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ORIGINAL ARTICLE

Suppression of Angiotensin-(1–7) on the Disruption of Blood-Brain Barrier in Rat of Brain Glioma

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Abstract

Glioblastoma multiforme (GBM) is the most primary brain tumor, specially characterized with the damage of blood-brain barrier (BBB). The Ang-(1–7) was proven to have an inhibitory effect on glioblastoma growth. However, its role on blood–brain barrier (BBB) and the underlying molecular mechanism remains unclear. In this study, Ang-(1–7) significantly relieved the damage of blood-brain barrier in rats with intracranial U87 gliomas as evaluated by magnetic resonance imaging (MRI). Furthermore, its treatment attenuated BBB permeability, tumor growth and edema formation. Similarly, Ang-(1–7) also decreased U87 glioma cells barrier permeability in vitro. Further analysis showed that Ang-(1–7) could effectively restore tight junction protein (claudin-5 and ZO-1) expression levels both in rats and U87 glioma cells by affecting the activation of JNK pathway. SP600125, an inhibitor of JNK, significantly enhanced the expression of Claudin-5 and ZO-1, and decreased the disruption of BBB and enhanced the efficiency of Ang-(1–7) in glioma rats. Taken together, this study demonstrated a protective role of Ang-(1–7) in glioma-induced blood-brain barrier damage by regulating tight junction protein expression. Accordingly, Ang-(1–7) may become a promising therapeutic agent against glioma.

Keywords Ang-(1–7) · Glioma · Blood–brain barrier · Claudin-5 · ZO-1

Background

Glioblastoma multiforme (GBM) is the most primary brain tumor with high mortality. According to the statistics, the prognosis of malignant glioma is poor with a median survival of 12 to 15 months. GBM accounts for the most of brain tumors and has a complex pathologic mechanism. Blood–brain barrier (BBB) is a metabolic and physical barrier to serve protective role in the brain by separating the peripheral microenvironment from nervous system [1]. The integrity of BBB has been identified to be essential for the regulation of neural microenvironment homeostasis and the maintenance of brain function. Accumulation evidences suggest that the loss of BBB integrity is one of the main characteristic of impaired brain function, and can be caused by various factors, such as ischemia, hypoxia, inflammation, tumor, physical and chemical injury in brain [1, 2]. Although the exact mechanism is unclear, BBB leakage has crucial role in the progression of human brain tumor, including glioma [3, 4]. Hence, more knowledge to the mechanisms by which the BBB is disrupted in glioma are urgently necessary to improve the therapy of human glioma.

Microvascular endothelium and tight junctions are the major components and structure of BBB [5]. Tight junction proteins play important roles in the endothelial junctions and the integrity of BBB. In cerebral endothelial cells, claudins protein family and zonula occludens (ZO) protein family have been identified to be the main tight junction proteins, which are transmembrane protein or subsidiary cytoplasmic protein. Previous studies demonstrated that the dysregulation of claudin-5 and ZO-1 in endothelial cells of brain is significantly associated with the structure of BBB, and the specific mark of BBB damage [6].

Angiotensin-(1–7) [Ang-(1–7)], generated from endo- or carboxy-peptidases of Ang II, is a bioactive fragment of renin-angiotensin system (RAS) [7]. By acting with the receptor, Ang-(1–7) has regulatory role in the control of blood pressure and sodium-water reabsorption, and is closely associated

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with the hypertension-related disasters [8]. Ang-(1–7) has been found to regulate different redox signaling and be involved in the regulation of oxidative stress, inflammatory response, cell apoptosis and edothelial dysfunction [9]. It has also been confirmed that Ang-(1–7) plays protective role in the modulation of BBB permeability as well as cerebral infarction and neurological functions [10]. However, whether Ang-(1–7) is involved in the regulation of BBB permeability in glioma are still unknown.

In present study, we constructed a rat model of glioma in vivo and a co-culture model of RBE4 endothelial cells with U87 glioma cells in vitro to explore the role of Ang (1–7) in the regulation of BBB function in glioma. These findings might provide a novel therapeutic target for human glioma.

