# PTP1B inhibitor alleviates deleterious microglial activation and neuronal injury after ischemic stroke by modulating ER stress-autophagy axis via PERK signaling in microglia

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**Research**

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# Abstract

Background : Cerebral ischemia/reperfusion (IR) after ischemic stroke causes microglial activation which lead to neuronal injury. Protein tyrosine phosphatase 1B (PTP1B) emerges to be a positive regulator of neuroinflammation, yet the effect of its inhibition on microglial activation as well as cerebral IR injury is largely unknown. Here we explored whether PTP1B inhibitor sc-222227 attenuates microglial activation and mitigates neuronal injury after cerebral IR injury.

Methods : Cerebral IR injury rat model was induced by transient middle cerebral artery occlusion (MCAO) and reperfusion. PTP1B inhibitor sc-222227 was administered intracerebroventricularly 0.5 h before IR injury. Neurological deficits, infarct volume and brain water content were examined. In vitro IR injury model were established by oxygen glucose deprivation/reoxygenation (OGD/R) in rat primary microglia. PTP1B protein level, microglial activation, neuroinflammation, endoplasmic reticulum (ER) stress, autophagy and neuronal apoptosis were detected in vivo and/or in vitro using western blot, immunohistochemistry, immunofluorescence, ELISA and real-time PCR assay. Protein interaction were assessed by proximity ligation assay.

Results : PTP1B expression were significantly increased after cerebral IR injury in vivo, and the enhancement was most prominent in microglia. PTP1B inhibitor reduced IR-induced microglial activation both in vitro and in vivo, and further attenuated IR-induced microglial ER stress and autophagy in rat. In vitro experiment showed PTP1B inhibitor mitigated OGD/R-induced microglial activation through inhibiting ER stress-dependent autophagy, whose effect was partly abolished by PERK activator CCT020312. The protein interaction between PTP1B and phosphorylated PERK were significantly increase in response to OGD/R in primary microglia. Finally, PTP1B inhibitor reduced neuronal apoptosis and improved neurologic function after cerebral IR injury in rat.

Conclusions : PTP1B inhibitor ameliorated neuronal injury and neurologic deficits following cerebral IR injury via attenuating deleterious microglial activation and subsequent neuroinflammation through modulating ER stress-autophagy axis in microglia. Treatment targeting microglial PTP1B might be a potential therapeutic strategy for ischemic stroke treatment.


# Background

Ischemic stroke is a world-wide neurological disorder causing severe mortality and morbidity[1, 2]. While restoring cerebral bleed supply by vessel recanalization is the current treatment for ischemic stroke, the reperfusion process can induce inflammation and is a major cause of neuronal injury and unfavorable prognosis[3, 4]. Microglia, as the major resident immune cells of the central nervous system, play important role in mediating inflammatory response and brain injury upon diverse insults. After ischemic stroke, microglia become activated and release multipole proinflammatory cytokines which further result in deleterious and neurotoxic consequences[5–7]. While reducing microglial activation and inhibit

neuroinflammatory response is considered to be a promising therapeutic strategy for ischemic stroke, the underlining mechanisms of microglial activation after ischemic stroke is far from clear.

Protein tyrosine phosphatase 1B (PTP1B) is a member of protein tyrosine phosphatase family which recently attracted much attention as a regulator of a variety of processes within the central nervous system. In addition to early findings of PTP1B's roles in mediating insulin signaling to regulate energy expenditure and adiposity[8, 9], recent studies further revealed that PTP1B is highly expressed in microglia[10] and is a positive regulator of neuroinflammation[11]. However, the roles of PTP1B in both ischemic stroke and microglia are still unclear, and based on current evidences it is possible that inhibition of PTP1B after ischemic stroke may exert neuroprotective effects by reducing neuroinflammation.

Endoplasmic reticulum (ER) stress has been demonstrated to be involved in the neuronal injury after ischemia/reperfusion (IR) injury, and inhibition of ER stress effectively protected neuronal injury after ischemic stroke[12–14]. Several studies have also demonstrated the role of PTP1B in positively regulating ER stress, and inhibition of PTP1B significantly alleviated ER stress-induced neurotoxicity[15, 16]. Moreover, recent researches revealed that ER stress is involved in the microglial activation process which causes neuroinflammation[17, 18].

Autophagy is a lysosome-mediated self-degradation process which eliminates damaged or aged proteins and organelles and is recognized as an important element of innate immune response[12]. Newly emerged evidences showed complicated roles of autophagy to be both neuroprotective or destructive in neuronal cell death[19], and various factors has been found to be involved in modulating autophagy. Studies have revealed the role of ER stress as an upstream trigger for autophagy induction[20–23], and recent researches further showed that ER stress-autophagy axis is involved in the acute neuronal injury caused by ischemic stroke-induced neuroinflammation[24] and particularly, the microglial ER stress-autophagy axis was found to plays critical role in regulating microglial activation and subsequent neuroinflammation in response to external cocaine stimulation[18]. The exact role of PTP1B in the ER stress and autophagy remained elusive, yet a recent study showed protective effects of PTP1B deletion for myocardial injury by obliterating ER stress through regulation of autophagy[25], indicating a potential involvement of PTP1B in the ER stress-autophagy axis.

Based on these findings, in the present study we investigated whether pharmacological inhibition of PTP1B might be able to modulate microglial ER stress-autophagy axis, and we explore the effect of PTP1B inhibition against cerebral ischemia /reperfusion (IR) injury.

# Methods

## Reagents

PTP1B inhibitor sc-222227 was purchased from Santa Cruz (Santa Cruz, TX, USA). Autophagy inhibitor N(3)-methyladenine (3-MA), ER stress inhibitor 4-phenylbutylamine (4-PBA) were from Sigma (Sigma, St. Louis, MO, USA). protein kinase R-like endoplasmic reticulum kinase (PERK) activator CCT020312 was from Merck Millipore (Millipore, Billerica, MA, USA). Antibodies were obtained from the following sources: CD11b/c, PTP1B, PERK, p-PERK, inositol requiring enzyme-1 (IRE1), p-IRE1, activating transcription factor 6 (ATF6), neuronal nuclear antigen (NeuN), ionized calcium binding adapter molecule 1 (Iba-1), microtubule-associated protein 1-light chain 3 (LC3)B, Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) and β-actin were from Abcam, LC3-I/II and Beclin-1 were from Cell Signaling (Cell Signaling, Danvers, MA, USA); glial fibrillary acidic protein (GFAP) was from Santa Cruz; primary antibody for p-PERK (Thr982), PERK and secondary antibodies were purchased from Invitrogen (Invitrogen, Carlsbad, CA, USA).

