ARTICLE

Tranilast Treatment Attenuates Cerebral Ischemia-Reperfusion Injury in Rats Through the Inhibition of Inflammatory Responses Mediated by NF-κB and PPARs

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Ischemia-reperfusion injury (IRI) occurs when blood supply returns to tissue after interruption, which is associated with life-threatening inflammatory response. Tranilast is a widely used antiallergic agent in the treatment against bronchial asthma and keloid. To study the function of tranilast, we used IRI in rat models. The brain tissues of IRI rats with or without tranilast treatment were collected. Neuronal apoptosis in the brain was detected by terminal deoxynucleotidyl transferase nick end labeling assay, and proinflammatory cytokine levels were measured by quantitative real-time polymerase chain reaction and enzyme-linked immunosorbent assay. The expression levels of nuclear factor-kappa B (NF-κB), inhibitor of κB (IκB) and peroxisome proliferator-activated receptors (PPARs) were detected by Western blot. The results showed that tranilast treatment reduced neuronal apoptosis in the brain of IRI rats. Tranilast enhanced the short-term memory and long-term memory to novel object recognition paradigm. Tranilast treatment decreased the messenger RNA (mRNA) and protein levels of multiple proinflammatory cytokines, and affected NF-κB and inhibitor of kappa B protein expressions. Tranilast promoted the expressions of PPAR-α and PPAR-γ. Our findings demonstrate that tranilast treatment could attenuate cerebral IRI by regulating the inflammatory cytokine production and PPAR expression. Tranilast is a potential drug for IRI treatment in the clinic.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?
☑ Tranilast is a widely used antiallergic agent in the treatment against bronchial asthma and keloid.

WHAT QUESTION DID THIS STUDY ADDRESS?
☑ It presented a way to study the function of tranilast in ischemia-reperfusion injury (IRI).

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?
☑ Tranilast treatment could attenuate cerebral IRI by regulating the inflammatory cytokine production and proliferator-activated receptor expression.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?
☑ This study implies the potential of tranilast as a potential drug for IRI treatment in the clinic.

Ischemia-reperfusion injury (IRI) is serious tissue damage arising from the restoration of the blood supply after ischemia.[1] The lack of oxygen, adenosine triphosphate, and nutrients from the blood leads to functional and metabolic disorders of the organs. As a consequence, restoration of the blood flow results in the oxidative damage and inflammation rather than recovery. IRI is mainly caused by calcium overload, pH paradox, oxidative stress, immune cell infiltration, and mitochondrial injury.[2] Oxygen-sensitive organs, such as the brain and myocardium, are particularly prone to the disease. Thus, it is a common occurrence following cerebral infarction, stroke, myocardial

infarction, organ transplantation, and trauma.[3] Clinical treatment of IRI includes therapeutic hypothermia, hydrogen sulfide treatment, cyclosporine, cannabinoids, superoxide dismutase, metformin, and stem cell therapy.[4] IRI is still a life-threatening risk to the patients because it causes inflammatory cytokine storm, arrhythmias, and infarction. However, the exact pathophysiological and biological mechanisms of IRI remain incompletely understood. Therefore, study of IRI pathogenesis and development of better therapeutic strategies are of high priority.

IRI is usually associated with life-threatening inflammatory responses, which is one of the leading causes of

brain injury and mortality. In human patients who undergo a stroke, previous studies have determined the proinflammatory cytokine profile after acute cerebral ischemia, including CD40L, interferon-r, interleukin (IL)-17, IL-1, IL-6, IL-8, and tumor necrosis factor alpha (TNF-α),[5] which indicated that the protein levels of IL-6, IL-8, and TNF-α were significantly increased in the plasma of patients who undergo a stroke. In addition, elevation of IL-6 is correlated with the disease severity and the poor prognosis.[5,6] In mice, the messenger RNA (mRNA) level of TNF-α was induced as early as 4 hours after both transient and permanent central neuron system ischemia.[7] Subsequently, the mRNA levels of IL-6 (16–18 hours) and TGF-β1 (96 hours) were also increased.[7] Mechanically, several signaling pathways were reported to exert critical roles in modulating the IRI-induced inflammatory responses, such as nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinases.[8] Moreover, the peroxisome proliferator-activated receptor-γ (PPAR-γ) also has an important role in modulating the inflammatory response and oxidative stress during reperfusion.[9] Two PPAR-γ agonists, rosiglitazone and pioglitazone, protected the rats against the cerebral IRI-induced inflammation.[10] These evidences suggest that the modulation of acute inflammatory responses has a critical role in the control of IRI. Thus, there is an imperative need to develop novel anti-inflammation agents for the treatment of IRI.