Methods

Drugs and Animal Model

Ang-(1–7) (Bachem, Switzerland) was dissolved in artificial cerebrospinal fluid. Male Sprague–Dawley rats of 280–320 g were purchased from National Rodent Laboratory Animal Resources, and housed under a photoperiod of 14 h of light as well as food and water ad libitum. The protocol was performed as institutional guidelines for animal welfare and approved by the Animal Care and Use Committee of The Fifth Affiliated Hospital of Zhengzhou University. After ketamine/xylazine anesthesia, nude rats ($n = 20$) were stereotactically implanted with 5×10^4 U87 glioma cells into right striatum.

Drug Application

Rats were implanted with minipump, and delivered cerebrospinal fluid (Ctrl, 0.5 μl/h) or Ang-(1–7) (1 pmol/0.5 μl/h, 100 pmol/0.5 μl/h or 10 nmol/0.5 μl/h) 3 times per week starting on day 5 after model. The MRI experiments were conducted on day 12 (1 week of treatment) or on day 19 (2 weeks of treatment) after tumor inoculation.

Magnetic Resonance Imaging

MRI assay was performed as described previously [11]. Briefly, rats were anaesthetized with isoflurane. A circulating water pad was used to keep the body temperature of rats at 37 °C. For CA administration, a catheter was inserted into tail vein, and the respiration of rats was continuously monitored. MRI measurements were performed on a scanner according to the operation manual. T1 and T2-weighted multislice RARE scans were conducted to localize the tumor. Images at each inversion pulse were acquired to obtain T1-relaxation rates. The dynamic changes in 12 min (120 scans) were monitored. 10 baseline scans was performed 1 min before CA administration to determine baseline measurement without CA. And the CA was manually injected into tail vein catheter to further measure. After CA injection, the initial area under CA administration was calculated at the time 0 to 60 s in contralateral brain. Total volumes of tumor and edema were calculated by tracing tumor and peritumoral edema areas on T2-weighted multislice RARE scans.

Brain Edema Assay

Brain edema assay was evaluated by examining brain water content. In brief, the hemispheres were dried at 100 °C for 48 h, then the wet and dry weights of hemispheres were weighed. Brain water content was calculated as (wet weight/dry weight) 100/wet weight.

Blood–Brain Barrier Permeability Assay

Blood–brain barrier permeability assay was examined by using Evans Blue. After tumor inoculation, Evans blue were injected into rats (100 μL) and kept for 3 h, followed by perfusing with 150 mmol/L NaCl. The brains were dissected and centrifuged. 500 μL trichloroacetic acid was then used to dilute the supernatants overnight at 4 °C. After centrifugation, the Evans blue was measured at 620 nm absorbance. Blood–brain barrier permeability was expressed as the percentage of Evans blue in contralateral cortex.

Cell Culture

Human U87 glioma cells and brain RBE4 endothelial cells were obtained from ATCC (Rockville, MD, USA). U87 cells were cultivated in DMEM medium supplemented with 10% FCS, and RBE4 cells were cultured in MEM/Ham's F-10 medium supplemented with 10% FBS and penicillin/streptomycin (Invitrogen, Darmstadt, Germany).

Barrier Function Assay

Barrier function was assessed using transendothelial electrical resistance (TEER) as described previously [12]. Briefly, U87 cells were cultured with RBE4 cells on collagen-coated transwell filter. The resistance of empty collagen-coated transwell was used to correct the resistance of transwell insert cells, followed by multiplying with surface area. Measurements were standardized to 0 h time point.

Quantitative RT-PCR

Total RNA was isolated by Trizol reagent (Invitrogen), and reversely transcribed into cDNA. Then, quantitative RT-PCR was conducted to evaluate the expression of claudin-5 and ZO-1. The primers were shown as follows: claudin-5: sense

5'-CACGGGAGGAGCGCTTTAT-3′ and antisense 5'-GGCACCGTTGGATCATAGAACT-3′; ZO-1: sense 5'-CGGTCCTCTGAGCCTGTAAG-3′ and antisense 5'-GGATCTACATGCGACGACAA-3′. The Ct of GAPDH was used to normalize the expression of claudin-5 and ZO-1.

Western Blotting

Total proteins of tissue or cell samples were isolated using lysis buffer. Equal amounts of protein were separated by SDS-PAGE, and transferred into PVDF membrane. The brands were blocked for 1 h in blocking buffer, then incubated with primary antibodies (mouse anti-claudin-5 and anti-ZO-1 antibody, 1:1000, BD, San Jose, CA; mouse anti-β-actin, 1:3000 dilution, ABclonal) overnight at 4 °C. The blots were reacted with secondary antibodies conjugated with horseradish peroxidase for 1 h at room temperature, and detected with enhanced chemiluminescence (ECL, Amersham Pharmacia, NJ).

