

# Thrombin aggravates hypoxia/reoxygenation injury of astrocytes by activating the autophagy pathway mediated by SPRED2

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**Abstract.** Autophagy plays an important role in ischemia/reperfusion brain injury, however, the signaling pathways involved in cell autophagy are not fully understood. The present study aimed to investigate the roles and molecular mechanisms of thrombin and Sprouty-related EVH1 domain-2 (SPRED2) on autophagy in hypoxia/reoxygenation (H/R) induced astrocytes. Reverse transcription-quantitative PCR and western blot analyses were performed to detect the expression levels of thrombin and SPRED2. Western blot analysis was also performed to detect the protein expression levels of Beclin 1, microtubule-associated protein light chain 3 (LC3)-II and LC3-I. The MTT assay was performed to assess cell viability, while ELISA was performed to determine the supernatant levels of interleukin (IL)-1 $\beta$ , IL-6 and tumor necrosis factor- $\alpha$ . The results demonstrated that the effects of H/R induction on inflammatory factor secretion, oxidative stress, autophagy and cell viability in astrocytes were aggravated by thrombin, the effects of which were reversed following SPRED2 knockdown. Taken together, the results of the present study suggest that thrombin aggravates H/R injury in astrocytes by activating the SPRED2-mediated autophagy.

## Introduction

Ischemia/reperfusion (I/R) brain injury is one of the leading causes of mortality in cardiovascular and cerebrovascular diseases, with high morbidity and mortality rates worldwide (1). Cerebral ischemic events impact brain physiology (2). An acute ischemic stroke may occur when an artery supplying blood to the brain becomes occluded, leading to excitotoxicity and cell death, ultimately leading to brain tissue death and focal neurological deficits (3).

Prothrombin is cleaved to form thrombin, which acts as a serine protease and belongs to the chymotrypsin family (4). Thrombin activates blood coagulation factors and the anticoagulant protein C (5). Previous studies have demonstrated that the expression and activity of thrombin increases in tissues and cells following cerebral ischemic injury, and brain cell toxicity is also induced by thrombin (6,7). Bushi *et al* (7) reported increased thrombin activity in the brain following acute ischemic stroke and demonstrated a close association between thrombin levels and progression of brain damage. Furthermore, thrombin expression is commonly upregulated around the lesion site in intracerebral hemorrhage (8).

Autophagy is a cellular degradation process in which unnecessary or damaged cytoplasmic contents are removed to maintain cellular homeostasis (9). Moderate autophagy has been reported to promote cell survival rate, while excessive autophagy may induce cell cytotoxicity, leading to cell death (10); for example, I/R injury enhanced autophagy (11). Thrombin levels are higher in the ischemic stroke model compared with the control group (12). However, the regulatory association between thrombin and autophagy remains unclear.

Sprouty-related EVH1 domain (SPRED) proteins bind to Ras and Raf-1 and inhibit cytokines, growth factors and the ERK-MAPK pathway (13). A recent study reported that SPRED2 is an essential regulator of cardiac autophagy, whereby its deficiency suppresses autophagy (14). However, the role of SPRED2 in autophagy and the association between SPRED2 and thrombin is yet to be investigated.

‘Proper’ autophagy is considered to protect against I/R injury, while excessive autophagy contributes to the injury (15,16). For astrocytes, activation of autophagy protects, as well as causes injury (17-19). I/R injury has been reported to activate autophagy in astrocytes, causing astrocyte cell death (17). However, a few studies have demonstrated that the autophagic flux in astrocytes protects brain I/R injury (18,19). Generally, thrombin induces autophagy in astrocytes (20,21). Given that thrombin may contribute to brain I/R injury, the association between thrombin-mediated autophagy in astrocytes and hypoxia/reoxygenation (H/R) conditions requires investigation.

The present study aimed to investigate the roles and molecular mechanisms of thrombin, SPRED2 and autophagy in H/R induced astrocytes and to provide a novel target therapy against cerebral I/R injury.

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**Key words:** thrombin, hypoxia/reoxygenation, Sprouty-related EVH1 domain-2, autophagy, inflammatory factor, oxidative stress

## Materials and methods

**Cell culture and H/R.** Rat primary astrocytes were purchased from the American Type Culture Collection and maintained in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Cytiva), 100 IU/ml penicillin and 100  $\mu$ g/ml streptomycin (Sigma-Aldrich; Merck KGaA), at 37°C with 5% CO<sub>2</sub> in a humidified atmosphere.

