Neurochemical Research (2019) 44:2606–2618
https://doi.org/10.1007/s11064-019-02880-8

ORIGINAL PAPER

TGF-β2/Smad3 Signaling Pathway Activation Through Enhancing VEGF and CD34 Ameliorates Cerebral Ischemia/Reperfusion Injury After Isoflurane Post-conditioning in Rats

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Received: 8 February 2019 / Revised: 26 August 2019 / Accepted: 16 September 2019 / Published online: 25 September 2019

Abstract

Evidence has shown the therapeutic potential of isoflurane (ISO) in cerebral stroke. The present study investigated the mechanism of ISO on vascular endothelial growth factor (VEGF) and CD34 expression in a rat model of stroke. Transient focal cerebral ischemia was established by middle cerebral artery occlusion (MCAO) for 1 h followed by reperfusion for 24 h in rats. ISO was administered for 1.5 h when the reperfusion was initiated. Neurologic deficit scores, infarct volumes, HE staining, Nissl staining, and TUNEL staining were evaluated at 24 h after reperfusion. The levels of transforming growth factor (TGF)-β2, Smad3, p-Smad3, VEGF, and CD34 proteins were detected by immunofluorescence (IF) staining and Western blot assay. Administration of ISO significantly reduced the neurologic deficit scores, infarct volumes, and damaged and apoptotic cells after cerebral ischemia/reperfusion (I/R) injury ($P < 0.05$). Meanwhile, ISO post-conditioning significantly increased the expression levels of TGF-β2, p-Smad3, VEGF, and CD34 ($P < 0.05$), whereas the expression of Smad3 showed no difference ($P > 0.05$). However, Pirfenidone, a TGF-β2 inhibitor, decreased the expression levels of TGF-β2, p-Smad3, VEGF, and CD34 ($P < 0.05$). Moreover, the protective effects of ISO post-conditioning were negated by the inhibitor. The present study indicated that ISO attenuates brain damage by activating the TGF-β2/Smad3 signaling pathway and increasing the protein expression of VEGF and CD34 in the rat MCAO model.

Keywords Focal cerebral ischemia · TGF-β2 · Signal transduction · VEGF · CD34 · Isoflurane

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Introduction

Stroke is a cerebrovascular disease with high mortality and disability due to sudden circulatory disturbance of cerebrovascular blood flow. It can be divided into two categories: ischemic stroke and hemorrhagic stroke, the former accounts for 80% [1]. At present, stroke has become the leading cause of death among Chinese residents [2]. So far, only thrombolysis has received a clinical approval from the Food and Drug Administration [3]. However, after a certain period of time, the recovery of blood flow may aggravate local brain damage and dysfunction. This phenomenon is called cerebral ischemia/reperfusion (I/R) injury. Therefore, the study of drugs with neuroprotective effects has great clinical value and significance.

Transforming growth factor β (TGF-β) family is a superfamily of growth factors and plays important roles in regulating biological processes [4]. Its members include many cytokines, such as TGF-β1, TGF-β2, TGF-β3, bone morphogenetic

proteins (BMPs), activins and so on. The biological functions of TGF-βs are similar and TGF-βs have an extreme high homology [5]. The extensive and profound research has been conducted on TGF-β1, while TGF-β2 and its role in cerebral ischemia have rarely been reported. What's more, among the TGF-β subtypes, both TGF-β1 and TGF-β2 expression enhance the proliferative signal. Our previous study found that the TGF-β1-Smad2/3 signaling pathway is involved in ISO post-conditioning for cerebral I/R injury [6]. However, whether the TGF-β2/Smad3 signaling pathway is involved in the protective mechanism of ISO post-conditioning for cerebral I/R injury have not been reported.

Angiogenesis is critical for the recovery of ischemic penumbra function after acute cerebral infarction. Research shows that the extent of capillary perfusion in the ischemic area directly affects the prognosis and survival time of patients [7]. Phenotypic and molecular characterizations of knock-out mice demonstrated that the TGF-β signaling pathway plays a critical role in angiogenesis [8]. Moreover, several cardiovascular syndromes are directly linked to mutations in their genes [9]. TGF-β family members have now gained a prominent spot among other key cytokines that control vascular function. After ischemic stroke, the body's compensatory angiogenesis begins, and new capillaries can improve the perfusion of blood around the ischemic area, provide a good microenvironment for the repair of nerve cells, and promote the recovery of neurological function after cerebral infarction; however, compensatory angiogenesis is often insufficient [10].

Studies have shown that inhaled anesthetics [11–14], such as xenon, halothane and desflurane, can provide brain protection. At present, most studies are about ISO and sevoflurane, and research on ISO is even more extensive [15–18]. Emerging evidence has demonstrated that ISO can increase the expression of vascular endothelial growth factor (VEGF) to promote proliferation and angiogenesis [19]. Our previous studies also demonstrated that 1.5% ISO post-conditioning produced a satisfactory protective effect [6]. Although the neuroprotective effect of ISO has been confirmed by literatures, its mechanism remains unclear. As research progresses, the exact mechanism becomes very complicated. Many biological factors in the body and related signaling pathways may be involved in this protection process.

Considering these researches, our study aims to elucidate the role of the TGF-β2/Smad3 pathway and the neuroprotective mechanism of ISO in cerebral I/R injury after stroke.

Materials and Methods

Animals and Ethics

Adult male Sprague–Dawley rats (220–280 g) were supplied by the Experimental Animal Center of Shihezi University, China. All animal procedures in this study were approved by the Animal Experimental Committee of the First Affiliated Hospital of the Medical College, Shihezi University, and proceeded in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Animal Model of Stroke

The model was prepared using the right internal carotid artery (ICA) suture ligation method, based on our previously reported method [20]. Rats were intraperitoneally injected with ketamine hydrochloride at 60 mg/kg. After anesthesia, the rats were fixed in the supine position. The muscles of the neck were separated bluntly, and the right common carotid artery (CCA), external carotid artery (ECA), and ICA were exposed. Then an 18 mm 3-0 monofilament nylon thread was inserted into the ICA until a slight resistance was felt. The ICA was ligated, and approximately 5 mm of the outer end of the wire plug was left. After 1.5 h ischemia, the plug was removed to start perfusion.

Drug Treatment and Animal Grouping

ISO (1.5%) was administered for 1 h after immediate reperfusion [6]. Pirfenidone, a TGF-β2 inhibitor (250 mg/kg) (Selleck Chemicals, USA) [21], was administered intraperitoneally. The inhibitor was administered 30 min before ischemia.

One hundred rats were randomly assigned into five groups (n = 20): sham-operated control group (Sham group), middle cerebral artery occlusion (MCAO) group (I/R group), MCAO rats treated with 1.5% ISO post-conditioning group (ISO group), MCAO rats treated with Pirfenidone group (I/R + Pir group), and the MCAO rats treated with Pirfenidone after ISO post-conditioning group (ISO + Pir group).

Evaluation of Neurologic Deficit Scores

After 24 h of reperfusion, neurological function scores were performed [17] to observe the effects of cerebral ischemia on neurological function. The scoring criteria are shown in Table 1:

The rat's scores range from 1 to 3 are successfully modeled for further experiments.

Measurement of Infarct Volumes

The rats were deep anesthetized and sacrificed. Then, the brains were cut into 2 mm-thick sections and incubated with TTC staining solution in the dark at 37 °C for 30 min.

