 72 hours, and 14 days after SE induction. The control group animals were sacrificed at 24 hours and 14 days after saline administration.

Real-time quantitative PCR analysis

The experimental animals were anesthetized and sacrificed at 24 hours and 72 hours after SE induction. According to manufacturer's instructions, total RNA was extracted from all hippocampus samples using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription reactions were performed using PrimeScript^{RT} reagent Kit with gDNA Eraser (Takara Biotechnology, Dalian, China). Primer sequences, designed by GeneCreate Biotechnology Company (Wuhan, China), were as follows: GAPDH (5'-CGCTAACATCAAATGGGGTG-3' and 5'-TTGCTGACAATCTTGAGGGAG-3'), TNF-α (5'-CACCACGCTCTTCTGTCTACTG-3' and 5'-GCTACGGGCTTGTCACTCG-3'), IL-1β (5'-GTGGCAGCTACCTATGTCTTGC-3' and 5'-CCACTTGTTGGCTTATGTTCTGT-3'), IL-6 (5'-GCCAGAGTCATTCAGAGCAAT-3' and 5'-CTTGGTCCTTAGCCACTCCT-3'), HIF-1α (5'-AAGCCCAGAGTCACTGGGACT-3' and 5'-GTACTCACTGGGACTGTTAGGCTC-3'). Real-time quantitative PCR was performed using StepOne Real-Time PCR System (Life Technologies, Carlsbad, CA, USA). Quantitative PCR data were calculated using the $2^{-\Delta\Delta CT}$ method by normalizing gene expression against GAPDH.

Western blotting analysis

Rats were anesthetized and sacrificed at 24 hours and 72 hours after SE induction. Hippocampal samples of SD rats were removed and stored at -80°C. Frozen tissues were then homogenized in RIPA lysis buffer. Total protein was extracted, in accordance with manufacturer's instructions, and protein concentrations

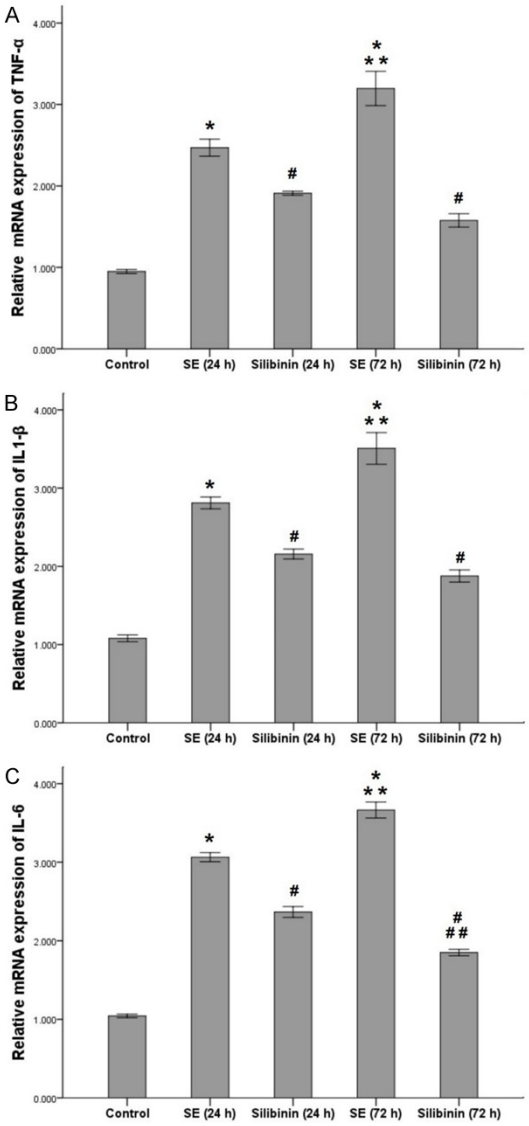

Figure 1. Silibinin inhibited mRNA expression of pro-inflammatory cytokines. A-C: PCR analyses showed that mRNA levels of TNF-α, IL-1β, and IL-6 were significantly increased in the hippocampus at 24 h and 72 h after SE induction compared to levels in the control group. Expression levels of PICs at 72 h were higher than those at 24 h after SE induction. Administration of silibinin significantly reduced expression of TNF-α, IL-1β, and IL-6 at 24 h and 72 h after the onset of seizures. In the silibinin-treated group, expression level of IL-6 at 72 h was significantly decreased compared to its expression level at 24 h. *P < 0.05, compared with the control group. **P < 0.05, compared with the SE group at 24 h. #P < 0.05, compared with the SE group. ##P < 0.05, compared with the silibinin-treated group at 24 h.