Statistical Analysis

All data were expressed as mean ± SEM, and analyzed using GraphPad Prism 6 (La Jolla, CA, USA). The difference between groups were measured by *t*-test, and the statistical comparisons were evaluated by ANOVA. $P < 0.05$ was considered statistically significant.

Results

Ang-(1–7) Abates the Damage of Blood-Brain Barrier in Glioma Rats

The integrity of BBB in glioma rats were disrupted compared with those in normal rats, as evaluated by Evans blue assay (Fig. 1a). After Ang-(1–7) administration, the destruction of BBB in glioma rats were relieved with varying degrees. And more decrease in Evans blue leakage was induced by higher concentration of Ang-(1–7). Additionally, similar changes in the integrity of BBB in glioma rats treated with Ang-(1–7) was observed by MRI scan, as evaluated by the decrease in tumor and edema volumes (Fig. 1b). This implied that Ang-(1–7) might have dose-dependent protective effect on BBB permeability in glioma. Further, we also found that Ang-(1–7) significantly reduced the content of brain water in glioma rats (Fig. 1c). Taken together, these results suggested that Ang-(1–7) may abate the damage of blood-brain barrier in glioma rats in a dose-dependent manner.

Ang-(1–7) Relieves the Disruption of Barrier Function in Endothelial Cells

Endothelial cells are the major component of blood-brain barrier. We then clarified the effects of Ang-(1–7) on disruption of barrier function in endothelial cells to further investigate the role of Ang-(1–7). After co-culture of U87 glioma cells and RBE4 endothelial cells, the disruption of barrier function in endothelial