Table 1 Zea-Longa's five-level four-point scale [22]

Nerve defect symptom	Score
No neurological deficits	0
Slight neurological deficit, unable to fully extend the contralateral forepaw	1
Moderate neurological deficit, manifested as a contralateral rotation	2
Severe neurological deficit, manifested as dumping to the contralateral side	3
Failed to walk independently and exhibited depressed level of consciousness	4

After staining, the brains were fixed in 4% paraformaldehyde (PFA) (Sigma, USA) overnight for imaging and analyzing.

HE Staining

The rats were anesthetized and then transcardially perfused with normal saline followed by 4% PFA (Sigma, USA) at 24 h after MCAO. Four-micrometer sections were cut serially with a microtome (KEDEE, China) and then, the sections were stained with hematoxylin for 3 min and eosin for 1 min. The paraffin sections were also utilized for further experiments.

Nissl Staining

The sections were subjected to conventional dewaxing to water. Then, the sections were stained with thionine (Solarbio, China) for 1 h at 37 °C. The cell morphologies of the cerebral cortex and hippocampal CA1 were observed under a microscope (Olympus, Japan) to assess brain damage.

TUNEL Assay

Paraffin sections were placed in an oven at 60 °C for 2 h before staining. TUNEL assay was performed using the In Situ Cell Death Detection Kit (Roche, Germany) in accordance with the manufacturer's instruction. The apoptotic index (AI) was the number of apoptotic nuclei in 100 nuclei and was calculated as follows: AI = (number of TUNEL-positive cells/total cells) × 100%.

Immunofluorescence Staining

The sections were treatment with 3% H_2O_2 for 20 min after a standard histochemical procedure. Then, they blocked with PBS containing 0.3% Triton X-100 and 10% bovine serum albumin, the sections were incubated with anti-TGF-β2 (1:100, Santa Cruz Biotechnology, USA), anti-VEGF-A (1:100, Abcam, UK), and anti-CD34 (1:100, Santa Cruz Biotechnology, USA) overnight at 4 °C. Next, the cellular nuclei were stained with propidium iodide solution for 5 min in the dark. Finally, the images were captured using a confocal laser scanning microscope (Olympus, Japan), and the mean fluorescence density was analyzed using the Image-Pro Plus 6.0 software, Mean Density = (IOD SUM)/(area SUM).

Western Blot Analysis

The proteins were isolated from the ischemic brain tissue and its concentration was examined using a BCA protein assay kit (Beyotime, China). Then, they were loaded on a 6–12% SDS-PAGE gel and then transferred onto PVDF membranes. After blocking with 5% skimmed milk, the membranes were then incubated with anti-TGF-β2 (1:100, Santa Cruz Biotechnology, USA), anti-p-Smad3 (1:100, Santa Cruz Biotechnology, USA), anti-Smad3 (1:100, Santa Cruz Biotechnology, USA), anti-VEGF-A (1:100, Abcam, UK), anti-CD34 (1:100, Santa Cruz Biotechnology, USA), and anti-β-actin (Sigma, USA) overnight at 4 °C. The expression levels of proteins were detected with an enhanced chemiluminescence ECL reagent (Thermo Fisher, USA) and densitometric quantification was carried out using Image J software.

Statistical Analysis

All data were expressed as the mean ± SD. Data with normal distribution were analyzed by one-way ANOVA followed by the Tukey's post hoc test among multiple groups. Statistical analyses were conducted using SPSS 19.0 software, and $P < 0.05$ was considered to be statistically significant.

Results

ISO Reduced Infarct Volumes and Improved Neurological Outcomes After I/R Injury in Rats

Rats subjected to MCAO exhibited significant infarct volumes (Fig. 1a, b). The ISO group showed extensively reduced the infarct volumes compared with the I/R group at 24 h after MCAO/R injury in rats ($P < 0.05$, Fig. 1a, b). Conversely, the TGF-β2 inhibitor significantly attenuated the protective effect of ISO ($P < 0.05$, Fig. 1a, b). Moreover,

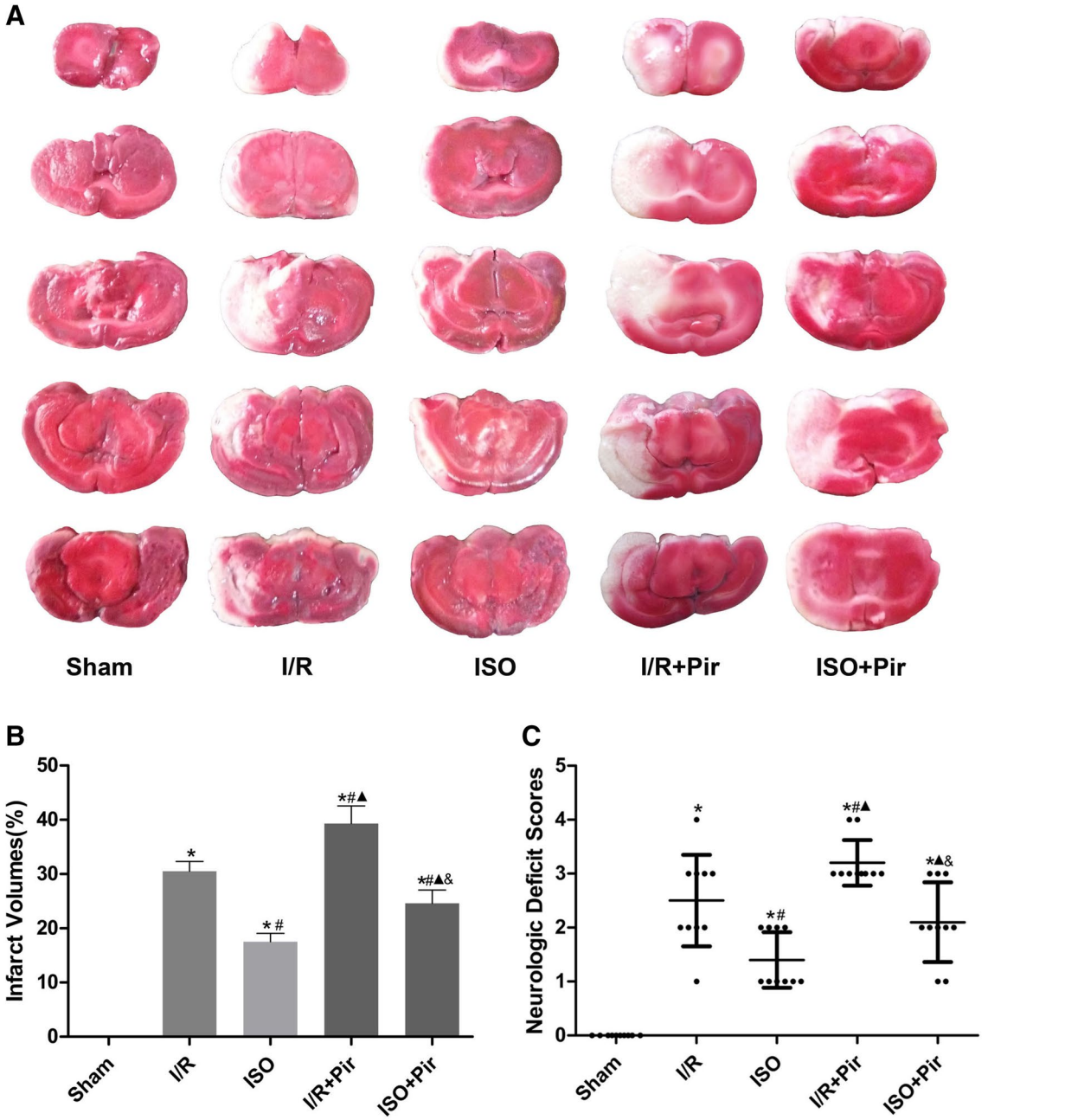

Fig. 1 ISO Reduced the infarct volume and Improved the Neurological Outcomes, whereas Pirfenidone inhibited the improved infarct volumes and neurologic deficit scores associated with ISO post-conditioning. **a** Infarct volumes assessed by TTC. Red represented normal tissue, and white represented infarct tissues. **b** Quantitative data of infarct volumes. **c** Neurologic deficit scores with the Zea-Longa score. Data are presented as the mean ± SD (n = 10). $^{*}P < 0.05$ vs. Sham; $^{\#}P < 0.05$ vs. I/R; $^{\blacktriangle}P < 0.05$ vs. ISO; $^{\&}P < 0.05$ vs. I/R + Pir (Color figure online)

