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Effects of Integrin β1 on behavior and neurovascular regeneration in rats with cerebral ischemia-reperfusion injury

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Abstract. – OBJECTIVE: The aim of this study is to investigate the effect of Integrin β1 on neurological behavior and neurovascular regeneration in rats with a cerebral ischemia-reperfusion injury.

MATERIALS AND METHODS: Rat middle cerebral artery occlusion (MCAO) was performed with a modified suture embolization method. Neurological function score of each rat was recorded. Cerebral infarct volume was calculated by Image J after TTC stain. Subsequently, behavioral tests were performed to evaluate neuronal damage, including griping strength test, corner test, cylinder test and sucrose preference test. The expression levels of VEGF, HIF-1α, Claudin5, and ZO-1 in rat brain tissues were detected by Western blot and quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR), respectively.

RESULTS: Neurological function score of the rat was remarkably decreased after cerebral ischemia-reperfusion. Anti-Integrin β1 administration aggravated neurological deficit and increased cerebral infarct volume of I/R rats. Symptoms of hemidysesthesia, dyskinesia, and affective disorder of rats were worse after anti-Integrin β1 administration in I/R rats. Anti-Integrin β1 administration downregulated VEGF and HIF-1α in rat brain tissues ($p<0.05$). However, no significant differences in Claudin5 and ZO-1 expressions were found before and after Integrin β1 treatment.

CONCLUSIONS: The inhibition of Integrin β1 pathway during cerebral ischemia-reperfusion aggravates the behavior and neurovascular regeneration of I/R rats. In the process of cerebral ischemia-reperfusion, Integrin β1 plays a key role in the repair and protection of neurovascular units by promoting angiogenesis.

Key Words:
Integrin β1, Ischemia-reperfusion, Neuroprotection, Angiogenesis.

Introduction

Cerebral ischemia (CI) is one of the cardio-cerebrovascular diseases that causes heavy social and economic burdens[1,2]. Cerebral ischemia-reperfusion injury (I/R) is a condition that cerebrovascular recanalization fails to improve the symptoms of cerebral ischemia, but even worsens neurological deficits at the lesion side. I/R would result in a series of pathological changes, mainly including inflammation, cell apoptosis, destruction of the blood-brain barrier, oxidative stress, and calcium overload[3,4].

Integrin, as an integral membrane protein complex, was first proposed by Tamkun et al in 1986 (Tamkun JW, DeSimone DW, Fonda D, Patel RS, Buck C, Horwitz AF, Hynes RO. Structure of integrin, a glycoprotein involved in the transmembrane linkage between fibronectin and actin. Cell 1986; 46: 271-282). Integrin is an essential member in cell adhesion molecule family. It is greatly involved in cell adhesion, differentiation, growth, migration, neural development and angiogenesis *via* regulating intracellular pathway with adhesion proteins of cytoskeleton[5,6]. Integrin is a non-covalent heterodimer formed by an α subunit (120-185 kD) and two β subunits (90-110 kD). It has been found that there are 18 α subunits and 9 β subunits, which could constitute more than 20 integrins[7-9].

Accumulating evidence has shown that integrins are closely related to different stages of neural development and neuropathological processes[10,11]. Under normal conditions, a large amount of Integrin β1 expressed by endothelial cells could enhance the adhesion between endothelial cell membranes. Integrin β1 exerts a crucial role in cell junctions, stable maintenance of the blood-

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brain barrier and selective filtration function[12]. After cerebral ischemic injury, Integrin β1 inhibits neuronal apoptosis and protects nerve cells via multiple pathways. Loss of vascular and neuronal matrix nutritional function after ischemia results in substantial damage. Functional repair after cerebral ischemia requires for the reconstruction of both nerves and blood vessels[13].

Scholars[14,15] have shown that Integrin β1 contributes to repair central nervous system injury with a large number of extracellular matrix growth factor receptors and other cell membrane protein factors. Integrin β1 is also related to the proliferation of endothelial cells and the reconstruction of the blood-brain barrier *via* upregulating VEGFR. In the present work, we mainly explored the role of Integrin β1 in regulating I/R and its underlying mechanism.