were quantified using BCA Protein Assay Kit (Aspen biological, Wuhan, China). For each sample, 40 μg of protein were loaded and resolved using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then transferred to polyvinylidene fluoride (PVDF) membranes by electrophoresis. The membranes were blocked with 5% skim milk at room temperature for 1 hour and incubated overnight at 4°C with primary antibodies rabbit anti-HIF-1α (1:1000), rabbit anti-Caspase-3 (1:1000), rabbit anti-cleaved Caspase-3 (1:1000) (Abcam, Cambridge, UK), and rabbit anti-β-actin (1:10000, TDY BIOTECH, Beijing, China). After rinsing three times in Tris-buffered saline with Tween (TBST), the membranes were incubated with diluted horseradish peroxidase-linked (HRP) secondary antibodies (1:10000, HRP-conjugated goat anti-rabbit, Aspen Biological) for 30 minutes at room temperature. Protein bands were visualized by electrochemiluminescence (ECL) reagents (Aspen Biological) and densities of the protein bands were determined using AlphaEaseFC software (Alpha Innotech, San Leandro, CA, USA).

TUNEL staining

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining was performed to assess cell apoptosis in the hippocampus by using an *in situ* cell death detection kit (Roche, USA). Rats were sacrificed at 24 hours after SE induction for TUNEL staining. According to manufacturer instructions, brain sections were deparaffinized and then incubated in Proteinase K (Roche) for 15 minutes. After immersing in 0.1% Triton X-100 solution for 10 minutes, brain slices were incubated in a TUNEL reaction mixture for 60 minutes at 37°C. After rinsing three times with PBS, the slices were treated with 4', 6-diamidino-2-phenylindole (DAPI) fluorescence counterstain. Finally, TUNEL positive cells in the CA1 region were observed using a fluorescence microscope (Olympus IX51, Japan) at low magnification (100 ×).

Immunohistochemistry

Rats were sacrificed on day 14 after SE induction. After perfusion with 300 mL of 0.9% NaCl and 400 mL of 4% paraformaldehyde, the brains of SD rats were removed and immersed in paraformaldehyde solution for 48 hours. Paraffin brain sections were deparaffinized and then incubated in 3% H_2O_2 at room temperature for 10 minutes. 5% bovine serum albumin was then used to block nonspecific binding for 20 minutes. The sections were then incubated

