Arctigenin Protects Focal Cerebral Ischemia-Reperfusion Rats through Inhibiting Neuroinflammation

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Received May 20, 2012; accepted July 31, 2012; advance publication released online August 22, 2012

Stroke is the third leading cause of death in industrialized countries and the most important cause of acquired adult disability.5) About 15 million people worldwide suffer from stroke annually, out of which about 87% are ischemic strokes.2) Previous studies have demonstrated that inflammation is a critical determinant of the outcome and might be the secondary injury mechanism of cerebral ischemia.3,4) Microglial cell activation is a rapidly occurring cellular response to cerebral ischaemia. Microglia is considered as resident immunocompetent and phagocytic cells in the central nervous system, which can be activated by cerebral ischemia. Activated microglia will synthesize and release cytotoxins, inflammatory mediators, and cytokines, such as tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β).5,6) IL-1β is generally thought to be the initial factor of the inflammatory cascade reaction, it can up-regulate the expression of adhesion factors in coordination with nuclear factor κB (NF-κB), TNF-α and interferon γ (IFN-γ) which involved in the inflammatory reaction after ischemia.7) Based on previous observations, many inflammation inhibitors have been tested for their ability to delay neuronal death. However, no satisfactory outcomes were obtained in ischemic brain injury.9)

In China, Arctium lappa L. has been widely used as traditional Chinese medicine to treat diabetes, diuretic and cerebral ischemia injury.9–12) Arctigenin is confirmed to be responsible for most activities of Arctium lappa L. including anti-inflammation, anti-apoptosis and anti-cancer effects. Arctigenin can decrease inflammatory reaction in animal models of acute inflammation and inhibit B-cell and T-cell mediated allergic inflammation.13,14) Previous studies reported that arctigenin ameliorated metabolic disorders via inhibition of mitochondria complex I.15) Most recently, arctigenin was reported to confer neuroprotective effect via inhibition of neuroinflammation.16)

However, the anti-inflammatory effect of arctigenin against ischemic stroke has not been examined. In our present study, we investigated whether arctigenin is neuroprotective against brain ischemia injury with focus on the anti-inflammation effect of arctigenin on rats subjected to middle cerebral artery occluded (MCAO).

MATERIALS AND METHODS

Preparation of Arctigenin Arctigenin and arctiin were isolated from the dried seeds of Arctium lappa L. by using reported methods.17) The further preparation of arctigenin were using the method of hydrolyzing arctiin with β-glucosidase.18,19) Highly purified arctigenin (99.0%, purity) was enriched by the method of pre-HPLC.

Animals Healthy male Sprague-Dawley rats (2 months old and weighing 225±25g) were purchased from SLAC Animals Laboratory Centre (Shanghai, China) and experiments were approved by the Animal Research Ethics Committee, School of Medicine, Shanghai Jiaotong University. Rats were housed under diurnal lighting condition and allowed food and water ad libitum. All efforts were made to minimize the number of animals used and their suffering.

Experimental Protocol All the rats were divided into 5 groups randomly: sham-operated group (Sham); vehicle-treated MCAO group (Vehicle); stroke groups treated with 6.25mg/kg per day, 12.5mg/kg per day, and 25mg/kg per day of Arctigenin (Arc (L) Arc(M), Arc(H)). The Arc group

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was administrated intraperitoneally once daily before MCAO procedure (seven times). For the vehicle group, saline was administrated in the same manner. After one week of pre-treatment, the rats of ARC and vehicle groups were subjected to the experimental middle cerebral artery occlusion 2 h and reperfusion 22 h. Brain tissues were then obtained for further research.

Dose Selection The effects of different doses of arctigenin on cerebral ischemia reperfusion injury were examined in pilot studies to determine the optimal dose of arctigenin that provided the best neuroprotection. A dose of 6.25, 12.5 and 25 mg/kg of arctigenin was selected. This 25 mg/kg of arctigenin dose has also shown maximal protective effect in memory deficits.20) According to these findings, rats were pretreated systemically with arctigenin once daily for 7 d.