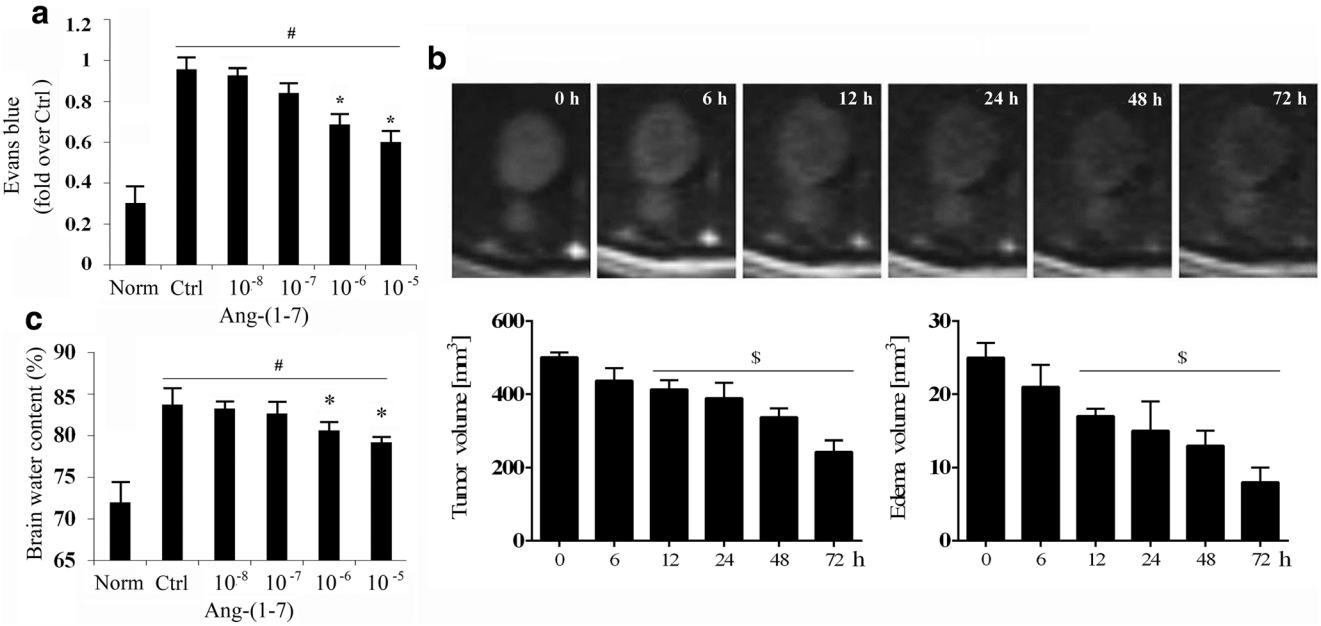

Fig. 1 Ang-(1–7) abates the damage of blood-brain barrier in glioma rats. a Evans blue assay was performed in normal rats (Norm) or in glioma rats treated with different concentration of Ang-(1–7) or not (Ctrl). **b** MRI scan was performed to test the tumor and edema volumes in glioma rats treated with Ang-(1–7) (10^{-5} mol/L). **c** The level of brain water was measured in glioma rats treated with Ang-(1–7) or not. ($^{\#}p < 0.05$ versus Norm; $^{*}p < 0.05$ versus Ctrl; $^{\$}p < 0.05$ versus 0 h)

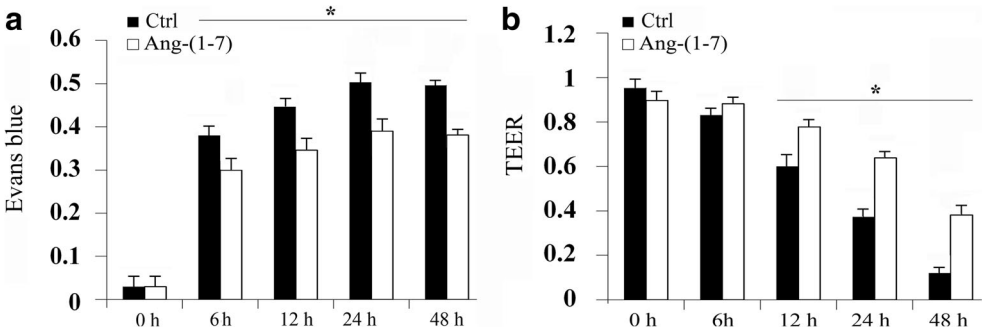

Fig. 2 Ang-(1–7) relieves the disruption of barrier function in endothelial cells. RBE4 cells co-cultured with U87 was treated with Ang-(1–7) (10^{-5} mol/L) or not (Ctrl). **a** Evans blue assay and **b** TEER assay were performed to evaluate the barrier function of RBE4 cells at different times. (*$p < 0.05$ versus Ctrl)

cells was observed as evaluated by Evans blue assay. Compared with the control, Ang-(1–7) treatment significantly decreased the leakage of Evans blue in RBE4 cells co-cultured with U87 (Fig. 2a). And TEER assay also showed that the decrease of barrier permeability in RBE4 cells co-cultured with U87 was obviously moderated by Ang-(1–7) in a time-dependent manner compared with control group (Fig. 2b). These results indicated that Ang-(1–7) relieves the disruption of barrier function in endothelial cells to contribute to the integrity of BBB in glioma.

Ang-(1–7) Increases the Expression of Tight Junction Proteins

Claudin-5 and ZO-1 are major cell adhesion molecule which play crucial roles in the tight junction in endothelia and brain. And the change of Claudin-5 and ZO-1 expression and rearrangement has been identified to be closely associated with the dysfunction of BBB [13]. We further investigate the expression of Claudin-5 and ZO-1 in glioma rats and RBE4 cells co-cultured with U87 to demonstrate the potentially mechanism by which Ang-(1–7) plays protective effect on the integrity of BBB in glioma. As seen in Fig. 3a and b, the decreased expression of Claudin-5 mRNA and protein was up-regulated by Ang-(1–7) in RBE4 cells co-cultured with U87 compared to those in control. And the similar change was observed in ZO-1 expression. Furthermore, Ang-(1–7) also significantly enhanced the expression of Claudin-5 and ZO-1 in glioma rats (Fig. 3c and d). This indicated that Ang-(1–7) may relieve the disruption of blood-brain barrier in glioma rats by activating the expression of tight junction proteins in endothelia cells.