Tranilast, with the chemical name N-(3′,4′-dimethoxycinnamoyl) anthranilic acid, is an analog of a metabolite of tryptophan.[11] Tranilast is an antiallergic agent widely used in the treatment for bronchial asthma, keloid and hypertrophic scar, arthritis, and allergic pink eye.[12–14] It has been shown to inhibit the production of various proinflammatory cytokines in different cell types. For example, it decreased the TNF-α–positive mast cells and reduced the mRNA level of TNF-α, IL-6, stem cell factor, cathepsin-K, and receptor activator of NF-κB in the paws of arthritic mice.[15] Moreover, it suppressed the production of nitric oxide, prostaglandin E2, TNF-α, and IL-1b in RAW264.7 macrophage cells upon lipopolysaccharide stimulation.[16] In addition, it inhibited the release of histamine by mast cells. However, the anti-inflammation function of tranilast on the neuronal system is poorly understood. Here, we reported that tranilast significantly reduced neuronal apoptosis and proinflammatory cytokine production after ischemia/reperfusion (I/R) in rats. Further study showed that tranilast protected the rats from IRI through regulating NF-κB and PPARs.

METHODS
Animal model
Male Sprague-Dawley rats (4–6 weeks) were ordered from Nanjing Model Animal Institute (Nanjing, China). The procedures of experimental animals were approved and complied with the Guide for the Care and Use of Laboratory Animals. The rats were kept for 2 weeks before the experiment initiation. The IRI model was established as described previously.[8] Briefly, rats were placed in a supine position while under anesthesia, and an incision was made on the right side of the neck to expose the right common carotid artery, external carotid artery, and internal carotid artery. A focal cerebral ischemia was then conducted in the internal carotid artery with a 50-mm nylon monofilament, which was pulled out carefully to induce reperfusion 2 hours later. The same procedure was performed in sham rats, only without nylon filament inserted into the right carotid artery to induce brain ischemia and reperfusion. Rats were randomly divided into four groups: group 1: sham; group 2: model group (I/R-induced injury); group 3: sham group with tranilast treatment; and group 4: model group with tranilast treatment. Tranilast was administered orally (50 mg/kg) every day for 6 weeks.

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling staining
For terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) staining, heads of rats were fixed by 4% paraformaldehyde and cryoprotected in 30% sucrose. Frozen brain sections were incubated in 0.25 U/mL terminal deoxynucleotidyl transferase and 20 mM biotinylated-16-dUTP (Sigma, St. Louis, MO) for 60 minutes at room temperature. After that, the sections were washed three times in phosphate-buffered saline and then blocked for 1 hour with 1% bovine serum albumin. The sections were then incubated with fluorescein isothiocyanate-coupled streptavidin 1:100 in phosphate-buffered saline for 30 minutes, rinsed, and then counterstained with the 4′,6-diamidino-2-phenylindole nuclear stain (1 mg/mL; Sigma).

Western blot analysis
To test the expression of inhibitor of kappa B (IκB) and NF-κB, cells were lysate by NP-40 lysis buffer. Antibodies used were against IκB (#9242; Cell Signaling), NF-κB (#9242; Cell Signaling), p-NF-κB (#3033; Cell Signaling), and β-actin (ab8227; Abcam, Cambridge, MA). Western blot analysis was performed as described previously.[17]