Materials and Methods

Experimental Rats

Adult male CD-1 rats weighing 20-30 g were randomly assigned into 4 groups, namely sham group, MCAO control group (vehicle group), ischemia-reperfusion (I/R group), and anti-Integrin β1 group. MCAO in rats was achieved using suture embolization method introduced by Longa et al[16]. Rats in sham group underwent the same procedures except for nylon suture. Intraventricular injection of 5 μL of 2% DMSO (dimethyl sulfoxide) was performed 15 min before ischemia and 15 min after reperfusion in rats of the vehicle group. For rats in the anti-integrin β1 group, intraventricular injection of 5 μL of 2% DMSO was performed 15 min before ischemia. 10 ng/mL anti-integrin β1 diluted in saline was administrated in tail vein 30 min before model construction. This investigation was approved by the Animal Ethics Committee of China-Japan Union Hospital of Jilin University Animal Center.

Behavioral Detections

Griping strength test: Muscle damage repair of rats was evaluated by griping strength meter (GSM). Rats were pulled back quickly in horizontal direction when their paws grabbed in the bar. Forelimb griping strength was recorded when the grip was released. Grip strengths of forelimbs were recorded. Three successful records were taken and the average grip strength was calculated.

Corner test: The rat was placed between two boards at a 30° angle facing the corner. Both sides of the vibrissae were stimulated when the rat reached deep into the corner, wherein the rat reared and turned either to the left or right to exit the corner. Turns involving a rearing movement were scored. A total of 10 proper turns were recorded for each animal in each session with an interval of 1 min. Lateral index (LI) = (turns to the right-turns to the left)/total turns.

Cylinder test: The rat was placed in a transparent cylinder (20 cm in diameter and 40 cm in height). Exploration of rats in the cylinder was observed for 5 min. A mirror can be placed when necessary to ensure that the forelimb activity of rat can be recorded even if the rat turns away from the tester.

Sucrose preference test: The rat was trained to adapt to a 1% sucrose solution (w/v) for 48 h at the beginning of the experiment; after the training session, the rats were deprived of water and food for 23 h, followed by the sucrose preference test, in which the rats were housed in individual cages for 4 h and had free access to two bottles that contained 1% sucrose or tap water. To prevent a preference for the position, the location of both bottles was changed every 2 h during the test. At the end of 1 h, the sucrose preference (SP) score was expressed as the percentage of the total liquid.

Immunohistochemistry

Brain tissues that were already fixed in paraformaldehyde were sliced into 2 mm sections. Brain sections were treated with 75% ethanol, 85% ethanol, 95% ethanol I, 95% ethanol II, 100% ethanol I, and 100% ethanol II, sequentially. Subsequently, sections were dehydrated and embedded with paraffin. HE (hematoxylin-eosin) staining was performed before the sections were treated with xylene and ethanol.

Western Blot

The total protein was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Protein samples were then separated by 10% SDS (sodium dodecyl sulphate) protein electrophoresis after the concentration of each sample was adjusted to the same level. Proteins were then transferred to a PVDF (polyvinylidene difluoride) membrane (Millipore, Billerica, MA, USA) and routinely immunostained at 4 °C overnight (diluted in 1: 500). Membranes were then incubated with the secondary antibody (l: 1000) at room temperature for 1 h. All membranes were exposed by enhanced chemiluminescence (ECL) method.

Statistical Analysis

SPSS11.0 software (Statistic Package for Social Science) was used for statistical analysis (SPSS Inc., Chicago, IL, USA). Continuous variables were expressed as mean ± standard deviation. The independent sample *t*-test was used to compare the data between the two groups. Data among different groups were compared using one way-ANOVA, followed by Student-Newman-Keuls (SNK) test. $p<0.05$ indicated the difference was statistically significant.

Results

Neuroprotective Effects of Integrin β1 on Focal I/R Rats

Neurological function score was remarkably lower in the vehicle group than that of the I/R group ($p<0.05$), indicating the successful construction of the MCAO rat model. Higher neurological function score was found in the anti-Integrin β1 group compared with that of I/R group ($p<0.05$, Figure 1A), suggesting that anti-Integrin β1 stimulates the neu-