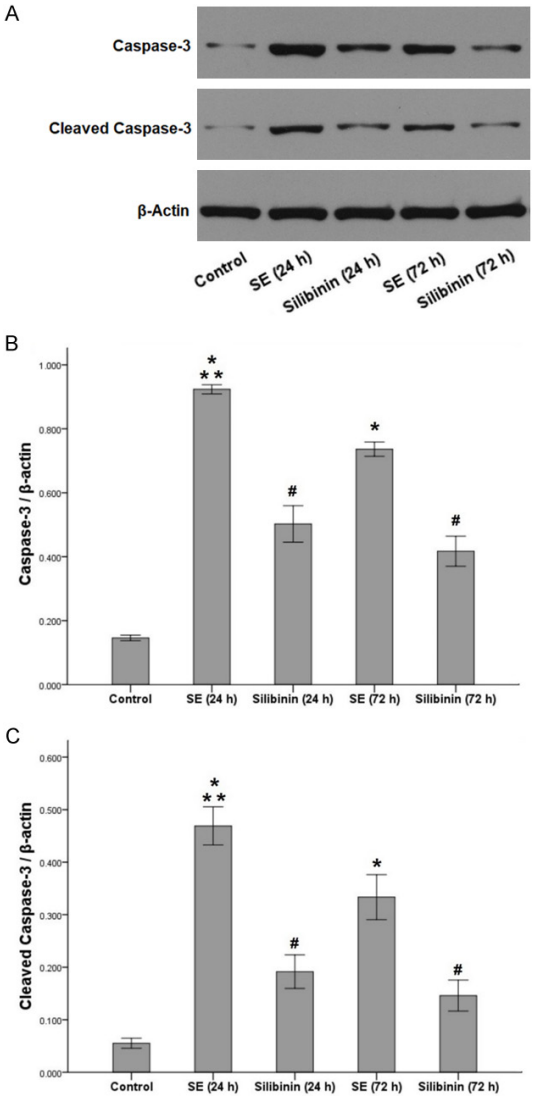

Figure 2. Silibinin inhibited expression of caspase-3 and cleaved caspase-3. A: Representative Western blotting results are shown. B, C: Western blot analyses demonstrated that protein levels of caspase-3 and cleaved caspase-3 were significantly increased at 24 h and 72 h in the hippocampus after SE induction, especially at 24 h. Silibinin significantly inhibited expression of caspase-3 and cleaved caspase-3 at 24 h and 72 h after SE induction. *P < 0.05, compared with the control group. **P < 0.05, compared with the SE group at 72 h. #P < 0.05, compared with the SE group.

with a primary mouse anti-NeuN (1:200, Abcam) antibody for 24 hours at 4°C. After rinsing three times in PBS, the slices were incubated in a secondary antibody (1:200, HRP-goat anti-mouse, Aspen biological) for 50 minutes at 37°C. The sections were then treated with Diaminobenzidine (DAB) Kit (ZSGB-BIO, Beijing, China) and counterstaining was performed by hematoxylin. Images were captured using an Olympus IX51 inverted microscope and immunopositive cells were quantified under 100 × visual fields.

Statistical analysis

All experimental data are presented as mean ± SEM. One-way analysis of variance (ANOVA) was used for analyzing quantitative data and multiple comparisons were performed using Student-Newman-Keuls test. *P* values < 0.05 were considered statistically significant.

Results

Evaluation of epilepsy model

Following pilocarpine injections, 72 rats had class IV or class V convulsive seizures and were included in this study. The mortality rate in SE group was 21.1% (8/38) in the five days following induction of epilepsy whereas the mortality rate in silibinin-treated group was 11.8% (4/34). No rats died on day 6 through day 14.

Silibinin inhibited expression of proinflammatory cytokines

PCR analyses (**Figure 1**) revealed that mRNA expression of TNF-α, IL-1β, and IL-6 was clearly upregulated in the hippocampus at 24 hours and 72 hours after induction of SE compared to the control group at 24 hours after saline injection (n=3 in each group and at each time point). Expression levels of PICs at 72 hours were higher than those at 24 hours after SE induction. Treatment with silibinin markedly attenuated expression of pro-inflammatory mediators at 24 hours and 72 hours after the onset of seizures. In the silibinin-treated group, expression of IL-6 at 72 hours after SE induction was significantly decreased compared to the level at 24 hours, however, there weren't significant differences between the two time points when observing levels of TNF-α and IL-1β (P>0.05).

Silibinin reduced apoptotic cell death

As indicators of cell apoptosis, caspase-3 and active caspase-3 (cleaved caspase-3) have a crucial role in apoptotic cascade [28]. In the present study, Western blotting analysis (**Figure 2**) showed that protein levels of caspase-3 and cleaved caspase-3 were significantly increased at 24 hours and 72 hours in the hippocampus