Real-time polymerase chain reaction analysis
Total RNAs were extracted from tissues by using TRIzol reagent (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions. The quantitative polymerase chain reaction (PCR) assay was performed on a 7,500 real-time PCR system with SYBR Green Real-time PCR Master Mix (Takara, Dalian, China). The quantitative value was expressed using the $2^{-\Delta\Delta Ct}$ method. The primers were as follows: TNF-α 5′-CCCTCACACTCAGATCATCTTCT-3′ (forward), 5′-GCTACGACGTGGGCTACAG-3′ (reverse); IL-1β 5′-GGCTGCTTCCAAACCTTTGA-3′ (forward), 5′-GAAGACACGGATTCCATGGT-3′ (reverse); IL-6 5′-TAGTCCTTCCTACCCCAATTTCC-3′ (forward), 5′-TTGGTCCTTAGCCACTCCTTC-3′ (reverse); IL-8 5′-AATTCTCGAGTCGCGAATGGCTGCTCAAGGCTG-3′ (forward), 5′-ATTACGGCCGTCGCGATTAGGCATCACTGCCTG-3′ (reverse); and β-actin 5′-TCACCAACTGGGACG-3′ (forward), 5′-GCATACAGGGACAACA-3′ (reverse).

Serum collection
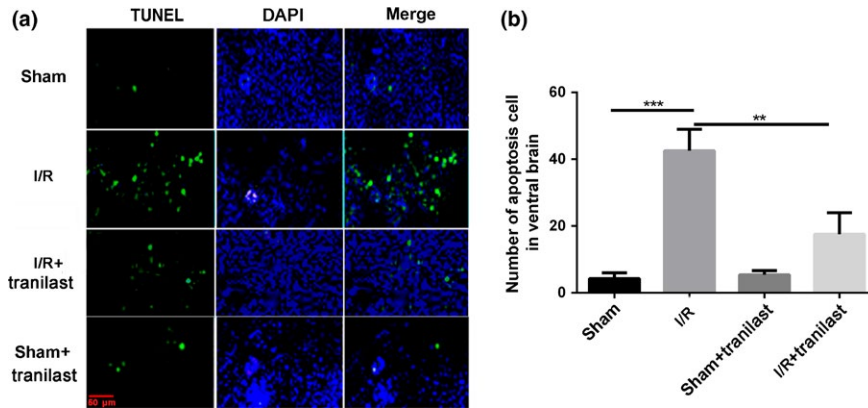The 0.5–1 mL-blood samples were collected by cardiac puncture from rats and were stored at room temperature

Figure 1 Effects of tranilast treatment on neuronal apoptosis 24 hours after ischemia/reperfusion (I/R) in ventral brain of rats. (**a**) Representative photomicrographs of triphosphate nick-end labeling (TUNEL) staining in the four experiments groups. Cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; blue). Brain sections were collected for TUNEL staining after 60 minutes of ischemia and 120 minutes of reperfusion (magnification ×200). Scale bar = 50 μm. (**b**) The quantification of TUNEL-positive cells. Values are represented as mean ± SD, $n = 8$ for each group. **$P < 0.01$, ***$P < 0.001$, one-way analysis of variance followed by a Tukey's *post hoc* test.

for 30 minutes to allow the blood to clot. Then the blood sample was centrifuged at 4,200 g for 5 minutes, and the serum was transferred to a new tube and stored at −80°C until further use.

Novel object recognition task
Rats were given a 30-minute habituation time in a plastic cage without objects. After that, objects were placed in the middle of the box. Rats were allowed to freely explore for 10 minutes. During the training, the mean speed and the distance traveled by rats were evaluated. Short-term memory (STM) and long-term memory (LTM) were evaluated after 1.5 and 24 hours after training, respectively. Rats were returned to the plastic box, which contained the original objects and a novel object. Exploration time was defined as the time spent by the rats sniffing or touching the object with the nose.

Enzyme-linked immunosorbent assay
TNF-α, IL-1β, IL-6, and IL-8 in the serum were assayed using the following enzyme immunoassay kits[18]: TNF-α (RTA00; R&D Systems, Minneapolis, MN), IL-1β (RLB00; R&D Systems), IL-6 (R6000B; R&D Systems), IL-8 (MBS282844; MyBioSource, San Diego, CA), according to the procedure described by the manufacturer.

Statistical analysis
All P values were calculated using the SPSS software. All error bars represent mean ± SD derived from three independent experiments. In all cases, any P values < 0.05 were considered to be statistically significant.