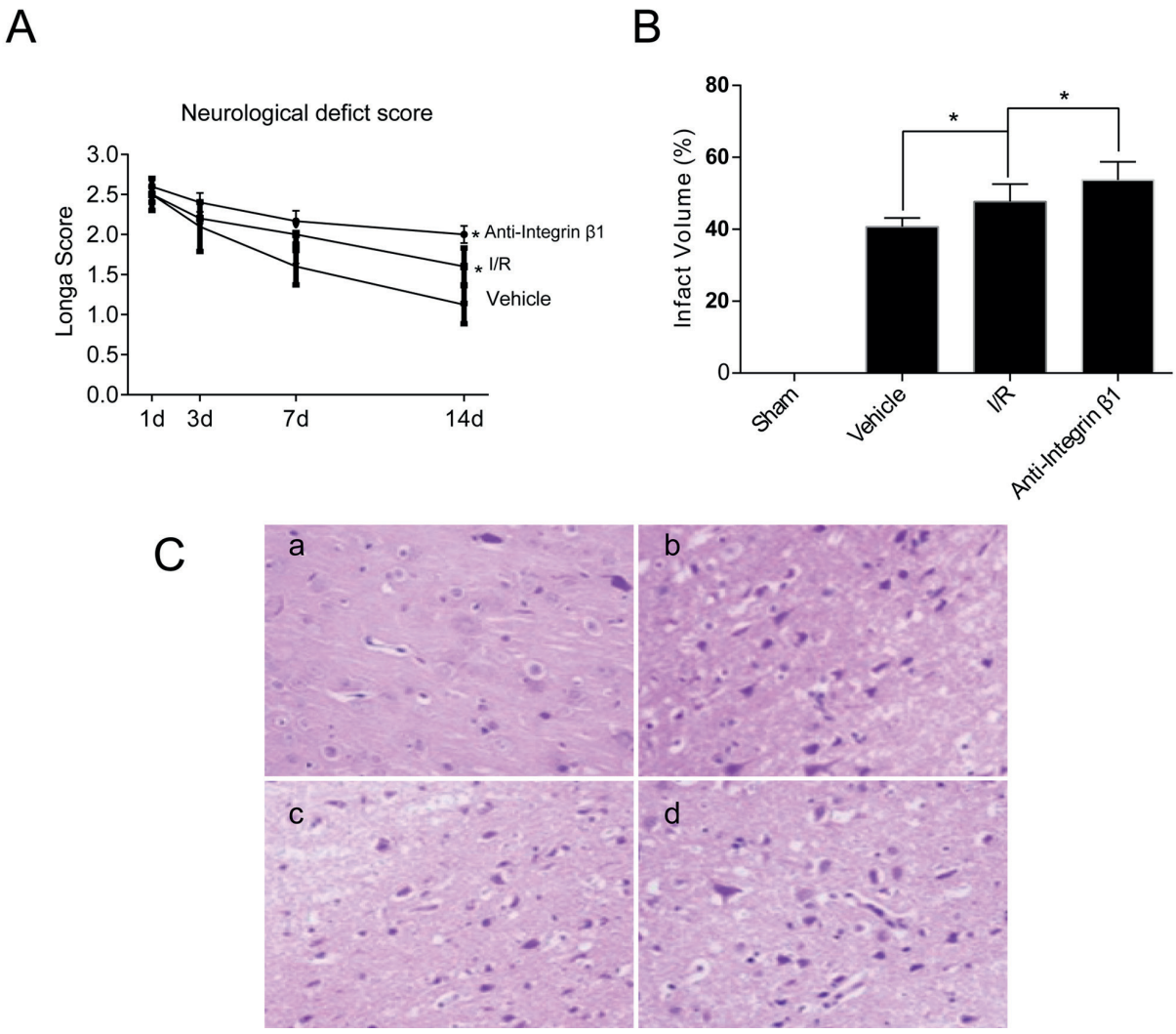

Figure 1. Neuroprotective effects of Integrin β1 on focal I/R rats. ***A,*** Neurological function score in the four groups. ***B,*** Cerebral infarct area in the four group. ***C,*** Effect of Integrin β1 on ischemic damages in neuronal cells. After MCAO/reperfusion, brain tissues were stained by H&E staining (magnification ×400). a, sham group; b, I/R group; c, vehicle group; d, anti-Integrin β1 group (*$p<0.05$).