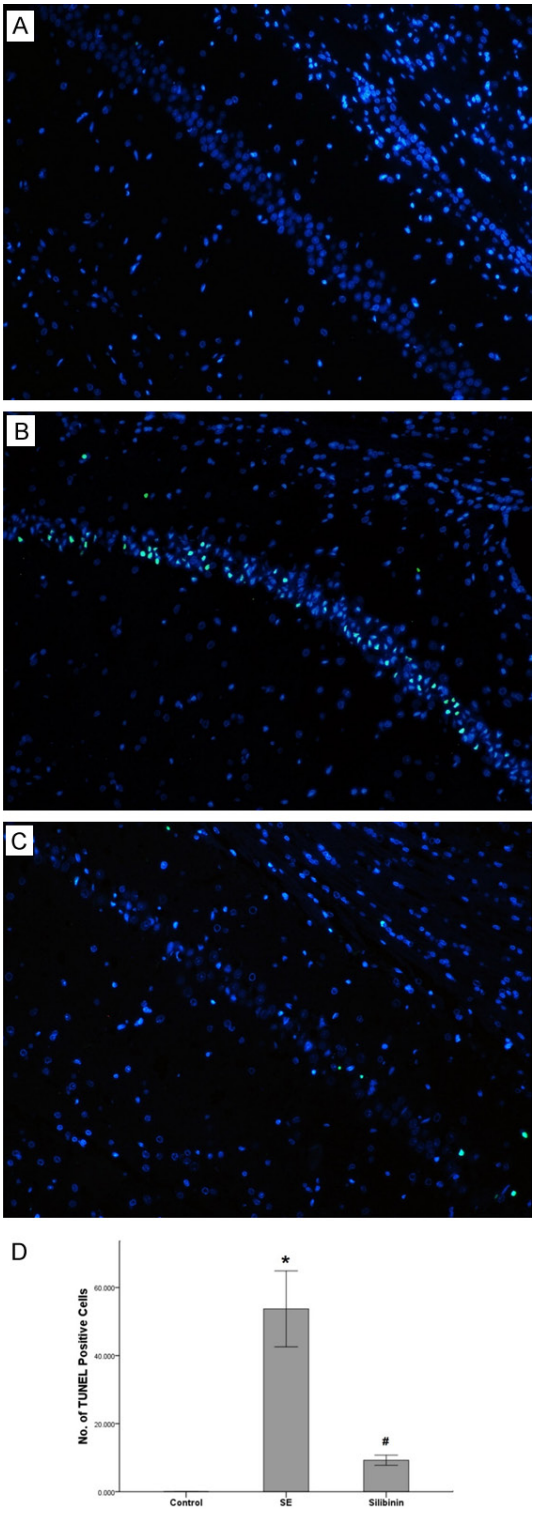

Figure 3. Silibinin reduced apoptotic cell death. A-C: Representative TUNEL staining images are shown (100 ×). A: control group. B: SE group. C: Silibinin-treated group. D: TUNEL staining showed that there were a large number of TUNEL-positive cells observed in the CA1 hippocampal subfield in the SE group. No TUNEL-positive cells were found in the control group at 24 h after drug administration. Treatment with silibinin significantly reduced the increased number of TUNEL-immunoreactive cells. *P < 0.05, compared with the control group. #P < 0.05, compared with the SE group.

after pilocarpine-induced seizures (n=4 in each group and at each time point), especially at 24 hours. Silibinin markedly inhibited expression of caspase-3 and cleaved caspase-3 at 24 hours and 72 hours after SE induction whereas no significant differences in protein levels of caspase-3 or cleaved caspase-3 between the 24 hour and 72 hour time points were observed in the silibinin-treated group (P>0.05).

TUNEL staining (**Figure 3**) revealed a large number of TUNEL-positive cells in the CA1 hippocampal subfield in SE group but no TUNEL-positive cells were observed in the control group at 24 hours after drug administration (n=4 in each group). Treatment with silibinin significantly reduced the increased number of TUNEL-immunoreactive cells.

Immunohistochemistry staining for NeuN (**Figure 4**) revealed that massive neuronal loss occurred in the CA1 hippocampal subfield of SE group compared to the control group on day 14 (n=4 in each group). Administration of silibinin clearly promoted neuronal survival in the CA1 region and no significant difference between the control group and silibinin-treated group was observed (P>0.05).

Silibinin downregulated expression of HIF-1α

PCR (n=3 in each group and at each time point) and Western blot (n=4 in each group and at each time point) analyses revealed a prominent increase in HIF-1α mRNA and protein levels at 24 hours and 72 hours after pilocarpine injection (**Figure 5**). After silibinin treatment, however, overexpression of HIF-1α was markedly inhibited. Moreover, mRNA expression level of HIF-1α in the silibinin-treated group at 72 hours was significantly lower than levels at 24 hours. However, comparison of HIF-1α protein expression levels in the silibinin-treated group at 24 hours and 72 hours showed no significant differences (P>0.05).

Discussion

In the present study, our data demonstrates that silibinin inhibits expression of PICs and reduces apoptotic cell death and neuronal loss in the hippocampus after SE induction.

