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ORIGINAL ARTICLE

The Total Flavones of *Abelmoschus manihot* Inhibit Reactive Proliferation of Astrocytes Following Cerebral Ischemia

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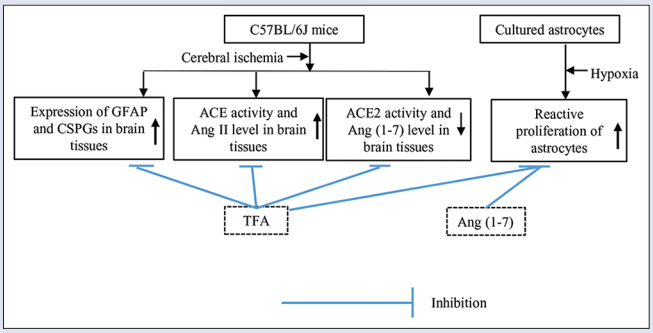
ABSTRACT

Objective: The aim of this study was to demonstrate the role of the total flavones of *Abelmoschus manihot* (TFA) in the reactive proliferation of astrocytes after cerebral ischemia in mice. **Materials and Methods:** The expression of glial fibrillary acidic protein (GFAP) and secretion of chondroitin sulfate proteoglycans (CSPGs) from astrocytes in brain tissues were used to evaluate the effect of TFA on the reactive proliferation of astrocytes after cerebral ischemia. Besides, we detected the activities of angiotensin-converting enzyme (ACE) and ACE2 and production of angiotensin (Ang)-II and Ang-(1–7) in the brain tissues. Furthermore, the role of Ang-(1–7) and TFA in GFAP expression and proliferation of primary cultured astrocytes under hypoxia induced by cobalt chloride ($CoCl_2$) was tested. **Results:** Cerebral ischemia induced a significant increase of GFAP expression and CSPGs secretion in mice brain tissues, which was inhibited by TFA treatment. In addition, TFA treatment inhibited the increment of ACE activity and Ang II production induced by ischemia in the brain tissues; likewise, TFA induced a significant upregulation of ACE2 activity and Ang-(1–7) production. Furthermore, TFA or Ang-(1–7) treatment markedly reduced reactive proliferation of astrocytes under hypoxia. **Conclusion:** TFA inhibits reactive proliferation of astrocytes in the brain tissues following cerebral ischemia by upregulating ACE2/Ang-(1–7).

Key words: ACE, ACE2, astrocytes, reactive proliferation, TFA

SUMMARY

- In this study, the role of the TFA in reactive proliferation of astrocytes after cerebral ischemia was investigated. The results revealed that TFA could inhibit the reactive proliferation of astrocytes in the brain tissues following cerebral ischemia. The mechanism may be related with the upregulation of ACE2/Ang-(1–7).



Abbreviations used: TFA: total flavones of *Abelmoschus manihot*; CCAs: common carotid arteries; GFAP: glial fibrillary acidic protein; ACE: angiotensin converting enzyme; ACE2: angiotensin converting enzyme 2; Ang II: angiotensin II; Ang-(1–7): angiotensin-(1–7); CSPGs: chondroitin sulfate proteoglycans

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INTRODUCTION

In the central nervous system (CNS), astrocytes are the most abundant cells and play vital regulatory roles in various physiological functions of the CNS, such as regulating blood flow, participating in neuron metabolism, regulating synaptic function as well as plasticity, and maintaining extracellular ion balance and fluid balance.[1] However, excessive activation of astrocytes is an important molecular event that causes pathological cascade reaction and accelerates ischemic brain injury.[2,3]

Cerebral ischemia results in an increased expression and phosphorylation of glial fibrillary acidic protein (GFAP,) which is regarded as an important index of activation and reactive proliferation of astrocytes. Within a few days after ischemic stroke, glial scar, primarily produced by reactive astrocytes, forms surrounding the necrotic brain tissues. On the one hand, the glial scar isolates the injured area and prevents its expansion; on the other hand, astrocytes in the scar extensively express chondroitin sulfate proteoglycans (CSPGs),[4] which inhibits the axon regeneration and neural function recovery following cerebral ischemia via activating RhoA/Rho kinase (ROCK) signaling pathway.[5] Therefore, moderate intervention of reactive proliferation of astrocytes is an important direction to treat brain injury after ischemic stroke and promote nerve repair.[4]