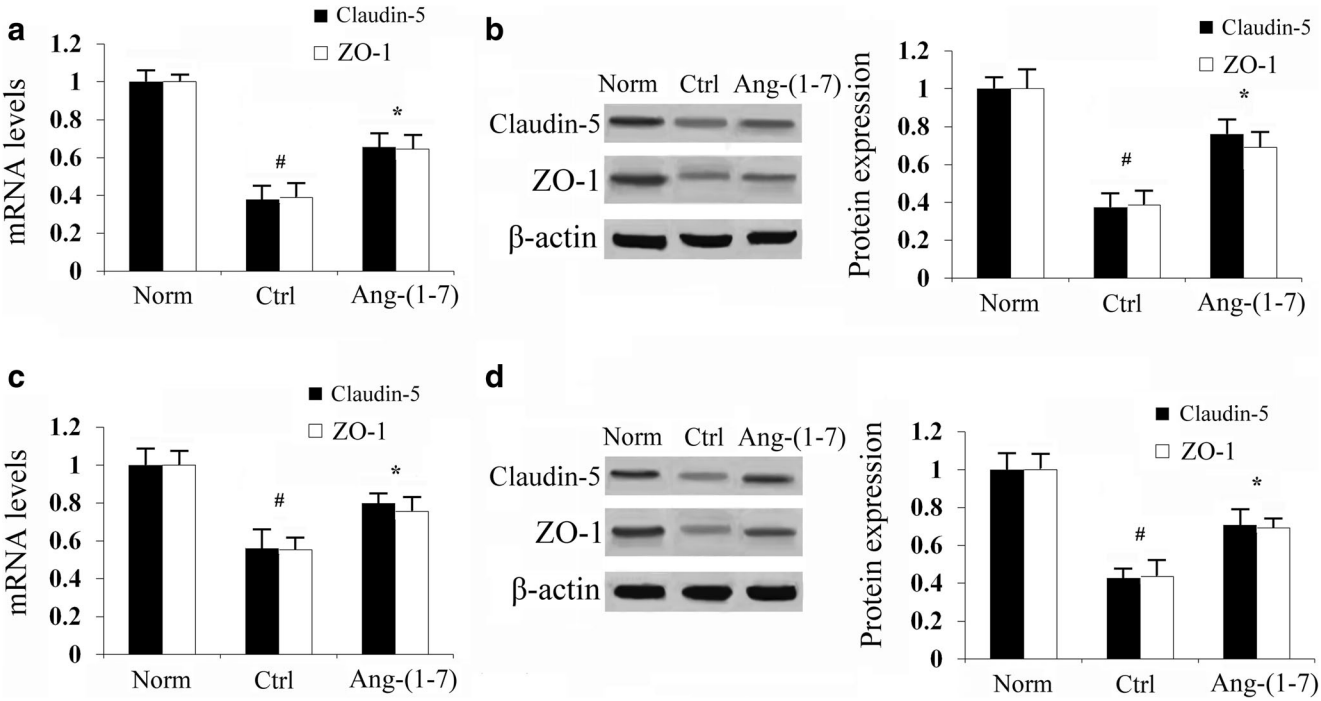

Fig. 3 Ang-(1–7) increases the expression of tight junction proteins. RBE4 cells co-cultured with U87 was treated with Ang-(1–7) (10^{-5} mol/L) or not (Ctrl). **a** RT-PCR and **b** Western blot were performed to detect the expression of Claudin-5 and ZO-1 in normal RBE4 cells (Norm) or in RBE4 co-cultured with U87 cells. Then, glioma rats were treated with Ang-(1–7) (10^{-5} mol/L) or not (Ctrl). **c** RT-PCR and **d** Western blot were performed to test the expression of Claudin-5 and ZO-1 in normal rats (Norm) or in glioma rats. ($^{\#}p < 0.05$ versus Norm; *$p < 0.05$ versus Ctrl)

JNK Pathway is Involved in the Regulation of Ang-(1–7) in Blood-Brain Barrier of Glioma Rats

JNK pathway has been reported to play key role in the mediation of tight junction in brain [14]. To further determine the underlying role of Ang-(1–7) in protecting integrity of BBB in glioma, we investigate the interaction between Ang-(1–7) and JNK pathway. Western blot showed that the levels of phospho-JNK and phospho-c-jun were up-regulated in RBE4 cells co-cultured with U87 compared with normal RBE4 cells. On the contrary, the phospho-JNK and phospho-c-jun levels were decreased in RBE4 cells co-cultured with U87 after treating with Ang-(1–7) (Fig. 4a). Additionally, SP600125, an inhibitor of JNK, could significantly enhanced the expression of Claudin-5 and ZO-1 in RBE4 cells, which were inhibited by glioma cells (Fig. 4b). And SP600125 treatment significantly decreased the disruption of BBB and enhanced the efficiency of Ang-(1–7) in glioma rats, as evaluated by MRI (Fig. 4c and d), indicating the involvement of JNK pathway in Ang-(1–7)-mediated protective role in blood-brain barrier of glioma rats.