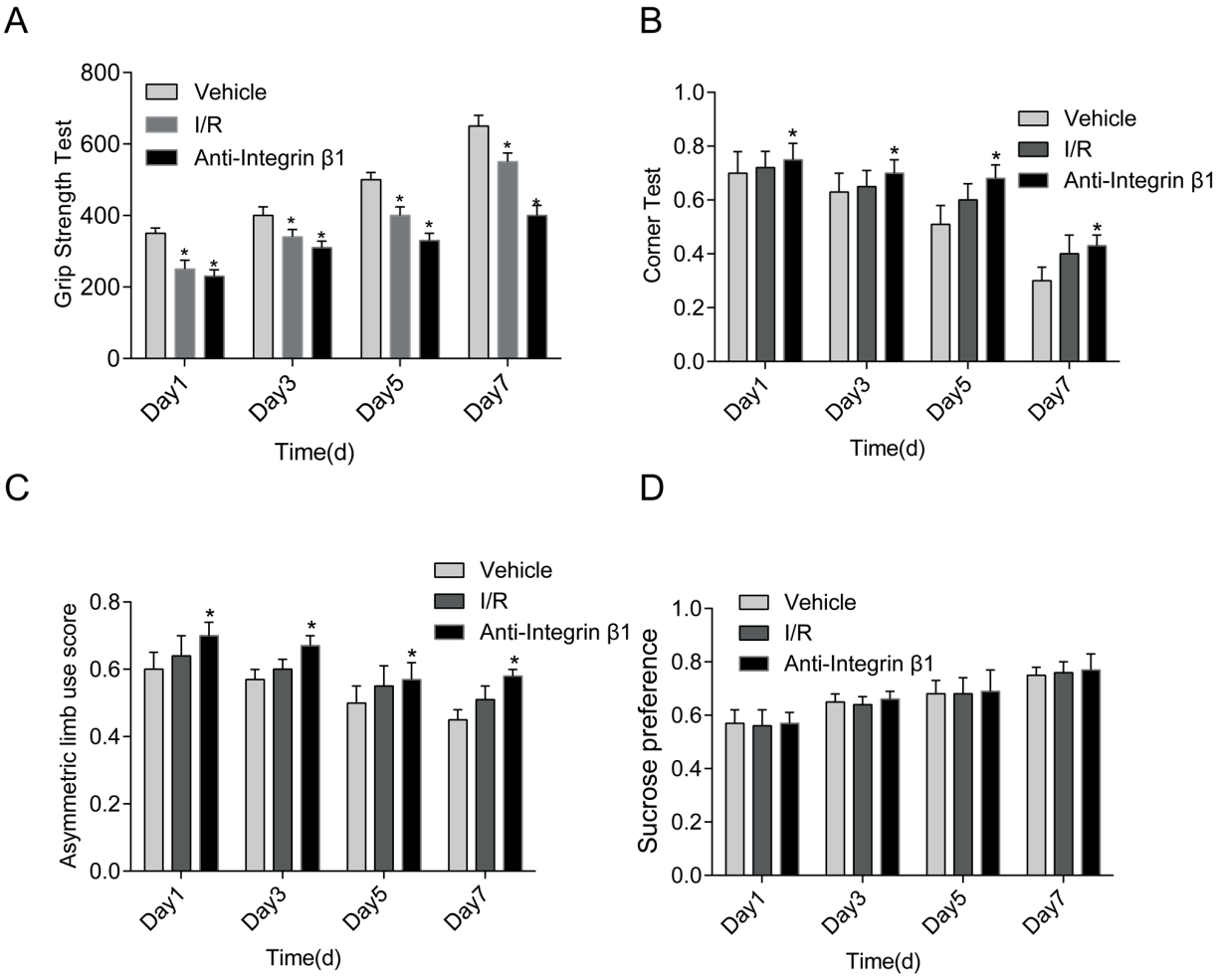

Figure 2. Effect of Integrin β1 on behavioral tests of I/R rats. ***A,*** Grip strength in the four groups. ***B,*** LI in the four groups. ***C,*** Limb asymmetry score in the four groups. ***D,*** SP in the four groups.

rological deficit in I/R. Subsequently, we detected cerebral infarct area in the four groups. The infarct area was remarkably larger in the anti-Integrin β1 group than that of I/R group ($p<0.05$, Figure 1B). To explore the effect of Integrin β1 on neurons of I/R rats, we investigated the morphological changes of neuronal cells in the ischemic hemisphere of MCAO-induced rats. No significant pathological changes were seen in brain tissues of the sham group. However, edema and necrosis were seen around the infarcted tissue with dark stained and pyknotic nucleus in I/R group and vehicle group. The condition of edema and necrosis were even worse in the anti-Integrin β1 group (Figure 1C).