Renin–angiotensin system (RAS) is a vital component of the circulation system and exerts a vital role in regulating blood pressure as well as adjusting the homeostasis of water and sodium.[6] Besides, an independent local RAS was found in the CNS and proven to be related to the pathogenesis of ischemic stroke.[7,8] Accumulated evidence suggested that upregulation of angiotensin converting enzyme (ACE)/Ang II/Ang II receptor axis in the brain tissues contributes to neuronal injury,[9] while ACE2 directly metabolizes Ang II to produce Ang-(1–7), which has a protective role in neurons against focal cerebral ischemia.[10] Therefore,

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we hypothesized that upregulation of Ang-(1–7) might contribute to the intervention of reactive proliferation of astrocytes.

Total flavones of *Abelmoschus manihot* (TFA), the major active component of *A. manihot*, is used as a traditional Chinese medicine for the therapy of vascular injury.[11] We previously revealed that TFA protects against cerebral ischemic/reperfusion injury in rats.[12,13] In view of the fact that flavonoids could reduce increase in the ACE activity in rat aorta induced by radiation,[14] the present research is based on the hypothesis that TFA mediates reactive proliferation of astrocytes via promoting the ACE2 activity and Ang-(1–7) production.

MATERIALS AND METHODS

Reagents

TFA was purchased from Nanjing University of Chinese Medicine. The content of flavone glycosides is over 99% in TFA. The flavone glycosides in TFA were identified by using high-performance liquid chromatography. TFA contains 43.2% of hyperoside, 27.1% of hibifolin, 13.7% of isoquercetin, 8.8% of quercetin-3′-*O*-glucoside, 3.8% of quercetin-3-*O*-robinobioside, 3.2% of myricetin, and 0.2% of quercetin. Ang-(1–7) (Catalog No. HY-12403) was purchased from MedchemExpress (Shanghai, China). Anti-GFAP (Catalog No. AF6166) and anti-β-actin (Catalog No. AF7018) were obtained from Affinity Biosciences LTD (Jiangsu, China). Anti-CSPGs (CS-56 [aggrecan], Catalog No. C8035) was obtained from Sigma Chemicals (St. Louis, MO, USA). Anti-mouse IgG (H + L) horseradish peroxidase (HRP) (Catalog No. S0002) was purchased from Affinity Biosciences LTD (Jiangsu, China). ACE (Catalog No. ab155452) and ACE2 (Catalog No. ab213843) enzyme-linked immunosorbent assay (ELISA) kits for determination of ACE and ACE2 were obtained from Abcam (Shanghai, China). Ang II and Ang-(1–7) assay kits were obtained from Kemin Biological Co (Shanghai, China). Cell counting Kit-8 (CCK-8) assay was purchased from Beyotime Institute of Biotechnology (Jiangsu, China).

Animals

Adult C57BL/6J mice (female to male: 1:1, 20–25 g) and newborn rats on the day of birth were obtained from the Animal Center of Anhui Medical University. Mice were housed under standard laboratory conditions (humidity 54% ± 2%, temperature 22°C ± 2°C) for 1 week to habituate to the novel environment with free access to food and water. All procedures of the experiment abided by the Ethics Review Committee of Hefei Technical College, which conformed to the protocol outlined in the Guide for the Care and Use of laboratory Animals published by the US National Institutes of Health (NIH publication number 86-23, revised 2011).

Experimental procedure

The mice were randomly divided into the following groups: sham group, model (cerebral ischemia) group, and TFA treatment groups (20, 40, 80 mg/kg, $n = 8$ animals per group). The dosage of TFA was determined according to our previous research.[12] TFA was administered by using intravenous injection for 7 days after cerebral ischemia.

Model of cerebral ischemia in mice

Bilateral common carotid arteries' (CCAs) occlusion was used to establish the model of cerebral ischemia in this study.[15,16] Briefly, bilateral CCAs were carefully separated after operation under anesthesia with 5% isoflurane and were fully ligated with threads for 7 days. Then, the mice were sacrificed under anesthesia with 5% isoflurane to collect the mice brain. The vessels of sham group were separated only without ligation. The body temperature of mice was maintained at 37°C throughout surgery. All efforts were taken to minimize suffering of animals.