Initially, cells were cultured in DMEM supplemented with 0.5% FBS for 12 h and placed in a hypoxia chamber with 5% CO<sub>2</sub>/95% N<sub>2</sub> and incubated at 37°C for 24 h. Subsequently, astrocytes were cultured in DMEM supplemented with 10% FBS at 37°C with 5% CO<sub>2</sub> for 4 h for reoxygenation.

**Cell treatment and transfection.** Dabigatran (1 nM; Selleck Chemicals) or thrombin (5 U/ml; Sigma-Aldrich; Merck KGaA) with/without dabigatran were used to treat the induced cells, or the induced cells were treated with thrombin (5 U/ml) with/without 3-MA (Sigma-Aldrich; Merck KGaA).

The H/R induced cells were treated with thrombin (5 U/ml) and transfected with small interfering (si)-SPRED2 or si-negative control (NC; 5 nM; all synthesized and purchased from Shanghai GenePharma Co., Ltd.) using Lipofectamine<sup>®</sup> 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. The Lipofectamine<sup>®</sup> 2000-si-SPRED2 complex was formed by mixing for 20 min at room temperature. Then, Lipofectamine<sup>®</sup> 2000-si-SPRED2 complex was added to the cells and the cells were cultured in serum-free Opti-MEM (Gibco; Thermo Fisher Scientific, Inc.) at 37°C for 4 h, followed by incubation in serum-containing medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) at 37°C for 72 h. After 48 h, the transfection efficiency was evaluated by reverse transcription-quantitative (RT-qPCR). The sequences were as follows: si-SPRED2, 5'-AUAGCAUCCAAUACAACCAG-3' and si-NC, 5'-AAUUGACCCAACAAUCACAU-3'.

**MTT assay.** The MTT assay was performed to assess cell viability. Briefly, astrocytes in different groups were seeded into 96-well plates at a density of 3x10<sup>3</sup> cells/well and were incubated for 48 h at 37°C, with 5% CO<sub>2</sub>. Subsequently, cells were incubated with 10  $\mu$ l MTT of 5 mg/ml (Roche Diagnostics) for 4 h at 37°C. Following the MTT incubation, the purple formazan crystals were dissolved using 180  $\mu$ l DMSO and viability was subsequently analyzed at a wavelength of 450 nm, using a spectrophotometer (Omega Bio-Tek).

**RT-qPCR.** RT-qPCR analysis was performed to detect the expression levels of SPRED2 and thrombin. Briefly, total RNA was extracted from cells using TRIzol<sup>®</sup> reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA was reverse transcribed into cDNA using the PrimeScript RT reagent kit (Applied Biosystems; Thermo Fisher Scientific, Inc.). RT conditions were as follows: 30°C for 10 min, 42°C for 50 min and 95°C for 5 min. PCR reactions were performed in an ABI 7500 Fast RealTime PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). qPCR was subsequently performed using the Fast Start Universal SYBR-Green Master (ROX, Takara Biotechnology Co., Ltd.) under the following thermocycling conditions: 95°C for 30 sec, 95°C for 5 sec

and 60°C for 35 sec for 40 cycles, then 72°C for 10 min. The following primer sequences were used for qPCR: SPRED2 forward, 5'-TGTGAGCACCGGAAGATTATACC-3' and reverse, 5'-CGCGGCGGCTTTGTGCTT-3'; thrombin forward, 5'-ATGGCTGCAATCCGAAGAAG-3' and reverse, 5'-ACAGTAGGGACGTAGACCTCC-3'; and GAPDH forward, 5'-ACCACAGTCCATGCCATCAC-3' and reverse, 5'-TCCACCACCCTGTTGCTGTA-3'. Relative expression levels were calculated using the 2<sup>- $\Delta\Delta$ C<sub>q</sub></sup> method and normalized to the internal reference gene GAPDH (22).

**Western blotting.** Western blot analysis was performed to detect the protein expression levels of SPRED2, microtubule-associated protein light chain 3 (LC3)-II, LC3-I and Beclin 1. Briefly, treated cell lines were harvested and washed with PBS. Total protein was extracted from cells using RIPA buffer (Vazyme Biotech Co., Ltd.). Protein was then quantified using the BCA<sup>™</sup> Protein Assay kit (Merck KGaA). Samples (30  $\mu$ g) were then separated by 10% SDS-PAGE (Thermo Fisher Scientific, Inc.). The separated proteins were subsequently transferred onto PVDF membranes (Amersham; Cytiva) and blocked with 5% non-fat milk at room temperature for 2 h. The membranes were incubated with primary antibodies against thrombin (cat. no. ab92621; 1:1,000), SPRED2 (cat. no. ab153700; 1:500), Beclin 1 (cat. no. ab210498; 1:1,000), LC3-II/I (cat. no. ab62721; 1:2,000) and GAPDH (cat. no. ab181602; 1:10,000) overnight at 4°C (all purchased from Abcam). Following the primary incubation, membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (cat. no. ab205718; 1:2,000; Abcam) at 37°C for 45 min. Protein bands were visualized by Pierce ECL Western Blotting Substrate (Pierce; Thermo Fisher Scientific, Inc.) using the Bio-Image Analysis System (Bio-Rad Laboratories, Inc.). GAPDH served as the internal control.

