Ferulic acid exerts neuroprotective effects against cerebral ischemia/reperfusion-induced injury via antioxidant and anti-apoptotic mechanisms in vitro and in vivo

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Abstract. Ferulic acid (FA) is a derivative of cinnamic acid. It is used in the treatment of heart head blood-vessel disease and exerts protective effects against hypoxia/ischemia-induced cell injury in the brain. This study investigated the potential neuroprotective effects of FA against ischemia/reperfusion (I/R)-induced brain injury in vivo and in vitro through hematoxylin and eosin (H&E) and Nissl staining assays, flow cytometry, Hoechst 33258 staining, quantitative PCR, western blot analysis and fluorescence microscopic analysis. In this study, models of cerebral I/R injury were established using rats and pheochromocytoma (PC-12) cells. The results revealed that treatment with FA significantly attenuated memory impairment, and reduced hippocampal neuronal apoptosis and oxidative stress in a dose-dependent manner. The results from in vitro experiments also indicated that FA protected the PC-12 cells against I/R-induced reactive oxygen species (ROS) generation and apoptosis by inhibiting apoptosis, Ca2+ influx, superoxide anion (O2−), malondialdehyde (MDA) and glutathione peroxidase (GSH-Px) production in a concentration-dependent manner. Moreover, FA inactivated the Toll-like receptor (TLR)/myeloid differentiation factor 88 (MyD88) pathway. MyD88 overexpression abolished the neuroprotective effects of FA. On the whole, we found that FA attenuated memory dysfunction and exerted protective effects against oxidative stress and apoptosis induced by I/R injury by inhibiting the TLR4/MyD88 signaling pathway. This study supports the view that FA may be a promising neuroprotective agent for use in the treatment of cerebral ischemia.

Introduction

Ferulic acid (4-hydroxy-3-methoxycinnamic acid, FA), a constituent isolated from Ferula foetida, is one of many of the main active ingredients of traditional Chinese medicine (TCM) used to promote blood circulation, and is also an effective constituent of some Chinese medicinal herbs, such as Ligusticum striatum (chuanxiong) (1). Chuanxiong has been extensively used in TCM in recent years. Pharmacological studies have suggested that FA exerts antioxidant (2), free radical-scavenging (3), anti-thrombotic (4), hematic fat lowering (5), antibacterial (6), antiviral (7), anti-mutagenesis and anticancer effects (8) and has been used in the prevention and treatment of coronary heart disease (9). It has also been reported that FA exerts anti-inflammatory and antioxidant effects in some cerebral models (10).

Cerebral ischemia/reperfusion injury (IRI), is characterised by an insufficient oxygen supply and restoration of blood flow, and involves complex and multi-factorial mechanisms, and can result in irreversible damage to tissue (11). IRI is one of the is a threat to human health and survival and is associated with a high morbidity. IRI often contributes to the interruption of blood flow, and leads to hypoxia and hypoglycemia, subsequently promoting neuronal cell death (12,13). There is increasing evidence to suggest that cerebral IRI leads to neurological deficits, including learning and memory impairment (14-17). Cerebral IRI can induce oxidative stress, which is characterized by decreased superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) levels, and increased malondialdehyde (MDA) levels (18). Hippocampal neurogenesis is involved in memory and learning (19). Pro-inflammatory cytokines released in the periphery and neuronal apoptosis induced by cerebral IRI may exert potential damage to the hippocampal cells, leading to learning and memory impairment (20,21).

Neurons are vulnerable to hypoxia and hypoglycemia. Cerebral ischemia induces involves types of biochemical mechanisms which can damage nervous system function via the generation of reactive oxygen species (ROS) and reactive
nitrogen species (RNS) (22). ROS have been shown to be involved in nervous system dysfunction and brain disorders. Following brain injury, cellular functions are impaired by the increased production of free radicals, which are produced through many different cellular signal pathways (23). To investigate the mechanisms involved in neuronal cell death induced by ischemic injury and identify potential protective components, a cell model of ischemia using PC-12 cells has been used in vitro (24). Oxidative stress and mitochondrial dysfunction are known to induce the activation of the apoptotic cascade (25). Thus, the regulation of intracellular ROS and the Ca\textsuperscript{2+} influx level may be necessary for the suppression of pathological apoptosis in cerebral ischemia. Oxidative stress induced by ischemia is also involved in apoptosis. It has been shown that oxidative stress can promote the activation of caspases, subsequently inducing apoptosis (26,27). Bcl-2 anti-apoptotic family proteins, including pro-apoptotic (Bax and Bad) and anti-apoptotic (Bcl-2 and Bcl-xL) proteins, play an important role in apoptosis (28). Previous studies have suggested that apoptosis and necrosis are characterized by neuronal death during cerebral ischemia/reperfusion-induced injury (29,30). ROS attack neuronal components and finally induce apoptosis (31). The balance between anti-apoptotic Bcl-2 and pro-apoptotic Bax plays a key role in the progression of cerebral IRI (32). IRI is associated with the accumulation of ROS and apoptosis. Extended periods of ischemia can result in significant cellular dysfunction and cell death. FA has been shown to exert anti-inflammatory and antioxidant effects and can be regarded as an eliminator of free radicals (33,34).