## Animals

Male Wistar rats weighing 200-250 g were obtained from Shanghai SLAC Laboratory Animal Co., Ltd (Shanghai, China). Animals were housed under conditions of constant temperature and humidity, kept on a 12 hours light and 12 hours dark cycle. Food and water were available ad libitum. All procedures for handling animals were performed according to the protocols approved by the Institutional Animal Care Committee of the First Affiliated Hospital, Zhejiang University School of Medicine.

## Transient middle cerebral artery occlusion (MCAO) and reperfusion

Transient MCAO model by intraluminal suture method was established as previously described[26, 27]. Briefly, rats were anesthetized with intraperitoneal injection of pentobarbital sodium (50mg/kg). A normothermic range of body temperature (37℃ to 38 ℃) was maintained with a temperature-controlled heating pad. A midline neck incision was made followed by isolation of right common carotid artery, internal carotid artery and external carotid artery. A 4/0 monofilament nylon with a silicone-beaded tip was inserted into the right internal carotid artery through the external carotid artery to block the blood supply of the right middle cerebral artery. Laser Doppler flowmetry (PeriFlux 5000; Stockholm, Sweden) was used to monitor the blood flow of right middle cerebral artery. Successful ischemia was defined as over 25% of reduction in blood flow from baseline. After 2 hours of occlusion, the monofilament was withdrawn to initiate reperfusion. The sham groups were objected to same operation without insertion of monofilament.

## Intracerebroventricular drug administration

PTP1B inhibitor sc-222227 (Santa Cruz) was dissolved in DMSO and diluted with 0.9 saline to a final concentration of <0.1% DMSO. Anesthetized rats were placed in a stereotaxic frame, and sc-222227 (5μM, 10μM) was injected in to the right cerebral ventricle with Hamilton syringe using the following coordinates: 3mm rostral to bregma, 2mm lateral to midline and 2mm ventral to the skull surface. The injection rate was 0.2μL/min, and the needle was left in place for 5 minutes after injection before being slowly withdrawn. After drug administration, MCAO and reperfusion were

performed immediately as described above. Both sham and IR groups were administered intracerebroventricularly with vehicle (DMSO).

### Neurological score and motor assessment scale

Neurological deficiency was assessed 24 hours after reperfusion according to a 21-point Garcia test score system as described previously[24]. Briefly, 7 tests were included in the score system: spontaneous activity, axial sensation, vibrissae proprioception, symmetry of limb movement, lateral turning, forelimb outstretching and climbing. Each test was scored between 0 (worst) and 3 (best) to make a total score of 21. Motor function was evaluated according to a 10-point score system (number of successful forepaw placements out of 10 consecutive vibrissae-elicited excitation) as previously reported[28].

### Triphenyltetrazolium chloride (TTC) staining and quantification of infarct volume

Rats were euthanized with overdose of anesthesia at 24 hours post-reperfusion and quickly decapitated. After immediately removed and weighed, the brain was further sectioned coronally at 2mm intervals. Then the slices were stained with 2% 2,3,5 TTC for 15 minutes at 37℃. Digitalized slice images and the infarct areas were analyzed by Image J (Version 1.49, NIH, USA). The viable part of brain slice was red, while infarct lesion was defined as complete lack of staining with TTC. The infarct volume was calculated by multiplying the added infarct areas of each slices, and the ratio of (infarct volume/whole brain volume) ×100% were expressed as results.

## Assessment of brain water content

Immediately after removal, the brain weight (wet weight) was assessed. Then brain slices after TTC staining were dried at 110℃ for 48 hours and the dry brain weight was assessed. The brain water content was calculated as (wet weight-dry weight)/wet weight×100%.

## Rat primary microglial cell isolation

Primary microglia cells were obtained from 1-to-3-days-old rat pups as previously reported[29, 30]. Briefly, neonatal rat cerebral cortices were minced into small pieces, digested in DMEM and centrifuged (300×g, 10min). The precipitate was resuspended in DMED with 5% fetal calf serum, 10% FBS and 0.05 mg/ml gentamycin (Invitrogen) in an incubator (37℃, 5% $CO_2$, 95% air). The cellular debris, nonadherent cells and the supernatant were removed after 2 days, and the mixed cells were cultured for 8-10 days. Then, by shaking flasks on an orbital shaker (65 rpm, 4-6 hours, 37℃), microglial suspensions were harvested. Trypan Blue test confirm the cell viability was greater than 95%. The purity of the microglial was more than 99% confirmed by immunostaining for microglia/macrophage marker CD11b/c (Abcam, Cambridge, MA, USA).

## In vitro Oxygen Glucose Deprivation/reoxygenation (OGD/R) and drug treatments

Primary microglial cells were maintained in glucose-free medium in an anaerobic chamber (Thermo Fisher Scientific, Waltham, MA, USA) filled with 94% N2, 1% O2 and 5% at 37℃ CO2 for 3 hours. Then cells were

returned to normal cell culture incubators (95% air and 5% CO2) with normal medium. After 24 hours of re-oxygenation, the OGD/R treated conditioned medium was collected, and the supernatant without OGD/R treatment served as sham control. PTP1B inhibitor sc-22227 (1μM, 2μM; Santa Cruz), PERK activator CCT020312 (200nM, Millipore), 4-PBA (4mM, Sigma) and 3-MA (1mM, Sigma) were added to microglial cultures 2 hours before OGD/R treatment.