Effect of Integrin β1 on Behavioral Tests of I/R Rats

Grip strength was lower in rats of I/R group than that of vehicle group at different time points. Besides, lower grip strength was observed in the anti-Integrin β1 group compared with that of I/R group on the 1st, 3rd, 5th, and 7th day, respectively ($p<0.05$, Figure 2A). Corner test results showed that LI was higher in the anti-Integrin β1 group compared with that of I/R group and vehicle group ($p<0.05$, Figure 2B). Cylinder test revealed that limb asymmetry score was the highest in the anti-Integrin β1 group at different time points ($p<0.05$, Figure 2C). Finally, sucrose preference test demonstrated that there was no significant difference in SP of the four groups even though SP was elevated in every group ($p>0.05$, Figure 2D). The above data showed that anti-Integrin β1 administration aggravates neurological deficit.

Effect of Integrin β1 on Neurovascular Regeneration

Compared with that of the preoperative level, VEGF expression was remarkably reduced on the

postoperative first day, which was gradually increased on the 3rd, 5th, and 7th day in the anti-Integrin β1 group (p<0.05, Figure 3A). No significant difference was found in VEGF expression between the anti-Integrin β1 group and I/R group at different time points (p>0.05, Figure 3B). Similar results were obtained when detecting protein expression of HIF-1α (Figure 3C and 3D), indicating that Integrin β1 promotes neurovascular regeneration *via* regulating VEGF and HIF-1α in I/R rats.

Effect of Integrin β1 on Maintaining Permeability and Integrity of Blood-Brain Barrier

Postoperative expressions of Claudin5 and ZO-1 were remarkably elevated than those of preoperative levels in the vehicle group, I/R group and anti-Integrin β1 group. However, no significant differences in Claudin5 and ZO-1 expressions were found among the three group at different time points (p>0.05, Figure 4A-4D), indicating that Integrin β1 could not change the permeability and integrity of blood-brain barrier.

Discussion

Cerebral ischemia-reperfusion injury is manifested as sensation, dyskinesia and emotional cognitive impairment. Behavioral tests are the most direct and effective ways to assess neural function[17]. Direct evaluation of nerve function injury can be determined *via* analyzing forelimb grip strength, coordination and integration capacities, and preference for sucrose[18]. In the present investigation, behavioral tests were rarely influenced by subjective factors, which could effectively simulate the clinical condition of CI patients.