Toll-like receptors (TLRs) also play an important role in cerebral IRI through the induction of inflammatory responses (35,36). The pathogenic role of TLRs in cerebral IRI processes was firstly implied in a mouse model of IRI, where it was found that mice lacking functional TLR4 had reduced IRI compared to wild-type mice (37). It has been reported that TLR2 can promote cellular dysfunction after ischemia, and TLR2 is also shown to aggravate ischemic damage in a myeloid differentiation factor 88 (MyD88)-dependent and/or -independent manner (38). In recent years, TLR activation has been shown to play a crucial role in IRI in the kidneys, gut and liver (39-41). TLR4 is a transmembrane receptor protein, which has a homology domain of intracellular Toll/interleukin-1 receptor (TIR). The TIR domain can interact with the adapter protein, which is named MyD88. It has been reported that MyD88-dependent signaling plays a crucial role in the pathogenesis of acute lung inflammation and injury following ischemia and reperfusion (42). It can also promote inflammatory responses induced by IRI in mice (43). Thus, we wished to examine the association between the protective effects of FA and the TLR4/MyD88 signaling pathway.

In the past, a few studies have reported that FA attenuates IRI in the brain both in vitro and in vivo. However, the underlying molecular mechanisms remain unclear. The association between FA and IRI-induced learning and memory impairment has not been reported to date, at least to the best of our knowledge. Thus, the aim of this study was to investigate the effects of FA on IRI-induced learning and memory impairment, and to elucidate the potential mechanisms involved as regards oxidative stress and apoptosis. Our results demonstrate that FA exerts protective effects against the impairment of learning and memory induced by IRI in vivo and in vitro via antioxidant and anti-apoptotic mechanisms. Our data may provide a possible therapeutic approach with which to prevent or suppress IRI and FA may be have potential for use as a neuroprotective agent in the treatment and/or prevention of cerebral ischemia.

Materials and methods

Ethics statement. All animal experiments were approved by the Ethics Committee of Kunming Medical University [no. SYXX (dian) K2015-0002], in accordance with the animal ethical guidelines of the Chinese National Health and Medical Research Council (NHMRC).

Animals and model of cerebral ischemia/reperfusion. Male Sprague-Dawley rats (7-8 weeks old) weighing 220±20 g were purchased from the Experimental Animal Center of Kunming Medical University, Kunming, China. After anesthesia, the rats were fixed on a table, and a midline incision was made on the neck, exposing the bilateral common carotid artery (CCA) and the vagus nerve. The CCA and vagus nerve were then separated and the bilateral CCA was blocked with a vascular clamp clip for 20 min. The bilateral vascular clamp was then loosened, allowing for the recovery of blood flow. The rats were divided into 5 groups with 10 rats in each. FA was obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). The rats in 3 of these groups received various doses of FA (28, 56 and 112 mg/kg; FA-L, FA-M and FA-H groups, respectively) after ischemia for 5 consecutive days, and one group was left untreated after ischemia. The rats in the sham-operated group were treated with saline and were not subjected to any surgery. After treatment for 5 days, samples in all groups are collect for the following tests.

Passive avoidance test. The passive avoidance test (44) was performed with the Gemini Avoidance system (San Diego Instruments, San Diego, CA, USA). The test included two identical chambers (25x20x17 cm) connected by a gate (9x7 cm); 14 stainless steel rods (6 mm in diameter) were included on the floor of each chamber with an interval of 1.8 cm, connected to a shock scrambler. The passive avoidance test was performed as follows:

Habitation: 1 day prior to being subjected to cerebral IRI, the rats were placed in the bright room of this system; 10 sec later, the right chamber was illuminated, and the gate opened simultaneously. Once the rats entered the left chamber (dark room), the gate closed instantaneously. After 30 sec, the rats were shifted from the dark room to the rat cage. If the rats stayed in the bright room for >100 sec, they were excluded from the experiment.