RESULTS
Tranilast treatment reduces I/R-induced neuronal apoptosis
Cerebral IRI in rats is a frequently used animal model. To investigate whether tranilast exerted an effect on I/R-mediated injury, we tested the neuronal apoptosis upon tranilast treatment. We collected the brain sections of rats

after I/R and then compared TUNEL staining between each group. The rats in the I/R group showed significantly increased neuronal apoptosis compared with the sham group, whereas tranilast treatment dramatically inhibited the neuronal apoptosis (**Figure 1a**). The quantification of TUNEL-positive cells also indicated that tranilast could remarkably reduce I/R-mediated cell apoptosis in the ventral brain of rats (**Figure 1b**).

Tranilast is beneficial for short-term and long-term memory of rats
In addition to the protection against neuronal injury, novel object recognition (NOR) paradigm is another important assessment of brain injury. To further examine the role of tranilast in NOR, we used a brain injury model to examine the STM and LTM of rats. The rats with head injuries were administrated with or without tranilast, and their exploration time was measured. After training the rats for 1.5 hours or 24 hours, respectively, we found that the exploration time was increased after I/R, whereas tranilast treatment resulted in a decreased exploration time (**Figure 2a,b**). Those results suggested that tranilast could enhance the NOR capacity of both STM and LTM.

Tranilast treatment inhibits mRNA and protein expression of proinflammatory cytokines
Considering that proinflammatory cytokines play a critical role in IRI, we next tested whether tranilast treatment could affect the expression of multiple cytokines. We collected the cortical tissues from indicated groups and then performed RT-PCR to test the mRNA levels of TNF-α, IL-1β, IL-6, and IL-8. The results showed that the mRNA expressions of TNF-α (**Figure 3a**), IL-1β (**Figure 3b**), IL-6 (**Figure 3c**), and IL-8 (**Figure 3d**) were upregulated in the I/R group compared with the sham group. However, their expressions were significantly decreased when the rats were treated with tranilast (**Figure 3a,b**), which indicated that tranilast suppressed I/R-mediated brain injury
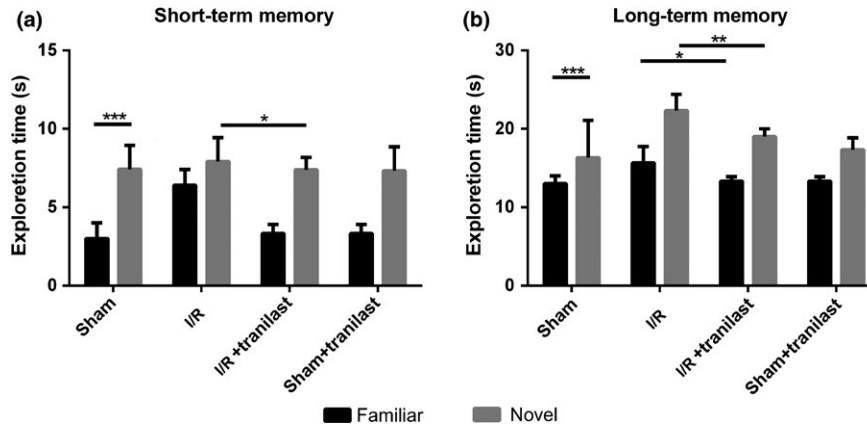
Figure 2 Effects of tranilast treatment on short-term memory (STM) and long-term memory (LTM) assessed by the novel object recognition paradigm (NOR). (a) Test phase of animals that underwent STM task. (b) Test phase of animals that underwent LTM task. Data were analyzed via two-way analysis of variance with repeated measures followed by Bonferroni *post hoc* and unpaired Student's *t*-test, when necessary. Values are represented as mean ± SD, *n* = 8 for each group. * *P* < 0.05,** *P* < 0.01, *** *P* < 0.001. I/R, ischemia/reperfusion.
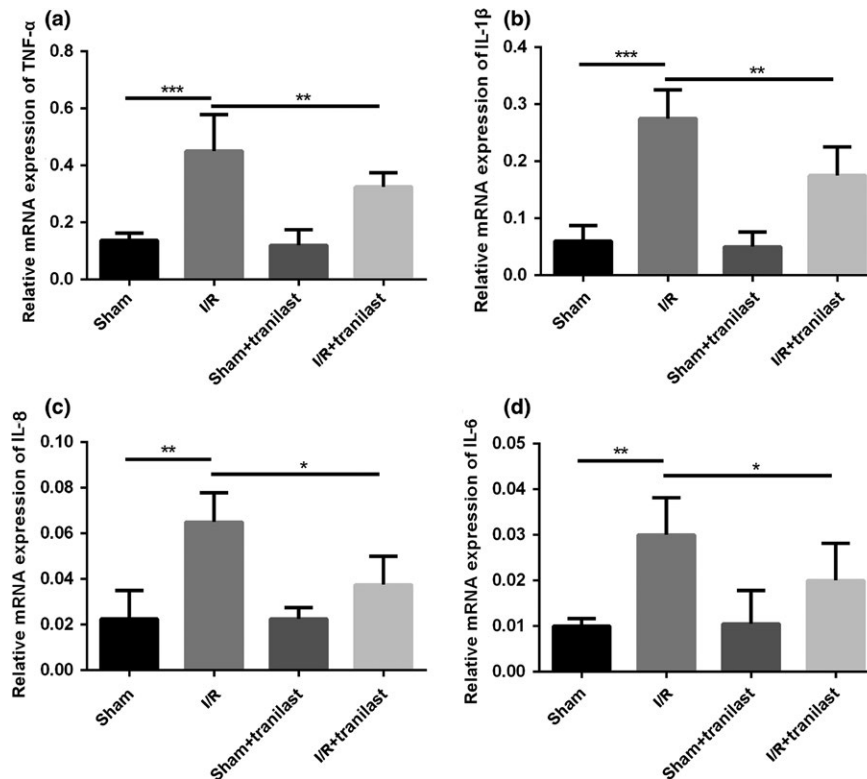