**ELISA.** ELISA kits were used to measure the protein expression levels of interleukin (IL)-1 $\beta$ , IL-6 and tumor necrosis factor (TNF)- $\alpha$  in the cell supernatant. The following ELISA kits were used: Mouse IL-1 $\beta$  ELISA kit (cat. no. ab197742), Mouse IL-6 ELISA kit (cat. no. ab100712) and Mouse TNF- $\alpha$  ELISA kit (cat. no. ab208348), according to the manufacturer's instructions (all purchased from Abcam).

**Measurement of superoxide dismutase (SOD), malondialdehyde (MDA) and reactive oxygen species (ROS) generation.** Following H/R treatment, SOD activity was detected using the xanthine oxidase method and MDA content was quantified via the thiobarbituric acid assay (TBA) using MDA (cat. no. A003-1-2) and SOD (cat. no. A001-3-2) kits (Nanjing Jiancheng Bioengineering Institute). ROS generation during reoxygenation was detected using CM-H2DCFDA (cat. no. C6827; Invitrogen; Thermo Fisher Scientific, Inc.).

**Statistical analysis.** Statistical analysis was performed using SPSS 20.0 software (IBM Corp.). All experiments were repeated in triplicate. Data are presented as the mean  $\pm$  standard deviation. Unpaired Student's t-test was used to compare differences between two groups, while one-way ANOVA followed by Tukey's post hoc test were used to compare differences between multiple groups. P<0.05 was considered to indicate a statistically significant difference.