Focal Cerebral Ischemia Focal cerebral ischemia was induced by right MCAO as described previously.21) In brief, rats were initially anesthetized with pentobarbital sodium (40 mg/kg, intraperitoneally (i.p.)). The right common carotid artery was exposed by operating the neck skin. A 4-0 nylon monofilament with a blunt end was introduced into the external carotid artery (ECA) and advanced into the middle cerebral artery via the internal carotid artery (20–22 mm), until a slight resistance was felt. At this point, the blood flow of the middle cerebral artery was occluded from the origin. Two hours after the occlusion, the filament was slowly withdrawn to restore the blood flow. Rats in the sham group, all surgical procedures were conducted except the reduction of blood flow. Body temperature was maintained at 37±0.5°C with a heating pad.

Quantification of Infarct Volume The infarct volume was assessed with 2,3,5-triphenyltetrazolium chloride (TTC) staining. Five coronal sections were made from the olfactory bulb to the cerebellum and stained with 0.1% TTC solution at 37°C for 30 min followed by 4% formalin solution preservation. The infarct volume was analyzed in a blinded manner using an image analyzer system (Scion image for windows) and converted by integration (including correction for edema and atrophy) to the true infarct size of ischemic damage in the whole hemisphere as previously described.22)

Neurological Deficit Testing Neurological deficit was evaluated by a set of modified neurological severity score at 24 h after MCAO blindly. Neurological function was graded on a scale of 0–5 (normal score, 0; maximum deficit score, 5).23) The higher the neurological deficit score, the more severe impairment of neurological function.

Measurement of Apoptotic Cells Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining was performed with the DNA Fragmentation Detection Kit (Promega, U.S.A.) according to the manufacturer’s instruction. Briefly, rats were perfused transcardially with 0.1x phosphate-buffered saline (PBS) (pH 7.4) followed by a fixative solution containing 4% paraformaldehyde (PF) in PBS (pH 7.4). The brains were removed and fixed in 4% PF at 4°C overnight. The brain slices (20 µm) were mounted onto slides and fixed for 20 min with 4% PF in PBS (pH 7.4), and then were pretreated with 3% H2O2 in metha-

Fig. 1. Protective Effect of Arctigenin on Infarct Volume and Behavior Function
(A) Representative TTC-stained coronal sections of each group. (B) The corrected edema percentage in the infarcted hemisphere. n=8–10 rats per group. (C) Neurological dysfunction 24 h after MCAO. n=6–10 per group. *p<0.05 vs. vehicle group. **p<0.01 vs. vehicle group. #p<0.05 vs. Arc (M) group.

Fig. 2. Apoptotic Staining in Infarct Area 24 h after MCAO (200× Magnification) for Each Group
(A) Positive cells were brown. Positive cells with TUNEL staining were markedly decreased by arctigenin pretreatment. (B) Apoptotic index indicated the percentage of TUNEL-positive cells. *p<0.01 vs. sham group. **p<0.01 vs. vehicle group, n=8. Bar=100 µm.
...and 0.1% Triton X-100. The TdT enzyme and nucleotide mix were then added at proportions specified by the kit and incubated for 60 min at 37°C. After three washes with PBS (pH 7.4), 75 µL diaminobenzidine (DAB) was added. Slides were incubated for 10 min at 37°C. The slides were mounted under glass cover slip and analyzed under light microscope.