the inhibitor also increased the infarct volumes in rats after MCAO and reperfusion ($P < 0.05$, Fig. 1a, b).

No infarct volume and neurological deficit were observed in the Sham group. After 24 h reperfusion, neurological deficit scores in the ISO group decreased compared with those of the I/R group ($P < 0.05$, Fig. 1c). The TGF-β2 inhibitor noticeably increased the scores ($P < 0.05$, Fig. 1c).

ISO Reduced MCAO Induced Histological Damage in the Cortex and Hippocampus After I/R Injury in Rats

HE staining showed that the cortical cell morphology was normal in the Sham group (Fig. 2a). The neurons were pyknotic and shrunken and had an irregular appearance

in the I/R group (Fig. 2a). Compared with the I/R group, the neuron morphology in the ISO group had less pyknosis (Fig. 2a). In the I/R + Pir and ISO + Pir groups, a large number of irregular and vacuolar neurons were found, and the gap around the cells was widened (Fig. 2a). Morphology changes in the hippocampal CA1 neurons were similar to those in the cortical neurons (Fig. 2b).

Nissl staining showed that the cortical neurons in the Sham group were deeply stained and exhibited a blue-violet color, and abundant Nissl bodies were found in the neurons with clear boundary (Fig. 2c). The structure in the I/R group was fuzzy, and a large number of Nissl bodies were absent in the neurons (Fig. 2c). Compared with the I/R group, the number of neurons in the ISO group was higher, and the Nissl bodies were more abundant in the neurons (Fig. 2c). Moreover, compared with the ISO group, the number of Nissl bodies in the I/R + Pir and ISO + Pir groups was significantly lower, and the staining was shallow (Fig. 2c). Similarly, pyramidal neurons in the CA1 region also showed this trend (Fig. 2d).

ISO Reduced MCAO Induced Apoptosis in the Cortex and Hippocampus After I/R Injury in Rats

In the Sham group, only a few TUNE-positive cells were found in the cerebral cortex (Fig. 3a, c). ISO post-conditioning decreased the cortical AI compared with the I/R group ($P < 0.05$, Fig. 3a, c). The TGF-β2 inhibitor noticeably increased the cortical AI ($P < 0.05$, Fig. 3a, c).

In the hippocampal CA1, I/R injury significantly increased the hippocampal AI compared with the Sham group ($P < 0.05$, Fig. 3b, d). The hippocampal AI in the

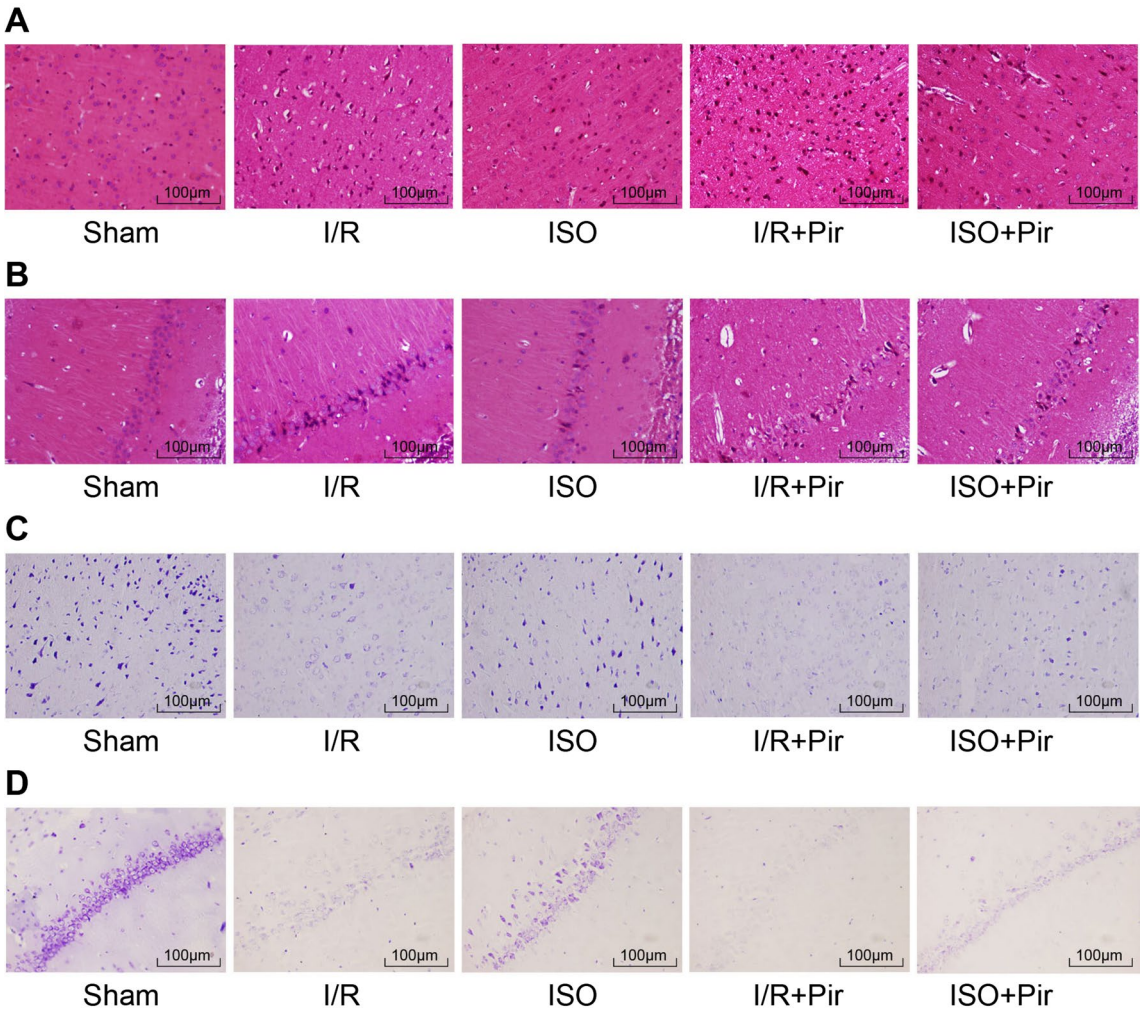

Fig. 2 Morphological evaluation of HE staining and Nissl staining in hippocampal CA1 and cortex after I/R injury. **a** Cell morphology by HE staining in the cortex. **b** Cell morphology by HE staining in the hippocampal CA1. **c** Nissl staining of the surviving cells in the cortex. **d** Nissl staining of the surviving cells in the hippocampal CA1. Data are presented as the mean ± SD (n = 5). Scale bars = 100 μm. $^{*}P < 0.05$ vs. Sham; $^{\#}P < 0.05$ vs. I/R; $^{\blacktriangle}P < 0.05$ vs. ISO; $^{\&}P < 0.05$ vs. I/R + Pir

ISO group decreased compared with that in the I/R group ($P < 0.05$, Fig. 3c). Similarly, the inhibitor increased the AI in the hippocampal CA1 ($P < 0.05$, Fig. 3b, d).