## Western blot

Western blot assay was performed to detect protein levels in ipsilateral cerebral cortex and primary microglia cells. The ipsilateral cerebral cortex tissues were homogenized and lysed with RIPA buffer (Thermo Fisher Scientific) with protease and phosphatase inhibitor cocktails (Abcam). and treated cells were lysed using the Mammalian Cell Lysis kit (Sigma). The extracted proteins were then separated by 10% sodium dodecyl sulfate (SDS)-polyacrylamide and electrically transferred to PVDF membrane (Millipore). The membranes were then blocked with TBST with 5% nonfat dry milk for 1 hour at room temperature. The western blots were probed with primary antibodies recognizing the indicated proteins: PTP1B (1:1000, Abcam), p-PERK (Thr982, 1:1000, Invitrogen), PERK (1:1000, Invitrogen), p-IRE1 (Ser724, 1:1000, Abcam), IRE1 (1:2000, Abcam), ATF6 (1:2000, Abcam), LC3-I/II (1:1000, Cell Signaling), Beclin-1 (1:1000, Cell Signaling), β-actin (1:5000, Abcam), GAPDH (1:5000, Abcam), and further incubated with corresponding secondary antibody (1:10000, Invitrogen). The protein expression levels were analyzed using Image J software (Version 1.49, NIH,

USA) normalized to the β-actin and GAPDH. Phosphorylated protein levels were evaluated compared to total protein levels.

### Immunohistochemistry, double immunofluorescence and Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining

For immunohistochemistry, rats were anesthetized and perfused through the ascending aorta with 0.9% saline followed by 4% paraformaldehyde 24 hours following cerebral IR injury. After decapitation, immunohistochemistry was performed in the ipsilateral hemisphere in paraffin-embedded coronal sections (6μm) as reported previously[31]. Briefly, after de-waxing, the sections were washed with PBS (pH 7.4), processed with 3% hydrogen peroxide and washed with PBS. Sections were then incubated with primary antibody to detect CD11b/c (1:1000, Abcam) overnight at 4℃. The sections were then retrieved and washed with PBS, followed by addition of secondary antibody (1:5000, ZSGB-BIO Company, Beijing, China) for incubation at room temperature for 30 minutes. Sections were then washed with PBS, and Diaminobenzidine (DAB) chromogenic reagent (ZSGB-BIO Company, Beijing, China) was used for developing sections. Reaction were terminated by tap water and Hematoxylin was used for counterstaining the nucleus followed by washing and bluing. Finally, sections were dehydrated and sealed with neutral gum, visualized and photographed using microscope (Leica, Heerbrugg, Germany).

For double immunofluorescence assay, sections were first blocked with 10% donkey serum for 2 hours to avoid the unspecific staining. Then sections were incubated with primary antibody against PTP1B (1:50,

Abcam), NeuN (1:300, Abcam), Iba-1 (1:500, Abcam), GFAP (1:1000, Santa Cruz), p-PERK (Thr982, 1:200, Invitrogen), p-IRE (1:100, Abcam), LC3B (1:200, Abcam) at 4℃ for overnight. Then they were incubated with secondary antibodies (Invitrogen) at room temperature for 2 hours after washed by PBS. Samples were counterstained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) for 5 min, then observed and photographed with a laser confocal microscope (Nikon, A1, Tokyo, Japan).

To detect apoptotic neurons cells, immunofluorescence and TUNEL staining were performed. Briefly, sectioned slides were washed in TBS (50mM Tris-HCl, pH 7.4); 150mM NaCl) plus 0.3% Triton X-100 with gentle agitation and block in 10% normal serum in TBS for 2 hours at room temperature. Slides were then incubated with anti-NeuN antibody (1:300, Abcam) overnight at 4℃. Then slides were incubated with secondary antibody for 2 hours at room temperature after rinsing with TBS. Sections were then stained using TUNEL kit (Keygenbiotech, Nanjing, China) according to the manufacturer’s instructions. After that, sections were incubated with DAPI for 10 minutes, and finally observed and photographed with a laser confocal microscope (Nikon, A1, Tokyo, Japan). Apoptotic neurons were identified by Green TUNEL dots located in red neurons with a blue nucleus.

In each of the experiments above, 5 views from the penumbra site were assessed by a pathologist blinded to the experimental conditions.

## Measurements of mRNA levels

Total RNA was extracted from ipsilateral cerebral cortex tissue and primary microglia cells by TRIzol reagent (Takara, Kyoto, Japan). Samples were then reverse transcribed to cDNA using cDNA Synthesis Kit (Takara, Kyoto, Japan). Then, cDNA was amplified by a SYBR Premix Ex Taq kit (Takara, Kyoto, Japan) on a 7500 Real-Time PCR System (Applied Biosystems, CA, USA). All procedures were performed according to the manufacturers' instructions. Data were normalized to β-actin, and expressed as fold-change compared to the control. The primers (Sangon BiotechCo., Ltd, Shanghai, China) used for the amplification are shown in Table 1.

**Table 1 Primers used for qRT-PCR**

| Primers | Forward (5'-3') | Reverse (5'-3') |
|---|---|---|
| **TNF-α** | TGTGGAACTGGCAGAGGA | ACAGAAGAGCGTGGTGGC |
| **IL-1β** | CAAATCTCACAGCAGCATCTC | AGGACGGGCTCTTCTTCA |
| **IL-6** | GCCACTGCCTTCCCTACT | CACAACTCTTTTCTCATTTCCA |
| **CCL2** | TGTTGTTCACAGTTGCTGCCTG | GTGCTGAAGTCCTTAGGGTTGAT |
| **β-actin** | CAAGTGGGTGGCATAGAGG | ATGACGAAGAGCACAGATGG |

## Enzyme-linked immunosorbent assay (ELISA)

The levels of secreted pro-inflammatory cytokines including Tumor Necrosis Factor-alpha (TNF-α) and Chemokine (C-C Motif) Ligand 2 (CCL2) in supernatants collected from rat primary microglia were detected by commercially available ELISA kit (eBioScience, San Diego, CA, USA) according to manufacturer's instructions. The data represent results obtained from 4 independent tests.

## Proximity ligation assays (PLA)

PLA assay was performed to detect protein interaction as previously described[32-34]. Briefly, after OGD/R insult, primary microglia were incubated with primary antibodies against PTP1B (1:100, Abcam) and p-PERK (Thr982, 1:200, Invitrogen) and then with a pair of PLA probes (Sigma); probe ligation (Sigma), signal amplification (Sigma) and mounting with DAPI (Sigma) were performed according to manufacturer's instructions. Representative images were obtained with Z1 inverted microscope (Carl Zeiss, German). Images were analyzed using ImageJ software (Version 1.49, NIH, USA).