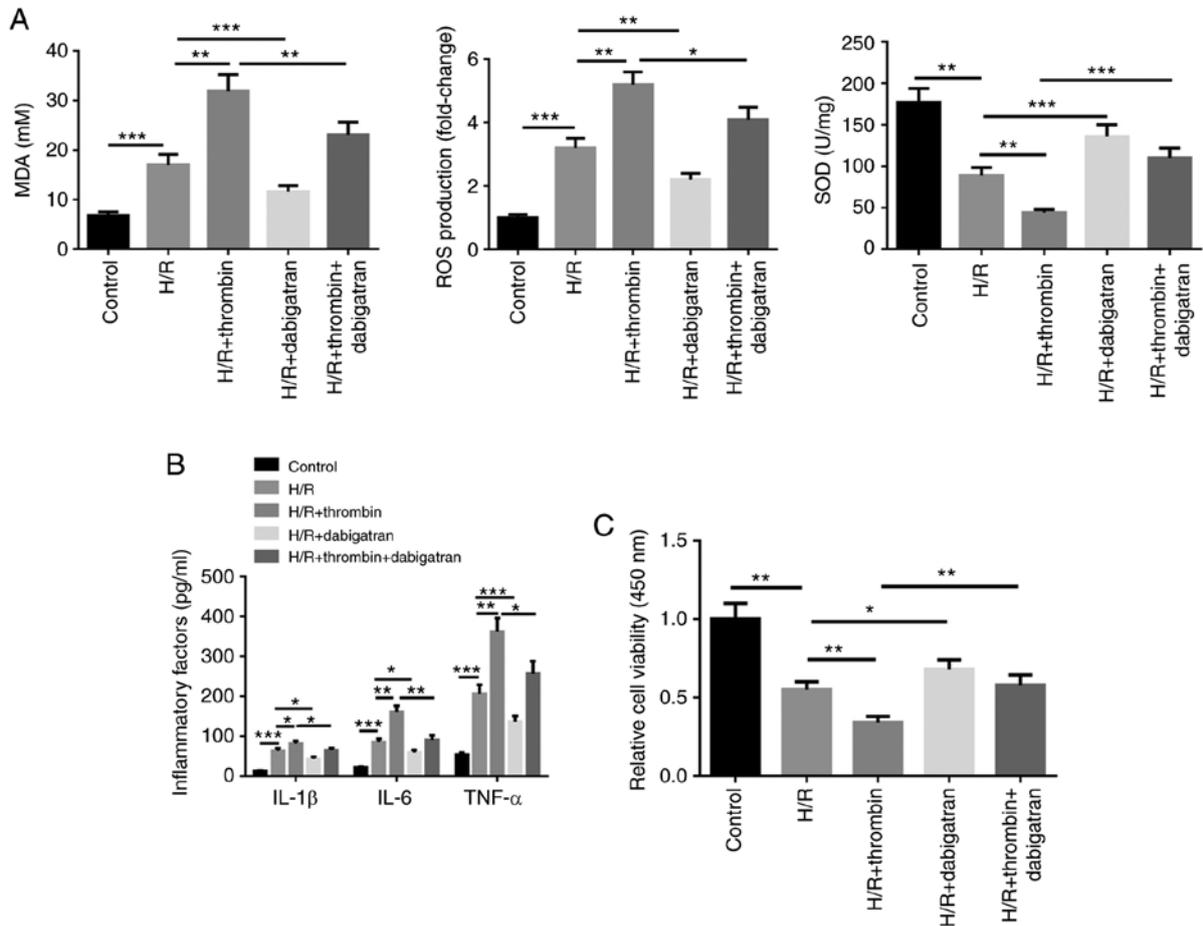


Figure 1. Thrombin aggravates inflammatory factor secretion and oxidative stress in H/R induced astrocytes. (A) The xanthine oxidase method was used to detect SOD activity, the thiobarbituric acid assay was performed to quantify MDA content and CM-H<sub>2</sub>DCFDA was used to detect ROS generation in H/R induced cells. H/R induced cells were treated with thrombin, dabigatran, and thrombin and dabigatran, respectively. (B) The cell supernatant levels of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  were detected via ELISA. (C) The MTT assay was performed to assess cell viability. \*P<0.05; \*\*P<0.01; \*\*\*P<0.001. H/R, hypoxia/reoxygenation; SOD, superoxide dismutase; MDA, malondialdehyde; ROS, reactive oxygen species; IL, interleukin; TNF, tumor necrosis factor.

## Results

**Thrombin aggravates inflammatory factor secretion and oxidative stress in H/R induced astrocytes.** The effect of thrombin on astrocytes induced by H/R treatment was assessed by detecting inflammatory factor secretion, oxidative stress and cell viability. The results demonstrated that SOD activity was inhibited following H/R treatment, whereas MDA content and ROS levels increased (Fig. 1A). In addition, the cell supernatant levels of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  increased following H/R treatment (Fig. 1B), whereas cell viability was suppressed, according to the MTT assay (Fig. 1C). In addition, the effect of H/R on astrocytes for SOD activity, MDA content, ROS level, the cell supernatant levels of IL-1 $\beta$ , IL-6 and TNF- $\alpha$ , and cell viability was promoted by thrombin, the effects of which were reversed following treatment with dabigatran (Fig. 1A-C). Taken together, these results suggest that thrombin can aggravate inflammatory factor secretion and oxidative stress in H/R induced astrocytes, and inhibit cell viability.

**Thrombin aggravates autophagy in H/R induced astrocytes.** The effect of thrombin on autophagy in H/R induced astrocytes was investigated. Western blot analysis demonstrated that the protein expression levels of thrombin, SPRED2 and

Beclin 1, and the ratio of LC3-II/I increased following H/R induction, which was promoted by thrombin but attenuated by dabigatran (Fig. 2A). In addition, the regulation of thrombin on the expression levels of SPRED2, Beclin 1 and the ratio of LC3-II/I was reversed by dabigatran. RT-qPCR analysis demonstrated that the mRNA expression levels of thrombin and SPRED2 increased following treatment with thrombin and decreased following treatment with dabigatran, and dabigatran reversed the effects of thrombin on the levels of thrombin and SPRED2 (Fig. 2B). Collectively, these results suggest that thrombin aggravates autophagy in H/R induced astrocytes.

**SPRED2 knockdown suppresses the effect of thrombin on inflammatory factor secretion and oxidative stress in H/R induced astrocytes.** The molecular mechanism of SPRED2 for thrombin on inflammatory factor secretion, oxidative stress and cell viability in H/R induced astrocytes was investigated. As presented in Fig. 3A, SPRED2 knockdown significantly decreased SPRED2 protein expression. MDA content and ROS levels increased following H/R treatment, whereas SOD activity was suppressed (Fig. 3B). In addition, the levels of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  in cell supernatant increased following H/R treatment (Fig. 3C), whereas cell viability was