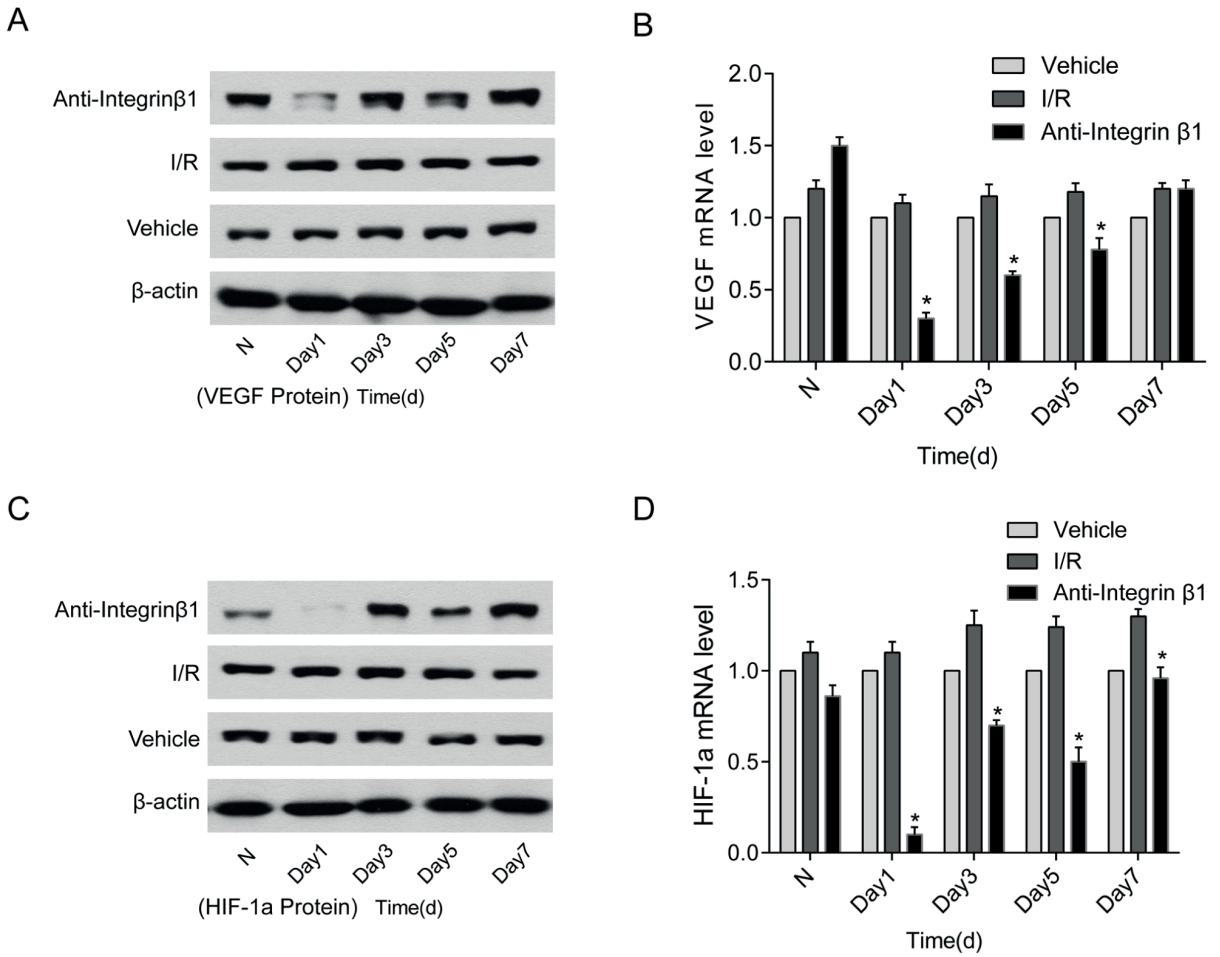

Figure 3. Effect of Integrin β1 on neurovascular regeneration. ***A,*** Protein expression of VEGF in the four groups. ***B,*** The mRNA level of VEGF in the four groups. ***C,*** Protein expression of HIF-1α in the four groups. ***D,*** The mRNA level of HIF-1α in the four groups.