### Statistical analysis

All data were analyzed by SPSS (Version 19.0, Michigan Avenue, Chicago, Il, USA). Values are expressed as means±SEM. The non-paired t test was used to determine the significance of differences between two groups. A value of $P<0.05$ was considered to be statistically significant.

## Results

#### Upregulation of PTP1B in microglia after rat ischemia/reperfusion injury

To determine whether the expression level of PTP1B were altered in response to cerebral IR injury, western blot assay was performed. Results showed that whereas the protein level of PTP1B in the rat ipsilateral cerebral cortex was relatively low in the sham-operated control group, significant increase occurred at 12h, reached a maximum at 24 hours, and declined at 72 hours after IR injury (12h and 24h group, $P<0.001$ vs sham group; 72h group, $P<0.01$ vs sham group; Figure 1A).

To investigate cell distribution of PTP1B expression after IR injury, double immunofluorescence was applied using antibodies targeting Iba-1, GFAP and NeuN to identify microglia, astrocytes and neurons, respectively. Results showed that PTP1B was localized in microglia, neurons and astrocytes in the sham cerebral cortex (Figure 1B). Twenty-four hours after cerebral IR injury, the number of PTP1B-postive cells was significantly increased in microglia and neurons but not astrocyte, and notably, this augmentation in PTP1B-positive cell percentage was more dramatic in microglia compared with neurons (Figure 1C). These findings implied that upregulation of PTP1B expression after rat IR injury occurred prominently in

microglia. As microglia are the main immune cells in the brain that mediate inflammatory response after cerebral IR injury, and considering the critical role of PTP1B in regulation neuroinflammation, the results above suggested that PTP1B might contribute to the pathophysiologic process via regulating microglia function following cerebral IR injury.

### PTP1B inhibitor reduced ischemia/reperfusion-induced microglial activation both in vitro and in vivo

To investigate the role of PTP1B in regulating microglial activation, we first explored the effect of a selective PTP1B inhibitor, sc-222227 (referred to as PTP1Bsc thereafter) on rat primary microglial cells activation after OGD/R injury. We found that 2uM of PTP1Bsc treatment significantly decreased the mRNA levels of IL-1β, IL-6 and TNF-α (relative mRNA expression level: IL-1β, $P<0.001$; IL-6, $P<0.01$; TNF-α, $P<0.01$; all compared to OGD/R groups; Figure 2A-C) in rat primary microglial cells after OGD/R insult. In vivo experiment (outlined in Figure 2 D) further showed that intraventricular administration of both 5μM and 10μM of PTP1B inhibitor 30 minutes prior to IR injury effectively decreased the number of activated microglia (CD11b/c+) in rat brain after cerebral IR injury ($P<0.001$ vs sham group, Figure 2D, E).

### PTP1B inhibitor attenuated cerebral ischemia/reperfusion-induced overall and microglial endoplasmic reticulum stress in rat

Endoplasmic reticulum (ER) stress is involved in microglial activation[17, 18, 35], and PTP1B recently emerged as an important regulator of ER stress in microglia[36]. To explore the involvement of ER stress pathway in cerebral IR injury and the role of PTP1B inhibitor in modulating microglial ER stress after IR injury, western blot assay was performed (Figure 3A) to detect ER stress proteins in sham surgery group, cerebral IR injury group and IR with PTP1Bsc treatment group in rats. Results showed that IR injury induced significant upregulation of p-PERK, p-IRE1 and ATF6 in the ipsilateral cerebral cortex (Figure 3B-D), indicating massive precipitation of ER stress after IR injury. PTP1Bsc administration dramatically attenuated IR-induced ER stress (p-PERK protein level, $P<0.01$; p-IRE1 protein level, $P<0.001$; AFT6 protein level, $P<0.001$; all compared to sham groups; Figure 3 B-D). The results from western blot were further supported by immunofluorescence staining (Figure 3E and H) that cerebral IR injury significantly enhanced overall fluorescence intensity of both p-PERK and p-IRE in ipsilateral rat cerebral cortex (Figure 3F and I) compared with sham groups.

To investigate cerebral IR-induced ER stress change in microglia, double immunofluorescence was performed and results showed significantly increased expression of p-PERK and p-IRE in Iba-1 positive cells (Figure 3G and J). Both overall and microglial ER stress were significantly weakened in both 5μM and 10μM PTP1Bsc treatment groups evidenced by decreased overall p-PERK IOD level ($P<0.001$; Figure 3F), p-IRE1 IOD level ($P<0.001$; Figure 3I) as well as reduced p-PERK/Iba-1 (5μM group, $P<0.01$; 10μM group, $P<0.001$; Figure 3G) and p-IRE1/Iba-1 double positive cell density ($P<0.001$; Figure 3J). Together, these results showed that PTP1B inhibitor treatment significantly attenuated overall and microglial ER stress induced by cerebral IR injury.

### PTP1B inhibitor alleviated ischemia/reperfusion-induced overall and microglial autophagy in rat

Autophagy has been demonstrated to be involved in the microglia-induced inflammatory response after ischemic stroke[37]. Reports showed that excessive autophagy contributes to neuronal death after ischemia injury[38], and suppression of autophagy in microglia effectively suppressed ischemia-induced inflammatory response[39]. To investigate the role of PTP1B inhibitor in overall and microglial autophagy after IR injury, western blot assay (Figure 4A) were performed and results showed that as cerebral IR injury obviously enhanced the ratio of LC3-II/I and beclin-1 expression level, treatment of PTP1Bsc significantly attenuated IR-induced increase in LC3-II/I ratio as well as beclin-1 level (LC3-II/I ratio, $P<0.001$ vs IR group, Figure 4B; beclin-1; $P<0.01$ vs IR group, Figure 4C).