Primary astrocytes' isolation culture

On the day of birth, newborn rats were killed by using CO_2 overdose, and then the primary astrocytes were isolated and collected from the cerebral cortex. The isolated brain cortexes were dissected and put in phosphate-buffered saline (PBS) including 100 μg/mL streptomycin and 100 U/mL penicillin. The tissues were cut into pieces and put in a solution containing 0.25% trypsin for digestion for 15 min at 37°C. The cells were collected after filtration and centrifugation and resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum (FBS), 100 μg/mL streptomycin, and 100 U/mL penicillin. The cells in DMEM were cultured in a 5% CO_2 incubator at 37°C. To promote cell survival and proliferation, forskolin (FSK) as well as glial cell-derived neurotrophic factor (GDNF) were added in the medium. The flasks were shaken at 260 rpm on a rotary shaker at 37°C for 18–20 h to remove the microglia and oligodendrocyte when the cells grew to confluence. Astrocytes were incubated with $CoCl_2$ (3 μM)[17] for 7 days to mimic hypoxic conditions. Ang-(1–7) (10 μmol/L)[18] or TFA (100 mg/L)[19] was added to the medium during hypoxic conditions.

Western blot

The total protein was prepared according to a previous study.[20] Total proteins (30 μg) derived from mouse brain tissues were separated on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane. Membranes containing proteins were successively blocked with skim milk (5%) and Tween-20 (0.05%) in Tris-buffer saline for 2 h at room temperature and incubated with monoclonal antibodies against CS56 (CSPGs), GFAP, or β-actin at 4°C overnight in Tris-buffer saline. Membranes were finally incubated with Tris-buffer saline containing biotin-conjugated secondary antibody at 37°C for 1 h. An enhanced chemiluminescence kit (Thermo, Massachusetts, USA) was used to observe and record the immunoreactive band on the membrane, and the relative intensity of the protein band was evaluated by densitometry of the target protein compared with β-actin, an internal control. Blot intensities were examined in a blinded manner.

Astrocytes' proliferation

Astrocytes' proliferation after hypoxic culture was evaluated by using CCK-8 assay kit (Beyotime Institute of Biotechnology). Briefly, the hypoxic astrocytes were prepared as mentioned earlier. Subsequently, CCK-8 solution (10 μL) and culture medium (90 μL) were added into each well of the plate. After incubation at a 37°C incubator for 2 h, the absorbance of each well was tested at 450 nm with a microplate reader to assess the astrocytes' proliferation.

Biochemical measurements

Mice were killed 7 days after cerebral ischemia, and the brain tissues were isolated and homogenized. For measurement of ACE and ACE2 activities and Ang-(1–7) and Ang II levels in the brain tissues, the lysate of brain tissues from each mouse was transferred to 96-well plates. ACE and ACE2 activities were measured by ELISA using the kit purchased from Abcam according to the methods offered by the assay kit. The Ang-(1–7) and Ang II contents in the brain tissues were detected by ELISA kits (Kemin Biological Co) according to the manufacturer's protocols.[21]

Data analysis

All results are presented as mean ± standard error of the mean (SEM). Data analysis was performed by a person blinded to the experiment design using Statistical Package for the Social Sciences (SPSS) statistics 16.0 software (IBM Corp., Armonk, NY, USA). The homogeneity of variance and normal distribution of data were analyzed by using one-way analysis of variance (ANOVA). All data conform to normal distribution and homogeneity of variance. The differences between groups were determined by Duncan test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Role of TFA in the reactive proliferation of astrocytes after cerebral ischemia

Expression of GFAP and CSPGs in the brain tissues at 7 days after cerebral ischemia was assessed by using western blot to assess the proliferation of reactive astrocytes. As shown in Figure 1, significant increment of GFAP and CSPG expression was detected in the brain tissues following cerebral ischemia, suggesting a significant proliferation of astrocytes. Interestingly, the increase of GFAP and CSPG expression was obviously inhibited by treatment of TFA. These data reveal that TFA blocked the reactive proliferation of astrocytes after cerebral ischemia.