Training: this was carried out 1 h prior to IRI. The procedure was similar to that of the habitation session. After the rats entered the dark room, the gate closed immediately, intermittent electric shocks (50 Hz, 2 sec, 0.5 mA), and the number of training sessions was recorded. After 10 sec, the rats were transferred back to the cage. The latency was then recorded if the rats did not enter the dark room. After 2 min, the aforementioned process was repeated once, and the longest latency (in seconds) that occurred first was selected. The process was repeated again after 2 min.
Retention trial: The procedure was similar to that of the training session. The time for which the rats stayed in the bright room was recorded. The retention trial was performed once a day during 3 days after reperfusion.

Morris water maze task. The standard Morris water maze task was used to evaluate the ability of hippocampal-dependent spatial navigation learning and memory in rats (45). The water maze was a 120-cm circular pool, filled 45 cm deep with 26°C water. The trials were video-recorded and scored for measures including the latency of finding the hidden platform. Rats were trained in the hidden platform of the Morris water maze system in a consistent manner. If a rat spent >20 sec to find the hidden platform, it was excluded from the experiment. The retention trial was performed once at 24 h after reperfusion and on at 7 days after reperfusion.

Hematoxylin and eosin (H&E) and Nissl staining. A total of 5 rats from each group were used for H&E and Nissl staining. After being anesthetized with choral hydrate (300 mg/kg), the rats were perfused with 0.9% NaCl and the brain tissues were removed and fixed with 4% formaldehyde. The brain tissues were dehydrated with various concentrations of xylene and were then embedded in paraffin. The hippocampal sections were observed under a microscope (Olympus, Tokyo, Japan).

Cell culture and treatment. The PC-12 cells were plated in a 6-well dish and cultured in RPMI-1640 completed medium in an incubator with 5% CO₂ and 37°C. The PC-12 cells were also divided into the following groups: the control group with no treatment; the ischemic injury group stimulated with Na₂SO₄ (30 nM; Pepro Tech, Rocky Hill, NJ, USA); the group subjected to ischemia and treated with a low concentration of FA (50 μM; termed the isch + FA-L group); the group subjected to ischemia and treated with a moderate concentration of FA (100 μM; termed the isch + FA-M group); the group subjected to ischemia and treated with a high concentration of FA (200 μM; termed the isch + FA-H group); and the group subjected to ischemia and treated with 200 μM FA and MyD88 overexpression plasmids (termed MyD88-Ov+ischemia+FA group). After 24 h, cells were harvested and processed for the following experiments.

Cell viability test. We examined PC-12 cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Beyotime, Haimen, China) assay. The cells were plated in a 96-well plate and tissue homogenate were treated as described above. The contents of SOD (xanthine oxidase assay method) and MDA (thiobarbituric acid assay method) were detected using respective kits which are purchased from Nanjing Jiancheng Bio-Engineering Institute Co., Ltd (Nanjing, China) (46,47). The GSH-Px activity was detected using a chemical assay kit following the manufacturer's instructions and as previously described (48). The changes in absorbance were determined using a microplate reader, and the results were expressed as nmol/mg for MDA and U/mg protein for SOD activity.

Measurement of ROS, superoxide anion (O₂⁻) and Ca²⁺ levels. ROS generation was detected using the DCFH-DA fluorescent probe (Beyotime). Intracellular H₂O₂ can oxidize DCFH-DA to the highly fluorescent compound, dichlorofluorescin (DCF). After the cells were treated as described above, 10 mM DCFH-DA were added to the cells for 30 min at 37°C. Finally, after washing, the fluorescence of the cells was observed under a fluorescence microscope (Nikon TE2000U; Nikon, Tokyo, Japan).

Intracellular O₂⁻ levels were measured using O₂⁻ fluorescent probe, dihydroethidium (DHE; Beyotime, Shanghai, China). DHE was added to the cells for 30 min, followed by washing 3 times with 1X PBS to remove the non-specific staining. The cells were observed under a fluorescence microscope.