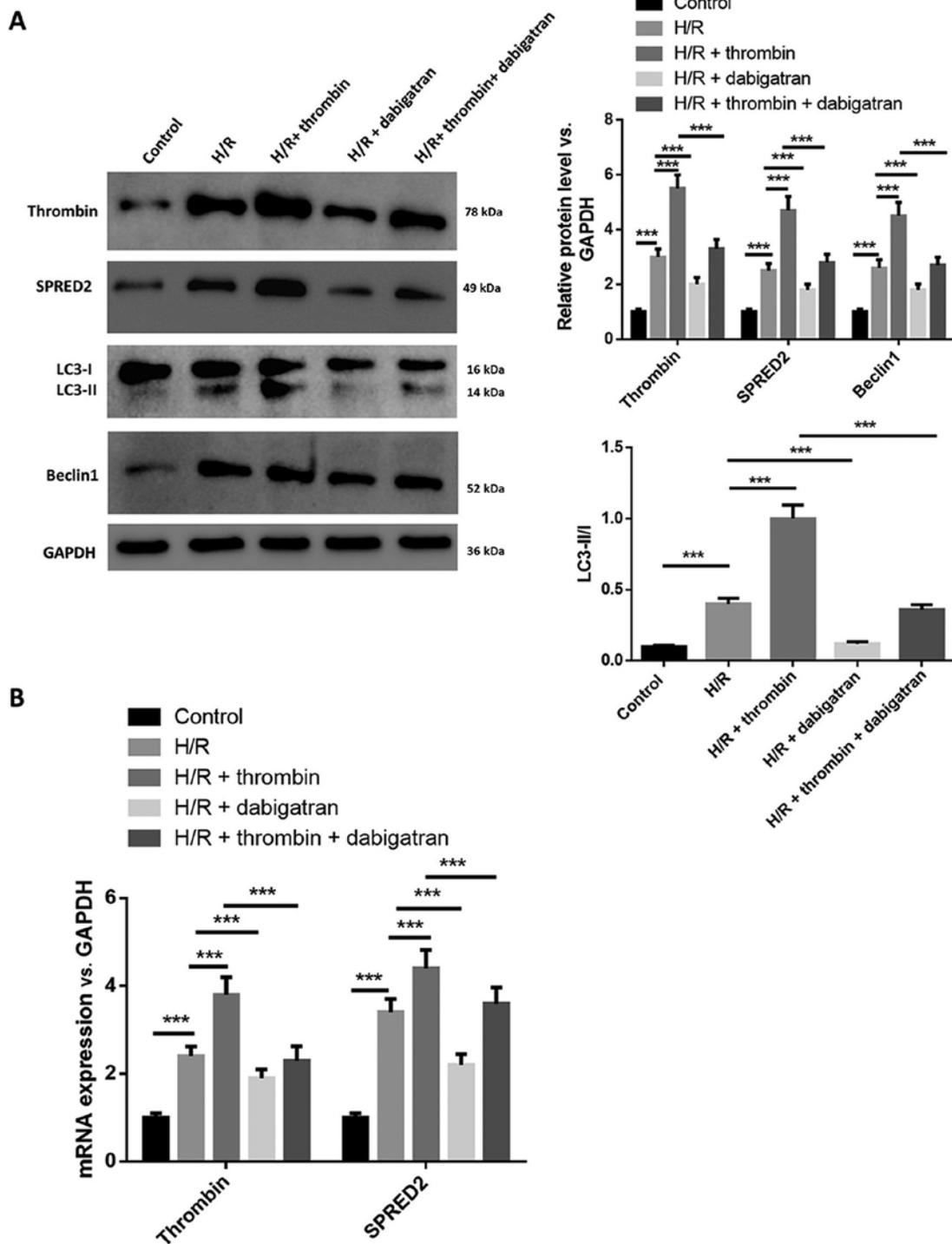


Figure 2. Thrombin aggravates autophagy in H/R induced astrocytes. (A) Western blot analysis was performed to detect the protein expression levels of thrombin, SPRED2, Beclin 1, LC3-II and LC3-I. (B) Reverse transcription quantitative PCR analysis was performed to detect the mRNA expression levels of SPRED2 and thrombin. \*\*\* $P < 0.001$ . H/R, hypoxia/reoxygenation; SPRED2, Sprouty-related EVH1 domain-2; LC3, microtubule-associated protein light chain 3.

inhibited (Fig. 3D). The effect of H/R induction on astrocytes was aggravated following addition of thrombin. However, the expression levels of the inflammatory factors (IL-1 $\beta$ , IL-6 and TNF- $\alpha$ ) and oxidative stress markers (MDA and ROS) in cells treated with thrombin were inhibited following SPRED2 knockdown, while the level of SOD was elevated (Fig. 3B and C). Furthermore, the expression levels of the inflammatory factors and oxidative stress markers in cells treated with thrombin were suppressed by autophagy inhibitor

3-MA. Taken together, these results suggest that SPRED2 knockdown suppresses the effect of thrombin on inflammatory factor secretion and oxidative stress in H/R induced astrocytes.