ISO Enhanced the Activation of the TGF-β2/Smad3 Signaling Pathway After I/R Injury in Rats

IF staining showed that TGF-β2 was located in the cytoplasm (Fig. 4a, b). Analysis of TGF-β2 optical density in the cortex showed that the expression level of TGF-β2 was low in the Sham group (Fig. 4a, c). After I/R injury, the expression level of TGF-β2 in the I/R group significantly increased, and ISO application increased the expression compared with the I/R group ($P < 0.05$, Fig. 4a, c). However, the TGF-β2 inhibitor attenuated the expression of TGF-β2 ($P < 0.05$, Fig. 4a, c). In addition, the expression level of TGF-β2 in the I/R + Pir group was the lowest among all groups ($P < 0.05$, Fig. 4a, c).

Analysis of TGF-β2 optical density in the hippocampal CA1 showed that TGF-β2 significantly increased after I/R injury, and ISO further promoted the expression. ($P < 0.05$, Fig. 4b, d). Fluorescence expression in the hippocampal CA1 of TGF-β2 also showed the same trend with the cortical expression after the application of inhibitor ($P < 0.05$, Fig. 4b, d).

Western blot analysis demonstrated a significant elevation of TGF-β2 ($P < 0.05$, Fig. 5a, b), p-Smad3 ($P < 0.05$, Fig. 5a, c) and p-Smad3/Smad3($P < 0.05$, Fig. 5a, e) in the ischemic tissue of the brain, whereas the expression level of Smad3 had no significant changes in all groups ($P > 0.05$, Fig. 5d). After treatment with ISO, the expression levels of TGF-β2 ($P < 0.05$, Fig. 5a, b), p-Smad3 ($P < 0.05$, Fig. 5a, c) and p-Smad3/Smad3 ($P < 0.05$, Fig. 5a, e) proteins were significantly upregulated compared with those in the I/R group.

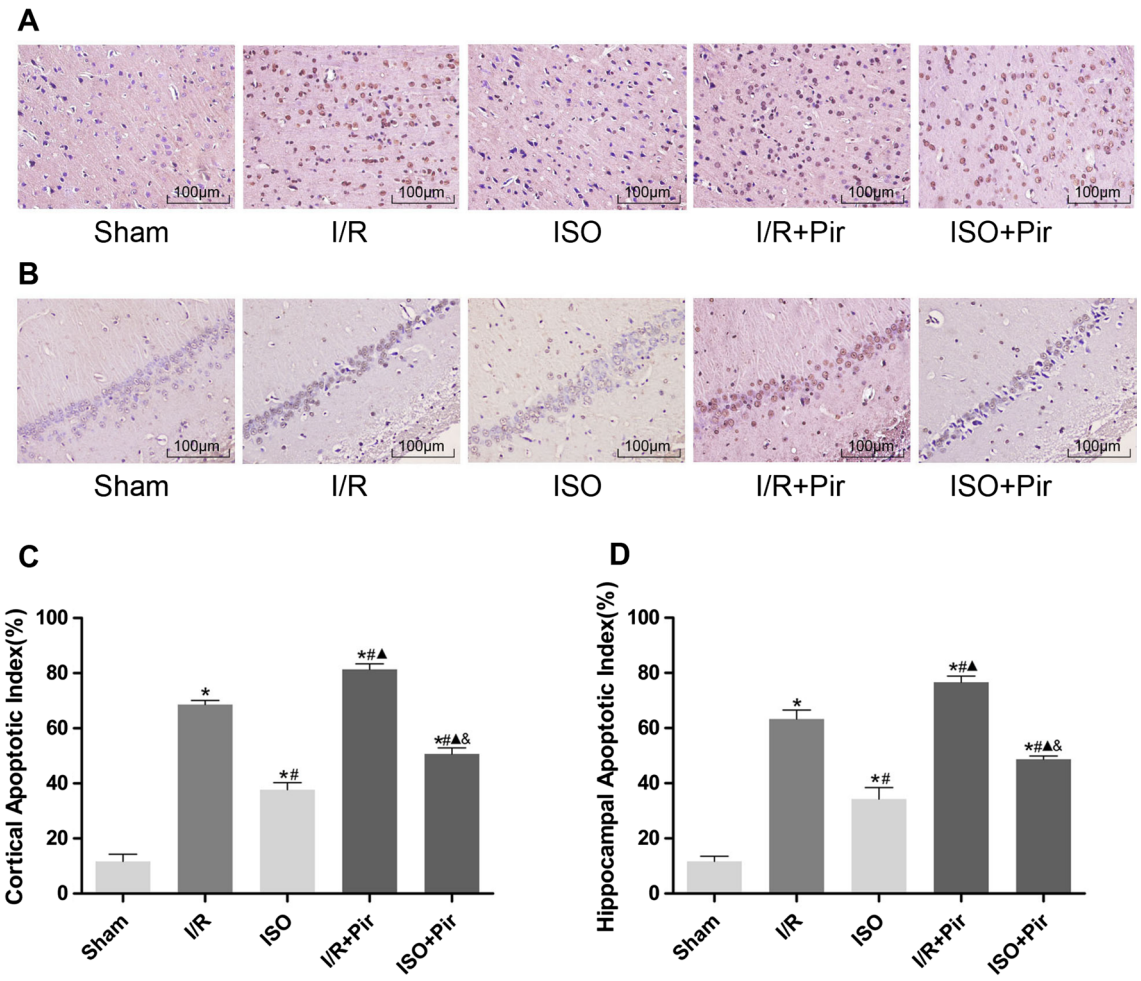

Fig. 3 TUNEL staining in the cerebral cortex and hippocampus after I/R injury in rats. **a** TUNEL staining in the cerebral cortex. **b** TUNEL staining in the hippocampus. **c** The TUNEL-positive cortical AI. **d** TUNEL-positive hippocampal AI. Data are presented as the mean ± SD (n = 5). Scale bars = 100 μm. $^{*}P < 0.05$ vs. Sham; $^{\#}P < 0.05$ vs. I/R; $^{\blacktriangle}P < 0.05$ vs. ISO; $^{\&}P < 0.05$ vs. I/R + Pir