The intracellular Ca²⁺ level was detected using Fluo-3/AM (Invitrogen, Carlsbad, CA, USA). Following treatment as described above, the PC-12 cells were incubated with Fluo-3/AM for 30 min in an incubator, then washed twice with 1X PBS. Finally, the cells were examined under a fluorescence microscope (Nikon TE2000U; Nikon).

Quantitative PCR (qPCR). Total RNA from the treated cells was extracted using TRIzol reagent (Takara, Dalian, China) according to the manufacturer's instructions. cDNA was synthesized from RNA (1 μg) of each group sample using a Script RT reagent kit (Takara). The relative gene mRNA levels were then evaluated by qPCR on an ABI 7300 PCR application (Applied Biosystems, Beijing, China) with the SYBR-Green PCR kit (Takara). PCR was performed under the following conditions: 95°C 5 min, (95°C 30 sec, 60°C 30 sec, 30 cycles). β-actin was used as the reference gene. Primers were designed
Table I. Latency period in the water maze following drug treatment.

| Group   | Dose (mg/kg) | 1 day     | 2 days    | 3 days    | 4 days     | 5 days     | 6 days     | 7 days     |
|---------|--------------|-----------|-----------|-----------|------------|------------|------------|------------|
| Sham    |             | 11.00±0.61| 8.80±0.62 | 7.20±1.02 | 7.60±0.42  | 5.20±0.63  | 3.80±0.16  | 2.80±0.21  |
| Ischemia|             | 37.00±0.31| 35.80±1.17| 39.06±0.12| 39.80±0.10 | 40.60±0.23 | 39.40±0.02 | 40.20±0.01 |
| FA      | 28          | 33.40±0.40| 31.20±0.19| 29.40±0.54| 28.00±0.42 | 27.40±0.21 | 26.40±0.35 | 25.00±0.13 |
|         | 56          | 31.00±0.09| 27.20±0.35| 28.40±0.51| 25.00±0.43 | 21.60±0.11 | 21.00±0.25 | 13.40±0.09 |
|         | 112         | 24.60±0.17b| 23.60±0.26b| 19.80±0.13b| 12.00±0.19b| 14.20±0.23b| 11.00±0.17b| 9.00±0.09b  |

Sham, sham-operated; aP<0.05 vs. sham-operated group; bP<0.05 vs. ischemia group. Data are the means ± SD (n=10 rats per group).

Table II. Latency period in the inhibitory avoidance task following drug treatment.

| Group   | Dose (mg/kg) | 1 day     | 2 days    | 3 days    | 4 days     | 5 days     | 6 days     | 7 days     |
|---------|--------------|-----------|-----------|-----------|------------|------------|------------|------------|
| Sham    |             | 304.40±2.75| 284.20±1.62| 288.40±1.56| 23.60±0.26b| 19.80±0.13b| 12.00±0.19b| 9.00±0.09b  |
| Ischemia|             | 99.40±1.35| 83.60±1.17| 68.60±1.12| 12.00±0.17  | 11.00±0.17  | 11.00±0.17  | 9.00±0.09b  |
| FA      | 28          | 108.80±1.21| 117.00±1.54| 132.20±1.51| 24.60±0.17  | 23.60±0.26  | 19.80±0.13  | 12.00±0.19  |
|         | 56          | 121.40±1.62| 132.20±1.51| 132.20±1.51| 24.60±0.17  | 23.60±0.26  | 19.80±0.13  | 12.00±0.19  |
|         | 112         | 129.00±1.37| 135.40±1.13b| 135.40±1.13b| 24.60±0.17  | 23.60±0.26  | 19.80±0.13  | 12.00±0.19  |

Sham, sham-operated; aP<0.05 vs. sham group; bP<0.05 vs. model group. Data are the means ± SD (n=10 rats per group).

using Primer Premier 5.0 software. All primers were synthesized by Invitrogen. The primers were as follows: Bax forward, 5'-CTGCAGAGATGATCGCA-3' and reverse, 5'-GATCAGTCCGGCCTATTTAG-3'; Bcl-2 forward, 5'-CGATGCAGATGTCCA-3' and reverse, 5'-ATGCAGGTGTTCAGTACTCAG-3'; caspase-3 forward, 5'-GGACCTGTGGACCTGAAA-3' and reverse, 5'-CATGCATATGATCGACGACGAG-3'; TLR4 forward, 5'-CTGTTTCTGCTGGTTGCGTA-3'; GAPDH forward, 5'-GATCAGCTCGGGCACTTTAG-3' and reverse, 5'-ATGCCGGTTACTGACATGAC-3'; Bcl-2 forward, 5'-GAGATCCGCGAGTTTGAGAC-3' and reverse, 5'-TCTAGATTTAATCTACCTAC-3'; Ischemia - 27.20±0.35 28.40±0.51 25.00±0.43 21.60±0.11 21.00±0.25 13.40±0.09 25.00±0.13