Effect of TFA on the changes in ACE activity and Ang II production in the tissues of mice brain

The role of TFA on the changes in ACE activity and Ang II content are shown in Figure 2. Cerebral ischemia induced a significant increase of ACE activity and Ang II production, which could be markedly blocked by TFA treatment. These findings indicate that TFA has a remarkable inhibitory effect on the increment of ACE activity and Ang II production induced by cerebral ischemia in the brain tissues.

Role of TFA in ACE2 activity and Ang-(1–7) production in the brain tissues

We then tested the role of TFA on the change of ACE2 activity and Ang-(1–7) production in the brain tissues at 7 days after cerebral

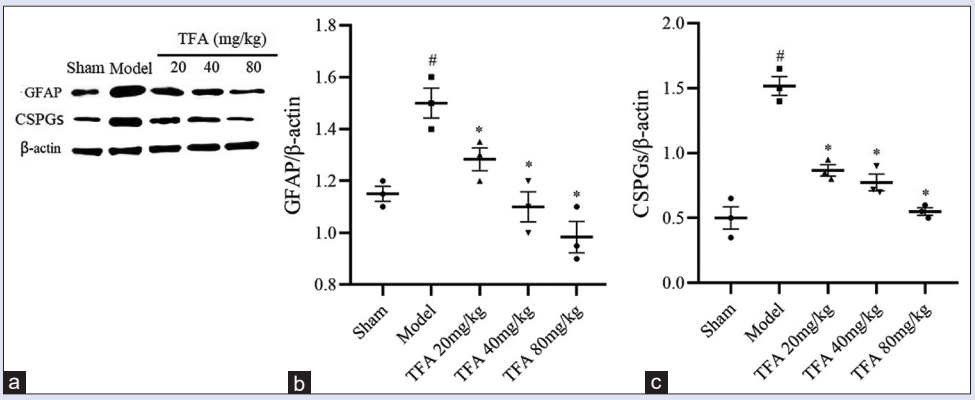

Figure 1: Effect of TFA on the expression of GFAP and CSPGs in the brain tissues after cerebral ischemia. (a) Expression of GFAP and CSPGs in the brain tissues (western blot); β-actin was used as the loading control. The relative expression of (b) GFAP and (c) CSPGs was quantified by calculating the densitometric ratio of the bands of GFAP and CSPGs to the same β-actins. $n = 3$ animals per group. Data are presented as means ± SEM. $^{\#}P < 0.05$ compared with the sham group, $^{*}P < 0.05$ compared with the model group. CSPGs = chondroitin sulfate proteoglycans, GFAP = glial fibrillary acidic protein, SEM = standard error of the mean, TFA = total flavones of *Abelmoschus manihot*