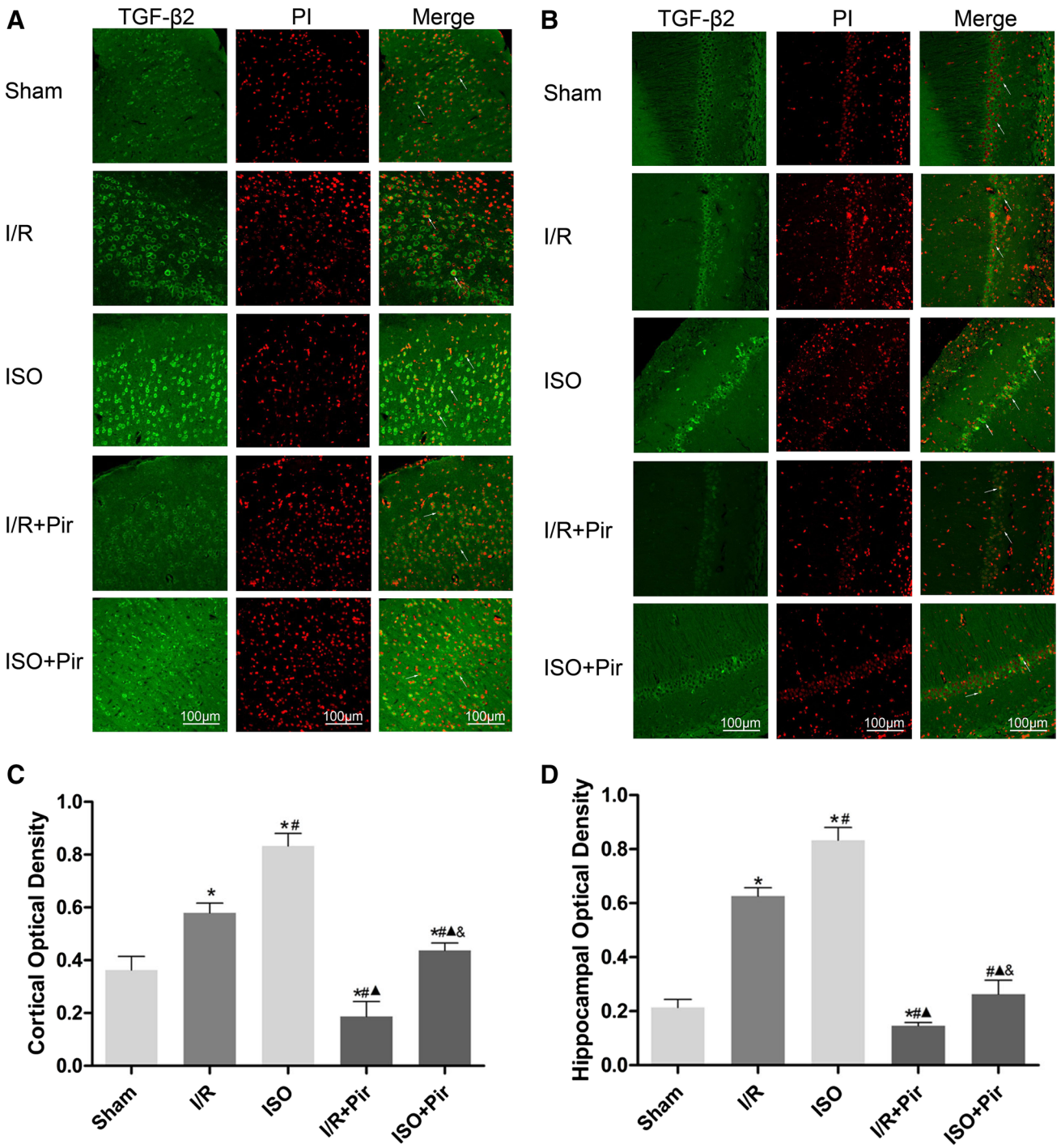

Fig. 4 Protein expression of the TGF-β2 in the ischemic cortex and hippocampus in rats. **a** IF of TGF-β2 in the cerebral cortex. **b** IF of TGF-β2 in the hippocampal CA1. **c** Cortical optical density analysis of TGF-β2 in each group. **d** Hippocampal optical density analysis of TGF-β2 in each group. The arrows (white) indicated TGF-β2 protein expression. Data are presented as the mean ± SD (n = 5). Scale bars = 100 μm. $^{*}P < 0.05$ vs. Sham; $^{\#}P < 0.05$ vs. I/R; $^{\blacktriangle}P < 0.05$ vs. ISO; $^{\&}P < 0.05$ vs. I/R + Pir

In the groups treated with TGF-β2 inhibitor, the expression levels of TGF-β2 ($P < 0.05$, Fig. 5a, b), p-Smad3 ($P < 0.05$, Fig. 5a, c) and p-Smad3/Smad3($P < 0.05$, Fig. 5a, e) were significantly downregulated.