Table II. Latency period in the inhibitory avoidance task following drug treatment.

| Group    | Dose (mg/kg) | 1 day     | 2 days    | 3 days    | 4 days     | 5 days     | 6 days     | 7 days     |
|----------|--------------|-----------|-----------|-----------|------------|------------|------------|------------|
| Sham     |             | 304.40±2.75| 284.20±1.62| 288.40±1.56| 23.60±0.26b| 19.80±0.13b| 12.00±0.19b| 9.00±0.09b  |
| Ischemia |             | 99.40±1.35| 83.60±1.17| 68.60±1.12| 12.00±0.17  | 11.00±0.17  | 11.00±0.17  | 9.00±0.09b  |
| FA       | 28          | 108.80±1.21| 117.00±1.54| 132.20±1.51| 24.60±0.17  | 23.60±0.26  | 19.80±0.13  | 12.00±0.19  |
|          | 56          | 121.40±1.62| 132.20±1.51| 132.20±1.51| 24.60±0.17  | 23.60±0.26  | 19.80±0.13  | 12.00±0.19  |
|          | 112         | 129.00±1.37| 135.40±1.13b| 135.40±1.13b| 24.60±0.17  | 23.60±0.26  | 19.80±0.13  | 12.00±0.19  |

Sham, sham-operated; aP<0.05 vs. sham group; bP<0.05 vs. model group. Data are the means ± SD (n=10 rats per group).

Western blot analysis. The proteins were isolated from the treated cells using lysis buffer (Beyotime), and the protein concentrations were examined using a BCA protein assay kit (Beyotime). The proteins (30 µg) were separated by denaturing 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto nitrocellulose membranes (Millipore). After the membranes were blocked with 5% milk for 2.5 h, they were incubated with the primary antibodies [caspase3 (sc-1225), Bcl-2 (sc-56015), Bax (sc-20067), TLR4 (sc-293072) and MyD88 (sc-74532); Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA] at 1:1,000 dilutions overnight. The membranes were then incubated with goat anti-mouse or rabbit HRP-conjugated secondary antibodies [goat anti-mouse (sc-2005) or rabbit (sc-2004) HRP-conjugated; Santa Cruz Biotechnology, Inc.] for 2 h at 4°C. The bands on the membranes were visualized using an ECL detection system (Thermo Fisher Scientific, Inc., Waltham, MA, USA). β-actin was used as a reference control.

Statistical analysis. All data are expressed as the means ± SD. Experiments were performed at least 3 times. Comparisons were assessed with one-way analysis of variance (ANOVA) (Tukey’s post hoc test) using GraphPad Prism software version 5.0a (GraphPad, La Jolla, CA, USA). A P-value <0.05 was considered to indicate a statistically significant difference compared with the control group.

Results

FA attenuates memory impairment induced by IRI. We performed the Morris water maze test and passive avoidance tests to examine hippocampus-related learning and memory abilities. The results revealed that the escape latencies of the rats in the sham-operated group were markedly decreased and the values obtained differed significantly from those of the ischemia group (P<0.05) in 5 days. However, no statistically significant difference was observed in the ischemia group over the successive trials. However, the rats with ischemia treated with FA exhibited a significant decrease in the times for escape latency compared with the ischemia group in a concentration-dependent manner (P<0.05). Furthermore, the FA-treated ischemia rats exhibited decreased times for escape latency and these times were shorter than those of the rats in the ischemia group (Table I).

As shown in Table II, the period of latency for the inhibitory avoidance task in the ischemia group was decreased compared to that of the sham-operated group in each session, and the values were statistically significant (P<0.05). Furthermore, with the passage of time, the rats in the ischemia group exhibited severe memory impairment and loss of memory for electrical stimulation. The period of latency in the ischemia model exhibited a steady decrease. Of note, the FA-treated rats exhibited increased escape latency times in a concentration-dependent manner compared with the ischemia group. These data indicated that FA significantly repaired the spatial cognitive and memory deficits induced by ischemia.