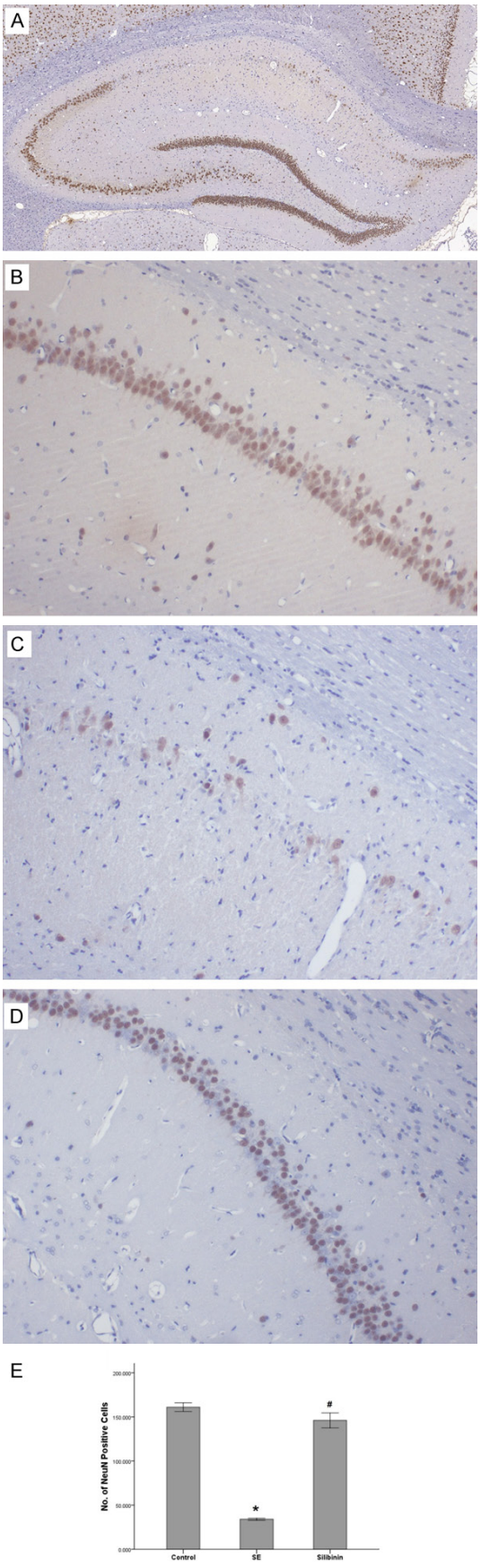

Figure 4. Silibinin reduced neuronal loss. A: Representative NeuN staining image of the hippocampus in the SE group is shown (20 ×). B-D: Representative NeuN staining image of the CA1 hippocampal subfield is shown (100 ×). B: Control group. C: SE group. D: Silibinin-treated group. E: Immunohistochemistry analyses for NeuN demonstrated that massive neuronal loss was observed in CA1 region of the hippocampus of SE-induced rats when compared to that of the control group on day 14. Treatment with silibinin significantly increased neuronal survival in the CA1 region and no significant difference betwen the control group and the silibinin-treated group was observed ($P > 0.05$). *$P < 0.05$, compared with the control group. #$P < 0.05$, compared with the SE group.

Moreover, overexpression of HIF-1α induced by SE is attenuated by silibinin administration. Overall, we show that silibinin has anti-inflammatory and neuroprotective effects during epileptogenesis and we conclude that these effects may result from inhibition of HIF-1α signaling.

Inflammation plays a key role during acute and chronic epilepsy. Significant upregulation of PICs (TNF-α, IL-1β, and IL-6) has been observed in astrocytes and microglia within three days after onset of SE [8, 29]. Different SE induction methods may yield different time points of peak PIC expression. In the current study, mRNA expression levels of PICs in the SE group at 72 hours were higher than those at 24 hours. Overexpression of PICs activates the transcription of certain inflammatory genes including prostaglandins (PGs) and cyclooxygenase-2 (COX-2), thereby causing a downstream cascade of inflammatory activity [4]. Studies have shown that TNF-α causes cellular damage and death and a neutralizing antibody against TNF-α has been shown to alleviate neuronal apoptosis in the kainic acid epilepsy model [30-32]. IL-1β may induce neuronal hyper-excitability and cell death by activating kinase signaling in epileptogenesis [33]. In TLE models, the activated IL-1β system can result in destruction of the blood brain barrier (BBB) and neurodegeneration [34]. Further, endogenous increases in brain IL-6 levels not only induce spontaneous neurodegeneration but also increase sensitivity to glutamatergic activity-induced seizures [35]. A previous study has shown that transgenic mice overexpressing IL-6 developed severe inflammatory and neurodegenerative diseases of the central nervous system (CNS) [36]. Therefore, inhibiting the secretion of PICs may