Immunofluorescence staining (Figure 4D) further demonstrated significant enhancement of total LC3B signal (Figure 4E) as well as microglial LC3B expression (LC3B+/Iba-1+ cells) after IR injury (Figure 4F), whereas these above increases were abolished by intracerebroventricular administration of both 5μM and 10μM PTP1Bsc (relative IOD of LC3B, $P<0.001$, Figure 4E; LC3B+/Iba-1+ cell number, $P<0.001$, Figure 4F). Together, these data suggested that PTP1B inhibitor treatment significantly alleviated IR-induced overall and microglial autophagy.

## PTP1B inhibitor mitigated OGD/R-induced microglial activation through inhibiting ER stress-dependent autophagy in primary microglia with involvement of PERK signaling

Studies reported that generation of autophagy can be triggered by upstream ER stress pathways[24, 40, 41], and the ER stress-autophagy axis is involved in cocaine-induced microglial activation[18]. First, to determine the role of ER stress in the induction of autophagy in the context of IR injury as well as its effect on OGD/R-induced microglial activation, specific ER stress inhibitor 4-PBA and specific autophagy inhibitor 3-MA were applied. Western blot assay showed that OGD/R injury resulted in significant upregulation of ER stress and autophagy activity (Figure 5A-D) in primary microglia, and suppression of ER stress by 4-PBA not only inhibited OGD/R-induced upregulation of p-PERK ($P<0.001$ vs OGD/R group, Figure 5B), p-IRE1 ($P<0.001$ vs OGD/R group, Figure 5C) and ATF6 ($P<0.001$ vs OGD/R group, Figure 5D) protein level, but also suppressed OGD/R-induced upregulation of autophagy as evidenced by the decreased ratio of LC3-II/I ($P<0.001$ vs OGD/R group, Figure 5E) and beclin-1 ($P<0.001$ vs OGD/R group, Figure 5F) protein level. However, 3-MA treatment only attenuated autophagy (LC3-II/I ratio: $P<0.001$ vs OGD/R group, Figure 5E; Beclin-1: $P<0.001$ vs OGD/R group, Figure 5F) with no significant effects on ER stress proteins($P>0.05$ vs OGD/R groups for p-PERK, p-IRE1 and ATF6, Figure 5B-D). Then we examined the role of microglial ER stress-autophagy axis on microglial activation after OGD/R insult. Results showed that both 4-PBA and 3-MA significantly attenuated IR injury-induced microglial activation as evidenced by significant decrease of TNF-α, IL-1β, IL-6 and CCL-2 mRNA expression level ($P<0.05$ vs OGD/R group, Figure 5G) as well as decline of both TNF-α ($P<0.001$ vs OGD/R group, Figure 5H) and CCL2 ($P<0.001$ vs OGD/R group, Figure 5I) protein level. These results indicated that microglial ER stress is the upstream event after OGD/R insult, and the ER stress-autophagy axis is positively involved in the OGD/R-induced microglial activation.

PTP1B has been demonstrated to be a negative modulator of both ER stress as well as the ER stress-autophagy axis, and PERK was indicated to be a critical factor triggering ER stress-autophagy axis[18, 25, 40]. To further evaluated the effect of PTP1B inhibitor on OGD/R-induced microglial activation, microglial ER stress-autophagy axis and its underlying signaling, PTP1B inhibitor PTP1Bsc and CCT020312, a selective PERK activator, were applied. Western blot assay (Figure 5A) showed that PTP1Bsc significantly decreased OGD/R-induced upregulation of p-PERK, p-IRE1 and ATF6 protein level ($P<0.001$ vs OGD/R group, Figure 5B-D), autophagy level (LC3II/I ratio: $P<0.01$ vs OGD/R group, Figure 5E; Beclin-1: $P<0.001$ vs OGD/R group, Figure 5F) as well as microglial activation (TNF-α, IL-1β, IL-6 and CCL-2 mRNA expression: $P<0.05$ vs OGD/R group, Figure 5G; TNF-α protein level: $P<0.001$ vs OGD/R group, Figure 5H; CCL2 protein level, $P<0.01$ vs OGD/R group, Figure 5I). However, these effects of PTP1B inhibitor to suppress OGD/R-induced ER stress and autophagy were partly abolished by co-administration of CCT020312 (p-PERK, $P<0.01$ vs PTP1Bsc group, Figure 5B; p-IRE1, $P<0.001$ vs PTP1Bsc group, Figure 5C; ATF6, $P<0.001$ vs PTP1Bsc group, Figure 5D; LC3II/I ratio, $P<0.01$ vs PTP1Bsc group, Figure 5E; Beclin-1, $P<0.05$ vs PTP1Bsc group, Figure 5F). Real-time PCR and ELISA further showed that co-administration of CCT020312 also partly abolished the PTP1Bsc's effects on inhibiting OGD/R-induced microglial activation (TNF-α, IL-1β, IL-6 and CCL-2 mRNA expression, $P<0.05$ vs PTP1Bsc group, Figure 5G; TNF-α and CCL2 protein expression, $P<0.05$ vs PTP1Bsc group, Figure 5H, I). These results indicated that PTP1B inhibitor effectively attenuated OGD/R-induced microglial ER stress, autophagy and microglial activation at least partly through PERK signaling.

Together, the above data suggested that PTP1B inhibition by PTP1Bsc mitigated microglial activation through inhibiting endoplasmic reticulum stress-dependent autophagy in primary microglia, and PERK signaling was involved in this process.

### OGD/R injury increased the interaction between PTP1B and phosphorylated PERK in primary microglia

As interaction between PTP1B has been reported to interact with PERK in several tissue types to trigger ER stress[33], we examined the protein interaction of PTP1B and PERK in the context of OGD/R injury by PLA probe assay. Results showed a basal weak interaction between PTP1B and p-PERK in primary microglia (Figure 6A), whereas the interactions were markedly enhanced after OGD/R insult evidenced by significant increase in the number of red fluorescent dots per cell ($P<0.001$ vs control group, Figure 6A, B). These data further supported the finding that PTP1B inhibition by PTP1Bsc trigger downstream events possibly by targeting PERK.