FA decreases ischemia reperfusion-induced neuronal death in the hippocampus. Hippocampal neurons have been reported
to be closely related to learning and memory (49). We thus investigated the effects of FA on cell survival in the rat hippocampus area following IRI. We used H&E staining to observe the morphological changes in the cells in the hippocampus. As shown in Fig. 1, in the sham-operated group, the hippocampus area was found to have multiple layers, and the cells were arranged neatly, with a clear structure and normal shape, and the nucleus was round or oval, stained light blue. However, in the ischemia group, the layers of cells in the hippocampus area were decreased and exhibited a disorganized cell arrangement. Part of the hippocampus area cell exhibited nuclear condensation and deep blue staining. Of note, with the increase in the concentration of the FA (in the FA-L, FA-M, FA-H groups), the cells in the hippocampus area had more layers and were arranged more closely compared to those of the ischemia group (Fig. 1A).

In addition, we observed the changes of Nissl bodies through Nissl staining. Histological evaluation revealed that the sham-operated group had round nuclei and integral Nissl bodies, whereas the Nissl bodies in the ischemia group exhibited marked nuclear deviation, and were pathologically changed into particles. IRI induced the severe loss of Nissl bodies in the hippocampus of the rats with IRI compared with rats in the sham-operated group. Nevertheless, cell injury, as well as the abnormalities in Nissl bodies induced by IRI were attenuated in a dose-dependent manner by treatment with FA (Fig. 1B). These data indicated that FA effectively reduced IRI-induced neuronal death.

**FA attenuates IRI-induced neuronal apoptosis in the hippocampus.** To examine the effects of FA on neuronal apoptosis, we examined the expression of cleaved caspase-3, Bcl-2 and Bax by qPCR and western blot analysis. Apoptosis was detected by measuring the levels of cleaved caspase-3 and Bax in the brain tissue homogenates. We found significantly increased levels of cleaved caspase-3 and Bax, and decreased levels of Bcl-2 in the ischemia group compared with the sham-operated group. On the contrary, treatment with FA (the FA-L, FA-M, FA-H groups) effectively increased the levels of Bcl-2, and inhibited the levels of cleaved caspase-3 and Bax in a concentration-dependent manner (Fig. 2). These results suggest that FA exerts neuroprotective effects against IRI-induced brain injury through anti-apoptotic mechanisms.

**FA exerts an antioxidant effect on rats with IRI.** We then investigated the functions of FA as an antioxidant in rats with IRI. We wished to identify enzymatic sources of excessive ROS production following IRI, such as SOD, MDA and GSH-Px. As shown in Fig. 3, the increased contents of MDA, and the decreased activities of SOD and GSH-Px in the brain tissues were detected in the ischemia group. However, FA decreased the MDA contents and promoted the activity of SOD, GSH-Px in a dose-dependent manner (Fig. 3).

**Protective effects of FA on PC-12 cells following ischemic injury.** The chemical structure of FA is shown in Fig. 4A. The effect of FA on the viability of the PC-12 cells was detected by MTT assay. Compared to the control group, the viability of the cells exposed to Na$_2$S$_2$O$_4$ markedly decreased (Fig. 4B and C). FA prevented the decrease in PC-12 cell viability induced by Na$_2$S$_2$O$_4$ in a dose-dependent manner.

**Anti-apoptotic effects of FA on PC-12 cells following ischemic injury.** It has been reported that apoptosis plays a crucial role in ischemic injury (50). Therefore, we further examined the effects of FA on ischemia-induced apoptosis by Annexin V/PI staining with flow cytometric analysis. As shown in Fig. 5A and B, the results from Annexin V/PI staining revealed a significant increase in the apoptotic cell population in the ischemia group compared with the control group, while treatment with FA...
significantly decreased the apoptotic cell population induced by ischemia compared with the ischemia group in a concentration-dependent manner. The difference in nuclear morphology were also detected by Hoechst 33258 staining. The number

Figure 2. Ferulic acid (FA) decreases ischemia/reperfusion-induced cell apoptosis in the hippocampus. (A and B) qPCR and western blot analysis of the levels of caspase-3, Bax and Bcl-2 in each group.

Figure 3. Effects of ferulic acid (FA) on the content of malondialdehyde (MDA), and SOD and glutathione peroxidase (GSH-Px) activities in vivo. (A) MDA content, and (B) SOD and (C) GSH-Px activities were detected using respective kits at 532, 560 and 422 nm on a microplate reader.
of condensed nuclei was increased in the cells in the ischemia group compared with those in the control group. Treatment with various concentration of FA (50, 100 and 200 µM) attenuated this effect in a dose-dependent manner (Fig. 5C and D).