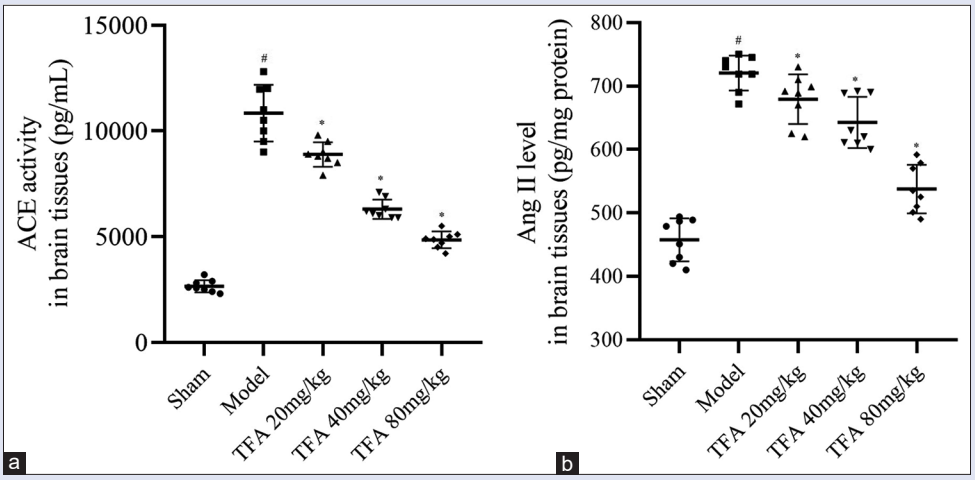

Figure 2: Changes in ACE activity and Ang II production in the brain tissues. (a) Individual data points indicate the role of TFA on the changes of ACE activity. (b) Individual data points indicate the role of TFA on the changes of Ang II production. $n = 8$ animals per group, $^{\#}P < 0.05$ versus sham, $^{*}P < 0.05$ versus model group. ACE = angiotensin converting enzyme, Ang II = angiotensin II, TFA = total flavones of *Abelmoschus manihot*

ischemia. As shown in Figure 3, contrary to changes in ACE activity and Ang II production, cerebral ischemia induced a remarkable reduction of ACE2 activity and Ang-(1–7) production in the brain tissues. But application of TFA could obviously upregulate the ACE2 activity and Ang-(1–7) production. These data further confirm that TFA could regulate RAS.

Role of TFA against hypoxia injury in the primary cultured astrocytes

The cell proliferation tested by CCK-8 assay was chosen to evaluate the role of TFA in astrocyte proliferation under hypoxic conditions. As shown in Figure 4, continuous hypoxic treatment could promote the proliferation of astrocytes. Both TFA and Ang-(1–7) treatment have inhibitory effect on the cell proliferation in a dose-dependent manner. Not surprisingly, Ang-(1–7) (10 μmol/L) or TFA (100 mg/L) could markedly reduce the expression of GFAP in astrocytes following hypoxia [Figure 5]. These results confirm that ACE2-mediated Ang-(1–7) production may be related to the inhibitory effect of TFA on the reactive proliferation of astrocytes.

DISCUSSION

Ischemic stroke, a complex brain injury, is induced by thrombus or embolus, which obstructs blood flow to the corresponding brain tissues. This leads to energy failure and neuronal death induced by deprivation

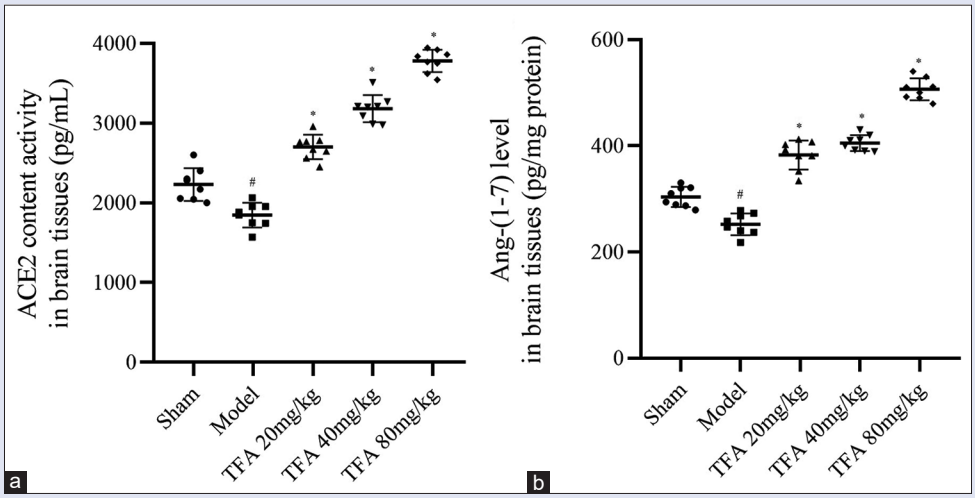

Figure 3: Changes in ACE2 activity and Ang-(1–7) production in the brain tissues. (a) Individual data points indicate the effect of TFA on the changes in ACE2 activity. (b) Individual data points indicate the effect of TFA on the changes in Ang-(1–7) production. $n = 8$ animals per group. $^{\#}P < 0.05$ versus sham, $^{*}P < 0.05$ versus model group. ACE2 = angiotensin converting enzyme 2, Ang-(1–7) = angiotensin-(1–7), TFA = total flavones of *Abelmoschus manihot*