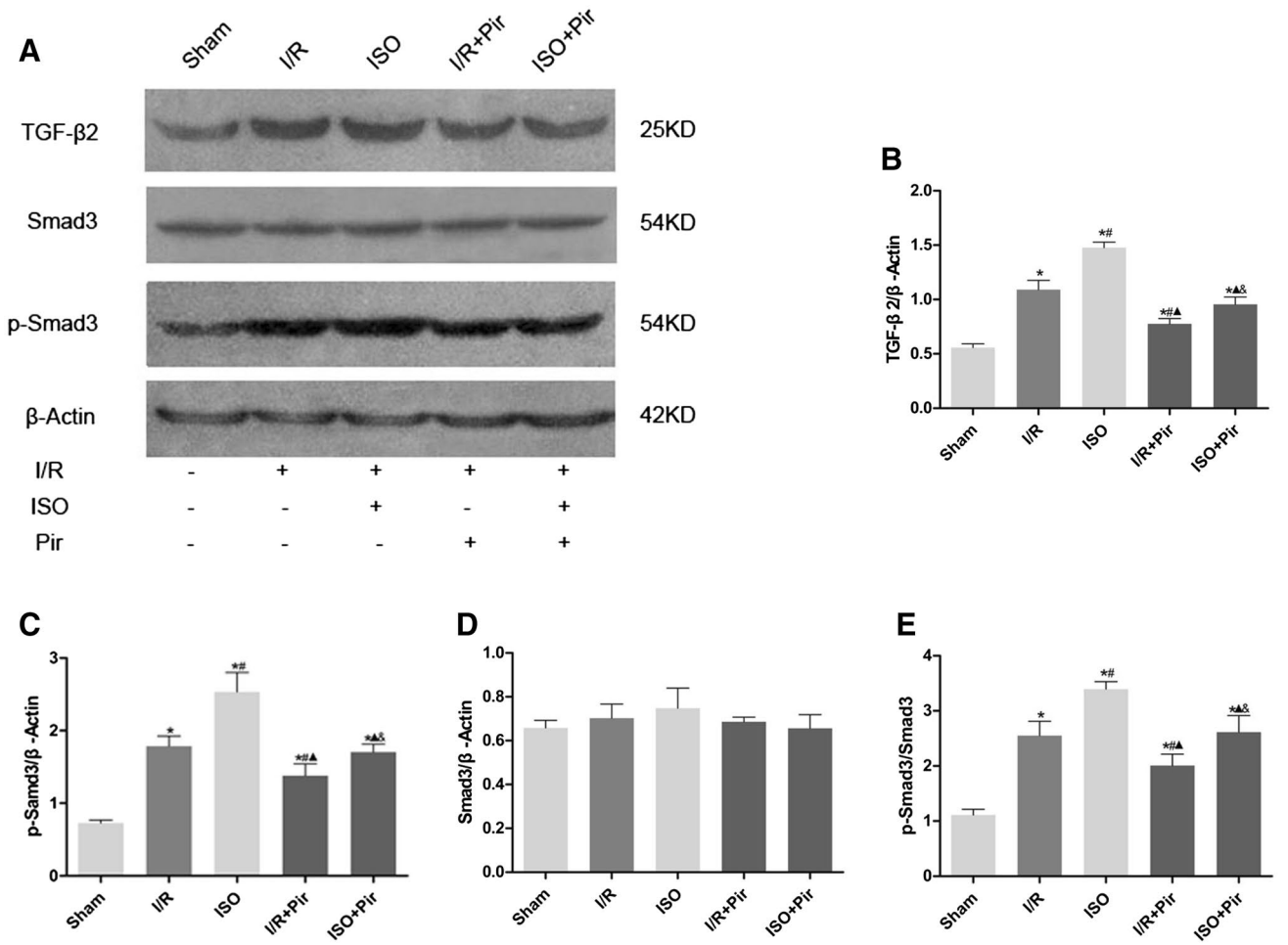

Fig. 5 Protein expression levels and Western blot analysis of TGF-β2/Smad3 signaling pathway in cortex. **a** Protein expression levels of TGF-β2, Smad3, p-Smad3, and β-Actin detected by western blot analysis. Quantitative analysis shows **b** TGF-β2/β-Actin, **c** p-Smad3/β-Actin, **d** Smad3/β-Actin, and **e** p-Smad3/Smad3. Data are presented as the mean ± SD (n = 5). $^{*}P < 0.05$ vs. Sham; $^{\#}P < 0.05$ vs. I/R; $^{\blacktriangle}P < 0.05$ vs. ISO; $^{\&}P < 0.05$ vs. I/R + Pir

ISO Increased Expression Levels of VEGF-A and CD34 Protein in the Brain After I/R Injury in Rats

In the IF assessment, we demonstrated that I/R injury significantly enhanced the expression levels of VEGF-A ($P < 0.05$, Fig. 6a, c) and CD34 ($P < 0.05$, Fig. 6b, d) in the cortical area compared with the I/R group, and 1.5% ISO further enhanced the expression levels of VEGF-A ($P < 0.05$, Fig. 6a, c) and CD34 ($P < 0.05$, Fig. 6b, d). The TGF-β2 inhibitor noticeably decreased the cortical expression of VEGF-A ($P < 0.05$, Fig. 6a, c) and CD34 ($P < 0.05$, Fig. 6b, d) induced by ISO post-conditioning. The inhibitor also reduced the expression of VEGF-A ($P < 0.05$, Fig. 6a, c) and CD34 ($P < 0.05$, Fig. 6b, d) after MCAO.

After 24 h reperfusion, I/R injury significantly enhanced the hippocampal optical density of VEGF-A ($P < 0.05$, Fig. 7a, c) and CD34 ($P < 0.05$, Fig. 7b, d) in the hippocampus compared with the I/R group, and 1.5% ISO further enhanced the expression levels of VEGF-A ($P < 0.05$, Fig. 7a, c) and CD34 ($P < 0.05$, Fig. 7b, d). The TGF-β2 inhibitor noticeably decreased the cortical expression of VEGF-A ($P < 0.05$, Fig. 7a, c) and CD34 ($P < 0.05$, Fig. 7b, d) induced by ISO post-conditioning. The inhibitor also reduced the expression of VEGF-A ($P < 0.05$, Fig. 7a, c) and CD34 ($P < 0.05$, Fig. 7b, d) after MCAO.

Western blot analysis demonstrated a significant elevation of VEGF-A ($P < 0.05$, Fig. 8a) and CD34 ($P < 0.05$, Fig. 8b) in the ischemic tissue of the brain. After ISO post-conditioning, the expression levels of VEGF-A ($P < 0.05$, Fig. 8a) and CD34 ($P < 0.05$, Fig. 8b) proteins were significantly upregulated compared with those in the I/R group. In the groups administered with the TGF-β2 inhibitor, the expression levels of VEGF-A ($P < 0.05$, Fig. 8a) and CD34 ($P < 0.05$, Fig. 8b) were significantly downregulated.

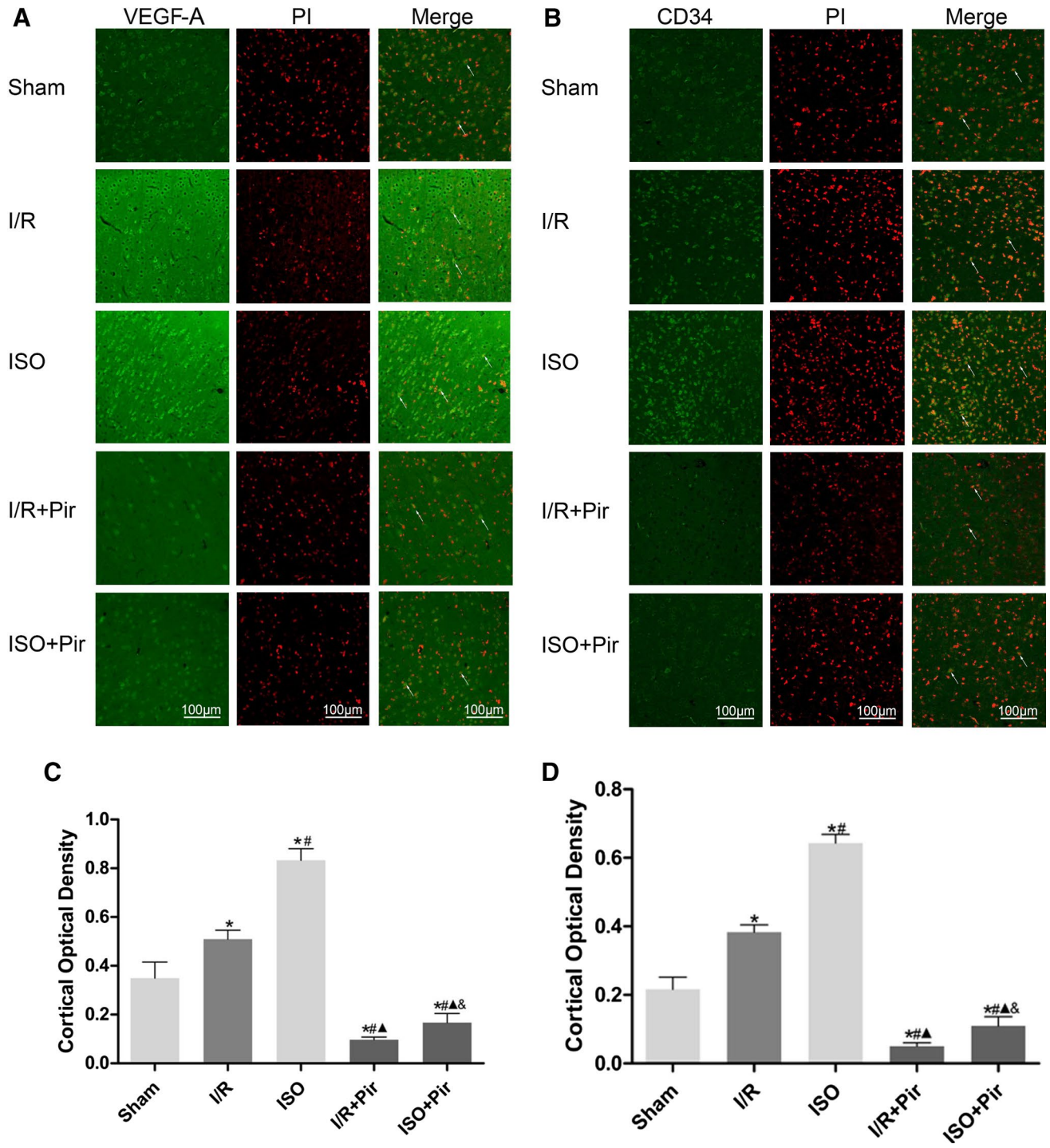

Fig. 6 Expression of the VEGF-A and CD34 in the ischemic cortex in rats. **a** IF of VEGF-A in the cerebral cortex. The arrows (white) indicated VEGF-A protein expression. **b** IF of CD34 in the cerebral cortex. The arrows (white) indicated CD34 protein expression. **c** Cortical optical density analysis of VEGF-A in each group. **d** Cortical optical density analysis of CD34 in each group. Data are presented as the mean ± SD (n = 5). Scale bars = 100 μm. $^{*}P < 0.05$ vs. Sham; $^{\#}P < 0.05$ vs. I/R; $^{\blacktriangle}P < 0.05$ vs. ISO; $^{\&}P < 0.05$ vs. I/R + Pir