Figure 3 Effects of tranilast treatment on the messenger RNA (mRNA) levels of inflammatory mediators of tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, IL-6, and IL-8 after ischemia/reperfusion (I/R) injury in rats. (**a**–**d**) The mRNA levels of TNF-α, IL-1β, IL-6, and IL-8 in cortical tissue were determined by real-time polymerase chain reaction. Values are represented as mean ± SD, *n* = 6 for each group. *P < 0.05, **P < 0.01, ***P < 0.001, one-way analysis of variance followed by a Tukey's *post hoc* test.

by inhibiting the expression of these proinflammatory cytokines.

To further investigate the role of tranilast on the regulation of inflammatory cytokine production, we tested the protein levels of these cytokines. We collected serum samples from each group and then performed enzyme-linked immunosorbent assay analysis. Consistent with the previous results on mRNA levels, tranilast could also decrease the protein levels of TNF-α (**Figure 4a**), IL-1β (**Figure 4b**), IL-6 (**Figure 4c**), and IL-8 (**Figure 4d**). The above results demonstrated that tranilast treatment inhibited the inflammatory cytokines' production.
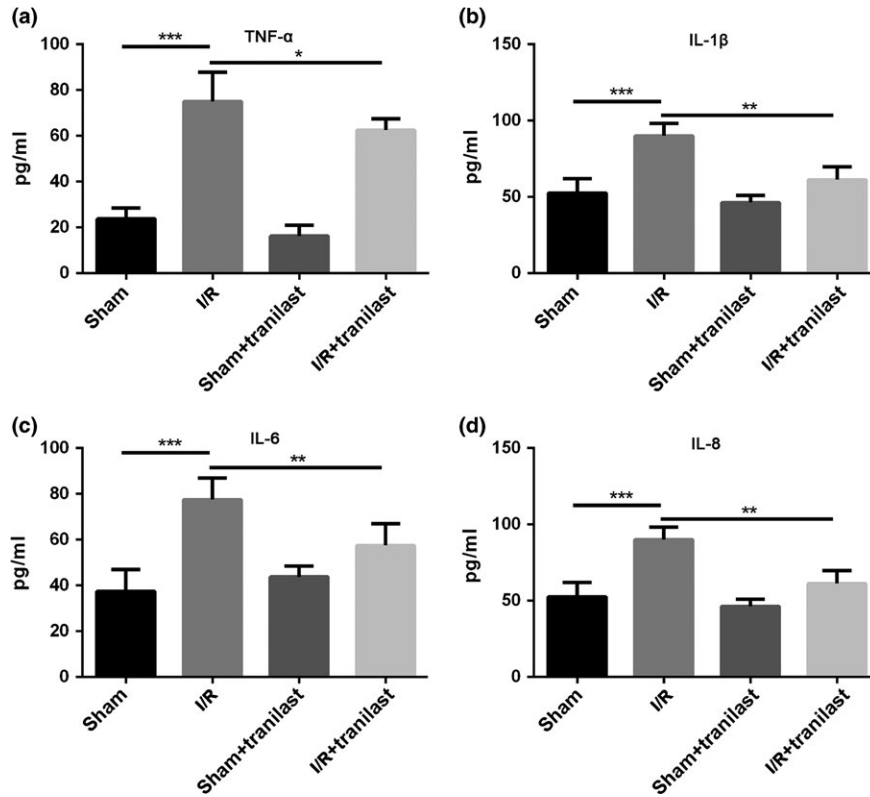
Figure 4 Effects of tranilast treatment on the protein levels of inflammatory mediators of tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β, IL-6, and IL-8 after ischemia/reperfusion (I/R) injury in rats. (**a–d**) TNF-α, IL-1β, IL-6, and IL-8 levels in the serum, as determined by enzyme-linked immunosorbent assay. Values are represented as mean ± SD, $n = 6$ for each group. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, one-way analysis of variance followed by a Tukey's *post hoc* test.
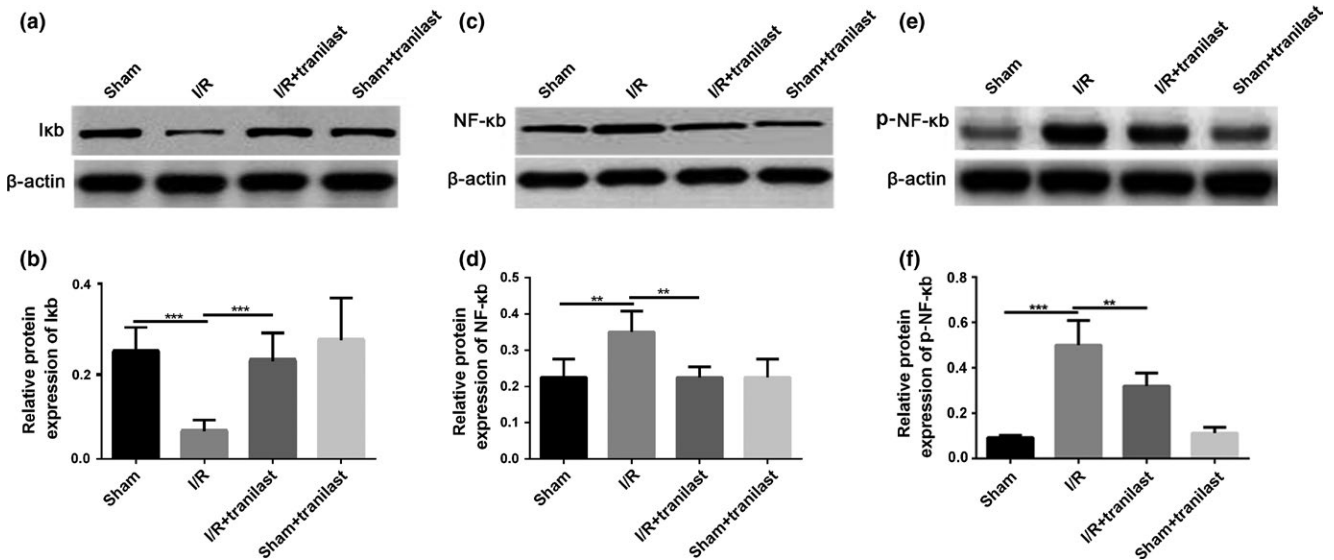