### PTP1B inhibitor protected against cerebral ischemia/reperfusion injury and confers neuroprotection in rat

Finally, we examined the therapeutic effects of intracerebroventricular administration of PTP1Bsc for cerebral IR-induced neuronal damage and neurological function deficits. To achieve optimal treatment, diverse administration time points as well as diverse doses of PTP1Bsc were tested, and intracerebroventricular injection 30 minutes prior to cerebral IR injury with 5μM and 10μM dose of PTP1Bsc were selected. Rats were killed for immunofluorescence, infarct volume assessment (TTC staining) and brain water content measurement at 24 hours, and behavioral tests were performed at 3

days after cerebral IR injury. TUNEL assay (Figure 7A) showed that PTP1Bsc significantly attenuated neuronal death at 24 hours after IR injury compared with IR group ($P<0.05$ vs IR group, Figure 7B). Results also showed that both 5μM and 10μM of PTP1Bsc administration significantly diminished infarct volume ($P<0.001$ vs IR group, Figure 7C, D) and brain water content ($P<0.001$ vs IR group, Figure 7E). Further, behavioral tests showed that PTP1Bsc administration significantly increased motor assessment scale ($P<0.01$, 10μM group vs IR group, Figure 7F) and neurological score ($P<0.05$, 5μM group vs IR group; $P<0.001$, 10μM group vs IR group; Figure 7G). Together, these data demonstrated that PTP1Bsc treatment has a neuroprotective effect against cerebral IR injury.

# Discussion

Microglia play crucial roles in the pathophysiology of cerebral ischemia, and deleterious microglial activation have been demonstrated to cause neuroinflammation that exacerbates neuronal damage. Diverse treatment aiming at inhibition of microglial activation were associated with improved outcome after ischemic stroke[42–44]. In this study, we demonstrated, using both in vitro and in vivo approaches, that pharmacological inhibition of PTP1B in microglia effectively reduced detrimental microglial activation, attenuated inflammatory response and protected neuronal death after cerebral IR injury. We also provided evidence that ER stress-autophagy axis via PERK signaling is involved in the protective effect of PTP1B inhibition in microglia, indicating a novel mechanism for PTP1B in regulation of microglial activation and neuroinflammation.

PTP1B has been demonstrated to be involved in inflammatory responses to a variety of injuries, including spinal cord injury and radiation injury[45, 46]. However, whether PTP1B participates in cerebral IR injury-induced neuroinflammation as well as its relevant cell types were poorly defined. In the current study we first observed significant upregulation of PTP1B level after cerebral IR injury, and the most prominent enhancement of PTP1B expression were particularly found in microglia, which are the resident macrophages of the brain that regulates neuroinflammation. The expression of PTP1B in the ipsilateral cortex reached a peak at 24 hours after cerebral IR injury followed by a decrease at 72 hours post-IR, indicating a relatively short-term participation of PTP1B in the acute pathophysiological process of cerebral IR injury. Further, the upregulation of PTP1B were most significant in microglia, and as deleterious microglial activation causing excessive inflammatory response were also demonstrated to occur in the acute and sub-acute phase after IR injury[47, 48], these results preliminarily indicated a potential role of PTP1B in regulating IR-induced deleterious microglia activation.

Several studies have reported the role of PTP1B as a positive regulator of microglial activation, and inhibition or deficiency of PTP1B effectively attenuated neuroinflammation[11, 49, 50]. To investigate the possible effect of PTP1B inhibition in suppression of detrimental microglial activation after cerebral IR injury, a selective PTP1B inhibitor sc-222227 was used as a pharmacological treatment both in primary microglia OGD/R model and rat cerebral IR model. The results showed that in both OGD/R-treated primary microglia and IR-treated rat, inhibition of PTP1B by sc-222227 effectively suppressed microglial activation and subsequent release of pro-inflammatory cytokines. Thus, current study further confirmed

the previously indicated role of PTP1B in regulating microglial activation, and particularly, the results of this study extended this crucial role of PTP1B in the context of cerebral IR injury.

ER stress has been demonstrated to be a critical event to cause cerebral IR injury, and recent studies showed extensive involvement of ER stress in microglial activation to participate in diverse cerebral injuries including Alzheimer's Disease[51], spinal cord injury[52] and LPS/cocaine-induced neuroinflammation[17, 53]. PTP1B was reported to be a positive regulator of ER stress[15, 16], yet its role in microglial ER stress has not been well illustrated. In the current study, both western blot and immunofluorescence assay showed significant increase of ER stress protein levels in the ipsilateral cerebral cortex after cerebral IR injury, confirming the critical involvement of ER stress in ischemic stroke-induced injury. Moreover, intracerebroventricular administration of PTP1B inhibitor significantly attenuated the IR injury-induced ER stress, demonstrating a crucial role of PTP1B in the cerebral ER stress pathophysiology after IR injury. Finally, the double immunofluorescence assay labeling ER stress protein expression in microglia also showed significant increase of microglial ER stress, which was effectively reversed by PTP1B inhibitor treatment. Together, these findings indicated an important role of PTP1B to regulate cerebral IR injury-induced microglial ER stress.

Autophagy is a crucial cellular catabolic pathway that plays important role in the maintenance of cellular homeostasis and cell survival. Accumulating evidence has revealed the involvement of autophagic activation in diverse cell types in response to cerebral IR injury, yet the exact role of autophagy in pathophysiological process of cerebral IR injury have yet to be elucidated[54]. While several studies showed protective effect of autophagy against cerebral IR injury[55], many studies demonstrated that excessive autophagic activation leads to exacerbated neuronal injury during cerebral ischemic stroke, and inhibition of autophagy effectively decreased infarct size and increased neurological score after cerebral ischemia[24, 56]. Further, the role of autophagy in microglia upon ischemic insult are largely unknown. In this study, we observed significant enhancement of autophagy proteins in the ipsilateral cerebral cortex as well as microglia cells of cerebral IR injured rats, these results were consistent with the reports of several previous studies using permanent MCAO mouse model[39, 57]. Recently, several studies indicated a positive role of PTP1B in regulating autophagy, yet whether PTP1B possesses similar function in microglia remained to be illustrated. In this study, we observed significant attenuation of autophagy in both ipsilateral cerebral cortex and microglia cells by intracerebroventricular treatment of PTP1B inhibitor after IR injury, indicating a vital function of PTP1B in regulation of cerebral IR injury-induced microglial autophagy.