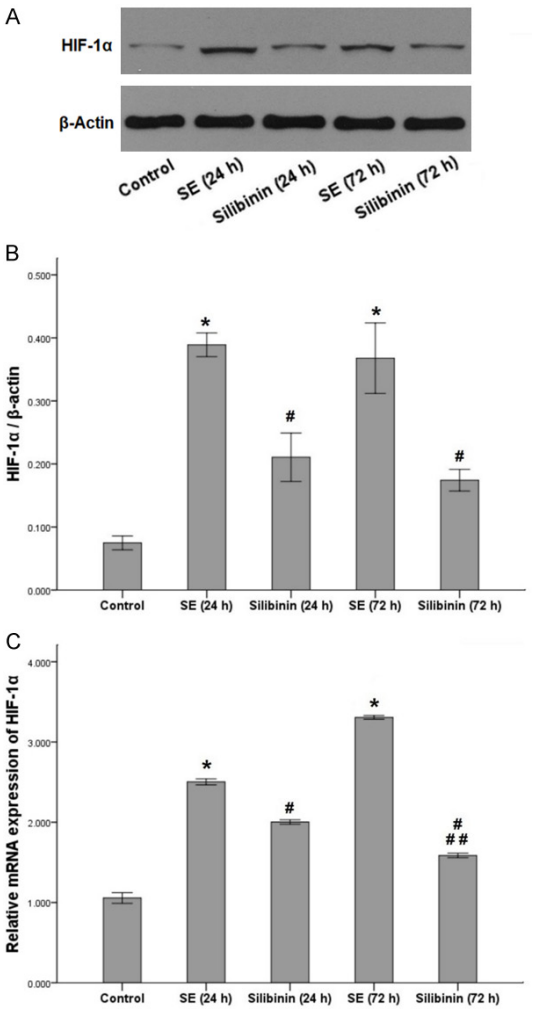

Figure 5. Silibinin inhibited overexpression of HIF-1α. A: Representative Western blotting results are shown. B, C: Western blotting and PCR analyses demonstrated significant increases in HIF-1α mRNA and protein levels at 24 h and 72 h after SE induction. In the silibinin-treated group, overexpression of HIF-1α was significantly inhibited and mRNA expression level of HIF-1α at 72 h was significantly lower than that at 24 h. *P < 0.05, compared with the control group. #P < 0.05, compared with the SE group. ##P < 0.05, compared with the silibinin-treated group at 24 h.

have neuroprotective effects and, in our present study, silibinin substantially attenuated induction of PICs.

In agreement with a previous study [16], our Western blot and TUNEL staining data herein showed that apoptosis in the hippocampus was significantly increased after induction of epilepsy, especially at the 24 hour time point. Immunohistochemistry analyses also revealed that neuronal loss was predominantly found in the CA1 region, consistent with previous research [4]. After silibinin treatment, apoptosis, and neuronal loss in the hippocampus were significantly reduced. Furthermore, expression of IL-6 at 72 hours after SE induction was significantly decreased compared to the level at 24 hours in the silibinin-treated group. Though no significant differences were observed herein, expression levels of TNF-α, IL-1β, caspase-3, and activated caspase-3 at the 72 hour time point also tended to be lower than those at the 24 hour time point in the silibinin-treated group. These results indicate that consecutive treatment with silibinin may improve anti-inflammation and anti-apoptosis outcomes because of spontaneous recurrent seizures (SRSs) that may occur after SE.

HIF-1α is a fundamental and crucial signaling molecule in the pathophysiologic processes of various diseases including carcinoma, hypoxic-ischemic encephalopathy, chronic lung disease, and myocardial ischemic disease [13]. However, the effects of HIF-1α in CNS diseases are still widely debated. It has been reported that HIF-1α exerts neuroprotective effects on cerebral ischemia in rats [37] whereas Koh and colleagues found that HIF-1α activates microglia and astrocytes to induce an inflammatory response in the hypoxia-ischemia model. Further, neutrophil migration and subsequent brain damage are reduced in HIF-1α-deficient mice [38]. Recent studies have demonstrated that overexpression of HIF-1α induces inflammation and hippocampal apoptosis in the epilepsy model [16, 17]. HIF-1α also modulates multidrug resistance gene 1 (MDR1) expression in refractory epilepsy [39] and may be modulated by miR-153 and miR-199 in intractable TLE patients [40, 41]. In our current study, consecutive administration of silibinin significantly inhibited overexpression of HIF-1α mRNA and protein levels.

In conclusion, our study demonstrates that silibinin can exhibit anti-inflammatory and anti-apoptotic effects in the TLE model. Moreover, this anti-epileptic and neuroprotective role of silibinin may be connected with its inhibition of HIF-1α signaling. Therefore, silibinin may be a potential candidate drug for the treatment of TLE.

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Disclosure of conflict of interest

None.

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