Discussion

To the best of our knowledge, this study is the first in vivo mechanistic study to demonstrate that the TGF-β2/Smad3 signaling pathway mediates post-stroke ISO-induced neuroprotective effect in I/R injury, which was associated with the elevation of VEGF-A and CD34. We demonstrated that ISO can significantly lower infarct volumes

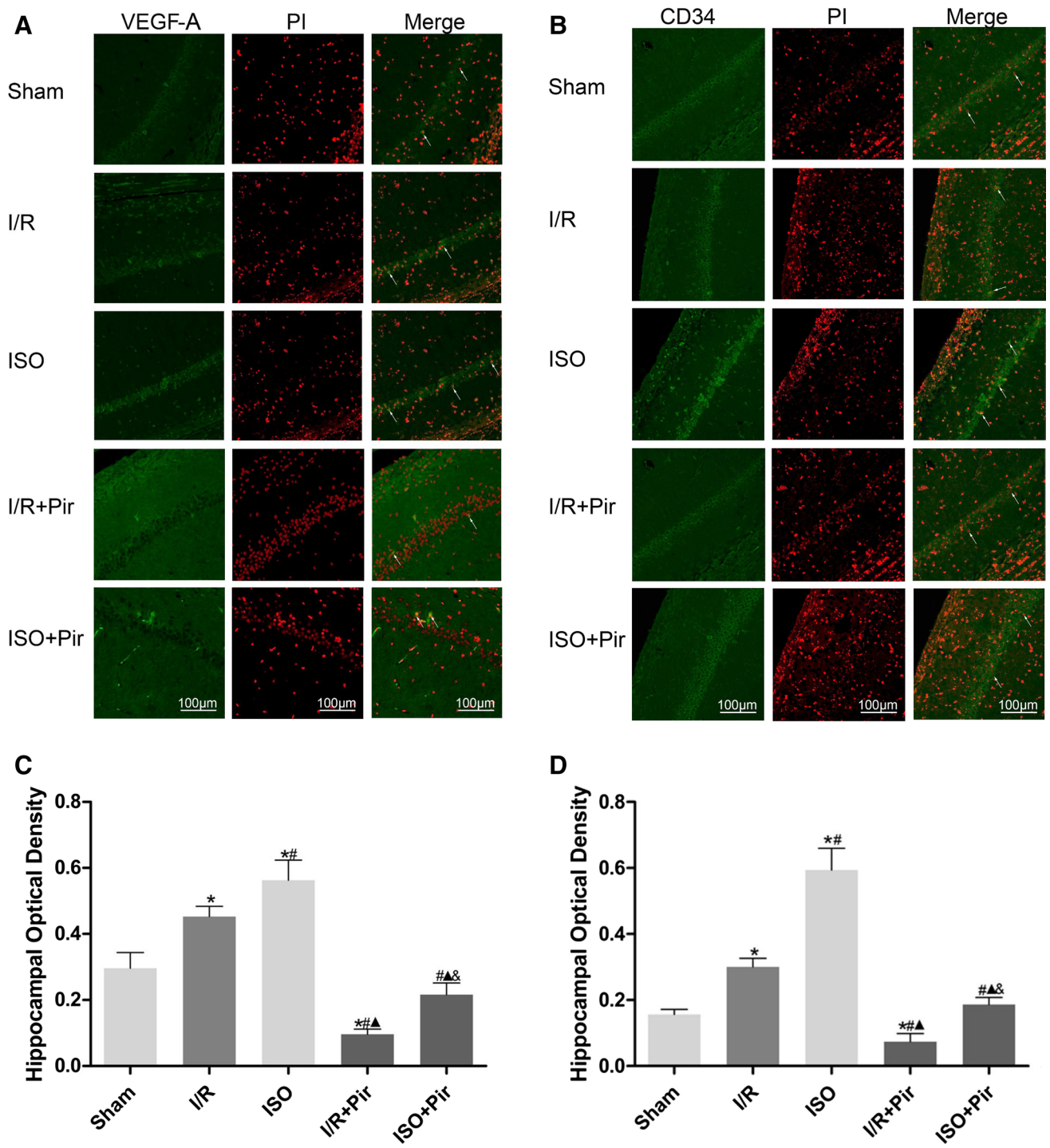

Fig. 7 Expression of the VEGF-A and CD34 in the ischemic hippocampus in rats. **a** IF of VEGF-A in the hippocampal CA1. The arrows (white) indicated VEGF-A protein expression. **b** IF of CD34 in the hippocampal CA1. The arrows (white) indicated CD34 protein expression. **c** Hippocampal optical density analysis of VEGF-A in each group. **d** Hippocampal optical density analysis of CD34 in each group. Data are presented as the mean ± SD (n = 5). Scale bars = 100 μm. $^{*}P < 0.05$ vs. Sham; $^{\#}P < 0.05$ vs. I/R; $^{\blacktriangle}P < 0.05$ vs. ISO; $^{\&}P < 0.05$ vs. I/R + Pir

and neurologic deficit scores, increase surviving neurons, and minimize damaged and apoptotic cells after cerebral I/R injury.

The present study revealed the protective effects of ISO in terms of ameliorating the ischemic insult. In line with our findings, Gaidhani, demonstrated that 90 min ISO