Studies has demonstrated involvement of ER stress in the induction of autophagy, and this ER stress-autophagy axis was further shown to contribute to microglial activation. In this report, we provide evidence that cerebral IR-induced upregulation of ER stress preceded the autophagic response in primary microglia, and the ER stress-autophagy axis was associated with microglial activation and subsequent neuroinflammation in response to OGD/R insult in primary microglia. Further, the current study showed that PTP1B inhibitor administration effectively suppressed both cerebral IR-induced ER stress and downstream autophagy in primary microglia. PERK signaling is an important pathway regulating ER

stress, and several studies has shown regulatory role of PTP1B in PERK-mediated ER stress. To further investigate the underlying mechanisms of the effects of PTP1B inhibition, a selective PERK activator, CCT020312 was used. Our study showed that PERK activator partly abolished the effects of PTP1B inhibition on both ER stress and autophagic activation, as well as subsequent neuroinflammatory responses. Previous studies have revealed that PTP1B interact with PERK to participate in modulation of ER stress in mesenteric arteries and brown adipose tissue[33, 58]. in this study the PLA probe experiment also showed a significant enhanced interaction between PTP1B and activated PERK in the primary microglia cells after OGD/R insult. Together, our data suggests that ER stress is the upstream event of autophagy in microglia cells in response to IR injury, and this ER stress-autophagy axis contributes to microglial activation process during cerebral IR insult. Our study also indicated that PTP1B is a crucial regulator of the ER stress-autophagy axis-mediated microglia activation and neuroinflammation after IR insult, and PTP1B inhibitor mitigated ER stress, autophagy and microglial activation possibly through targeting PERK signaling.

Finally, we investigated whether the effective inhibition of cerebral IR injury-induced microglial activation by PTP1B inhibitor administration would after all alleviate neuronal apoptosis, reduce neuronal damage and in general protect neurological function after cerebral IR injury in vivo. Results showed that intracerebroventricular administration of PTP1B inhibitor significantly decreased cerebral IR injury-induced neuronal apoptosis. Moreover, in agreement with the results of a previous study[59], our data also demonstrated a protective role of PTP1B inhibitor treatment against cerebral IR injury, evidenced by reduced infarct volume, brain water content as well as significantly improved neurologic function after cerebral IR injury.

# Conclusion

In summary, as illustrated in Fig. 8, our findings suggest that PTP1B inhibitor sc-222227 is able to reduce cerebral IR injury-induced deleterious microglial activation and subsequent neuroinflammation through modulating ER stress-autophagy axis in microglia. Intracerebroventricular administration of PTP1B inhibitor effectively protects against cerebral IR injury. These findings provide novel insights into the molecular association between PTP1B, ER stress-autophagy axis, microglial activation and neuroinflammation, suggesting PTP1B is an important factor in regulating microglia function. Moreover, our study shed light on the novel therapeutic strategies which target microglial PTP1B for cerebral ischemic stroke treatment.

# List Of Abbreviations

PTP1B:Protein tyrosine phosphatase 1B (PTP1B); PTP1Bsc:PTP1B inhibitor sc-222227; ER:endoplasmic reticulum; IR:ischemia/reperfusion; MCAO:middle cerebral artery occlusion; OGD/R:oxygen glucose deprivation/reoxygenation; ELISA:Enzyme-linked immunosorbent assay; PCR:polymerase chain reaction; PERK:protein kinase R-like endoplasmic reticulum kinase; IRE1:inositol requiring enzyme-1; ATF6:activating transcription factor 6; NeuN:neuronal nuclear antigen; Iba-1:ionized calcium binding

adapter molecule 1; LC3:microtubule-associated protein 1-light chain 3; GFAP:glial fibrillary acidic protein; GAPDH:Glyceraldehyde-3-Phosphate Dehydrogenase; TNF-α:Tumor Necrosis Factor-alpha; CCL2:Chemokine (C-C Motif) Ligand 2; IL-1β:Interleukin-1beta; IL-6:Interleukin-6; PLA:proximity ligation assays; IOD:integrated optical density; 4-PBA:4-phenylbutylamine; 3-MA:N(3)-methyladenine; TUNEL:Terminal deoxynucleotidyl transferase dUTP nick end labeling.

# Declarations


### Acknowledgements

Not applicable.


### Authors' contributions

YZ, JG, DY, DC and ZX performed experiments. YZ and RZ wrote and provided the final manuscript. JY, JZ, JS HZ and YW analyzed the data. YZ, JP and RZ contributed in study planning.


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### Availability of data and materials

The datasets and/or analyzed in the current study are available from the corresponding author on reasonable request.

### Ethics approval and consent to participate

All experiments were approved by the Institutional Animal Care Committee of the First Affiliated Hospital, Zhejiang University School of Medicine.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interest.

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# Figures

**Figure 1**

Upregulation of PTP1B protein expression after cerebral ischemia/reperfusion (IR) injury. (a) PTP1B protein level were detected by western blot in the rat ipsilateral cortex at 12 h, 24 h, and 72 h after cerebral IR injury, and was normalized to GAPDH. Quantitative results of relative band density are presented as mean ± SD (n=4 per group). (b) Double immunofluorescence staining to detect cell type distribution of PTP1B in microglia (Iba-1), astrocyte (GFAP) and neuron (NeuN) in ipsilateral cerebral cortex. Scale bar=50μm. (c) Quantitative analysis of the percentage of PTP1B positive cell in microglia, astrocytes and neurons after cerebral IR injury compared with sham group, results are presented as mean ± SEM (n=5 per group). *p < 0.05; **p < 0.01; ***p < 0.001 compared with sham group.

**Figure 2**

Microglial activation after oxygen glucose deprivation/reoxygenation (OGD/R) and cerebral ischemia/reperfusion (IR) injury were attenuated by PTP1B inhibitor treatment both in vitro and in vivo. (a-c) mRNA level IL-1β, IL-6 and TNF-α after OGD/R insult were tested by real-time PCR in rat primary microglia. Fold changes were normalized to β-actin, and quantitative results are presented as the mean ± SD (n=5 per group). (d) Outline of in vivo experiment to detect the effect of intracerebroventricular administration of PTP1B inhibitor after cerebral IR injury. (e) Immunohistology to detect CD11b/c cell in ipsilateral cerebral cortex, and quantitative analysis of CD11b/c positive cell number are presented as mean ± SEM (n=5 per group). Scale bar=50μm. *p < 0.05; **p < 0.01; ***p < 0.001 compared with IR group. PTP1Bsc/sc=PTP1B inhibitor sc-222227. i.c.v=intracerebroventricular injection. R=Reperfusion.