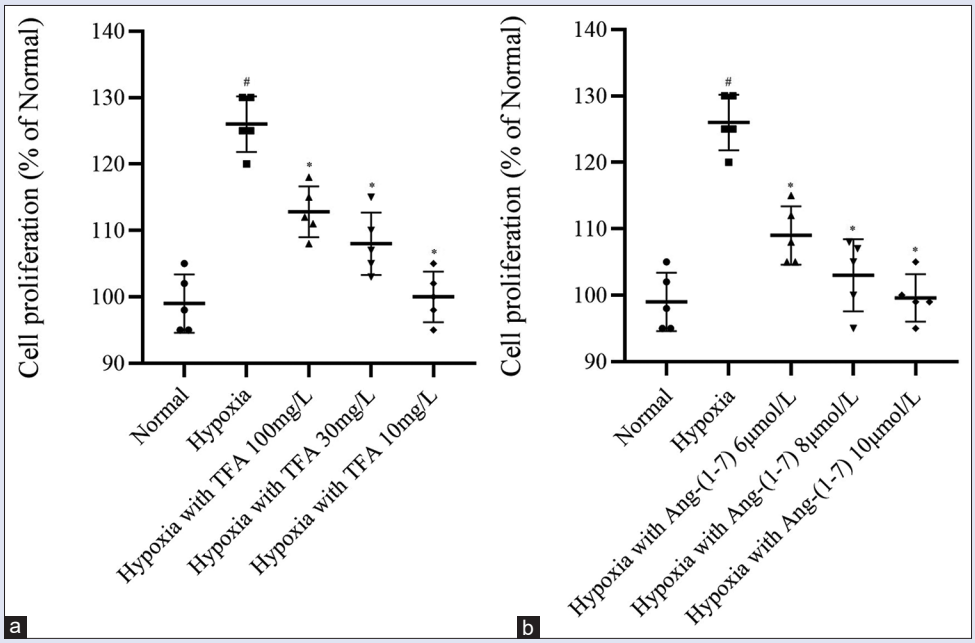

Figure 4: Role of TFA and Ang-(1–7) in the proliferation of astrocytes under hypoxic conditions. (a) Individual data points indicate the effect of TFA on the changes in astrocytes' proliferation. (b) Individual data points indicate the effect of Ang-(1–7) on the changes in astrocytes' proliferation. $n = 5$ animals per group, $^{\#}P < 0.05$ versus sham. $^{*}P < 0.05$ versus model group. Ang-(1–7) = angiotensin-(1–7), TFA = total flavones of *Abelmoschus manihot*

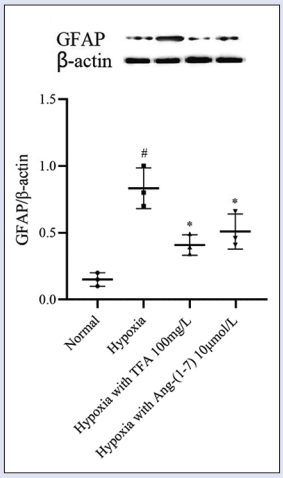

Figure 5: Effect of TFA and Ang-(1–7) on the GFAP expression in astrocytes under hypoxic conditions. Changes in GFAP expression were assessed using western blot. $n = 3$ per group. Data are shown as individual data points. $^{\#}P < 0.05$ versus sham group, $^{*}P < 0.05$ versus model group. Ang-(1–7) = angiotensin-(1–7), GFAP = glial fibrillary acidic protein, TFA = total flavones of *Abelmoschus manihot*

of oxygen and glucose.[22] Besides, astrocytes become reactive and proliferate in the peri-ischemic area, with an increased expression of GFAP and CSPG secretion, and these changes are widely used as a marker for reactive astrocytes.[23] Therefore, the changes in GFAP and CSPG expression in the brain tissues after ischemia were used in the present study to evaluate the reactive proliferation of astrocytes after ischemic stroke.