Figure 5 Effects of tranilast treatment on the protein level of nuclear factor-kappa B (NF-κB) signal in ischemia/reperfusion (I/R) rats. (**a**) Western blotting bands of inhibitor of κB (IκB). (**b**) The relative protein expression of Iκb. (**c**) Western blotting bands of NF-κb. (**d**) The relative protein expression of NF-κb. (**e**) Western blotting bands of phosphor-NF-κb NF-κb (p-NF-κb). (**f**) The relative protein expression of p-NF-κb. Values are represented as mean ± SD, $n = 8$ for each group. $**P < 0.01$, $***P < 0.001$, one-way analysis of variance followed by a Tukey's *post hoc* test.
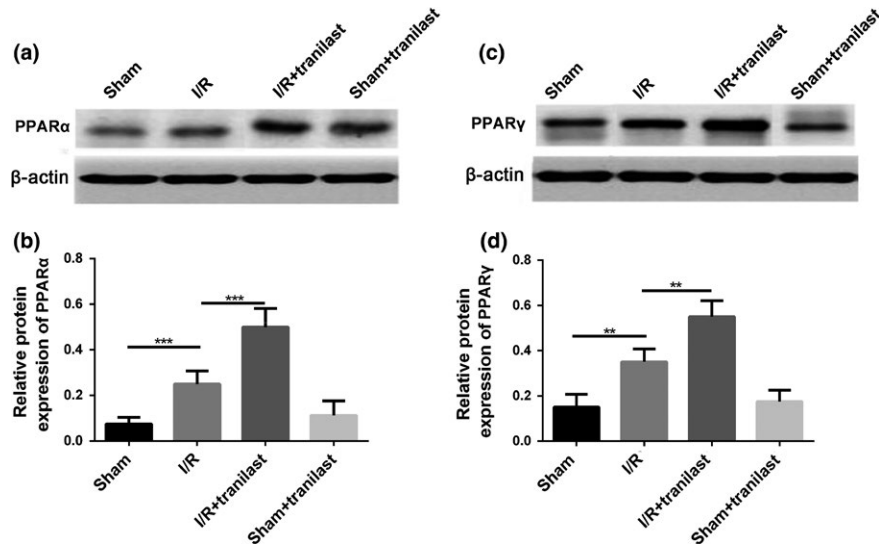
Figure 6 Effects of tranilast treatment on the protein levels of peroxisome proliferator-activated receptor (PPAR)-α and PPAR-γ in ischemia/reperfusion (I/R) rats. (**a**) Western blotting bands of PPAR-α. (**b**) The relative protein expression of PPAR-α. (**c**) Western blotting bands of PPAR-γ. (**d**) The relative protein expression of PPAR-γ. Values are represented as mean ± SD, $n = 8$ for each group. **$P < 0.01$, ***$P < 0.001$, one-way analysis of variance followed by a Tukey's *post hoc* test.

Tranilast treatment affects the expression of IκB and NF-κB

It is well known that NF-κB signaling is involved in the regulation of multiple inflammatory cytokines' production; we, therefore, investigated the effect of tranilast on the expression levels of IκB and NF-κB. We detected the expression of IκB and NF-κB in the brain tissues collected 24 hours after IRI, with or without tranilast treatment. The results showed that the protein level of IκB was reduced after IRI, whereas IκB expression was restored by tranilast treatment (**Figure 5a,b**). The degradation of IκB is a key step for the activation of NF-κB signaling. Thus, we also detected the protein level of NF-κB after IRI. We found that NF-κB expression was upregulated in the brains of IRI rats, whereas tranilast could impair this effect (**Figure 5c,d**). In addition, we also detected the expression levels of phosphor-NF-κB (p-NF-κB) in different groups. We found that I/R could significantly upregulate the p-NF-κB, whereas tranilast could reduce the expression of p-NF-κB (**Figure 5e,f**). Those results indicated that tranilast inhibited the production of inflammatory cytokines by regulating the expression of IκB, NF-κB, and p-NF-κB.

Tranilast treatment enhances the expression of PPAR-α and PPAR-γ

PPARs were reported to be the key regulators of inflammatory responses. In order to further explore whether PPARs were involved in the neuroprotection against I/R, the expression levels of PPAR-α and PPAR-γ were detected in the brain tissues with or without tranilast treatment. We found that tranilast treatment increased the protein level of PPAR-α in IRI rats (**Figure 6a,b**). Consistently, the expression of PPAR-γ was also upregulated by tranilast when compared with IRI rats without tranilast administration (**Figure 6c,d**). In conclusion, our data demonstrated that tranilast treatment enhanced the protein levels of PPAR-α and PPAR-γ, leading to the neuroprotection against IRI in rat model.