We also examined the expression of caspase-3, Bax and Bcl-2 by qPCR and western blot analysis. As observed in Fig. 5E and F, the Bax expression level was significantly increased in the ischemia group; however, the expression of Bcl-2 was significantly inhibited. FA decreased the Bax expression level and increased Bcl-2 expression (Fig. 5E and F). These results implied that FA inhibited the apoptosis of PC-12 cells subjected to ischemic injury.

Antioxidant effects of FA on SOD, MDA and GSH-Px. As shown in Fig. 6, after the PC-12 cells were subjected to ischemic injury, we also found that the content of MDA was elevated, and the activities of SOD and GSH-Px were decreased in the PC-12 cells. Treatment with FA significantly decreased the MDA content, and promoted the activities of SOD and GSH-Px in a dose-dependent manner.

Antioxidant effects of FA on the ROS, O\textsuperscript{2-} and Ca\textsuperscript{2+} contents. In this study, the contents of ROS, O\textsuperscript{2-} and Ca\textsuperscript{2+} were measured using DCFH-DA, DHE and Flou-3/AM fluorescent probes with a fluorescence microscope. As shown in Fig. 7, the PC-12 cells subjected to ischemic injury exhibited an increase in DCF fluorescence; however, FA inhibited this increase in DCF fluorescence. A similar effect was observed for O\textsuperscript{2-} generation; FA effectively reduced O\textsuperscript{2-} generation in the PC-12 cells subjected to ischemic injury. The fluorescence intensity of Flou-3/AM also was increased in the ischemia group compared to the control group. However, FA treatment decreased this fluorescence intensity. These results suggested that FA exerted the antioxidant effects by decreasing the accumulation of ROS, O\textsuperscript{2-} and Ca\textsuperscript{2+}.

Effects of FA on the TLR/MyD88 signaling pathway. We also examined the TLR4 and MyD88 expression levels by qPCR and western blot analysis. As shown in Fig. 8A and B, ischemic injury promoted TLR4 and MyD88 expression; however, FA significantly decreased TLR4 and MyD88 expression in a concentration-dependent manner (Fig. 8A and B). These data implied that FA inhibited the activation of the TLR/MyD88 signal pathway.

To investigate whether the TLR/MyD88 pathway plays an important role in ischemic injury, the effects of MyD88 overexpression on the anti-apoptotic of FA on PC-12 cells subjected to ischemic injury were determined by qPCR and western blot analysis. As shown in Fig. 8C and D, the expression levels of cleaved caspase-3 and Bax significantly increased in the ischemia group compared with the control group; however, treatment with FA decreased the expression of cleaved caspase-3 and Bax. However, these expression levels were markedly increased in the FA + MyD88-Ov group compared with the FA group. Moreover, Bcl-2 expression in the ischemia group was decreased compared to that of the control group, and the Bcl-2 level was increased following treatment with FA. However, Bcl-2 expression was markedly decreased in the FA + MyD88-Ov group compared with the FA group. Taken together, these results demonstrate that FA suppresses the apoptosis of PC-12 cells subjected to ischemic injury by inhibiting the activation of the TLR/MyD88 signaling pathway.

Discussion

Fa (4-hydroxy-3-methoxycinnamic acid) is an element isolated from Ferula foetida. FA is regarded as a free radical scavenger, and exerts antioxidant and anti-inflammatory effects in the brain. However, the protective effects of FA against cerebral IRI have not been fully explored. The aim of this study was to
Figure 5. Effect of ferulic acid (FA) on ischemia-induced apoptosis of PC-12 cells. (A) Annexin V/PI staining was used to determine apoptosis by flow cytometry. (B) Histograms showing the ratio of dead cells to the total nuclei. (C) Nuclear morphology was determined by Hoechst 33258 staining; the white arrows indicate the apoptotic cells, at x400 magnification. (D) Histograms showing the ratio of condensed nuclei to total nuclei. (E and F) The expression of apoptosis related proteins was examined by qPCR and western blot analysis.
investigate the effects of FA on learning and memory impairment induced by IRI, and to elucidate the potential underlying mechanisms responsible for the neuroprotective effects of FA against apoptosis and oxidative stress induced by IRI.