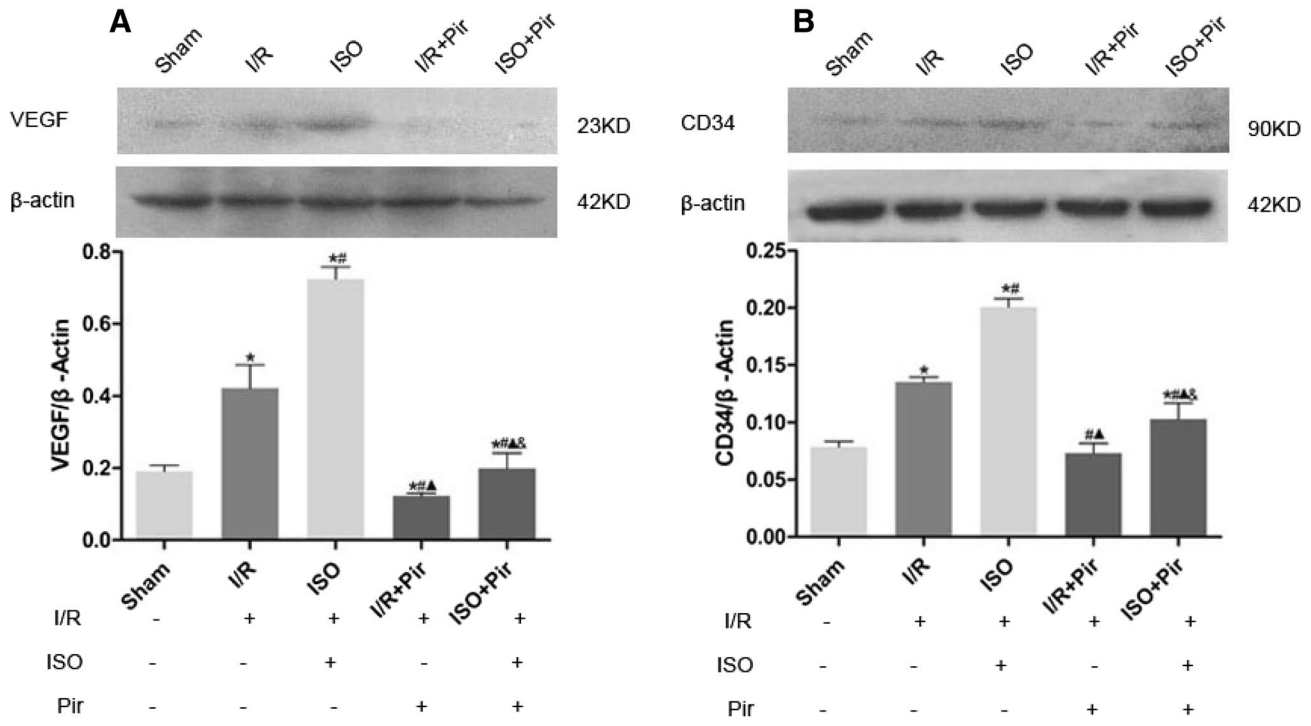

Fig. 8 Protein expression levels and Western blot analysis of VEGF-A and CD34 in cortex. **a** Protein expression levels of VEGF-A and quantitative analysis shows VEGF-A/β-Actin. **b** Protein expression levels of CD34 and quantitative analysis shows CD34/β-Actin. Data are presented as the mean ± SD (n = 5). $^{*}P < 0.05$ vs. Sham; $^{\#}P < 0.05$ vs. I/R; $^{\blacktriangle}P < 0.05$ vs. ISO; $^{\&}P < 0.05$ vs. I/R + Pir

anesthesia nearly completely protected brain tissues from tMCAO-induced injury [23]. Zhang et al. found that emulsified ISO improves the survival and neurological outcomes [24]. The results of IF manifested that ISO upregulated the expression of TGF-β2 in the ischemic cortex and hippocampal CA1 region. At the same time, TGF-β2 and p-Smad3 were upregulated after ISO post-conditioning at the protein level by Western blot. These results suggested that the activation of the TGF-β2/Smad3 signaling pathway was induced by ISO after I/R injury.

Conditioned medium from hippocampal cells proved that the proliferation and differentiation of hippocampal granule neurons was regulated by TGF-β2 [25]. In line with our results, various experimental studies revealed the neuroprotective effect of TGF-β2. An in vitro model identified that TGF-β2 was an essential neuroprotective factor [26]. Dhandapani et al. reported that 17 beta-estradiol increased the expression of the neuroprotective cytokine TGF-β2 to provide a mechanism of neuroprotection [27]. Moreover, TGF-β2 protein expression has been reported to increase persistently in neurons in ischemic animals and beneficial for transient cerebral ischemia [28]. Therefore, both previous reports and our study have indicated that the activated TGF-β2/Smad3 signaling pathway is critical to neuroprotection for ischemic cerebral injury.

VEGF promotes angiogenesis to ameliorate neurological function and neurovascular remodeling following I/R injury [29]. CD34 is an indirect marker of angiogenesis [30]. The present study showed that ISO post-conditioning significantly increased the expression levels of VEGF-A and CD34. The findings in this study are in line with the findings of recent studies. Zhao et al. observed that the increased VEGF expression was consistent with the decreased infarct volumes, improved neurological recovery and increased numbers of BrdU/NeuN-positive cells after cerebral I/R injury [31]. Ischemic injury increases the expression level of VEGF in cell cultures, and ischemic brains contribute to angiogenesis in experimental ischemic stroke [32]. Furthermore, Zheng et al. reported that the elevated expression levels of mRNA and protein of VEGF against cerebral ischemic injury [33]. Similarly, Zhang et al. showed that hyperforin treatment significantly increased the expression levels of VEGF and CD34 to against stroke [34]. In conclusion, ISO enhanced the expression of VEGF-A and CD34, which promoted angiogenesis and attenuated the I/R injury.

Next, we further determined whether the TGF-β2/Smad3 signaling pathway was involved in the angiogenesis induced by ISO. IF and Western blot analysis were performed to examine the expression levels of VEGF-A and CD34 after treatment with Pirfenidone. Our results

indicated that the expression levels of VEGF-A and CD34 were significantly downregulated accompanied with higher neurobehavioral scores, greater cerebral infarct volumes, and more damage and apoptotic neurons. This study suggested that the TGF-β2/Smad3 signaling pathway may regulate the expression of VEGF-A and CD34, which participate in the formation of the neurovascular unit post-stroke. Neurovascular units, the functional units in the brain, are composed of neurons, glial cells, and blood vessels, involving the complex interplay of biochemical and molecular mechanisms [35]. Currently, the focus of research on stroke pathophysiology has shifted from pure vascular concepts to the neurovascular unit. In recent years, neurovascular units have received increasing attention and have been considered to play critical roles in stroke recovery [36].

TGF-β is a multi-functional cytokine and regulates morphogenesis, proliferation, metabolism, apoptosis, and repair [37]. TGF-β is stored in the extracellular matrix and regulates the vascular matrix, resulting in cellular changes and angiogenesis [38]. Additionally, suberoylanilide hydroxamic acid pretreatment potently suppressed TGF-β2-driven cell proliferation, differentiation, ECM production, and angiogenic cytokine expression via inhibiting the Smad and non-Smad pathway [39]. However, contrary to the stimulated angiogenic factors and angiogenesis, Jeong et al. reported that TGF-β2 can decrease the expression of VEGF receptor-2 to inhibit the angiogenesis [40]. Indeed, the TGF-β signaling pathway is important in the development of the cardiovascular system and plays an unclear role in stimulating or inhibiting the formation of new blood vessels [41]. The molecular mechanism of pro- and anti-angiogenic properties in TGF-β are attributed to the stage and location of the pathological process [42]. However, unfortunately, there is currently no specific inhibitor of TGF-β2. According to the literature, the downregulation of TGF-beta is mediated at multiple levels. Pirfenidone leads to a reduction of TGF-beta2 mRNA levels and of the mature TGF-beta2 protein due to decreased expression and direct inhibition of the TGF-beta pro-protein convertase furin [19]. Therefore, before the start of the study, we finally selected Pirfenidone by consulting the literature and reagents.

Collectively, the protective role of ISO might be mediated by the TGF-β2/Smad3 signaling pathway and related to the enhancement of VEGF and CD34 after I/R injury in rats. The mechanism of ISO in attenuating stroke and brain ischemia warrants further investigations.

Acknowledgements This study was performed at the Key Laboratory of Xinjiang Endemic and Ethnic Diseases of Xinjiang Provincial Department of Physiology, School of Medicine, Shihezi University. This work was supported by the National Natural Science Foundation of China (Grant Number: 81860249, 81360203).

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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