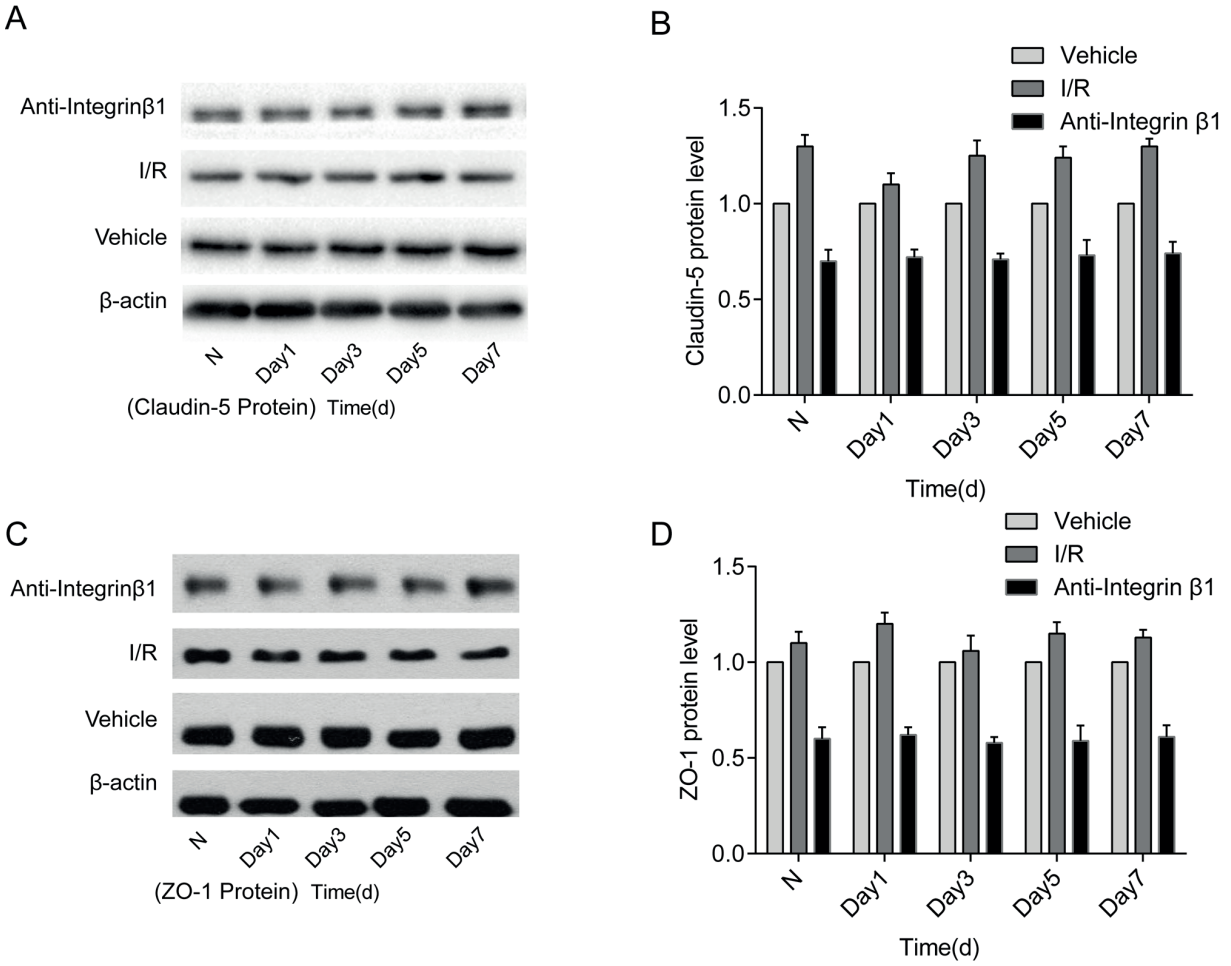

Figure 4. Effect of Integrin β1 on maintaining permeability and integrity of blood-brain barrier. ***A,*** Protein expression of Claudin5 in the four groups. ***B,*** Protein expression of ZO-1 in the four groups.

Clinical trials and animal experimental studies of I/R have found that brain tissue is extremely sensitive to hypoxia. Adaptive changes of brain tissues are observed after cerebral ischemia is caused by local vessel obstruction[19,20]. Such pathological change is verified in the neurovascular regeneration of brain tissue at the late stage of injury[21]. VEGF is a vascular endothelial growth factor that regulates angiogenesis under various pathophysiological conditions. It is an effective vasoactive polypeptide and neurotrophic factor with specific biological effects[22,23]. VEGF secretes relevant collagenases and tissue factors by selectively acting on endothelial cells. It also regulates extracellular matrix of endothelial cells and eventually induces neovascularity[24-26]. In this experiment, VEGF and HIF-1α expression were remarkably reduced on the first day after treatment of anti-Integrin β1 and then gradually increased with the prolonged intervention time. The anti-Integrin β1 was treated 30 minutes before modeling, the Integrin β1 was neutralized and VEGF and HIF-1α expression were remarkably reduced on the first day. However, the Integrin β1 was continuously synthesized and secreted in the brain tissue, VEGF and HIF-1α were upregulated with the increasing level of Integrin β1. So it was suggested that VEGF and HIF-1α were regulated in brain tissue after I/R *via* Integrin β1 pathway.

Some studies[27,28] demonstrated that neuronal-astrocyte-cerebral vascular endothelial cell pathway regulates the microenvironment dynamics in the brain. However, the specific regulatory mechanism still remains unclear. Occludin is one of the most important factors in maintaining the integrity of the blood-brain barrier. It is reported that decreased transcriptional and translational levels of Occludin disintegrate the tight junc-

tion structure, thereafter destroying the integrity of blood-brain barrier[29]. Claudin3 and Claudin5 were overexpressed on the membrane of brain vascular endothelial cells.

Claudin5 expression was remarkably decreased in ischemic brain tissue, which was positively correlated with the permeability of the blood-brain barrier damage of tight junction[30]. There are three kinds of cytoplasmic attachment proteins, namely ZO-1, ZO-2, and ZO-3, which act as cytoplasmic attachment proteins connecting to the intracellular matrix. Relative researches have pointed out that ZO-1 was downregulated in ischemic brain endothelial cells[31]. Our study elucidated that protein expressions of Claudin5 and ZO-1 did not alter on the postoperative 1st, 3rd, 5th, and 7th day, which may be explained by the activation of other pathways during I/R process.

Conclusions

We observed that the inhibition of the Integrin β1 pathway during cerebral ischemia-reperfusion aggravates the neuronal behaviors of I/R rats. In the process of cerebral ischemia-reperfusion injury and repair, Integrin β1 plays a key role in the repair and protection of neurovascular units by promoting angiogenesis.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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