DISCUSSION

Tranilast is an orally active antiallergic and anti-inflammatory agent. In humans, it is widely used in atopic dermatitis, allergic rhinitis, and asthma treatment.[12,15] In rats, the tranilast and methylprednisolone combination therapy has exhibited an effective anti-inflammation function during acute spinal cord injury.[19] Tranilast administration also reduced the serum levels of IL-6 and IL-13 in rats, which protected the rats from acute liver injury and hepatic encephalopathy.[20] However, the role of tranilast in neuronal systems remains poorly understood. IRI is one of the leading causes of the disability and death in patients after stroke, cerebral infarction, or organ transplantation.[21] It is usually associated with severe acute inflammation, such as systemic cytokine storm and substantial lymphocytes infiltration.[22,23] Thus, the development of anti-inflammation drugs for IRI treatment is of high priority. Our results have shown that tranilast exerts a neuronal protective effect during I/R in rats. The tranilast-administrated rats have significantly reduced neuron apoptosis and enhanced STM and LTM compared with the control group. We have further demonstrated that the levels of proinflammatory cytokines, including IL-6, IL-1b, IL-8, and TNF-α, were significantly decreased by tranilast administration during IRI. Taken together, our study reveals a previously unrecovered role of tranilast in neuronal inflammation treatment. Further studies and clinical data of patients are required for the medical translation in human diseases.

The NF-κB signaling pathway has an important role in regulating inflammatory responses, cell proliferation, and

differentiation, as well as cell death and function.[24] NF-κB activation is widely implicated in various inflammatory and autoimmune diseases, such as inflammatory bowel disease, multiple sclerosis, asthma, and arthritis, making it a critical target for new anti-inflammatory drugs.[25] At steady state, the NF-κB dimers are sequestered by IκB in the cytoplasm. Upon stimulation, IκB protein is degraded to release the NF-κB, which then enters into the nucleus where it activates the expression of proinflammatory cytokines, such as IL-1, IL-6, and TNF-α.[25] PPARs are members of the nuclear hormone receptor family, which have three isotypes: PPAR-α, PPAR-β, and PPAR-γ.[9,26] It has been reported that PPARs inhibit the production of pro-inflammatory cytokines through limiting NF-κB activation. PPARs could interact with NF-κB and inhibit NF-κB binding to their target genes.[9] In line with a previous study, our results showed that IκB expression in the brain tissue was significantly decreased in IRI rats compared with the sham group. However, after tranilast administration, the IκB level in the brain tissue of IRI rats was increased compared with the control group, suggesting that tranilast plays an important role in downregulation of NF-κB signaling during IRI. Besides, our study has provided evidence that both PPAR-α and PPAR-γ are involved in the neuron-protective effect of tranilast. Tranilast treatment significantly increased the protein expressions of PPAR-α and PPAR-γ in the brain of IRI rats. Consistent with our results, it has been reported that an anti-inflammation Chinese herb component Icariin alleviated IRI in rats through the regulation of NF-κB and PPARs.[26] Further analyses are needed to investigate whether other signaling pathways are also involved in the neuron-protective effect of tranilast.

In summary, our findings reveal a neuron-protective role of tranilast in IRI, and suggest it as a promising drug for IRI treatment. Tranilast inhibits the proinflammatory cytokine productions via regulating NF-κB signaling pathway and PPARs. These results help us better understand the molecular mechanisms of IRI, which will open a new door for more targeted therapies.

CONCLUSIONS

Our findings demonstrated that tranilast treatment could attenuate cerebral IRI by regulating the inflammatory cytokine production and PPARs expression. Tranilast is a potential drug for IRI treatment in the clinic.

Conflict of Interest. The authors declared no competing interests for this work.

Author Contributions. Y.Z. and J.Z. wrote the manuscript. Y.Z. and J.Z. designed the research. Y.Z. and J.Z. analyzed the data.

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