Measurement of IL-1β and TNF-α Level After 22 h of reperfusion, six rats from each group were sacrificed under 10% chloralhydrate anesthesia. The infarcted cortex (1–5 mm posterior to bregma and 1–5 mm medial to the sagittal suture) was harvested, weighed and homogenized (10%) in ice cold radio immunoprecipitation assay (RIPA) buffer (Beyotime Institute of Biotechnology, Shanghai, China), followed by centrifugation at 12000 × g at 4°C for 30 min. Supernatants were collected as total proteins, and proteins were electrophoresed through a 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by electrical transfer to a polyvinylidene difluoride (PVDF) membrane (Millipore Corp.). The membrane was incubated at 4°C overnight in tris-(hydroxymethyl)aminomethane-buffered saline, containing 5% milk, and protein expression was detected with primary rabbit-anti-rat polyclonal antibodies against TNF-α, and IL-1β (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). After washing with tris-(hydroxymethyl)aminomethane-buffered saline, the membranes were incubated with secondary antibodies (horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (IgG)) at room temperature for 1 h. Membranes were developed using the enhanced chemiluminescence system (No. RPN2108; GE Healthcare, Bethesda, MD, U.S.A.) and images were captured on autoradiography films (Kodak, New York, NY, U.S.A.). Films were scanned and densitometric analysis of the bands was performed using the Labworks 4.6 Analysis System (UVP, Upland, CA, U.S.A.). β-Actin (1:2000, Santa Cruz Biotechnology) served as the loading control.

Measurement of Microglia Activation Brains were removed after 12 and 24 h occlusion and immersed in with 4% PF in PBS for 24 h at 4°C. Brain sections (5 µm-thick) were blocked in 3% H2O2, 3% normal goat serum, and incubated with centrifugation at 12000×g at 4°C for 30 min. Supernatants were collected as total proteins, and proteins were electrophoresed through a 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by electrical transfer to a polyvinylidene difluoride (PVDF) membrane (Millipore Corp.). The membrane was incubated at 4°C overnight in tris-(hydroxymethyl)aminomethane-buffered saline, containing 5% milk, and protein expression was detected with primary rabbit-anti-rat polyclonal antibodies against TNF-α, and IL-1β (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). After washing with tris-(hydroxymethyl)aminomethane-buffered saline, the membranes were incubated with secondary antibodies (horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (IgG)) at room temperature for 1 h. Membranes were developed using the enhanced chemiluminescence system (No. RPN2108; GE Healthcare, Bethesda, MD, U.S.A.) and images were captured on autoradiography films (Kodak, New York, NY, U.S.A.). Films were scanned and densitometric analysis of the bands was performed using the Labworks 4.6 Analysis System (UVP, Upland, CA, U.S.A.). β-Actin (1:2000, Santa Cruz Biotechnology) served as the loading control.

Statistical Analysis Statistical data were represented as mean±S.D. of at least three independent preparations. Statistical analysis was performed by independent sample t-test and one-way analysis of variance (ANOVA) followed by post hoc test. A difference with p<0.05 was considered statistically significant.
RESULTS

Infarct Volume and Neurological Deficit To examine the neuroprotective effect of arctigenin on MCAO rats, TTC staining was used to measure the infarct volume. As is shown in Figs. 1A, and B, arctigenin pretreatment reduced infarct volume in a dose dependent manner. Twelve and half milligram per kilogram per day of arctigenin treatment could statistically reduce the infarct volume from 26.45±2.97% to 18.55±2.58%, whereas 25 mg/kg per day of arctigenin treatment statistically reduced the infarct volume to 14.50±2.74% (Fig. 1B). When evaluating neurological function, modified neurological deficit grading system was carried out 24 h after MCAO. As is shown in Fig. 1C, arctigenin pretreatment improved neurological function from 2.80±0.31 to 2.59±0.37 (Arc 6.25) and 2.1±0.25 (Arc 12.5, p<0.05) and 1.65±0.23 (Arc 25, p<0.05), which is in agreement with the results of infarct volume variation.

The Effect of Arctigenin on Apoptosis Induced by MCAO The apoptotic cells were identified through TUNEL staining. Arctigenin significantly reduced the apoptotic cell caused by stroke. Arctigenin pretreatment decreased the number of TUNEL-positive cells in the penumbra area (Fig. 2A). The proportion of TUNEL positive cells was 60.3±7.5% and 30.7±3.9% in the penumbra area of vehicle- and arctigenin-pretreated animals, respectively (Fig. 2B).