A. manihot belongs to the Malvaceae family. Accumulated findings have revealed that the active constituents and extracts of *A. manihot* have diverse biological activities such as antioxidant,[24] anti-inflammatory,[25] neuroprotective,[12] and so on. Among the components of *A. manihot* extracts, flavonoids have been considered as the active components according to the Chinese Pharmacopoeia, which was edited in 2015. Therefore, flavonoids are the most widely studied.[26] Although the TFA have been found to possess neuroprotective effect,[12] the role of TFA in reactive proliferation of astrocytes in ischemic brain tissues is still unclear. Therefore, we aim to demonstrate the changes in proliferation of reactive astrocytes after ischemic stroke and clarify the role of TFA in the reactive proliferation of astrocytes.

Bilateral CCAs occlusion was used to establish global cerebral ischemia.[15] The reactive astrocytes' proliferation is associated with an increase of astrocyte-specific marker, GFAP.[27] CSPG expression was also detected because the increased release of CSPGs from reactive astrocytes leads to dilapidated axon regeneration and blocks the recovery of neurons.[4] The results showed that the expression of GFAP and CSPG was significantly increased in the brain tissues at 7 days after cerebral ischemia, which is similar to a previous research reporting that cerebral ischemia/reperfusion (I/R) could induce increase of GFAP expression[28] and CSPGs' release.[29] Not surprisingly, the increment of GFAP expression and CSPGs release could be inhibited by TFA treatment, suggesting that TFA could inhibit the reactive proliferation of astrocytes.

ACE is a key metabolic enzyme in RAS.[30,31] Mnafgui *et al.* found that ischemic stroke induced remarkably elevated levels of ACE in the brain tissues, and inhibition of ACE activity was related to the neuroprotective mechanism of oleuropein. ACE2, the homologous gene product of ACE, is a new member of RAS. It can directly hydrolyze Ang II to produce Ang-(1–7). Ang-(1–7) plays a prominent role as it Possesses vasodilation, anti-inflammation, and antiproliferation effects by antagonizing the biological effects of Ang II.[32] Tao *et al.*[10] found that the ACE inhibitor captopril not only inhibited ACE activity, but also upregulated the expression of ACE2 and Ang-(1–7) production, thereby protecting against cerebral ischemia. All these findings suggested that activation of ACE2 and upregulation of Ang-(1–7) production may be a new treatment strategy for the ischemic stroke.[33]

Therefore, we sought to explore whether the inhibitory effect of TFA on the proliferation of reactive astrocytes is related to ACE and ACE2. We found that cerebral ischemia induced a significant increment of ACE activity and Ang II production in the brain tissues, which is similar to the results of a previous research which showed that upregulation of ACE is related to cerebral ischemia injury.[34] Treatment with TFA could reduce the increment of ACE activity and Ang II production, as well as increase the ACE2 activity and Ang-(1–7) production in the brain tissues.

To further confirm the role of TFA in the reactive proliferation of astrocytes and explore whether ACE2/Ang-(1–7) is involved in the role of TFA, the primary cultured astrocytes treated with $CoCl_2$ were used as a model of cell hypoxia and were associated with upregulation of cell proliferation and GFAP expression.[35] The results revealed that the upregulation of cell proliferation and GFAP expression in astrocytes under hypoxic conditions were blocked by TFA or Ang-(1–7) treatment. These data confirm that TFA could inhibit the reactive proliferation of astrocytes following cerebral ischemia. Besides, we found that TFA-mediated upregulation of ACE2 activity and Ang-(1–7) production is related to the inhibitory role of TFA in reactive astrocytes' proliferation.

CONCLUSION

In conclusion, our study showed the role the TFA in reactive proliferation of astrocytes following ischemic stroke. The following were our findings: (1) TFA had an inhibitory effect on reactive proliferation of astrocytes; (2) TFA could inhibit the cerebral ischemia-induced increase of ACE activity and Ang II production in the brain tissues, and upregulated the ACE2 activity and Ang-(1–7) production; (3) the mechanism of inhibitory effect of TFA on reactive astrocytes' proliferation is related to the upregulation of ACE2 activity and Ang-(1–7) production.

Compliance with ethical standards

All experiments on animals were reviewed and approved by the Ethics Review Committee of Hefei Technology College, which comply with the Guide for the Care and Use of laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 2011).

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Conflicts of interest

The authors have no conflicts of interest to disclose.

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