Discussion

GBM is a severe brain disorder as the top health risks with high mortality. It has been found that BBB leakage has crucial role in the progression of human glioma [15–17]. However, it remains unclear that the mechanism by which the BBB is disrupted in glioma, and whether the alleviation of BBB damage is beneficial for the treatment of glioma [18, 19]. In the present study, we determined the role of Ang-(1–7) in the maintenance of BBB function in glioma.

Ang-(1–7) is a bioactive fragment of renin-angiotensin system (RAS). By acting with the receptor, Ang-(1–7) has also been confirmed to plays protective role in the modulation of BBB permeability as well as cerebral infarction and neurological functions [20]. Herein, we found that Ang-(1–7) can decrease the Evans blue leakage to abate the damage of blood-brain barrier, and reduced the content of brain water in glioma rats in a dose-dependent manner. Besides, in RBE4 endothelial cells co-cultured with U87 glioma cells, Ang-(1–7) administration also decreased the leakage of Evans blue and relieved the disruption of barrier permeability in vitro. The protective role of Ang-(1–7)

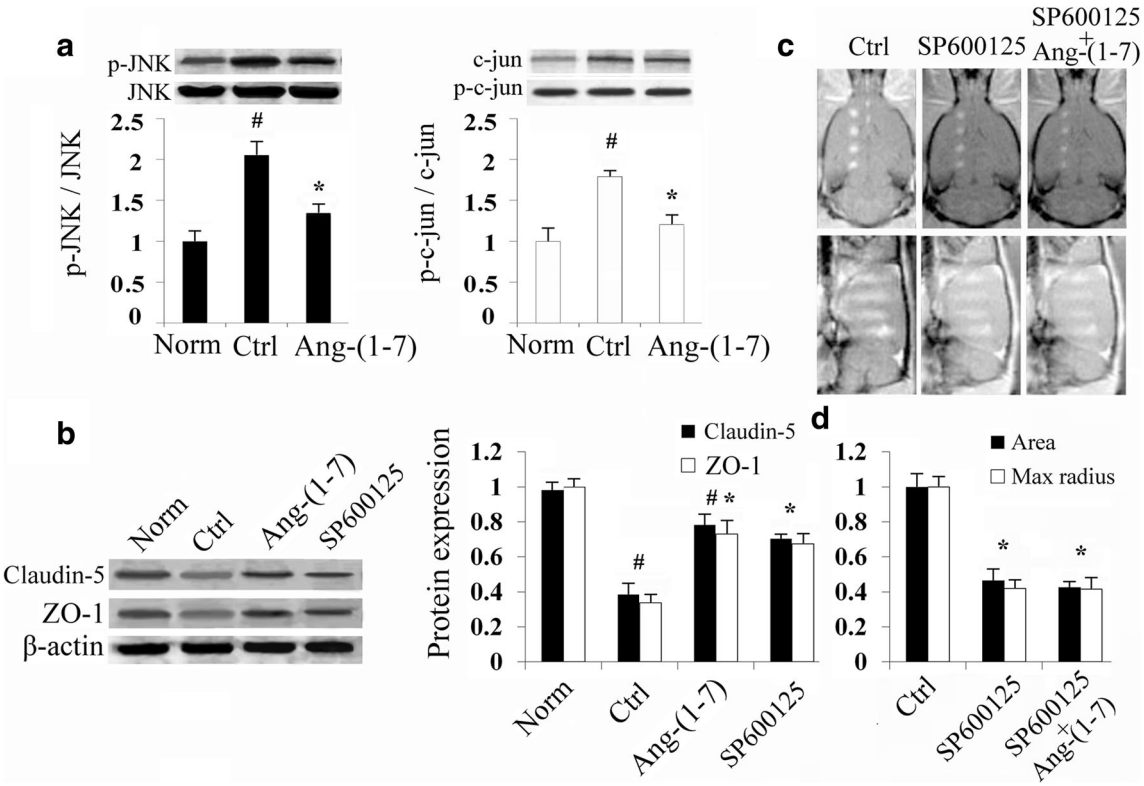

Fig. 4 Ang-(1–7) increased the expression of Claudin-5 and ZO-1 by regulating JNK pathway. a The levels of phospho-JNK (p-JNK) and phospho-c-jun (p-c-jun) were measured by Western blot in normal RBE4 cells (Norm) or in RBE4 cells co-cultured with U87 treated with Ang-(1–7) (10^{-5} mol/L) or not (Ctrl). **b** SP600125 was administrated into RBE4 cells co-cultured with U87, and the expression of Claudin-5 and ZO-1 was then detected. **c** SP600125 was administrated into glioma rats in the presence or absence of Ang-(1–7), and BBB disruption was measured by MRI. Up column, sagittal images; down column, coronal images. **d** The area and degree of BBB disruption were counted. ($^{\#}p < 0.05$ versus Norm; $^{*}p < 0.05$ versus Ctrl)

to BBB function may, at least in part, be associated with the structure and chemical composition of BBB. There is a neuronal structure, called "milieu", in brain, which has key roles in the processes of angiogenesis, synaptic remodeling and neurogenesis in the development of brain [21, 22]. The integrity of BBB is essential to maintain the function of neuronal "milieu". Additionally, brain edema has been considered to be another important symbol of central pathophysiological events and BBB injury [23]. The disruption of BBB permeability stimulates the flow of water from blood into the brain and the secretion of Na^+, which results in the formation of cerebral edema [20]. Accumulation of cerebral edema aggravates the injury of brain in patients with glioma and enhance the morbidity and mortality [24]. We further confirmed that Ang-(1–7) significantly reduced the content of brain water in glioma rats, suggesting the protective effect of Ang-(1–7) on brain in glioma.