*SPRED2 knockdown suppresses the regulation of thrombin on autophagy in H/R induced astrocytes.* The regulation of SPRED2 on autophagy in H/R induced astrocytes was investigated. The results demonstrated that thrombin increased

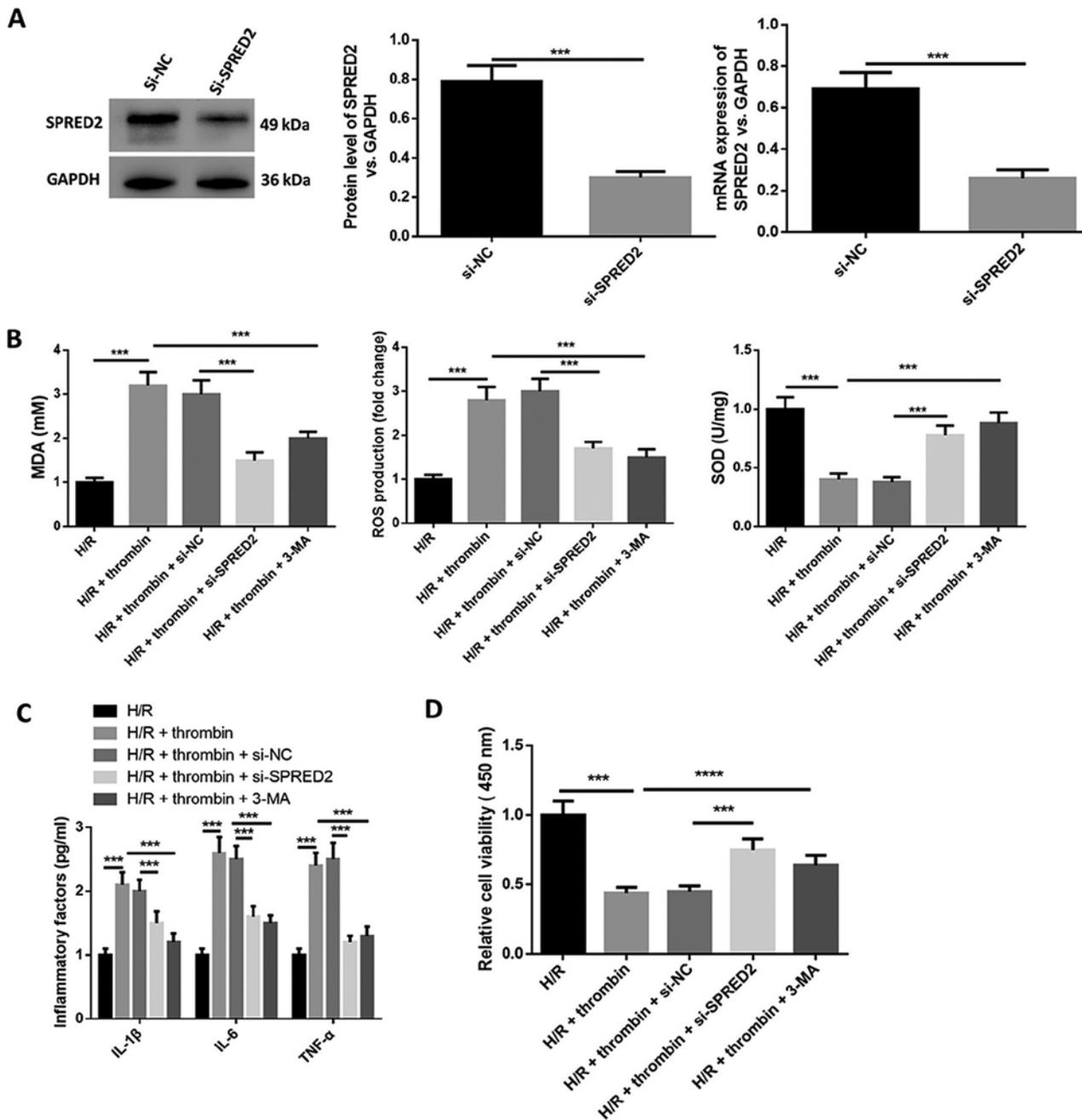


Figure 3. SPRED2 knockdown suppresses the effect of thrombin on inflammatory factor secretion and oxidative stress in H/R induced astrocytes. (A) Western blot and RT-qPCR analysis were performed to determine transfection efficiency of si-SPRED2. (B) The xanthine oxidase method was used to detect SOD activity, the thiobarbituric acid assay was performed to quantify MDA content and CM-H2DCFDA was used to detect ROS generation in H/R induced cells. H/R induced cells were treated with thrombin, and thrombin and 3-MA, and transfected with si-SPRED2 or si-NC. (C) The cell supernatant levels of IL-1 $\beta$ , IL-6 and TNF- $\alpha$  were detected via ELISA. (D) The MTT assay was performed to assess cell viability. \*\*\*P<0.001. SPRED2, Sprouty-related EVH1 domain-2; H/R, hypoxia/reoxygenation; si, small interfering; SOD, superoxide dismutase; MDA, malondialdehyde; ROS, reactive oxygen species; IL, interleukin; TNF, tumor necrosis factor; NC, negative control.