Cerebral ischemia and reperfusion can cause neuronal injury and death in the hippocampus area, subsequently inducing behavioral defects, including learning and memory impairments. Studies have reported that cognitive ability disorders are associated with the number of damaged neuronal cells in the hippocampus region, the damage of which is induced by the IRI (51-53). Importantly, the hippocampus region is the one of the most important regions for learning
and memory abilities, and is vulnerable to IRI injury (54,55). Therefore, in this study, we first constructed an animal model of ischemic injury by surgery. We detected the spatial learning and memory deficits in rats in response to IRI using the passive avoidance tests and Morris water maze task. H&E and Nissl staining were also used to examine neuronal damage in the hippocampus region. Our results implied that FA exerted neuroprotective effects.

IRI is involved in ROS accumulation and induces apoptosis (56). Free radical production has also been widely reported in cerebral ischemic injury (57). Ischemia can induce a disadvantageous cascade reaction, including inflammatory processes, excitotoxicity and oxidative stress, and finally results in neuronal cell death (58-60). Oxidative stress is one of the primary factors in IRI (61-62). Following oxidative stress, antioxidant enzymes, such as SOD, GSH-Px (63) are included in the defensive system for protection against oxidative stress; these play a crucial role in protecting neurons against reactive oxygen species-induced cell apoptosis (64). MDA is an oxidative stress marker, which is also a product of lipid peroxidation reaction. The content of MDA can indicate the degree of lipid peroxidation in tissues (65). SOD is an
important factor for the defense against the tissue damage induced by ROS, and it also catalyzes the superoxide anion transferred to hydrogen peroxide and inhibits the generation of hydroxyl radicals (66). GSH is an endogenous antioxidant, and it can react directly with ROS and/or regarded as a co-factor with the enzyme, GSH-Px to decrease hydrogen peroxide and lipid peroxide levels in tissues (67). In this study, the activities of SOD and GSH-Px were significantly decreased, while the MDA content was increased following IRI. This phenomenon indicated that oxidative stress occurs in rats suffering from IRI. Treatment with FA significantly inhibited the increased levels of MDA induced by IRI, and increased the generation of the cellular antioxidants, SOD and GSH.

We also constructed cell model of ischemic injury using sodium dithionite (Na2S2O4). In recent years, PC-12 cells are used in experiments as they constitute a widely used neuronal model system (68). The PC-12 cell line was obtained from a rat pheochromocytoma, and it is a useful cell line for studying various neuronal functions and is also widely used for the study of cellular ischemic injury (69,70). The findings of our study suggested that FA significantly decreased ischemia-induced apoptosis, as well as the contents of MDA, ROS, O2·− and Ca2+ accumulation, and promoted SOD and GSH-Px activities compared to the ischemia group in vivo and in vitro. FA was suggested to exert marked neuroprotective effects. Taken together, our data suggest that FA may have potential for use in the treatment of cerebral ischemia and reperfusion injury. Our results revealed that IRI contributed to severe neuronal damage in the hippocampus, and subsequently damaged the function of spatial learning and memory in rats. However, FA reversed these effects and attenuated pathological and neurological deficits, in a dose-dependent manner.

TLRs can capture ‘danger signals’ coming from necrotic cells, and participate in promoting the inflammatory reaction and cell apoptosis-related processes (71,72). It has been reported that IRI, surgical injury and systemic stress can induce an inflammatory reaction in part via the TLR signaling pathway (73). In this study, we also used an in vitro model to investigate the effects and mechanisms of action of FA against Na2S2O4-induced IRI in PC-12 cells. Our findings study implied that FA exerted protective effects on the PC-12 cells subjected to ischemic injury by inhibiting the activation of the TLRs/MyD88 signaling pathway.

In conclusion, this study suggested that FA has a clearly protective function against ischemic injury in vivo and in vitro by suppressing the Ca2+ influx, the generation of O2·− and ROS production, and inhibiting apoptosis. There is evidence to indicate that FA has a function of protecting neurons by confronting oxidative stress and related apoptotic processes following cerebral IRI in rats (74). In this study, the neuroprotective effects of FA were shown to be associated with the downregulated expression of TLR4 and MyD88. Above all, FA may have an anti-inflammatory effect. FA may have potential for use as a neuroprotective agent for protection against oxidative stress and apoptosis induced by ischemia. Some of the molecular mechanisms and related signaling pathways involved in the neuroprotective effects of FA were identified in this study. However, further studies are required to further elucidate and confirm the potential mechanisms.

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