Tight junctions, the major components and structure of BBB, have key structural and functional roles in the endothelial junctions and the integrity of BBB [25]. Among them, claudin-5 and ZO-1 have been confirmed to be the most common protein associated with tight junction, and involved in the maintenance of structure and function of endothelial junctions. Claudin-5 is a transmembrane protein and can regulate the paracellular permeability of small molecule, which is specifically important to the integrity and function of BBB [26]. ZO-1, a subsidiary cytoplasmic protein, belongs to the family of membrane-associated guanylate kinase homologs, and is key recognition protein in tight junctions [27]. The absence of ZO-1 protein may result in the disorganization of endothelial junctions and the BBB, contributing to the damage of brain [6, 28]. In this study, we found that the decreased expression of Claudin-5 and ZO-1 proteins was up-regulated by Ang-(1–7) in RBE4 cells co-cultured with U87 as well as glioma rats, indicating that Ang-(1–7) may relieve the disruption of blood-brain barrier in glioma rats by activating the expression of tight junction proteins in endothelia cells.

To determine the underlying role of Ang-(1–7) in protecting integrity of BBB in glioma, we further investigate the interaction between Ang-(1–7) and JNK pathway. JNK pathway has been reported to play key role in the mediation of tight junction in brain [14, 29]. We determined that the levels of phospho-JNK and phospho-c-jun were up-regulated in brain endothelial cells induced by U87 in vitro, but decreased by Ang-(1–7) treatment. And the inhibition of JNK signaling could enhanced the expression of Claudin-5 and ZO-1 in RBE4 cells. SP600125 treatment significantly decreased the disruption of BBB and enhanced the efficiency of Ang-(1–7) in glioma rats. These suggest the involvement of JNK pathway in Ang-(1–7)-mediated protective role in blood-brain barrier of glioma rats.

Conclusion

Our studies demonstrated the protective role of Ang-(1–7) in glioma-induced blood-brain barrier damage by regulating tight junction protein expression. Accordingly, Ang-(1–7) may become a promising therapeutic agent against human glioma.

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Compliance with Ethical Standards

Disclosures The authors have no financial conflicts of interest.

Conflicts of Interest The authors declare no conflict of interest regarding the publication of this paper.

Ethical Approval No.

Informed Consent No.

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