SPRED2 expression, the ratio of LC3-II/I and Beclin 1 expression (Fig. 4A), the effects of which were reversed following transfection with si-SPRED2. The effect of thrombin on cells induced by H/R was suppressed following treatment with 3-MA. RT-qPCR analysis demonstrated that SPRED2 expression decreased following transfection with si-SPRED2, and 3-MA reversed the effect of thrombin on cells with H/R induction (Fig. 4B). Collectively, these results suggest that thrombin regulation on autophagy in H/R induced astrocytes can be inhibited by silencing SPRED2.

## Discussion

Irreversible damages, such as oxidative stress, death associated with inflammation, neuronal injury and excitotoxicity, are the results of I/R brain injury (23). Oxidative stress plays an important role in the development of the ischemic cascade. Thus, the intervention of oxidative damage may prevent diseases induced by oxidative stress (24). Furthermore, inflammation plays an important role in the pathophysiology of cerebral ischemia, as well as ischemic cerebrovascular diseases (25). The roles and

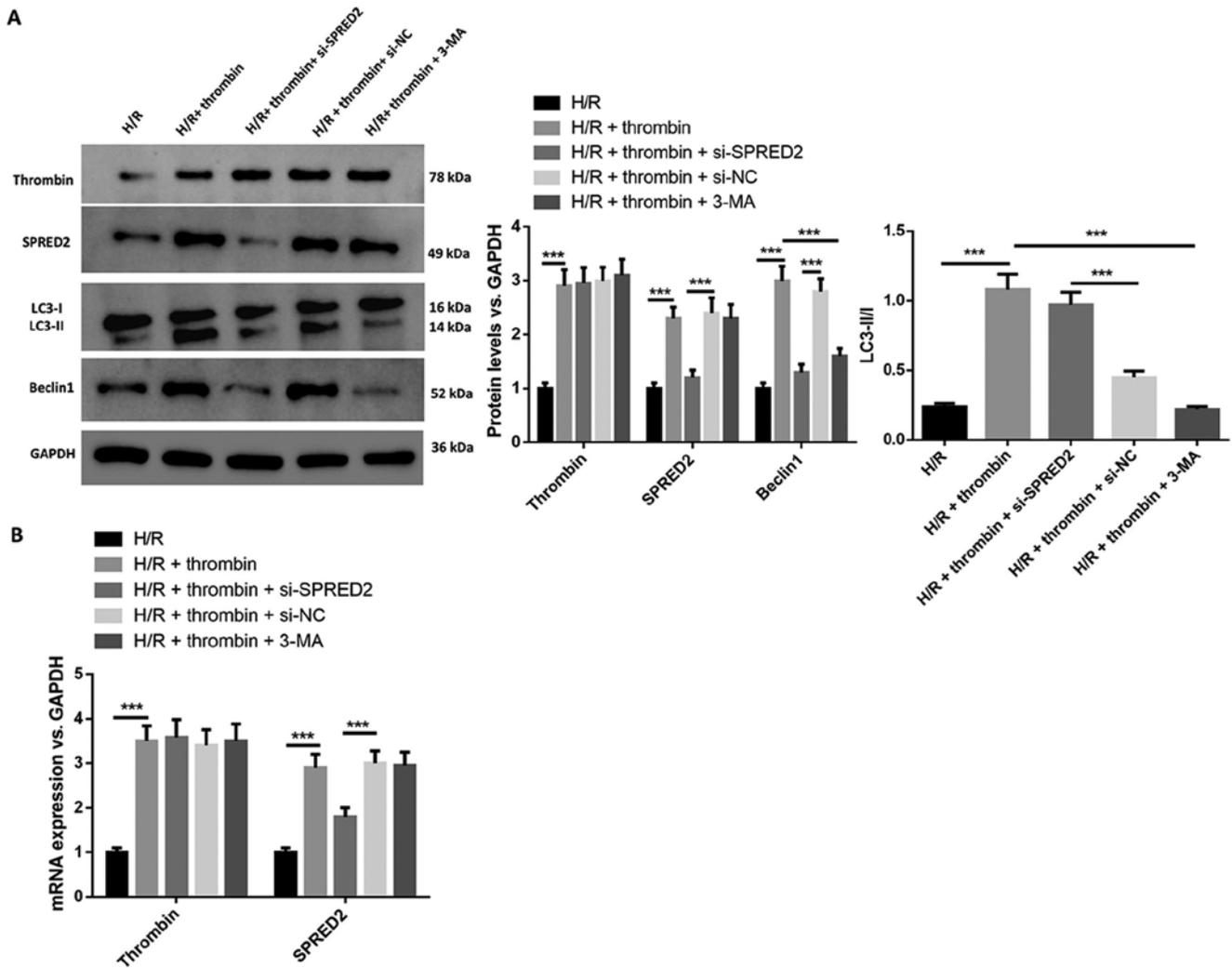


Figure 4. Regulation of thrombin on autophagy in H/R induced astrocytes is suppressed by silencing SPRED2. (A) Western blot analysis was performed to detect the protein expression levels of thrombin, SPRED2, Beclin 1, LC3-II and LC3-I. (B) Reverse transcription-quantitative PCR analysis was performed to detect the mRNA expression levels of SPRED2 and thrombin. \*\*\*P<0.001. H/R, hypoxia/reoxygenation; SPRED2, Sprouty-related EVH1 domain-2; LC3, microtubule-associated protein light chain 3; si, small interfering; NC, negative control.

molecular mechanisms of thrombin and SPRED2 in the I/R induced rat astrocytes model were investigated in the present stud.

Increasing evidence suggest that thrombin is a mediator for cerebrovascular inflammation and hypoxia, with obvious value in hypoxia, inflammation and oxidative stress (26,27). A previous study reported that thrombin promotes macrophage differentiation into an M1-like phenotype, closely associated with the expression of classical pro-inflammatory markers (28). Another study demonstrated that thrombin promotes inflammatory gene expression and enhances sustained signaling in astrocytes (29). The results of the present study demonstrated that thrombin aggravates inflammatory factor secretion and oxidative stress induced by H/R in astrocytes, which is consistent with the aforementioned reports (28,29). Autophagy usually occurs in response to cellular stresses, including oxidative stress, infection and nutrient starvation (15,30). However, the molecular mechanism of autophagy remains unknown. Hu *et al* (21) reported that thrombin increases the ratio of LC3-II/LC3-I and the level of cathepsin D; thus, thrombin activates autophagy and plays an important role in