The Effect of Arctigenin on the Activation of Microglia To investigate the effect of arctigenin pretreatment on the local inflammatory cells in brain, OX-42 and ED-1 were used to examine activated microglia and phagocytic macrophages respectively. As is shown in Fig. 3, Arctigenin significantly decreased the number of activated microglia by 31% (n=8, p<0.05) and 35% (n=8, p<0.05) with respect to the vehicle group 12 h and 24 h after occlusion, respectively. When microglia were activated and further converted to fully phagocytic macrophages, the morphological changes from the typical ramified morphology to rod, round, and ameboid-like microglia took place. In the vehicle-treated group, the number of phagocytic microglia was further increased 24 h after occlusion compared with 12 h. However, in the arctigenin-treated group, the inhibition of conversion of microglia to macrophage was observed, and the number of phagocytic microglia was decreased by 22% (n=8, p<0.01) and 36% (n=8, p<0.05) 12 h and 24 h after MCAO, respectively, compared with vehicle treated group (Fig. 4).

The Effect of Arctigenin on the Level of IL-1β and TNF-α Pro-inflammatory cytokines, including IL-1β and TNF-α released by activated microglia, promote systemic inflammation following the ischemic insults. As is shown in Fig. 5, IL-1β and TNF-α expression were found to be remarkably high in ischemic hemisphere of the rats in MCAO group rats. A noticeable reduction in IL-1β and TNF-α expression was observed in arctigenin pretreated MCAO group as compared to the control group.
DISCUSSION

Ischemic brain injury is associated with inflammatory reaction, which consists of inflammatory cells and inflammatory mediators. In active state, microglia produce pro-inflammatory cytokines (such as IL-1 and TNF-α), chemokines, adhesion molecules, and then amplify the inflammatory response. Previous studies have indicated that arctigenin reduced neuronal cell death induced by LPS through inhibiting the secretion of IL-1β, TNF-α. Given the prominent role of inflammation in ischemic stroke, we hypothesized that identifying drugs that target inflammation in acute phase may be broadly neuroprotective. In the present study, we investigated whether the anti-inflammatory effect of arctigenin in the rat model of stroke is associated with neuroprotection. Our data demonstrated that arctigenin could significantly reduce the infarct volume, ameliorated the apoptosis-like cell and improve the neurological impairment, which could be attributed to the anti-inflammatory potential of arctigenin.

It is well documented that inflammatory reaction, which consists of inflammatory cells and inflammatory mediators, is one of contributing factors in brain ischemic injury. In this study, we observed that microglial activation was well developed in the penumbra, and arctigenin significantly inhibited the activation of microglia and its further conversion into phagocytic microglia 24h after MCAO. We also found that in the MCAO/reperfusion rats, the protein expression of TNF-α and IL-1β synthesized and released by activated microglia were inhibited by arctigenin. However, direct evidence is not obtained. Further study is needed to clarify this point. Previous studies has evidenced that inhibition of TNF elicits neuroprotection against cerebral ischemia. The over-elevated expression of TNF-α induced by ischemic insult is largely considered to be detrimental. Activated microglia and macrophages are thought to be the major source of pathophysiologically important TNF in acute phase of ischemic stroke.

In this study, activation of microglia and macrophages were inhibited by arctigenin, which may explain the inhibition of TNF-α expression within 24h after ischemia onset. IL-1β primarily neurotoxic and is involved in the pathogenesis of hypoxic ischemic brain damage. We founded that ischemia induced the expression of IL-1β, and neuroprotection of arctigenin was accompanied by significant inhibition of IL-1β protein. This might indicate that in cerebral ischemia/reperfusion rats, IL-1β might act as a pro-inflammatory cytokine, and arctigenin might exert neuroprotection by inhibiting its detrimental action.

To the best of our knowledge, this is the first demonstration of the therapeutic potential of arctigenin on brain injury induced by ischemia/reperfusion. Modulation of local inflammation, as demonstrated by suppression of microglia activation, reduction of pro-inflammatory cytokines, may be potential underlying mechanisms of this neuroprotection. This study suggests that pretreatment of arctigenin has neuroprotective effects and carries a potential to be a preventive measure for a population with a higher risk of having stroke.

Acknowledgements The current study was mainly supported by research Grants from Natural Fund of Jiangsu Province (20110091220068).

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