## Figure 3

Microglial endoplasmic reticulum (ER) stress was mitigated by PTP1B inhibitor treatment after cerebral ischemia/reperfusion (IR) injury in rat. (a) Western blot to detect the effect of PTP1B inhibitor on phosphor-PERK, PERK, phospho-IRE1, IRE1 and ATF6 protein expression level in rat ipsilateral cortex 24 hours after IR injury (n=5 per group). (b-d) Quantitative results of relative band density are normalized to GAPDH and presented as mean ± SEM (n=4 per group). (e) Double immunofluorescence to detect phospho-PERK expression in microglia (Iba-1) cells 24 hours after IR injury. Scale bar=50μm. (f, g) Quantitative analysis of overall integrated optical density (IOD) value of phospho-PERK and phospho-PERK+/Iba-1+ cell density in ipsilateral cerebral cortex (n=5 per group). (h) Double immunofluorescence to detect phosphor-IRE1 expression in microglia (Iba-1) cells 24 hours after IR injury. Scale bar=50μm. (I, J) Quantitative analysis of overall integrated optical density (IOD) value of phospho-IRE1 and phospho-IRE1+/Iba-1+ cell density in ipsilateral cerebral cortex (n=5 per group). *p < 0.05; **p < 0.01; ***p < 0.001 compared with IR group. p-PERK= phospho-PERK, p-IRE1= phospho-IRE1, sc= PTP1B inhibitor sc-222227.

## Figure 4

Microglial autophagy was mitigated by PTP1B inhibitor treatment after cerebral ischemia/reperfusion (IR) injury in rat. (a) Western blot to detect the effect of PTP1B inhibitor on LC3-I/II and Beclin-1 protein expression level in rat ipsilateral cortex 24 hours after IR injury. (b, c) Quantitative results of relative band density are normalized to β-actin and presented as mean ± SEM (n=4 per group). (d) Double immunofluorescence to detect LC3B expression in microglia (Iba-1) cells 24 hours after IR injury. Scale bar=50μm. (e, f) Quantitative analysis of overall integrated optical density (IOD) value of LC3B and LC3B+/Iba-1+ cell density in ipsilateral cerebral cortex (n=5 per group). *p < 0.05; **p < 0.01; ***p < 0.001 compared with IR group. sc= PTP1B inhibitor sc-222227.

## Figure 5

PTP1B inhibitor mitigated oxygen glucose deprivation/reoxygenation (OGD/R)-induced microglial activation through inhibiting ER stress-dependent autophagy via PERK signaling. (a) PTP1B inhibitor sc-222227, 4-PBA and 3-MA were used to specifically inhibit PTP1B, ER stress and autophagy in primary microglia. CCT020312 was used to activate PERK. Western blot to detect p-PERK, PERK, p-IRE1, IRE1, LC-3I/II, and beclin-1 in primary microglia after OGD/R insult. (b-f) Quantitative results of relative band density are normalized to β-actin and presented as mean ± SEM (n=4 per group). (g) Real-time PCR results showing relative expression of TNF-α, IL-1β, IL-6 and CCL2 in primary microglia in response to treatment of PTP1B inhibitor, PTP1B inhibitor+PERK activator, 4-PBA and 3-MA after OGD/R insult. Data are normalized to β-actin and presented as means ± SEM (n=4 per group). (h, I) ELISA assay to detect expression of secreted TNF-α and CCL2 in primary microglia supernatant 24 hours after OGD/R insults. Data are expressed as means ± SEM (n=4 per group). *p < 0.05; **p < 0.01; ***p < 0.001 compared with IR

group. #p < 0.05; ##p < 0.01; ###p < 0.001 compared with PTP1B inhibitor group. sc=PTP1B inhibitor sc-222227. cc=PERK activator CCT020312. p-PERK= phospho-PERK. p-IRE1=phosphor-IRE1.

## Figure 6

PTP1B interacts with phospho-PERK in primary microglia under oxygen glucose deprivation/reoxygenation (OGD/R) condition. (a) Proximity ligation assays (PLA) probe was used to examine the proximity between PTP1B and phospho-PERK. Scale bar=20μm. (b) The number of red fluorescent dots per cell was quantified as mean ± SEM (n=16 per group). ***p < 0.001 compared with Sham group. p-PERK= phospho-PERK.

## Figure 7

PTP1B inhibitor treatment reduced neuronal apoptosis, cerebral infarct volume, brain water content and improved neurologic function after ischemia/reperfusion (IR) injury. (a) Immunofluorescence/TUNEL assay were performed to detect neuronal apoptosis in rat ipsilateral cerebral cortex 24 hours after IR injury. Scale bar=50μm. (b) The density of NeuN+/TUNEL+ cell was quantified as mean ± SEM (n=5 per group). (c) TTC staining were performed to determine infarct volume 24 hours after cerebral IR injury. Infarct area was defined as white area. (d) The infarct volume was quantified as the ratio of (infarct volume/whole brain volume) ×100%. (e) Brain water content. (f) Neurological score 3 days after IR injury. (g) Motor assessment score 3 days after IR injury. Data were presented as mean ± SEM (n=5 per group). *p < 0.05; **p < 0.01; ***p < 0.001 compared with IR group. PTP1Bsc/sc= PTP1B inhibitor sc-222227.

## Figure 8

Schematic diagram of the indicated molecular mechanisms underlying the protective effects of PTP1B inhibitor treatment against cerebral ischemia/reperfusion injury. PTP1B inhibitor treatment alleviated cerebral IR-induced microglial ER stress as well as its down-stream autophagy, and ultimately mitigated deleterious activation of microglia. PTP1B inhibitor attenuated microglial ER stress possibly through inhibiting PERK signaling. PTP1Bsc=PTP1B inhibitor scc-222227.