ICH-induced autophagy. Another report demonstrated that the level of perihematomal neuron autophagy was correlated with thrombin-antithrombin plasma levels in patients with intracerebral hemorrhage (31). The present study demonstrated that thrombin aggravated autophagy in H/R induced astrocytes.

SPRED2 belongs to a family of proteins containing a cysteine-rich domain and is widely expressed in several tissues, including the brain (32). SPRED2 regulates adipose tissue inflammation and metabolic abnormalities induced by a high-fat diet in mice (33). Downregulated SPRED2 expression attenuates epithelial cell injury and inflammation in dextran sulfate sodium-induced acute colitis in mice (34). Itakura *et al* (35) reported that inflammatory responses decrease by inhibiting SPRED2 from protecting mice from polymicrobial sepsis by activating the ERK/MAPK pathway. The results of the present study demonstrated that the promotion of inflammatory factor secretion and oxidative stress in H/R induced astrocytes by thrombin was suppressed following SPRED2 knockdown. However, very few studies have investigated the role of SPRED2 in autophagy (32-35). In addition, autophagy may be a promising target for promoting

SPRED2-mediated antitumor activity (36). The results of the present study demonstrated that SPRED2 knockdown suppressed the promotional effect of thrombin on autophagy in H/R induced astrocytes.

The present study is not without limitations. First, deeper insights on the regulatory association between SPRED2 and autophagy in the I/R process were not investigated in the present study. Secondly, it is unclear whether other signaling pathways are involved in thrombin aggravated I/R injury. Thus, further studies are required to confirm the results presented here.

In conclusion, the results of the present study demonstrated that inflammatory factor secretion, oxidative stress and autophagy in H/R induced astrocytes were aggravated by thrombin, which was inhibited by SPRED2 knockdown. To the best of our knowledge, the present study is the first to demonstrate H/R injury of astrocytes aggravated by thrombin by activating the autophagy pathway mediated by SPRED2. This research will help to identify novel targets against I/R brain injury.

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### Availability of data and materials

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

### Authors' contributions

XW designed and performed the experiments, analyzed data and wrote the manuscript. WL and BL performed experiments and collected and analyzed data. YX performed data analysis and reviewed and revised the manuscript. WL and YX confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

Not applicable.

### Patient consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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